GM CROPS & FOOD
2024, VOL. 15, NO. 1, 336–351
https://doi.org/10.1080/21645698.2024.2420412

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Risk assessment of homologous variants of biotech trait proteins using a bridging approach

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ABSTRACT

A transgenic protein is frequently expressed as different homologous variants in genetically modified crops due to differential processing of targeting peptides or optimization of activity and specificity. The aim of this study was to develop a science-based approach for risk assessment of homologous protein variants using dicamba mono-oxygenase (DMO) as a case study. In this study, DMO expressed in the next-generation dicamba-tolerant maize, sugar beet and soybean crops exhibited up to 27 amino acid sequence differences in the N-terminus. Structure modeling using AlphaFold, ESMFold and OpenFold demonstrates that these small N-terminal extensions lack an ordered secondary structure and do not disrupt the DMO functional structure. Three DMO variants were demonstrated to have equivalent immunoreactivity and functional activity ranging from 214 to 331 nmol/min/mg. Repeated toxicity studies using each DMO variant found no test substance-related adverse effects. These results support that homologous protein variants, which have demonstrated physicochemical and functional equivalence, can leverage existing safety data from one variant without requiring additional *de novo* safety assessments.

ARTICLE HISTORY
Received 4 June 2024
Revised 9 October 2024
Accepted 18 October 2024

KEYWORDS
Bridging; functional equivalence and DMO; homologous protein variants; safety assessment

1. Introduction

The safety assessment of newly expressed proteins (NEPs) in genetically modified (GM) crops is described in an internationally recognized framework established by the Food and Agriculture Organization of the United Nations (FAO)/the World Health Organization (WHO) Codex Alimentarius Commission in 2009.[1] Under this framework, proteins are assessed using a weight of evidence and case-by-case approach,[1–6] which has been extensively used to determine the safety of NEPs in GM crops for human and other vertebrate animal consumption. The International Food Biotechnology Committee of the International Life Sciences Institute (ILSI) described a two-tiered approach to optimize the safety assessment of NEPs.[2] Tier I identifies potential hazards through assessment of information and studies: (a) history of safe use/consumption in food, (b) source organism from which the protein is derived and its mode of action, (c) bioinformatics analysis providing a structural comparison (e.g., primary amino acid sequence homology) to proteins with a known history of safe use, (d) bioinformatics analysis providing a structural comparison (e.g., primary amino acid sequence homology) to known allergens, toxins or other biologically active proteins known to have adverse effects in mammals, (e) characterization of the physicochemical and functional properties of the protein, and (f) stability of the protein to degradation by gastrointestinal digestive proteases such as pepsin and pancreatin and to heat treatment as would occur during processing and/or cooking.[2] If a potential hazard is identified in Tier I, the hazard is characterized in Tier II through assessment of the following on a case-by-case basis: acute toxicity study and/or repeated dose toxicity study.[2,7] If no hazard is identified as a part of Tier I evaluations, it should be concluded that the NEP in the GM crop is safe for consumption.[2,7]

To date, little hazard has been identified from any NEP since the first GM crop was commercialized in 1996. During product development,

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rigorous safety assessments including bioinformatics analysis and early allergen testing are conducted and any undesired events including off-types are discontinued.[6,8–15] Despite the risk assessments not having identified any hazards from NEPs thus far, some regulatory agencies continue to require Tier II hazard characterization animal studies such as *in vivo* acute or repeated dose toxicity studies for the safety assessment of every NEP in GM crops. These required studies use a few to hundreds of grams of proteins and large numbers of animals. The welfare of animals in scientific research, as outlined by the three Rs of responsible animal use (Replacement, Reduction and Refinement), has been increasingly incorporated into regulations and regulatory agencies have announced plans to reduce or altogether eliminate mammalian study requirements.[16–21] Applying a hypothesis-driven or case-by-case approach in the safety assessment of NEPs and GM crops can significantly reduce animal testing without compromising either the quality or integrity of NEP and GM crop risk assessment[2,22–24] as requested by EPA[20] and the European Union.[25]

A common practice in the development of GM crops is to use targeted gene expression of the NEP with the help of chloroplast transit peptides (CTPs) to protect the protein from degradation by cytosolic proteases or protein turnover resulting in adequate accumulation of the protein *in planta*.[26–28] This approach also allows some NEPs, such as dicamba mono-oxygenase (DMO) to utilize chloroplast-localized partners or cofactors for its enzymatic activity.[14,29,30] These CTPs are processed during import of the NEP into the chloroplast, but in some cases this processing is incomplete, leaving a small fragment of the CTP at the N-terminus of the NEP.[6,14] The same NEPs with different N-termini in different plants are hereafter referred to as homologous variants. These N-terminal differences between homologous variants usually do not impact protein structure and activity as they involve only a few amino acids.[6,14] In the case of DMO, homologous variants display characteristics that are physicochemically and functionally equivalent to the non-CTP containing protein (hereafter referred as the mature protein) despite N-terminal differences. No potential hazards were identified in Tier I evaluations for these homologous variants; however, some regulatory agencies continue to request full safety data packages including animal studies for each new DMO homologous variant.

Both developers and global regulatory agencies have significant experience and knowledge in the safety assessment of NEPs. Safety assessments can be improved by leveraging this collection of scientific data and knowledge. Tier I studies (also referred as core studies) are important for protein safety assessment; however, need for hypothesis-driven Tier II studies (also referred as supplementary studies) can be determined on a case-by-case basis.[22,24] The use of a bridging approach can be an additional tool for risk assessment of NEPs for which multiple homologous variants exist.

In this study, mature proteins expressed in GM crops along with their respective homologous variants were compared side-by-side according to a strategy proposed by Brune et al.[22] for their physicochemical and functional properties. Our hypothesis is that homologous variants expressed across different GM crops have physicochemical and functional properties equivalent to the mature protein. If this hypothesis is valid, Tier II studies, including animal studies, should not be necessary.

2. Materials and methods

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (Burlington, MA, United States of America), and all materials were purchased from Vendors within the United States of America.

2.1. Development of next-generation dicamba-tolerant GM crops

The development of herbicide-tolerant crops has greatly advanced weed management. DMO, a terminal Rieske oxygenase in a three-component system with ferredoxin and reductase, enables transgenic plants that express DMO to degrade the broadleaf herbicide dicamba, thereby providing herbicide tolerance. For optimal functionality, DMO must be targeted to the chloroplast with the aid of CTP enabling co-localization with endogenous NADH-dependent reductase and ferredoxin, which facilitate electron transfer for the

DMO oxidative reaction.[29,30] The first-generation of DMO GM crops has been commercially available since 2015, establishing a history of safe use for the DMO protein.

