



## Safety assessment of dicamba mono-oxygenases that confer dicamba tolerance to various crops



Cunxi Wang<sup>\*</sup>, Kevin C. Glenn, Colton Kessenich, Erin Bell, Luis A. Burzio, Michael S. Koch, Bin Li, Andre Silvanovich

Monsanto Company, 800 North Lindbergh Blvd, St. Louis, MO 63167, USA

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### ABSTRACT

Dicamba tolerant (DT) soybean, cotton and maize were developed through constitutive expression of dicamba mono-oxygenase (DMO) in chloroplasts. DMO expressed in three DT crops exhibit 91.6–97.1% amino acid sequence identity to wild type DMO. All DMO forms maintain the characteristics of Rieske oxygenases that have a history of safe use. Additionally, they are all functionally similar *in vivo* since the three DT crops are all tolerant to dicamba treatment. None of these DMO sequences were found to have similarity to any known allergens or toxins. Herein, to further understand the safety of these DMO variants, a weight of evidence approach was employed. Each purified DMO protein was found to be completely deactivated *in vitro* by heating at temperatures 55 °C and above, and all were completely digested within 30 s or 5 min by pepsin and pancreatin, respectively. Mice orally dosed with each of these DMO proteins showed no adverse effects as evidenced by analysis of body weight gain, food consumption and clinical observations. Therefore, the weight of evidence from all these protein safety studies support the conclusion that the various forms of DMO proteins introduced into DT soybean, cotton and maize are safe for food and feed consumption, and the small amino acid sequence differences outside the active site of DMO do not raise any additional safety concerns.

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### 1. Introduction

Weeds growing in planted fields represent one of the main limiting factors to crop yields, since the weeds compete with the crops for much-needed nutrients, sunlight and access to available water resources (Gaddeyya and Ratna Kumar, 2014). The development of herbicide tolerant crops has led to striking advancements in weed management (Behrens et al., 2007; Cao et al., 2011). Crops tolerant to herbicides, especially glyphosate, have been rapidly adopted worldwide. Herbicide-tolerant adoption has been particularly rapid in soybeans, with U.S. farmers planting herbicide-tolerant soybeans on 93% of all soybean acreage in 2013. Similarly, herbicide-tolerant cotton occupied 82% of cotton acreage and herbicide-tolerant maize 85% of maize acreage in U.S. in 2013 (Fernandez-Cornejo et al., 2014). While GM crops tolerant to herbicides such as glyphosate and glufosinate have proven valuable in a commercial setting, crops tolerant to other herbicides are needed to avoid over reliance on any single herbicide and to increase

options for managing difficult to control weed species. Dicamba-tolerant (DT) crops, including soybean, cotton and maize, have been developed through the insertion of dicamba mono-oxygenase (*dmo*) from *Stenotrophomonas* (formerly *Pseudomonas*) *maltophilia*. The expressed DMO protein is targeted into chloroplasts with the help of chloroplast transit peptides (CTP) for co-localization with the endogenous NADH-dependent reductase and ferredoxin that supply electrons for the DMO oxidative reaction (Chakraborty et al., 2005; Herrmann, 1995; Klee et al., 1987). Plants producing DMO protein are able to degrade dicamba, a broadleaf herbicide that has been safely used for weed management in crops such as maize and sorghum since the 1960s (EPA, 2006). Although dicamba has been used for more than 50 years for the effective control of broadleaf weeds in maize and other monocot crops, its application is limited to specific development stages of these monocot crops and affected by environmental factors (Cao et al., 2011). The availability of DT crops, including maize, will provide farmers with additional choices for effective weed management.

The guidance for assessing the safety of proteins introduced into a GM crop has been developed and harmonized for more than 20 years (Delaney et al., 2008; Hammond et al., 2013; Herouet et al.,

<sup>\*</sup> Corresponding author.

E-mail address: [cunxi.wang@monsanto.com](mailto:cunxi.wang@monsanto.com) (C. Wang).

2005; Kier and Petrick, 2008). A weight of evidence approach to protein safety is founded on risk assessment principles that include data from both hazard identification studies and exposure assessments. The Codex Alimentarius commission, which maintains standards and codes of practice for international food trade, recommends that protein safety assessment include: (1) the evaluation of the history of safe use of the donor organism and the protein itself, (2) examination of whether the introduced protein has structural similarity to known protein toxins, allergens and anti-nutrients, (3) characterization of the physicochemical and functional properties of the protein, (4) susceptibility to degradation by gastrointestinal proteases, (5) evaluation of heat stability, and (6) appropriate oral toxicity studies in cases where the introduced protein is not similar to proteins that have previously been consumed safely in food (Codex Alimentarius, 2009).

Plant and animal-derived proteins are routinely consumed as an essential nutritional part of our diets and have a long history of safe consumption. Each protein's unique sequence of amino acids establishes  $\alpha$ -helices,  $\beta$ -strands, or other secondary structures that in turn combine to form tertiary macromolecular shapes that confer a specific function to the protein: structural, enzymatic, immunologic, neuronal, or hormonal (Hammond and Jez, 2011). Collectively a protein's sequence, structure and function can be used to group proteins into evolutionarily related families (Hammond and Jez, 2011). Higher order structures are a relevant measure of homology since structure is more conserved than amino acid sequence. Changes in amino acid sequence are, evolutionarily, mostly conservative, meaning that the changes do not necessarily affect the structure which also determines function (Caetano-Anollés et al., 2009; Illergård et al., 2009). This conservation of structure is predominant within important functional and structural domains of proteins in similar classes (Illergård et al., 2009). Therefore, it is necessary to understand the different levels of protein structure to properly assess homology and determine if homologs of a protein of interest are widely distributed in nature or are present in sources that have been consumed by humans and animals.

Rieske non-heme iron oxygenases are a family of proteins that, while retaining well conserved secondary and tertiary structures that confer similar enzymatic functionality, vary substantially at the primary amino acid sequence level (Ferraro et al., 2005). One member of the Rieske non-heme iron oxygenases, DMO, was discovered to allow transgenic plants to degrade the popular herbicide dicamba, thereby enabling crops to be tolerant to this herbicide when DMO is expressed (Behrens et al., 2007; Cao et al., 2011; Herman et al., 2005). The *dmo* gene was cloned from *S. maltophilia*, strain DI-6 which was found at the site of a dicamba manufacturing plant (Krueger et al., 1989). DMO is the terminal Rieske oxygenase of a three component system that includes ferredoxin and reductase. The crystal structure of DMO has been solved (D'Ordine et al., 2009; Dumitru et al., 2009) and demonstrated that the DMO monomer contains a Rieske [2Fe-2S] cluster domain and a non-heme iron center, a structure that is conserved across many Rieske-type monooxygenases. Functional DMO is a homotrimer with an arrangement of neighboring inter-subunit Rieske domains and non-heme iron sites similar to other structurally characterized Rieske oxygenases, and this arrangement of neighboring inter-subunit enables electron transport.