While the first-generation of DMO GM crops continues to be commercially valuable, new herbicide tolerant traits are needed to prevent overreliance on a single herbicidal mode of action and to enhance options for managing difficult-to-control weed species. Therefore, next-generation GM products were developed that express the DMO protein alongside multiple other herbicide-tolerant proteins. The next-generation GM soybeans tolerate glufosinate, mesotrione, FOPs (aryloxyphenoxypropionate herbicides), 2,4-D and dicamba herbicides. The next-generation GM maize tolerates glyphosate, glufosinate, FOPs, 2,4-D and dicamba while the next-generation GM sugar beet tolerates glyphosate, glufosinate, and dicamba. In contrast, the first-generation dicamba-tolerant GM soybeans express only DMO for dicamba tolerance, the first-generation GM sugar beet tolerates only glyphosate, and the first-generation GM maize tolerates both dicamba and glufosinate. Developing crops with multiple herbicide tolerant traits is crucial for weed management. DMO, as part of a trait stack with different proteins, is integral to the development of these next-generation dicamba-tolerant (DT) crops.

The *dmo* gene was originally cloned from *Stenotrophomonas maltophilia*, which expresses DMO protein (hereafter "wild type" DMO, accession ID: Q5S3I3.1, Figure 1(b)). Next-generation DT GM soybean, maize, and sugar beet were developed by fusing different CTP-coding sequences with the *dmo* gene. Additionally, a leucine codon was added at position 2 of wild type *dmo* to aid the cloning process during plant vector construction (Figure 1(b)).[31] The DMO proteins in the GM soybean and maize were fused with a CTP from the *Albino and pale green 6* (*Apg6)* gene from *Arabidopsis thaliana* encoding a HSP101 (heat shock protein) homologue and acts as a transit peptide that directs transport of the protein to the chloroplast.[32] Next-generation DT sugar beet was developed using the same *dmo* gene, and the CTP used was from pea (*Pisum sativum*) Rubisco small subunit as for the first-generation DT soybean DMO.[14] Figure 1 illustrates the variants of DMO present in the transformed crops, in which the DMO in next-generation GM soybean, maize, and sugar beet are designated nsDMO, nmDMO and sbDMO, respectively, in aligning with the previous DMO publication.[14]

2.2. Protein production

2.2.1. Plant protein purification for N-terminal determination

The DMO variant present in next-generation GM soybean (nsDMO) was purified in a series of steps. GM soybean seeds were harvested from field-grown plants, ground, and the resulting flour (100 g) was defatted with n-hexane (three 15 min extractions with 400 ml per extraction) and dried overnight in the fume hood before protein extraction. The defatted soybean flour (50 g) was suspended in 500 ml of Extraction Buffer (EB) (50 mM Tris pH 8.0, 1 mM DTT, 2 µM E-64, 1 mM benzamidine, 1 mM PMSF, and 1× EDTA free complete protease inhibitors (one pill per 50 ml)) and mixed for 2 hr at 4°C. After incubation, the sample was centrifuged at 20,000 x*g* for 10 min at 4°C. The clarified supernatant was collected and 1 M $CaCl_2$ added to a final concentration of 10 mM for precipitation for 30 min at 4°C. The sample was then centrifuged at 20,000 x*g* for 10 min at 4°C and the supernatant was collected. Solid ammonium sulfate was added to bring the concentration up to 0.75 M. Twenty-five ml of Q Sepharose Fast Flow resin (Cytiva, Marlborough, MA) was packed into a XK26/20 column (Cytiva). After filtration using a 45 µm filter, the clarified extract was further cleaned by passing through the Q Sepharose Fast Flow column. The column was flushed with EB plus 0.75 M ammonium sulfate until the absorbance at 280 nm (A280) reached baseline readings. The flow through was collected in one large fraction and loaded onto a DMO mAb affinity column. Three mg of the DMO mAb (Inotiv, Maryland Heights, MO) were crosslinked to one ml of the MabSelect Prism A resin (Cytiva) using bis-(sulfosuccinimidyl)suberate. One ml of the crosslinked resin was packed into an XK16/20 column (Cytiva). After washing with 50 column volumes of 50 mM Tris pH 8.0, 1 M ammonium sulfate, DMO was eluted using a buffer containing 50 mM Tris, pH 8.0, 0.8 M ammonium sulfate, 40% propylene

glycol. The purified DMO protein was further separated by SDS-PAGE gel for mass spectrometry analysis or transferred to a PVDF membrane for Edman degradation. nmDMO and sbDMO were purified using a similar procedure as described above, but $CaCl_2$ based precipitation and defatting were not needed.

2.2.2. DMO expression in *E. coli*

To produce sufficient amounts of DMO protein for analysis, surrogate proteins were produced utilizing *Escherichia coli* (*E. coli*). The coding sequence corresponding to nsDMO was cloned into a pET24 vector (Novagen, Madison, WI) and expressed in BL21 (*DE3*) *E. coli* (Invitrogen, Carlsbad, CA). The plant-produced nmDMO consists of a mixture of two forms. To achieve a similar mixture in *E. coli*, the coding sequences corresponding to each nmDMO form were simultaneously cloned into the pETDuet™-1 DNA (Novagen) and co-expressed in BL21 (*DE3*) *E. coli* (Invitrogen, Carlsbad, CA). To streamline and expedite the process, the coding sequence corresponding to sbDMO was cloned into the pET SUMO vector and expressed in Rosetta 2 (DE3) *E. coli*. The molecular cloning was conducted using the hot fusion method.[33]

E. coli cells were grown at 37°C in Terrific Broth medium containing 5.0 g/L glycerol, 12.0 g/L peptone, 24.0 g/L yeast extract, 2.3 g/L KH_2PO_4, and 12.5 g/L K_2HPO_4. Once the OD_{600} (optical density at 600 nm) reached 1–2, induction was carried out at 15 to 16°C with 1 mM IPTG, along with 50 µM Iron (II) sulfate heptahydrate and 50 µM Iron (III) chloride hexahydrate. After 24 hr, the cells were harvested. All three cell lines were fermented under these conditions, with differences in antibiotics: 50 mg/L Kanamycin for nsDMO, 100 mg/L Carbenicillin for nmDMO, and a combination of 50 mg/L Kanamycin and 30 mg/L Chloramphenicol for sbDMO. The DNA sequencing encoding each DMO protein was confirmed both prior to and following fermentation.