Previously conducted searches of publicly available databases using the DMO amino acid sequence from *S. maltophilia* DI-6 as a query yielded homologous proteins from many different species with amino acid sequence identity ranging up to as much as 42% (D'Ordine et al., 2009). Rieske non-heme iron oxygenases are ubiquitous in bacteria and plant species (Darrouzet et al., 2004; Ferraro et al., 2005; Gray et al., 2004; Hibino et al., 2002). No food safety concern has been reported to be associated with Rieske

non-heme oxygenases and functionally conserved proteins from this superfamily are widely present in plants that have a history of safe consumption.

DMO expressed in three DT crops (soybean, cotton, maize) exhibit 91.6–97.1% amino acid sequence identity to wild type DMO due to differential processing of CTP in the various plant species and different vector cloning processes during development of each DT crop. In the present report we describe the results from studies assessing the safety of the specific forms of DMO expressed in three different DT crops using the Codex weight of evidence approach. Specifically, the goal is to see whether the safety assessment results are affected by having up to 8.4% amino acid sequence differences across these three forms of DMO, or are consistent with the shared structural and functional similarities of these proteins.

## 2. Materials and methods

### 2.1. Development of DT crops

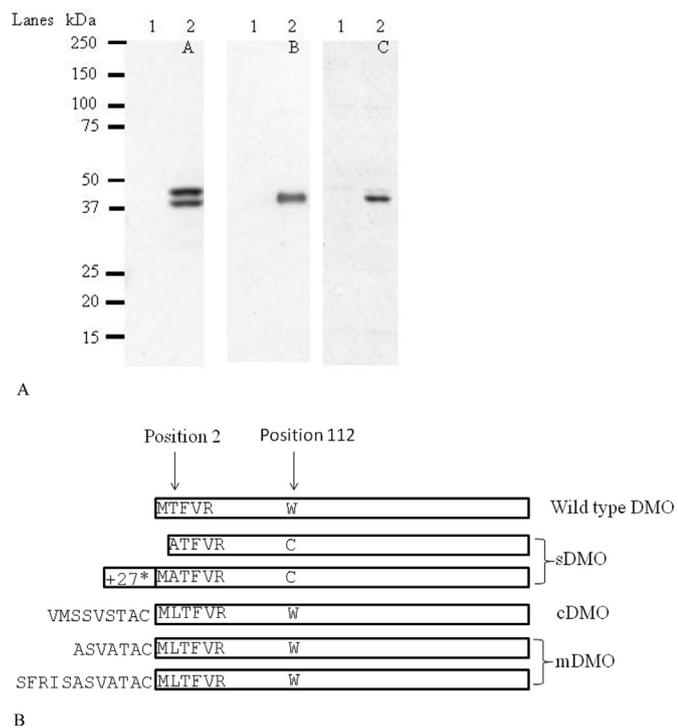
DT cotton and DT maize were developed by expressing the *dmo* gene from *S. maltophilia* (hereafter called “wild-type DMO”) fused with a CTP from *Arabidopsis* 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and a CTP from petunia hybrid EPSPS (Feng and Brinker, 2014), respectively. A leucine codon was added at position 2 of wild type *dmo* to aid the cloning process during plant vector construction (Feng and Brinker, 2014). DT soybean was developed in a similar way, but with three differences (Behrens et al., 2007): 1) the CTP was from pea (*Pisum sativum*) Rubisco small subunit, 2) the N-terminal 27 amino acids from the coding region of the pea Rubisco small subunit/intervening sequence was located between the CTP and the amino terminus of the coding region of *dmo* to potentially stabilize expression of this protein *in planta*, and 3) an alanine codon was introduced at position 2 to facilitate vector cloning and a cysteine replaced tryptophan at position 112 compared to wild type DMO. Fig. 1 illustrates the various forms of DMO present in the transformed crops, in which the DMO in DT soybean, DT cotton and DT maize are designated sDMO, cDMO and mDMO, respectively.

### 2.2. Production of DMO proteins

The DT soybean-produced DMO was purified from DT soybean grain involving a multi-step process including extraction, diafiltration, hydrophobic chromatography, anion exchange chromatography, concanavalin A-based chromatography, and ceramic hydroxyapatite chromatography. About 70 mg of sDMO was obtained.

The coding sequence corresponding to cDMO was cloned into a pET20 vector (Novagen, Madison, WI) and expressed in BL21 (*DE3*) *E. coli* (Invitrogen, Carlsbad, CA). The procedure to isolate cDMO from *E. coli* cells involves a multi-step process including ammonium sulfate precipitation, hydrophobic interaction chromatography, anion exchange chromatography and ceramic hydroxyapatite chromatography. Using the same protocol, a small amount of cDMO was also purified from DT cotton grain to assess its equivalence to the *E. coli*-produced protein.

The coding sequence corresponding to mDMO (DMO+12, the longer one, Fig. 1B) was cloned into a pET19 vector (Novagen, Madison, WI) and expressed in BL21 (*DE3*) star *E. coli* (Invitrogen, Carlsbad, CA). The procedure for mDMO production in *E. coli* was the same as for cDMO, except that the ammonium sulfate precipitation was omitted. A small amount of the plant-produced mDMO protein was purified from DT maize grain to assess its equivalence to the *E. coli*-produced protein. The mDMO was isolated from DT maize grain using the DMO specific monoclonal antibody-based



**Fig. 1.** Various forms of DMO. **A: Western analyses of DMO expressed in various DT crops.** Grain extracts were separated on Tris-glycine 4–20% polyacrylamide gels under denaturing and reducing conditions and transferred to nitrocellulose membranes. Blots were probed with goat anti-DMO specific polyclonal antibodies and developed using an enhanced chemiluminescence system. A: soybean grain extract, B: cotton seed extract, C: maize grain extract; 1: conventional crops, 2: DT crops. **B: Amino acid sequence comparison of DT crop-produced DMO.** The wild type *dmo* coding region introduced into soybean, cotton and maize to confer the identical function of dicamba tolerance to their respective GM crop (DT soybean, DT cotton, DT maize) is the *dmo* gene cloned from *S. maltophilia* (Herman et al., 2005). The number and amino acid residues listed at N-terminus indicate extra amino acids added to the DMO coding sequence. Arrows refer the positions with different amino acid. The DMO in DT soybean (sDMO) has three differences: 1) in approximately 60% of the expressed DMO, it includes 27 additional amino acids (\*MQVWPPIGKKKFETLSYLPLTRDSRA) from the N-terminal coding region of the pea Rubisco small subunit and an intervening sequence at the N-terminus, 2) an additional alanine in position 2 for cloning purpose, and 3) a cysteine instead of tryptophan at position 112. In DT cotton, the introduced DMO (cDMO) includes: 1) nine extra CTP-derived N-terminal amino acids and 2) an additional leucine in position 2 added for cloning purpose. For DT maize, the introduced DMO (mDMO) includes: 1) a mixture of 7 and 12 extra CTP-derived N-terminal amino acids and 2) the additional leucine in position 2 like in cDMO. sDMO, cDMO and mDMO sequences were reported previously (Behrens et al., 2007; Feng and Brinker, 2014).

affinity chromatography where the antibody was raised using wild type DMO protein.

### 2.3. Characterization of DMO proteins

All methods used to characterize proteins that are introduced into GM crops have previously been reported (Wang et al., 2015) and were similarly used to characterize sDMO, cDMO and mDMO in this study. The concentration of total protein was determined using quantitative amino acid compositional analysis. Purity and apparent molecular weight of the various DMO proteins were determined using densitometric analysis of stained SDS–PAGE gels. For immunoblot analysis, each DMO protein was subjected to SDS–PAGE and transferred to a nitrocellulose membrane. The blot was probed with a goat anti-DMO specific polyclonal antibody raised against the *E. coli*-expressed wild type DMO protein. Glycosylation analysis was conducted following ECL Glycoprotein

Detection method (GE Healthcare, Piscataway, NJ) and transferrin was used as a positive control. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to confirm the identity of each DMO form by tryptic digest mapping (Wang et al., 2015). N-terminal sequence analysis was performed for 15 cycles using an Applied Biosystems 494 Precise Sequencing System (Hunkapiller and Hood, 1983; Wang et al., 2015). DMO activity assay was performed according to the method reported previously (D'Ordine et al., 2009). The equivalence between plant- and *E. coli*-produced proteins was evaluated using approaches described previously (Wang et al., 2015).

### 2.4. Bioinformatic assessment of potential DMO allergenicity and toxicity, and assessment of DMO susceptibility to pepsin and pancreatin

Bioinformatic assessments of potential allergenicity, toxicity and antinutrients, were derived from those described previously by Wang et al. (2015), the exceptions being that 2016 versions of databases were used and the full 35% over 80 amino acid threshold search was able to be conducted (Codex Alimentarius, 2009). The all protein database used was GenBank release 209, the updated toxin database (TOX\_2016) contains 18,554 sequences, and the allergen database (AD\_2016) contains 1956 (FARRP, 2016). A BLAST (v 2.2.32+) search of the non redundant protein database from GenBank release 211 using DMO as a query was conducted (Altschul et al., 1997). FASTA (v36.3.5e) was used to conduct all pair wise comparisons (Pearson, 2000). Assessment of protein susceptibility to pepsin and pancreatin were conducted as described previously (Wang et al., 2015).

### 2.5. Heat stability study

Purified DMO protein was placed in the appropriate heat treatments at 25, 37, 55, 75, or 95 °C ± 2 and incubated for 15 min, respectively. All samples were returned to wet ice immediately following the heat treatments. The control sample was maintained on wet ice throughout the treatment period. Following the heat treatments, all samples were subjected to functional activity analysis (D'Ordine et al., 2009).

### 2.6. Acute oral toxicity assessment

Separate acute oral toxicity studies were conducted in CD-1 mice with each DMO form. The study designs were adapted from the EPA OPPTS Guideline 870.1100, were compliant with Good Laboratory Practices (GLPs), and were conducted at Charles River Laboratories, Spencerville, OH (an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility). The sDMO dose formulation was prepared with a vehicle buffer (20 mM potassium phosphate, pH 8.0) at a dose concentration of 4.2 mg/ml for targeting 140 mg sDMO/kg body weight, and the cDMO and mDMO dose formulations were prepared in the same vehicle buffer at dose concentrations of 8.5 mg/ml and 33.7 mg/ml for targeting 283 and 1000 mg/kg body weight, respectively. The dosing formulations were analyzed to confirm concentration, homogeneity, and stability. Briefly, mice were dosed once by oral gavage with a dose volume of 33.3 ml/kg on Day 0 and observed for 14 days thereafter (Wang et al., 2015). Five male mice (Day 0 fasted body weight range 29.2–31.7 g) and 5 female mice (Day 0 fasted body weight range 23.4–27.7 g) were administered sDMO while 10 male and 10 female mice were administered cDMO (males, Day 0 fasted body weight range 25.7–31.5 g; females, Day 0 fasted body weight range 20.5–25.3 g) or mDMO (males, Day 0 fasted body weight range 28.9–33.5 g; females, Day 0 fasted body weight range

22.3–26.1 g), respectively. Endpoints evaluated during the dosing and observation periods included: survival, clinical observations, body weights, body weight changes, and food consumption. Following the observation period, all surviving animals were humanely euthanized and subjected to a necropsy. The necropsy included macroscopic examination of the carcass and musculo-skeletal system; all external surfaces and orifices; cranial cavity and external surfaces of the brain; and thoracic, abdominal, and pelvic cavities with their associated organs and tissues under the supervision of a board-certified veterinary pathologist.

Body weight, body weight change, and food consumption data were statistically analyzed. Each data set was analyzed for homogeneity of variance (e.g., Levene's test). If the homogeneity of variance analysis was not significant, a single-factor parametric ANOVA was applied ( $p < 0.05$ ) and Dunnett's test was used to identify statistically significant differences between the control group and the test substance-treated group ( $p < 0.05$ ). If the homogeneity of variance analysis was significant, a Kruskal-Wallis non-parametric ANOVA was applied ( $p < 0.05$ ) and Dunn's test was used to identify statistically significant differences between the control group and the test substance-treated group ( $p < 0.05$ ).