2.2.2.1. *E. coli* nsDMO purification. The nsDMO protein was purified from *E. coli* cell paste expressing the DMO protein. All purification steps were performed at 4°C, except where specifically stated. The thawed cell paste was resuspended at a ratio of 1 kg cell paste/10 L of Extraction Buffer (50 mM Tris, 2 mM benzamidine, 1 mM PMSF, 1 mM $MgCl_2$, pH 8.0 with ~ 100,000 units of benzonase/L). The resuspension was mixed thoroughly with a polytron homogenizer to obtain a homogeneous solution. The cell paste resuspension was lysed by passing once through a homogenizer (SPXFLOW R5–10.38, SPX FLOW, Inc., Charlotte, NC) at ~ 12,000 psi (pounds per square inch). An additional 40,000-50,000 units/L of benzonase was added post homogenization. The cell lysate was clarified by centrifugation at 15,000 x*g* for 75 min in a Beckman Coulter centrifuge equipped with a JLA-8.1000 rotor (Beckman Coulter, Inc., Brea, CA). The supernatant was decanted and filtered through Miracloth (EMD-Millipore catalog# 3475855) and allowed to sit overnight at 4°C (12–-16 hr). The pellet was discarded. Ammonium sulfate was then added (233.4 g/L supernatant) to reach the final concentration at 40% saturation by slow addition with gentle stirring over 1 hr. After the final addition, the solution was incubated at 4°C for an additional 2 hr with slow stirring. The ammonium sulfate solution was then clarified by continuous flow centrifugation (CEPA Z61, Carl Padberg Zentrifugenbau GmbH, Germany) at ~ 17,000 x*g* at room temperature (RT) with a feed rate of ~35 L/hr to recover the ammonium sulfate pellet. The pellet was stored at −80°C prior to further processing. The supernatant was discarded. The ammonium sulfate pellet was thawed overnight at 4°C. The pellet was then resuspended in solubilization buffer (25 mM Tris, 1 mM benzamidine, pH 8.0). Typically, ~3 kg of pellet was resuspended in 100 L of solubilization buffer with gentle stirring at 4°C. This homogeneous solution was then further diluted by addition of chilled Milli-Q water so that conductivity of the solution was <6 mS/m. Each 100 L of solubilization solution required approximately 60 L of chilled Milli-Q water. The diluted solubilization buffer was then clarified by passing through a 5.0/10.0 µm Polycap HD filter cartridge (GE Healthcare, catalog# 2813) to remove a slight particulate.

The filtered ammonium sulfate solution (typically ~160 L) was then loaded onto a 20 L DEAE Sepharose column (BPG 300/500 Column, DEAE Sepharose Fast Flow, Cytiva) connected to a AKTA Process system (Cytiva) maintained at 4°C and pre-

equilibrated with DEAE Buffer A (50 mM Tris, 1 mM Benzamidine, pH 8.0). The sample was generally loaded at a flow rate of 8 L/hr overnight. After loading, the column was washed to baseline with 3 column volume (CV) of DEAE Buffer A. The DMO protein was eluted with a step gradient of 9% DEAE Buffer B (50 mM Tris, 1 mM Benzamidine, 1 M NaCl, pH 8.0) at a flow rate of 20–30 L/hr for 3 CVs. This was followed by a step gradient of 25% DEAE Buffer B at a flow rate of 30 L/hr. The DMO peak was collected based on absorbance at A280 nm.

The eluted DEAE Sepharose DMO pool was then directly loaded onto an 8 L Ceramic Hydroxyapatite (CHT) Type I 20 µm resin (Bio-Rad, Hercules, CA) packed in a BPG 200/500 column (Cytiva) that had been pre-equilibrated with DEAE Buffer A at a flow rate of 100–400 ml/min. The column was directly eluted with 6% CHT Buffer B (50 mM Tris, 50 mM potassium phosphate, pH 8.0) at a flow rate of 100–200 ml/min. The DMO protein was collected based on absorbance at A280 nm. The purified nsDMO was exchanged to a buffer containing 50 mM potassium phosphate, pH 8, 100 mM NaCl, 1 mM DTT, 1 mM benzamidine HCl, 10% glycerol, frozen in aliquots on dry ice and stored at −80°C.

2.2.2.2. E. coli **nmDMO purification.** Two forms of nmDMO were co-purified following the same method as nsDMO described above. The purified nmDMO was frozen in aliquots on dry ice and stored at −80°C. nmDMO was stored in a buffer containing 50 mM Tris, pH 8, 100 mM NaCl, 1 mM DTT, 1 mM benzamidine, 10% glycerol.

2.2.2.3. E. coli **sbDMO purification..** About 4 kg of *E. coli* cell paste expressing sbDMO was resuspended by homogenizer in 50 L of Extraction Buffer (50 mM Tris pH 7.4, 1 mM PMSF, 1 mM Benzamidine, 0.1% Triton X-114) and lysed by microfluidizer (Microfluidics International Corporation, Londonderry, NH) to a final volume of 100 L. The cell lysate was clarified at ~ 16,900 *xg* using a CEPA Z61, and the supernatant was loaded onto a BPG 200/500 column (Cytiva) packed with 1.5 L Ni-NTA resin (Cube Biotech, Monheim, Germany) at 4°C. Bound protein was washed with 3 CV of Wash Buffer (50 mM Tris pH 7.4, 50 mM Imidazole, 500 mM NaCl, 5 mM ATP, 5 mM $MgCl_2$) before being eluted with 3 CV Elution Buffer (50 mM Tris pH 7.4, 500 mM Imidazole). All chromatography was performed at 220 cm/hr (560 ml/min).

Eluted 6HIS-SUMO sbDMO was precipitated with 40% $(NH_4)_2SO_4$ and then the pellet was collected by centrifugation. The 6HIS-SUMO sbDMO containing pellet was re-suspended in 4 L Equilibration Buffer (50 mM Tris pH 7.4) and then dialyzed against 150 L dialysis buffer (50 mM Tris pH 7.4) over 72 hr. Dialyzed sbDMO was centrifuged to remove precipitants. SUMO protease was added to the clarified 6HIS-SUMO sbDMO at a ratio of 1:200 g/g and left to react for 16 hr at 4°C.

Cleaved sbDMO was purified by loading onto a series of BPG 200/500 column (CV = 1.5 L) packed with 1.5 L Ni-NTA resin (Cube Biotech) and a BPG 200/500 column packed with 1.5 L DEAE Sepharose Fast Flow (Cytica) at 4°C. The cleaved sbDMO was in the flowthrough of the Ni-NTA column and residual protein was washed from the Ni-NTA column with 3 CV Equilibration Buffer. The flowthrough and wash flowed directly into the DEAE Fast Flow column at 220 cm/hr (600 ml/min relative to Ni-NTA column) where sbDMO was bound and then eluted with 3 CV DEAE Elution Buffer (50 mM Tris pH 7.4, 135 mM NaCl) at 350 cm/hr (450 ml/min relative to DEAE column). The purified sbDMO was diafiltered using a 50 kDa MWCO hollow fiber unit (Cytica) to Final Buffer (50 mM potassium phosphate pH 8, 100 mM NaCl, 1 mM DTT, 10% glycerol) and concentrated to 3.5 L prior to being stored at −80°C.