### 2.7. Sequence alignment and structure homology analyses

To compile a protein dataset to compare with the wild type DMO from *S. maltophilia* (GI-55584974), the Rieske superfamily (Accession: cl00938) in NCBI's Conserved Domain Database (CDD) (Marchler-Bauer et al., 2015) was searched for members of known protein sequence or structure, with plant derived sequences being preferentially selected when possible. Using these criteria, 17 sequences were selected from the Rieske non-heme iron oxygenase family (the subfamily of the Rieske superfamily which contains DMO), and an 18th sequence (GI-402715431 from *Sulfolobus acidocaldarius*) was selected on the basis of having a known structure and belonging to a separate, but related subfamily, in order to serve as an out-of-group protein.

Amino acid alignments were generated with MUSCLE v3.8.31 (Edgar, 2004) and manually masked using Seaview v4.5.3 to ultimately retain 276 positions for tree-building. ProtTest v 3.4 (Darrriba et al., 2011) was used to select WAG + I + G as the best-fit model of amino acid replacement with both Akaike and Bayesian information criterion (AIC and BIC). The maximum likelihood tree was built using RAxML v8.1.3 (Stamatakis, 2014) under the calculated best fit model with 10,000 rapid bootstrap replicates. Final trees were drawn in FigTree v1.4.2 (Rambaut, 2012) and color coded to correspond to colors of contained members with a known protein structure. In the event a region of the tree did not have a publically available structure, the area was colored grey. Structural models were visualized and generated with Cn3D viewer v4.3 (Wang et al., 2000) and analogously colored to correspond to their respective regions of the phylogenetic tree (Fig. 2).

### 2.8. Dicamba tolerance injury ratings

DT crops and their respective isogenic conventional controls were grown in a greenhouse. There were 10 replicate pots with one plant in each pot of DT crop and the conventional control for each tested rate of dicamba application. The pots were randomly placed in a greenhouse. Two different application rates were applied to different replicate sets (see Table 2). Based on the U.S. herbicide labeled rates, the rates for the experiments were chosen and then adjusted for use on crops and for the optimal growing conditions in the greenhouse in order to achieve moderate to severe injury of non-DT plants. All plants were rated for percent injury. Ratings were based on visual assessment of chlorosis, necrosis,

malformation, stunting, and biomass reduction with 0 being no visible injury to 100 percent, completely dead. All 10 replicate ratings were averaged.

At the V2-V3 growth stage, soybean plants were sprayed with dicamba. Twenty to 21 days after application, all plants were rated for percent injury. At the 2–5 leaf growth stage, cotton plants were sprayed with dicamba. Twenty to 22 days after application, all plants were rated for percent injury. At the 2 to 3 leaf growth stage, maize plants were sprayed with dicamba. Fifteen days after herbicide application, all plants were rated for percent injury. The dicamba treatment rate used in the study is summarized in Table 2.

## 3. Results

### 3.1. Assessing the bioinformatic relationship of DMO to proteins in database

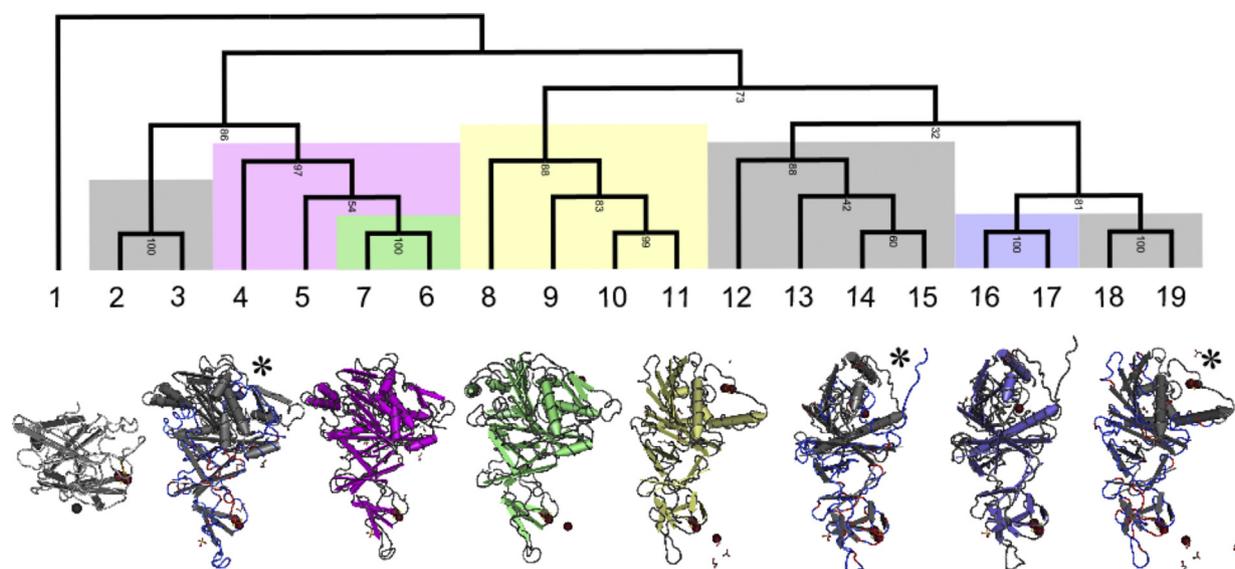
To evaluate the similarity of DMO to known proteins, a BLAST search of the non-redundant protein database from GenBank release 211 using wild type DMO as a query was conducted. This bioinformatic search resulted in several notable hits beyond the self-identification of DMO, one protein from *Sphingobium fuliginis* (AJS19061.1) with 99% amino acid sequence identity to DMO, a hypothetical protein from *Sphingomonas* sp. Y57 (WP\_047168602.1) with 97% sequence identity to DMO, a hypothetical protein from *Sphingomonas* sp. SRS2 (KKC23919.1) which displayed 58% sequence identity to DMO, and a hypothetical protein from *Herbaspirillum autotrophicum* (WP\_050460939.1) displaying 45% identity to DMO. Notably, each of these sequences exceeds the similarity of the closest recognized sequences which had been described previously at ~42% identity (D'Ordine et al., 2009), indicating that DMO homologs appear to be more abundant than originally reported.

For the sequence and structure homology comparison, eighteen protein sequences were selected from the Rieske superfamily on the basis of being proteins from bacteria or plants with solved structures and/or being closely related to proteins with known structures (Table 1). FASTA was used to conduct a pair-wise comparison between each of the 18 selected proteins with the wild type DMO and displays identities ranging from 24 to 41% and similarities ranging from 46% to 69% (Table 1).

The bacterial proteins naphthalene 1,2-dioxygenase, biphenyl 2,3-dioxygenase, and the oxygenase of 3-ketosteroid 9- $\alpha$ -hydroxylase all possess a similar three dimensional structure to the DMO protein (Fig. 2), although their amino acid sequences share less than 27% identity with DMO (Table 1). While three dimensional structures are not publically available for known Rieske proteins from food plants, the conserved regions of their structures can be estimated by superimposing their amino acid sequence to a neighboring known structure. These sequence and structure analyses showed that the DMO in DT crops is highly structurally similar to oxygenases present in crops with a history of safe consumption, such as sugar beet (*Betula vulgaris*), maize (*Zea mays*), tomato (*Solanum lycopersicum*) and rice (*Oryza sativa*) (Fig. 2). Evolutionarily the DMO protein appears to be structurally related to proteins that are involved in essential plant biochemical pathways such as chlorophyll and vanillate metabolism (Fig. 2), both of which are ubiquitous in the diet.

### 3.2. DMO expression and identity in DT crops

Transgenic soybean, cotton and maize expressing *dmo* each demonstrated dicamba resistance when dicamba was applied as compared to the respective conventional control (Table 2). Western blotting results showed that DT soybean contains a



**Fig. 2.** DMO phylogenetic tree and structure similarity analyses. A maximum likelihood phylogeny of members of the Rieske non-heme iron oxygenase family anchored by the Rieske iron-sulfur protein II from *Sulfolobus acidocaldarius*. Numbers at nodes represent maximum likelihood support. Colored regions of the tree designate common protein function and correspond to the protein structure of the same color. Grey regions represent proteins which do not have explicitly known structures. For these regions without a known structure, a member's amino acid sequence was overlaid on a known neighboring structure and designated with an asterisk (\*). These overlaid figures display regions of 100% identity in red and semi-conserved "block" regions in blue and have had their features colored grey. The numbers correspond to the numbers and descriptions provided in Table 1.

**Table 1**

Amino acid sequence identity and similarity between DMO and other proteins present in bacteria and plants.

Number	GI	Name	Protein	Identity	Similarity
<u>1</u>	402715431	<i>Sulfolobus acidocaldarius</i>	Rieske iron-sulfur protein II	25.70%	46.90%
<u>2</u>	20137680	<i>Betula vulgaris</i>	choline monooxygenase	26.20%	53.40%
<u>3*</u>	33300598	<i>Oryza sativa</i> Japonica Group	choline monooxygenase	24.20%	51.70%
<u>4</u>	37622229	<i>Mycobacterium</i> sp. JLS	Naphthalene 1,2-Dioxygenase	28.20%	54.10%
<u>5</u>	78101551	<i>Rhodococcus</i> Sp.	Naphthalene 1,2-Dioxygenase	25.70%	51.80%
<u>6</u>	1354284	<i>Cycloclasticus oligotrophus</i>	Biphenyl 2,3-dioxygenase	26.90%	51.30%
<u>7</u>	3023401	<i>Pseudomonas</i> sp.	Biphenyl 2,3-dioxygenase	25.20%	51.60%
<u>8</u>	57116474	<i>Comamonas testosteroni</i>	Isovanillate oxygenase	35.10%	60.60%
<u>9</u>	357938873	<i>Burkholderia</i> sp. Y123	vanillate monooxygenase**	41.00%	69.00%
<u>10</u>	55584974	<i>Stenotrophomonas maltophilia</i>	dicamba O-demethylase	100.00%	100.00%
<u>11</u>	766695610	<i>Sphingobium fuliginis</i>	dicamba O-demethylase**	99.70%	99.70%
<u>12*</u>	1935909	<i>Zea mays</i>	pheophorbide A oxygenase	34.70%	60.20%
<u>13</u>	30088964	<i>Vigna unguiculata</i>	pheophorbide A oxygenase	31.10%	51.90%
<u>14</u>	16973465	<i>Solanum lycopersicum</i>	pheophorbide A oxygenase	32.40%	58.00%
<u>15</u>	15230543	<i>Arabidopsis thaliana</i>	pheophorbide A oxygenase	33.50%	58.00%
<u>16</u>	21309823	<i>Rhodococcus erythropolis</i>	oxygenase of 3-ketosteroid 9-alpha-hydroxylase	26.30%	55.00%
<u>17</u>	89243566	<i>Mycobacterium smegmatis</i>	oxygenase of 3-ketosteroid 9-alpha-hydroxylase	25.30%	56.00%
<u>18*</u>	5669528	<i>Burkholderia pyrrocinia</i>	aminopyrrolnitrin oxygenase	29.40%	55.30%
<u>19</u>	68345267	<i>Pseudomonas protegens</i> PF-5	aminopyrrolnitrin oxygenase	30.30%	53.70%

Underlined numbers indicate that a corresponding structure or overlaid structure is given in the figure. Figures corresponding to numbers designated with an asterisk (\*) are of a neighboring structure overlaid with the given proteins amino acid sequence. In the case of overlaid figures, identities are shown as red and conserved blocks as blue. Percent identity and similarity were calculated using FASTA v36.3 to align the given protein against the DMO (GI-55584974) and retaining the most optimum alignment. \*\*the sequence showed up in top 500 alignments of the original blast search.

mixture of two forms of DMO peptides with apparent molecular weights of ~42.0 and 39.8 kDa (Fig 1A\_A). To further characterize these two forms of DMO protein, the DMO was partially purified through immunoprecipitation and SDS-PAGE. Edman sequencing and MALDI-TOF-MS analyses of the partially purified DMO sample confirmed that the 39.8 kDa peptide corresponds to the mature DMO protein with the N-terminal methionine cleaved, which is common in many organisms (Bradshaw et al., 1998), whereas the 42.0 kDa form is DMO plus 27 amino acids originating from the pea Rubisco small subunit and intervening sequence on its N-terminus starting with residues MQVWPPIGKKKFETL.

Similarly to DT soybean, two forms of DMO from DT maize were

detected by Edman sequencing and mass spectrometry analyses. Based on results of N-terminal sequence analyses, the smaller form has 7 extra amino acids on its N-terminus starting with residues ASVATAC and the larger form has 12 additional amino acids on its N-terminus starting with residues SFRISASVATAC compared to the wild type DMO (Fig. 1B). The two forms are indistinguishable by SDS-PAGE due to the small mass difference (Fig. 1A\_C). These extra amino acids originate from the CTP of *petunia hybrid* EPSPS (Fig. 1B).