2.3. Protein characterization

Methods to characterize the DMO protein have been reported previously[6,13] with minor modifications. Briefly, the purity-corrected protein concentration of the sample from seed or grain and total protein concentration of the sample from the bacteria fermentation product were determined using gel-based densitometry and amino acid compositional analysis, respectively. The identity of each DMO variant was confirmed by N-terminal

sequence determination and peptide mass fingerprint analysis using nano liquid chromatography tandem mass spectrometry (LC-MS/MS)[34,35] or N-terminal determination with Edman chemistry.[36] Purity and apparent molecular weight of each DMO homologue were determined using densitometric analysis of Coomassie-stained SDS–PAGE gels. For western blot analysis, each protein was subjected to SDS–PAGE and transferred to a nitrocellulose membrane. The blot was probed with either a mouse anti-DMO monoclonal or goat anti-DMO polyclonal antibody. DMO activity assay was performed according to the method reported previously.[37] Briefly, the functional activities of the DMO variants were assessed by HPLC (High-Performance Liquid Chromatography) (Agilent Technologies 1200 series) quantification of 3,6-dichlorosalicylic acid (DCSA) from the conversion of dicamba. Each assay, performed in quintuple, used a reaction mixture containing 25 mM potassium phosphate, pH 7.2, 4 µg ferredoxin, 15.4 µg reductase, 0.5 mM $FeSO_4$, 10 mM $MgCl_2$, 0.7 mM NADH, 0.3 mM dicamba, 2 µl (42.48 U/ml) of formaldehyde dehydrogenase and approximately 0.5 µg of the DMO protein or approximately 1 µg of his-tagged DMO protein as an assay positive control. The reactions (200 µl each) were incubated at 30.0°C for 15 min, initiated by addition of dicamba, and quenched with 50 µl of 5% H_2SO_4. The mixtures were filtered and analyzed by HPLC using a Phenomenex® Synergi 4 µm C18/ODS Hydro-RP column (150 × 4.6 mm ID, Torrance, CA). The mobile phase consisted of solvent A (21.5 mM phosphoric acid) and solvent B (100% acetonitrile) at a flow rate of 1.5 ml/min. DCSA was eluted using a linear gradient from 90% to 40% solvent A over 14 min, followed by a step to 10% solvent A for 1 min and then re-equilibration at 90% solvent A for 10 min before the next injection. DCSA production was monitored by fluorescent detection at 424 nm (excitation: 306 nm) and quantified against a standard curve of DCSA (0.1, 0.3, 0.6, 0.9, 1.2, 2.4, and 4.8 nmol/250 µl). The specific activity was defined as units per milligram of DMO protein (U/mg), where one unit (U) is 1 nmol of dicamba to DCSA conversion per min at 30°C. Variants were considered equivalent if their activities fell within the 95% prediction interval (PI) of the reference substance.

2.4. Prediction of DMO variant structures

The folded protein structures for nsDMO, nmDMO, sbDMO, along with wild type DMO (WT DMO) were obtained using locally-installed versions of the artificial intelligence-driven computational protein folding programs AlphaFold,[38] OpenFold,[39] and ESMFold.[40] Modeled structures were aligned using PyMOL v2.4.1 (https://pymol.org/). Supplementary Figure S1 displays a sequence alignment of the four different DMO protein sequences submitted to the protein folding calculations.

2.5. Acute oral toxicity assessment

Acute oral toxicity of nsDMO, nmDMO, and sbDMO was assessed in three separate studies in compliance with Good Laboratory Practice. The study designs were adapted from Environmental Protection Agency Health Effects Test Guideline OPPTS 870.1100. All three studies were conducted at Charles River Laboratories, Inc in Spencerville, Ohio. In brief, CD-1 mice, approximately eight weeks old, were allocated to three groups of 20 mice each (10 males and 10 females) using a stratified randomization scheme. They were fasted and administered a targeted dose of 5000 mg DMO/kg body weight on day one and observed for 14 days. Mice were dosed with either the test dosing solution (TDS) containing the relevant DMO protein, bovine serum albumin (BSA) formulated to a target dose of 5000 mg/kg as the control dosing solution (CDS), or a vehicle dosing solution (VDS). The permeate collected during TDS concentration was utilized as the VDS for nsDMO and nmDMO. The VDS utilized for sbDMO was 10 mM potassium phosphate at pH 8.

The nsDMO TDS was dialyzed against 10 mM potassium phosphate buffer at pH 8 using dialysis tubing [10 kDa molecular weight cutoff (MWCO), Spectra/Por] before being concentrated to 77.0 mg/ml using Amicon Ultra Centrifugal Filters 15 (50 kDa MWCO, Millipore Sigma). The nmDMO TDS in MilliQ water was concentrated to 78.7 mg/ml using Amicon Ultra Centrifugal Filters 15 (30 kDa MWCO). The sbDMO TDS, stored in 10 mM potassium phosphate buffer at pH 8, was concentrated to 71.2 mg/ml using Amicon Ultra

Centrifugal Filters 15 (30 kDa MWCO). The identity, protein concentration, purity, homogeneity, and ability of the substance to pass through the dosing needle were characterized prior to the start of each study.

During the 14-day observation period, PMI Nutrition International Certified Rodent Chow No. 5002 and water were available to the rodents *ad libitum* except during designated procedures. Animals were assessed for mortality twice daily. Detailed clinical observations were conducted once prior to the study start, once prior to dosing on Day 0, at least twice post dose on Day 0 and then once daily thereafter. Animal body weights were recorded on Days 0 (pre-fasted and fasted), 7 and 14. Food consumption was measured quantitatively on Days 0, 7, and 14. Study animals were subject to a complete necroscopy under the supervision of a board-certified veterinary pathologist.

The statistical significance was set at 5% for each of the studies and statistical tests. Two-sided tests were reported at 1% and 5%. Group homogeneity was assessed with Leverne's test. Either a two-sided t-test or Wilcoxon Rank sum test was used to compare datasets with two groups. Datasets with at least three groups were compared with either a one-way ANOVA F-test or Kruskal-Wallis test.

3. Results

3.1. DMO expression and identity in GM crops

Western blotting results demonstrated that the protein samples from the next-generation dicamba-tolerant GM soybean, maize and sugar beet displayed one immunoreactive band each with different molecular weights (Figure 1(a)). Both nsDMO and nmDMO have similar electrophoretic mobility at ~ 36 kDa while sbDMO has a slower mobility at ~ 38 kDa, indicating that sbDMO has additional amino acids as compared to nsDMO and nmDMO.