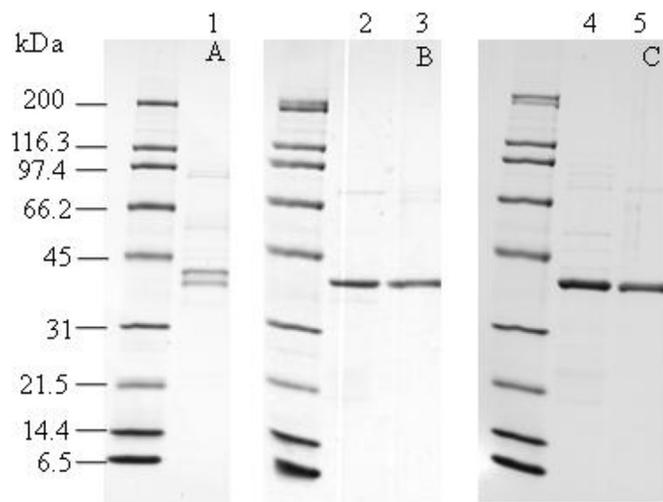
In contrast to DT soybean and maize, Edman sequencing and mass spectrometry analyses confirmed that DT cotton produced only one form (Fig. 1A\_B) that has 9 extra amino acids originating from the CTP of *Arabidopsis* EPSPS on its N-terminus starting with residues VMSSVSTAC. DMO expressed in these three DT crops

**Table 2**  
Dicamba tolerance injury ratings.

Crops	Labeled rate range (g/ha) <sup>a</sup>	Rates applied (g/ha) <sup>a</sup>	Injury ratings (%) <sup>b</sup>	
			Conventional control	DT crop
			Average	Average
Soybean	140–2242	561	100	0
		1120	100	1.8
Cotton	140–2242	561	87	0
		1120	92	5
Maize	128–4488	2244	8.5	0
		4488	30	0

<sup>a</sup> Crops were sprayed with Clarity<sup>®</sup> produced by BASF. Clarity<sup>®</sup> contains the salt form of dicamba. When determining the rate of application, the salt form is calculated back to the acid that is the active ingredient.

<sup>b</sup> Ratings were based on visual assessment of chlorosis, necrosis, malformation, stunting, and biomass reduction with 0 being no visible injury to 100 percent, completely dead. All 10 replicate ratings were averaged.



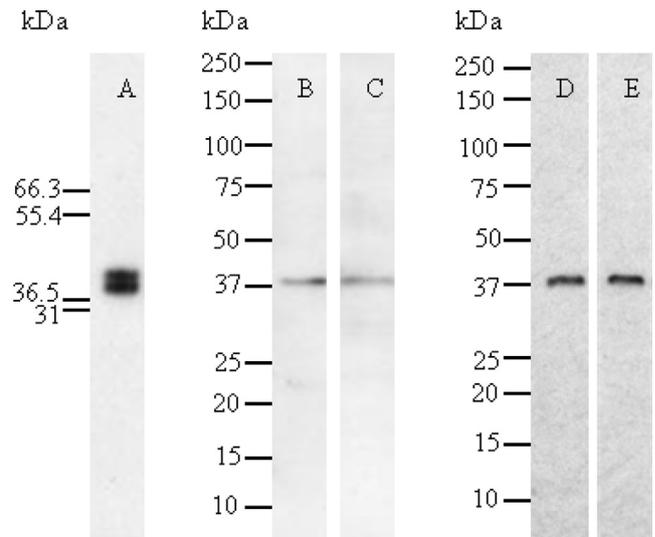
**Fig. 3.** SDS-PAGE analyses of purified DMO proteins. DMO protein samples were subjected to pre-cast Tris-glycine 4–20% (w/v) SDS-PAGE and stained with Brilliant Blue G-Colloidal stain. Approximate MWs (kDa) are shown on the left. Lane 1: 0.5 µg soybean seed-produced sDMO, lane 2: 0.5 µg *E. coli*-produced cDMO, lane 3: 0.5 µg cotton seed-produced cDMO, lane 4: *E. coli*-produced 1 µg mDMO, lane 5: 0.5 µg maize grain-produced mDMO.

possess 91.6–97.1% amino acid sequence identity to wild type DMO (Fig. 1B).

### 3.3. Purification and characterization of DMO proteins

DMO expression levels estimated by ELISA are 43, 20 and 0.19 ppm in grains of DT soybean, DT cotton and DT maize, respectively. To obtain DMO proteins from each DT crop for safety assessment, two different approaches were needed. sDMO, with a purity of 81%, was isolated directly from DT soybean seed to ensure that the natural ratio of the two different forms of DMO observed in DT soybean was retained and reflected in the safety assessment (Fig. 3A).

It is difficult to isolate sufficient quantities of cDMO and mDMO directly from plant tissues for safety assessment. Therefore, heterologous expression of these DMO proteins in *E. coli* was used for their production. For cotton, the DMO with additional 9 amino acids derived from CTP was expressed in *E. coli* and isolated with a purity of 88% (Fig. 3B). The DMO in DT maize is a mixture of two forms. Since the smaller form of mDMO (7 CTP-derived N-terminal amino acids) is encompassed in the larger form (12 CTP-derived N-terminal amino acids), the safety assessment of this larger form would cover the smaller one. Therefore, the longer form of mDMO



**Fig. 4.** Western blot analysis of DT crop and *E. coli*-produced DMO proteins. DMO protein samples were subjected to SDS-PAGE and electrotransferred to PVDF membrane (A, D and E) and nitrocellulose membrane (B and C). Proteins were detected using an anti-DMO specific polyclonal antibody. Immunoreactive bands were visualized using HRP-conjugated secondary antibodies and an ECL system. A: sDMO, 20 ng; B: the *E. coli*-produced cDMO, 0.5 ng; C: the DT cotton-produced cDMO, 0.5 ng; D: the *E. coli*-produced mDMO, 1 ng; E: the DT maize-produced mDMO, 1 ng.

was expressed and isolated from *E. coli* with a purity of 97% (Fig. 1B; Fig. 3C).

To demonstrate that heterologously expressed cDMO and mDMO are equivalent to the DMO proteins in DT cotton and DT maize, respectively, a small amount of DMO proteins were purified directly from their respective DT crops (Fig. 3). The apparent molecular weights of plant- and *E. coli*-produced cDMO, and plant- and *E. coli*-produced mDMO were similar, respectively, as assessed on SDS-PAGE gels (Fig. 3B and C). All purified DMO proteins were immunoreactive with the antibodies prepared with the wild type DMO (Fig. 4). Immunoreactivities of DT crop-produced cDMO and mDMO were comparable to that of their respective *E. coli*-produced DMO (Fig. 4, B and C; D and E). No glycosylation was detected for purified DMO proteins from any of the DT crops or *E. coli* (data not shown).

As shown in Table 2, all three DT crops exhibited reduced injury ratings by dicamba when compared to their respective conventional control, indicating that the expression of each of the respective forms of DMO inactivated dicamba. Similarly, the purified respective forms of DMO from the three DT crops and *E. coli* were shown to be active *in vitro*. Although batch to batch

differences in the protein purification used to produce the various components in the DMO activity assay significantly affect the overall reaction process (Herman et al., 2005), it is noteworthy that the specific activity for sDMO, cDMO and mDMO were similar at 62, 43 and 114 nmol DCSA/min/mg, respectively. Therefore, all preparations of DMO used for the various studies used for weight of evidence assessment of the overall safety of the DMO protein forms from these three DT crops were confirmed to be positive for DMO activity. These glycosylation, immunoreactivity, functional activity and apparent molecular weight analyses support that the *E. coli*-produced DMO were suitable surrogates for assessing the safety of plant-produced DMO from DT cotton and DT maize (Raybould et al., 2013; Wang et al., 2015), respectively.

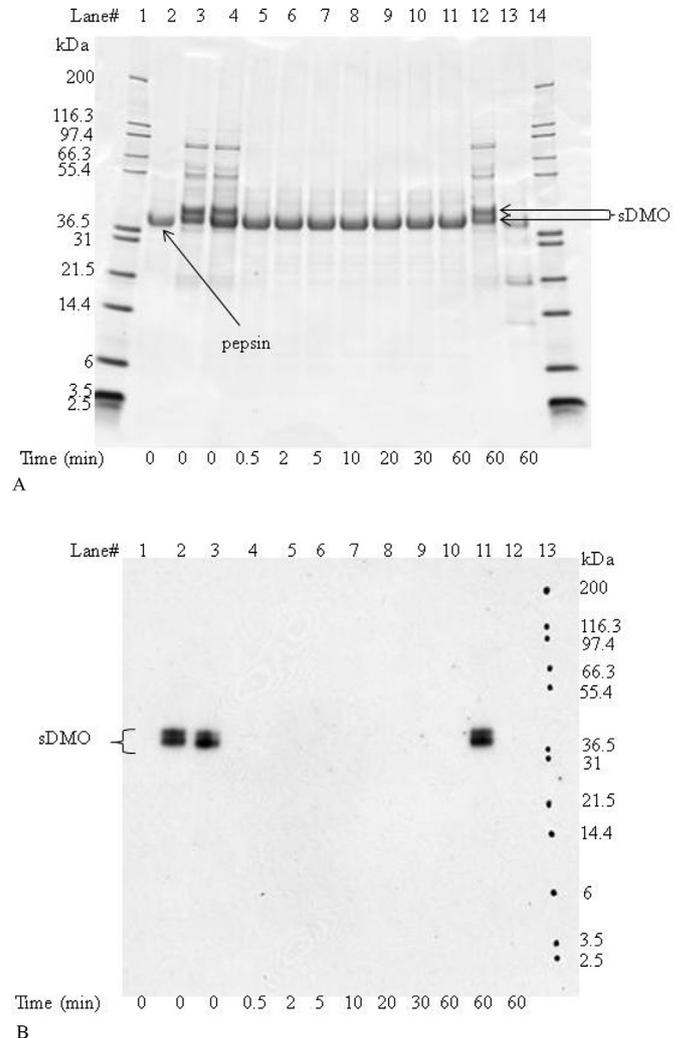
#### 3.4. Bioinformatics analyses of potential allergenicity and toxicity of DMO in DT crops

A key element of the safety evaluation for introduced proteins in GM crops is a bioinformatic analysis to assess whether the introduced protein shows similarity to known toxins or allergens (Hammond et al., 2013; Kier and Petrick, 2008). Bioinformatics analyses were performed on the translated sequence of the DMO coding sequence in these three DT crops to assess their structural relatedness with known allergens and toxins. The results of the search comparisons showed that no relevant alignments were observed against proteins in the allergen database (FARRP, 2016). Likewise, no relevant alignment was observed with the translated open reading frame derived from the various DMO genes in these three DT crops compared to proteins in a database of known protein toxins. These data demonstrate the lack of sequence similarities between the various forms of DMO sequences from these three DT crops to any known allergenic or toxic proteins and that the small differences in sequence have no impact on this bioinformatic assessment.

#### 3.5. Assessment of DMO susceptibility to digestive enzymes

Most dietary proteins are rapidly degraded by digestive proteases to aid absorption of nutritional amino acids, and as a result, minimizing exposure to intact proteins following consumption (Hammond et al., 2013; Kier and Petrick, 2008). Assessing susceptibility of a protein in a GM crop to proteolytic degradation, therefore, is a key study in the overall weight of evidence analysis of the safety of a protein (Codex Alimentarius, 2009). The DMO proteins in all three DT crops were assessed for their susceptibility to pepsin using a standardized assay (Thomas et al., 2004). Studies with sDMO are shown as representative for this group of DMO proteins.

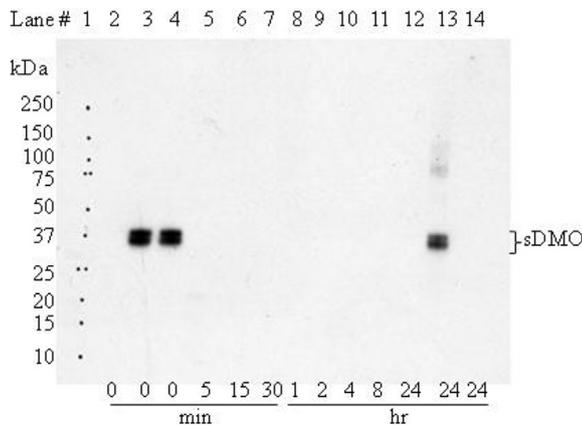
Cleavage of sDMO proteins by pepsin was evaluated by visual analysis of a stained polyacrylamide gel (Fig. 5A). The apparent molecular weights of pepsin (~38 kDa) and the mature form of sDMO with no CTP amino acids (~39 kDa) are similar (Fig. 5A, lanes 2 and 3, respectively); therefore, it is difficult to distinguish between the two on an SDS-PAGE gel (a situation that is similar for most other DMO forms). However, the intensity of the combined band at pepsin treated 0 min (lane 4) appears to be the combination of the intensity of both proteins run separately, which was further confirmed by running an 8% gel system that clearly separated both forms but did not allow for the observation of lower molecular weight bands, if present (results not shown). Visual examination of a stained gel showed that full-length sDMO proteins were rapidly degraded below the limit of detection (LOD) within 0.5 min of exposure to pepsin. On the stained gel, a transiently stable fragment with a molecular weight of ~21 kDa was observed (Fig. 5A, lanes 3 & 4), but it was not recognized by a western analysis