To further characterize these *in planta* produced DMO proteins, they were purified using an immunoaffinity purification method and Edman sequencing was used to determine the N-terminal sequence for each DMO variant (Figure 1(b)). Subsequently, DMO N-terminal sequences were further confirmed by mass spectrometry analysis. The next-generation soybean was determined to have a mature form of the DMO protein in which CTP was completely processed *in vivo* by the stromal processing peptidase[41,42] and this was designated as the mature DMO protein. However, the next-generation sugar beet accumulated mature DMO with an additional 27 amino acids (MQVWPPIGKKKFETLSYLPPLTRDSRA) at the N-terminus originating from the pea Rubisco small subunit and intervening sequence. In contrast to nsDMO and sbDMO, next-generation maize had two forms of DMO. One form was the mature DMO, while another form was a homologous variant with an extra cysteine at N-terminus derived from the CTP. The two forms of DMO were indistinguishable by SDS-PAGE /western blot due to the resolution of the separation method, which cannot differentiate the one amino acid difference (Figure 1). Alternative cleavage of CTP from DMO *in planta* by a general stromal processing peptidase is common.[42]

3.2. Characterization of *E. coli*-produced DMO variants for bridging

The expression level of DMO in GM crops is generally low (a few parts per million (ppm)); therefore, heterologous expression and purification of homologous DMO proteins in *E. coli* were used to produce the large quantities of active and high purity DMO that are required for safety assessments. The purity of each DMO variant produced exceeded 96% (Figure 2). Given the importance of functional characterization of NEPs for protein safety assessment and the lack of a standardized process for determination of homologous protein equivalence, Brune's approach[22] including determination of protein molecular weight, identity and functional activity was used for equivalence determination of homologous DMO variants produced from *E. coli*, facilitating bridging homologous proteins for safety assessment. Additionally, immunoreactivity and structure modeling were conducted to verify homologous protein equivalence or bridging. Although not a part of core studies used for bridging, acute toxicology studies were previously conducted to support global regulatory submissions and were in line with current requirements from regulatory agencies and are

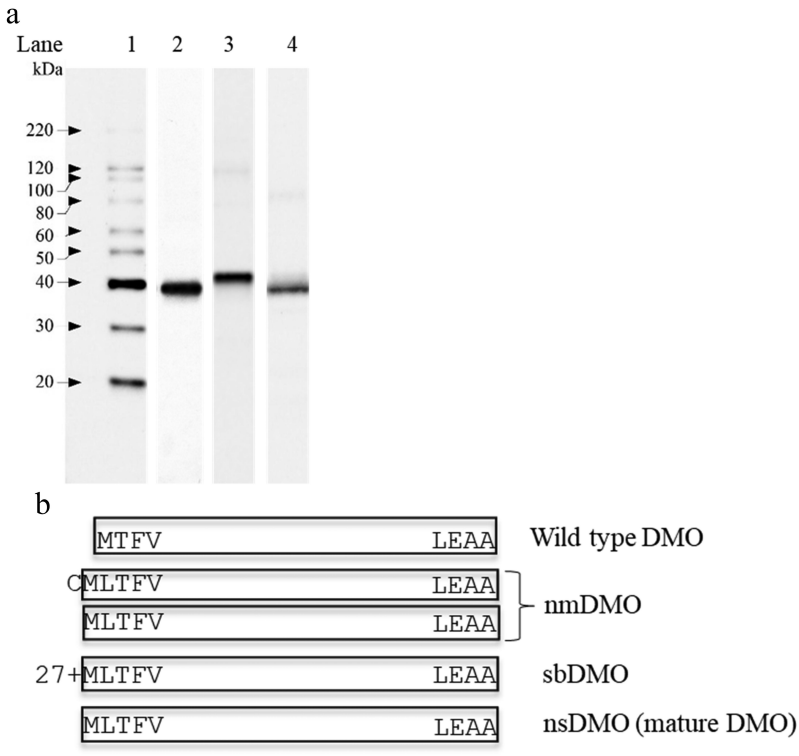

Figure 1. Various forms of DMO. (a) Western analyses of DMO expressed in various GM crops. Soybean and maize grain or sugar beet leaf tissue extracts were separated on Tris-glycine 4-20% polyacrylamide gels under denaturing and reducing conditions and transferred to nitrocellulose membranes. Blots were probed with a DMO specific monoclonal antibody and developed using an enhanced chemiluminescence system. 1: MagicMark XP Western Protein Standards (Thermo Fisher Scientific, Waltham, MA), 2: maize grain extract, 3: sugar beet leaf extract, 4: soybean seed extract. (b) Amino acid sequence comparison of GM crop-produced DMO. DMO in the next-generation of dicamba tolerant soybean (nsDMO, also known as MON 94313 DMO) has an identical amino acid sequence with wild type DMO except an additional leucine in position 2 for cloning purpose. The next generation of dicamba tolerant maize has two forms of DMO (nmDMO, also known as MON 87429 DMO): 1) extra cysteine in the N-terminal from incomplete processing CTP, 2) the same mature form as in soybean. Sugar beet has one form of DMO (sbDMO, also known as Sugar Beet KWS20-1 DMO) with 27 additional amino acids (*MQVWPPIGKKKFETLSYLPPLTRDSRA) from the N-terminal coding region of the pea Rubisco small subunit and an intervening sequence at the N-terminus.

included to further support the bridging approach for safety assessment of homologous proteins.

3.2.1. DMO molecular weight

For apparent molecular weight determination, the DMO homologous variants purified from *E. coli* were subjected to SDS-PAGE (Figure 2). Following electrophoresis, the gels were stained with Brilliant Blue G-Colloidal stain and analyzed. The apparent molecular weights of the nmDMO (Figure 2, lanes 2 & 3), sbDMO (Figure 2, lanes 3 & 4) and nsDMO (Figure 2, lanes 5 & 6) proteins were calculated to be 36.0, 38.6 and 36.2 kDa, respectively (Table 1). These analyses confirmed the expected theoretical molecular weights of the three DMO variants.

3.2.2. DMO identity confirmation

The identity of the DMO homologous variants produced from *E. coli* was determined with N-terminal sequence and peptide mass fingerprint analysis by mass spectrometry. The N-terminal sequence for each *E. coli*-produced DMO variant was consistent with the respective N-terminal sequences for the *in planta* GM crop-produced DMO variants (Table 1, Figure 1).

The identity of these recombinant DMO variants was further confirmed by LC-MS/MS analysis of peptide fragments produced by the trypsin digestion of DMO protein. The experimentally determined coverage for each DMO variant produced from *E. coli* was > 85% (Table 1). These analyses confirm the identity of *E. coli*-produced DMO protein variants.

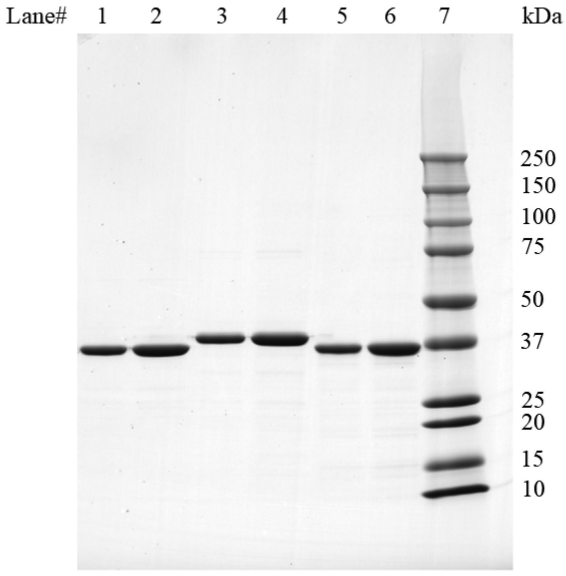

Figure 2. SDS-PAGE analysis of purified DMO proteins. DMO protein samples were separated on a Tris-glycine 4-20% (w/v) polyacrylamide gel and stained with Brilliant Blue G-Colloidal stain. Lanes 1 and 2: 1 and 2 µg *E. coli*-produced nmDMO, lanes 3 and 4: 1 and 2 µg *E. coli*-produced sbDMO, lanes 5 ad 6: 1 and 2 µg *E. coli*-produced nsDMO and lane 7: Molecular Weight Markers (Precision Plus Protein Dual color, Bio-Rad).

3.2.3. DMO functional activity

Prediction intervals (PI) is a well-established method to set acceptance limits for protein functional equivalence.[43–45] The DMO protein variants were considered to have equivalent functional specific activity if the results obtained were within acceptance limits of 93.8 to 735.1 U/mg (the 95% PI calculated from a data set of historically determined DMO protein activity) (Supplementary Table S1).

The experimentally determined specific activities were calculated as 331, 306 and 214, U/mg for nmDMO, sbDMO and nsDMO, respectively (Table 1). Because the specific activities of these DMO protein variants fall within the 95% PI, these DMO protein variants were considered to have equivalent functional activity,[46] confirming that the identified amino acid differences at their respective N-termini did not impact the DMO functional activity.

3.3. Immunoreactivity

Immunoblot analysis was conducted to determine the relative immunoreactivities of DMO protein variants. The results demonstrated that DMO protein variants migrated to expected positions on the blot (Figure 3) and showed comparable band intensities. This analysis demonstrated that DMO variants have equivalent immunoreactive properties, indicating that the identified amino acid differences at their respective N-termini did not impact immunoreaction to the DMO specific antibody.

3.4. Structure prediction of DMO variants

All four DMO protein structure models were in excellent alignment with the known crystal structure of wild type DMO molecule A from the PDB (Protein Data Bank ID:3GTE)[37] (root mean square deviation: r.m.s.d. <1 Å; Figure 4). All four DMO protein variants differ only at their N-termini, with two of the variants having no extension, one variant having a single amino acid addition, and one variant having a 27-residue N-terminal extension. Modeling reveals that the N-terminal extensions do not adopt an ordered secondary structure nor interfere with DMO functional domain structure.

Table 1. Bridging of DMO variants.

			Results		
Bridging		Methods	nsDMO	nmDMO	sbDMO
Apparent molecular weight[1] (MW, kDa)		SDS PAGE/ Densitometry	36.2	36.0	38.6
Amino acid sequence	N- terminal	Mass spectrometry	MLTFVRNAWYVAALP	MLTFVRNAWYVAA and CMLTFVRNAWYVAA	MQVWPPIGKKKFETL
	Mass fingerprint	Mass spectrometry	85% coverage of expected sequence	100% coverage of expected sequence	85% coverage of expected sequence
Activity[2] (nmol/min/mg)		HPLC	214	331	306

[1]The mean value was calculated from six lanes loaded in duplicate at 1, 2 and 3 µg based on total protein concentration, onto a Tris-glycine 4–20% polyacrylamide mini-gel.
[2]The mean value for each of nsDMO and sbDMO was calculated from eight DMO activity assays conducted by three analysts. The mean value for nmDMO was calculated from seven independent DMO activity assays, also performed by three analysts.

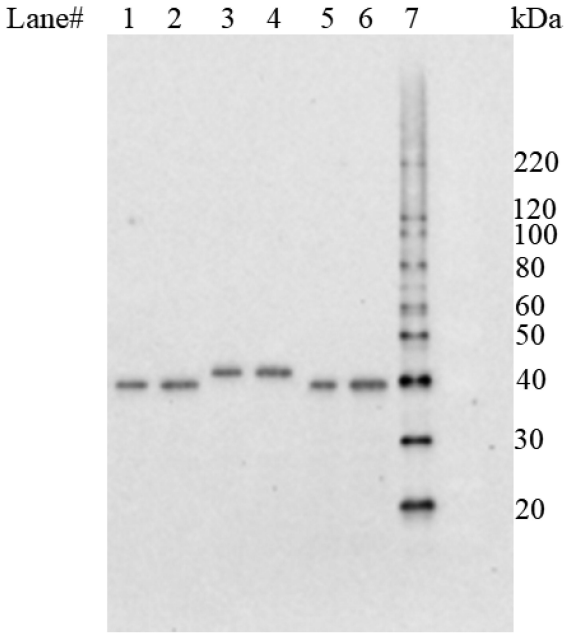

Figure 3. Western analysis of DMO produced from *E. coli*. Various DMO proteins were separated on a tris-glycine 4-20% polyacrylamide gel under denaturing and reducing conditions and transferred to a PVDF membrane. The blot was probed with goat anti-dmo specific polyclonal antibodies and developed using an enhanced chemiluminescence system. Lanes 1 and 2: 50 and 100 ng *E. coli*-produced nmDMO, lanes 3 and 4: 50 and 100 ng *E. coli*-produced sbDMO, lanes 5 and 6: 50 and 100 ng *E. coli*-produced nsDMO and lane 7: MagicMark XP Western protein standards (thermo fisher scientific).

3.5. Assessment of potential oral toxicity of DMO

Though a potential hazard was not identified for DMO in the Tier I evaluations, acute oral toxicity studies have been incorporated into the safety assessments of NEPs in GM crops in order to comply with regulatory requirements. Accordingly, three separate studies based on EPA Health Effects Test Guideline OPPTS 870.1100 were conducted to assess the acute oral toxicity of nmDMO, sbDMO and nsDMO. These studies are described in Supplementary S2. All CD-1 mice analyzed in each study survived to the end of the study (Day 14). The NOAELs (no observed adverse effect limits) were determined to be >5000 mg/kg body weight (bw)/day, >4742 mg/kg bw/day, and >5000 mg/kg bw/day, respectively (Table S2). Therefore, the results of the three acute toxicity studies found no test substance-related adverse effects of nmDMO, sbDMO, or nsDMO when administered to CD-1 mice at the highest doses tested.

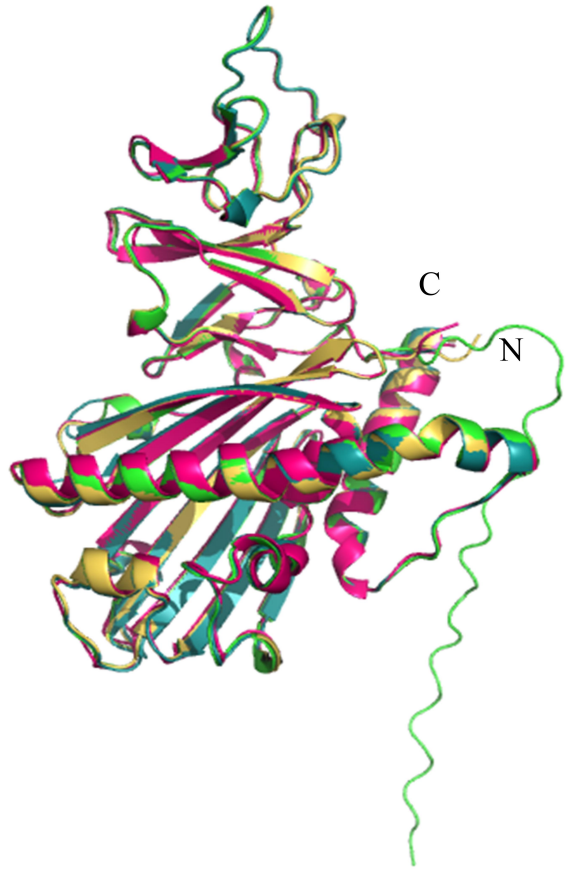

Figure 4. Structure alignment of DMO variants with wild type DMO. sbDMO (green), WT DMO (turquoise), nsDMO (magenta), and nmDMO (gold). The structures modeled using AlphaFold as representatives are presented. All align with r.m.s.d. of 0.2-0.7 Å. C: C-terminal; N: N-terminal.

4. Discussion

Next-generations of dicamba-tolerant soybean, maize, and sugar beet were developed through constitutive expression of the DMO protein targeted to chloroplasts with aid of CTPs. Due to the differential processing of CTPs during import of the DMO protein into the chloroplast, varying fragments of the CTPs remain at the N-terminus of the DMO proteins expressed in the next-generation soybean, maize and sugar beet crops. This results in expression of different homologous variants of the same DMO protein in these different crops, with DMO expressed with up to 27 additional amino acids at N-terminus. The focus of the present study is to determine whether homologous DMO proteins display structurally and functionally equivalent properties to a mature DMO protein and to assess if animal toxicity

studies provide additional value for the assessment of the homologous variants when safety of the mature protein has been confirmed and no hazard is identified.

DMO is expressed in the current commercialized Roundup Ready 2 Xtend® soybeans (referred to as sDMO). Dicamba-tolerant maize expressing DMO (referred to as mDMO) has also received global approval for safe consumption for humans and animals.[47] The DMO variants expressed in the next-generation of dicamba-tolerant crops differ slightly in amino acid sequence from the DMO variants expressed in the first-generation of dicamba-tolerant crops due to differences in processing of the CTP sequences. The first-generation of soybean expressing sDMO accumulates a mixture of two DMO variants including the mature form and DMO plus 27 amino acids originating from the CTP and intervening sequence on its N-terminus.[14] Additionally, sDMO has an amino acid W112C substitution and an additional alanine added at position 2 of the wild type DMO.[14] Similarly, mDMO in the first-generation of maize also accumulates two DMO variants, each of which has additional amino acids at its N-terminus due to incomplete processing of CTP. In developing the next-generation dicamba-tolerant crops discussed in this report, nsDMO accumulates only one mature variant in which the CTP was completely processed. Although nmDMO has two variants *in planta*, they differ by only one amino acid at the N-terminus, which was from incomplete processing of the CTP. Finally, sbDMO contains one form of DMO that is nearly identical to the longer form of sDMO except that sbDMO has an additional leucine at position 2 of the wild type DMO and does not have the W112C substitution (Figure 1(b)).

Modeling DMO variant structures using the DMO crystal structure as a template reveals that the N-terminal extensions do not adopt an ordered secondary structure nor interfere with DMO functional domain structure, which is consistent with previous observations.[37,48] The fact that all variants of DMO in the first- and next-generation of dicamba-tolerant crops were active *in vivo* by conferring dicamba tolerance, while having varying degrees of an extended N-terminal sequence due to incomplete processing of the CTP, demonstrates that these modifications at the N-terminus of DMO do not interfere with its functional activity. Indeed, an enzymatic assay confirmed that the differences in the N-terminal amino acid sequences have no impact on DMO functional activity, which was determined to be equivalent between nsDMO, nmDMO and sbDMO (Table 1) as well as DMO expressed in the first-generation of GM crops.[14]

Additionally, we compared predicted structures of DMO with additional N-terminal residues with three different prediction programs (AlphaFold,[38] OpenFold,[39] and ESMFold[40]) and compared these to the DMO experimentally determined crystal structure lacking the N-terminal additions. Protein structure prediction software has advanced rapidly and today provides very accurate predictions of protein tertiary structure.[49] All three programs predicted the native DMO fold with very low r.m.s.d (<1.0) to the experimentally determined crystal structure with and without N-terminal modifications. When N-terminal modifications were included in the prediction, it showed an extended protein backbone with no detected secondary structure that was conformationally restricted from interfering with the known DMO catalytic site. Experimental determinations of structure, structural predictions and the functional data presented demonstrate equivalency between the various DMO constructs *in planta* and the wild type.

Evolutionary changes within protein families used as food processing enzymes have not resulted in the enzymes becoming toxic to humans.[50] Homologous proteins share related amino acid sequences and three-dimensional structures, as well as common functions.[4] Similarly, it is highly unlikely that amino acid differences between homologous protein variants and the mature protein will turn a nontoxic protein into a toxic protein because any sequence changes would need to be consistent with a biological mechanism of toxicity.[4] Protein variants that are homologous to mature proteins, which have been confirmed to not be a hazard, should be considered to be "'as-safe-as'" the mature protein.[4] It should be noted that the various N-terminal sequence extensions do not have additional safety concerns because the CTPs

used for development of these GM crops are natively expressed in plants with a history of safe use and do not share any sequence similarity with known allergens and toxins.[14,47,51]

The weight of evidence from safety studies including animal acute toxicity studies support the conclusion that DMO protein variants introduced into different crops are safe for food and feed consumption, and the amino acid sequence differences outside the active site of DMO including N-terminal extensions and a W112C mutation do not raise any safety concerns.[14] Since DMO has been extensively assessed for safety,[14,47,51,52] the safety package conducted and submitted for one form of DMO should be applicable to the DMO homologous variants when their functional equivalence is determined. However, in order to address regulatory requirements, animal toxicity studies in mice were still conducted with each DMO variant. As expected, based on the absence of hazard identification in Tier I evaluations, these studies found no test substance-related adverse effects for any of the DMO variants.

While animal toxicity studies are a part of the protein safety package required by regulatory agencies, there is an increasing awareness of the 3 R's principle.[53] EPA remains committed to exploring alternatives to animal experiments.[17,20] Interest in reducing the requirement of animal testing to assess GM crop safety is increasing through employing a hypothesis-driven approach.[2,22–24] Although at least five animals per sex and per dose level are recommended for an acute toxicity study (EPA OPPTS Guideline 870.1100) and for a 28-day study (OECD 407), approximately 60 and 200 mice are needed for an acute toxicity and a 28-day toxicity study, respectively, to ensure sufficient animal samples and proper experimental design for various examinations such as in-life observations, hematology, clinical chemistry and gross necropsy and histopathology, and sample size for statistical analysis. Thirteen acute studies including three first-generation DMO variants and three next-generation DMO variants with different dosing levels and five 28-day studies including sDMO, mDMO, nmDMO, sbDMO and nsDMO have been conducted.[14,47,52,54] These studies used approximately 800 grams of DMO proteins and involved 1580 mice. The results fully supported the conclusion from the previously conducted Tier I studies that the various DMO protein variants introduced into different crops are safe for food and feed consumption. In addition to DMO, a number of other trait proteins such as Cry1A.105, Cry2Ab2, and FT_T accumulate in varying forms in different crops due to incomplete processing CTP or protein engineering (Table 2). The Cry1A.105 and Cry2Ab2 insecticidal crystal proteins, derived from *Bacillus thuringiensis*, provide protection in GM crops against feeding damage caused by targeted lepidopteran insect pests. The FT_T protein, a modified version of the *R*-2,4-dichlorophenoxypropionate dioxygenase derived from *Sphingobium herbicidovorans*, confers tolerance to FOPs and 2,4-D herbicides in GM crops. These proteins have been demonstrated to be physicochemically and functionally equivalent to their respective mature proteins (Table 2). Similar to DMO, although no hypothesis for hazard identification was evident from Tier I,

Table 2. Impact of small amino acid differences on the safety of homologous variants.

Trait protein	Amino acid (AA) sequence difference	Functional activity	Acute toxicity & 28 day toxicity studies	Reference
MON 89034 Cry1A.105 MON 87751 Cry1A.105	N-terminal 4 AA difference	5.8 ng Cry1A.105/ml diet 3.2 ng Cry1A.105/ml diet	Safety results from both forms of the protein are consistent	Cry1A.105 publication[6] US approval[55,56] EU approval[57,58]
MON 89034 Cry2Ab2 MON 87751 Cry2Ab2	N-terminal 18 AA difference	74 ng Cry2Ab2/ml diet 127.5 ng Cry2Ab2/ml diet	Safety results from both forms of the protein are consistent	US approval[55,56] EU approval[46,58]
MON 87429 FT_T MON 94313 FT_T.1	4 AA difference across full length sequence	771.2 U/mg 1585 U/mg	Safety results from both forms of the protein are consistent	US approval[59] EU approval[52]

supplementary Tier II animal feeding studies were conducted to satisfy regulatory requirements. Consistent with the results presented here, none of these proteins provided differing safety data from the mature protein (Table 2). Again, these animal feeding studies were conducted multiple times using the respective homologous protein variants such as Cry1A.105, Cry2Ab2, and FT_T and involved a total of 1206 mice and used approximately 500 grams of proteins (References listed in Table 2 and unpublished data).

The fact that all supplementary Tier II safety studies conducted using homologous proteins repeatedly demonstrated that their safety properties align with those of their respective mature proteins supports the conclusion that Tier II safety assessments should not be needed to address the safety of homologous variants when their mature protein safety has already been demonstrated. The question is how to establish criteria for characterization of homologous protein variants so that the safety data package conducted from the mature protein can be applied to its homologous variants, thereby achieving bridging in homologous protein safety assessments. In this study, we employed the characterization strategy described by Brune et al.[22] including molecular weight determination, amino acid sequence alignment and functional activity analysis (Figure 5). In addition, we conducted three supplementary experiments including immunoreactivity, structure prediction, and acute toxicity studies to further validate the bridging approach for homologous protein safety assessments. Our study confirms the strategy proposed by Brune et al.[22] for the characterization of NEPs is valid for bridging homologous protein variants with their mature protein safety package. In the proposed strategy, if amino acid sequence differences among homologous variants occur outside the active site, and these additional amino acids have no similarity to known toxins or allergens and do not impact functional activity, the safety package achieved using the mature protein can be applied to its homologous variants without requiring additional *de novo* safety assessment. This process, referred to as safety bridging, involves sharing a single *de novo* safety assessment package among homologous proteins where they are demonstrated to have physicochemical and functional equivalence. The three experiments prescribed by Brune et al,[22] namely: 1) molecular weight determination, 2) amino acid sequence alignment, and 3) functional activity analysis are sufficient for future bridging studies. Additional validation experiments, namely immunoreactivity and structural modeling, should only be considered for bridging if any of the primary bridging experiments are not feasible or if the data is inconsistent, particularly when an activity assay is unavailable for an intractable protein. This approach has been first-introduced by Bayer in context of an application to the European Food Safety Authority (EFSA) for

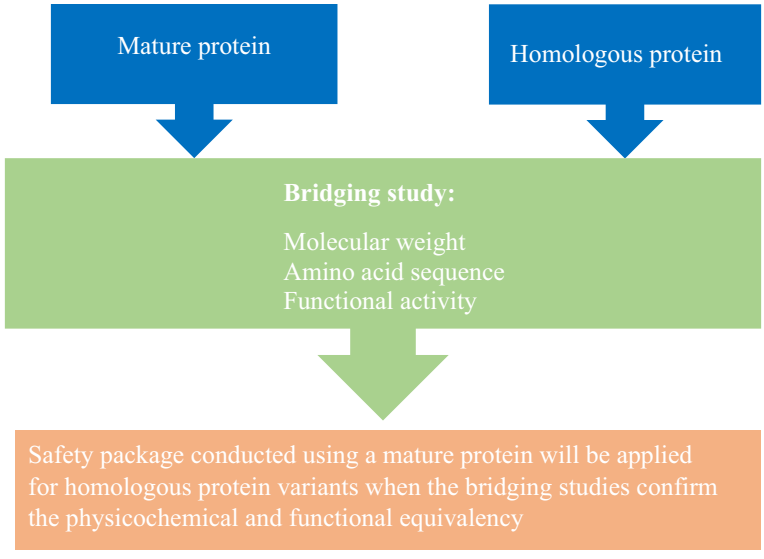

Figure 5. Bridging process.

a DMO-expressing maize, which has received a positive opinion from EFSA.[47] The continued use and expansion of this approach as part of future global protein safety assessments have the potential to significantly reduce the number of animals used in toxicity studies.

Acknowledgments

The authors would like to thank Monika VanGordon, Deepak G M, Jayachandra N, Lyudmyla Dymytrova, Michael Edmund Beck and Christy Taylor for developing ProteinFold_UI GUI which was used for modeling DMO structure, Sonya Franklin, John Vicini, Guangning Ye, Elizabeth Millard, Emily Scholting, Shuichi Nakai and Minsang Lee for critical reading of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The author(s) reported there is no funding associated with the work featured in this article.

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