**Fig. 5.** DMO degradation by pepsin. **A: SDS PAGE analysis of sDMO protein degradation by pepsin.** sDMO proteins were resolved by a pre-cast tricine 10–20% (w/v) polyacrylamide gradient mini-gels and tricine-SDS running buffer (Invitrogen, Carlsbad, CA), and stained by Colloidal Brilliant Blue G. One  $\mu$ g of sDMO proteins were loaded in each lane containing DMO protein. The incubation times are indicated. Lanes 1 & 14: molecular weight markers, lanes 2 & 13: pepsin only, lanes 3 & 12: sDMO only, lanes 4 to 11: a time course of pepsin digestion. **B: Western blot analysis of sDMO protein degradation by pepsin.** sDMO proteins were resolved by a pre-cast tricine 10–20% polyacrylamide gradient mini-gel and electrotransferred onto a nitrocellulose membrane. The blot was probed with an anti-DMO specific polyclonal antibody and developed using an enhanced chemiluminescence system. Twenty ng of sDMO proteins were loaded in each lane containing DMO protein. The incubation times are indicated in the Fig. Lanes 1 & 12: pepsin only, lanes 2 & 11: sDMO only, lanes 3 to 10: a time course of pepsin digestion. Lane 13: molecular weight markers.

(Fig. 5B). N-terminal sequencing (not shown) identified only 4 consecutive amino acid residues due to limited amount of the fragment, which did not match sDMO protein sequences. Most likely, the fragment originated from an endogenous soybean protein co-purified with sDMO. Such a fragment was not detected during pepsin digestion of cDMO or mDMO.

Pepsin treated sDMO protein samples were also analyzed by western blot analysis (Fig. 5B). The western blot used to assess the resistance of sDMO proteins to pepsin degradation (Fig. 5B) was run concurrently with a western blot to estimate LOD of sDMO proteins (not shown). The LOD was used to calculate the maximum relative amount of sDMO protein that could remain visually detectable after digestion, which is approximately 1.5% of the initial amount protein loaded. Therefore, based on the LOD, at least 98.5% of sDMO



**Fig. 6.** Western blot analysis of DMO protein degradation by pancreatin. Western blot probed with an anti-DMO specific polyclonal antibody was used to assess the degradation of sDMO proteins by pancreatin. Molecular weights (kDa) are shown on the left of the image. Twenty ng of sDMO proteins were loaded in each lane containing DMO protein. sDMO proteins were resolved by a pre-cast tricine 10–20% polyacrylamide gradient mini-gel and electrotransferred onto a nitrocellulose membrane. The blot was probed with an anti-DMO specific polyclonal antibody and developed using an enhanced chemiluminescence system. The incubation times are indicated. Lane 1: molecular weight markers, Lanes 2 & 14: pancreatin only, lanes 3 & 13: sDMO only, lanes 4 to 12: a time course of pepsin digestion.

proteins were degraded by pepsin within 0.5 min.

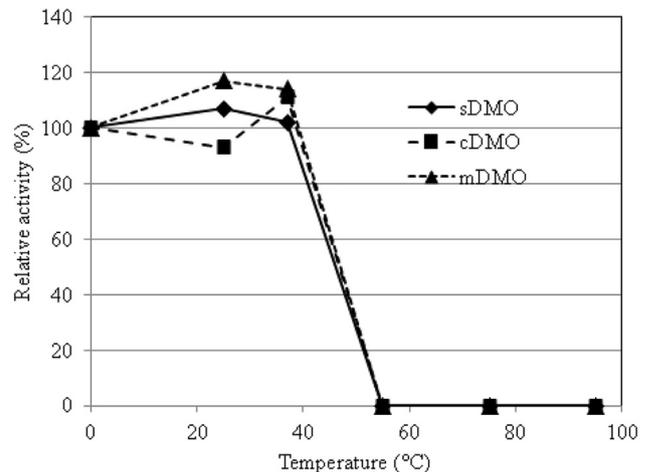
To better understand the digestive fate of DMO protein, an assessment of the susceptibility of sDMO to pancreatin at neutral pH was also conducted. Due to the many proteins from the pancreatin preparation that would be visualized on a stained SDS-PAGE, the susceptibility of sDMO to pancreatin degradation was evaluated by western blot analysis (Fig. 6). Visual examination of the western blot demonstrated that DMO was degraded by pancreatin within 5 min, the first time point assessed with no visible fragments (Fig. 6, lane 5). These results demonstrated that sDMO proteins are readily susceptible to degradation by digestive enzymes. cDMO and mDMO were also found to be rapidly degraded by digestive enzymes, similar to the results shown in Figs. 5 and 6 for sDMO (data not shown). Again, the differences in sequence among the three DMOs did not result in any differences in their susceptibility to these digestive enzymes.

### 3.6. Thermal stability of DMO protein

The thermal stability of DMO was evaluated by determining the percent residual enzyme activity following heat treatment compared with the activity of untreated DMO protein. The enzymatic activity of all forms of DMO was stable at incubation temperatures up to 37 °C, which is comparable to previous reports (Chakraborty et al., 2005; Herman et al., 2005). However, enzymatic activities of all forms of DMO were not detected following incubation at temperatures equal or above 55 °C (Fig. 7), indicating that they were all equally heat labile.

### 3.7. Assessment of potential oral toxicity of DMO

The assessment of protein safety is largely based on the “weight of evidence” studies already presented (e.g., history of safe use, bioinformatic comparison of amino acid sequence to known allergens, protein toxins and antinutrients, stability to heat or processing and susceptibility to degradation by pepsin), and is in large part consistent with the tiered testing strategy advocated by the International Life Science Institute's International Food Biotechnology Committee Task Force on Protein Safety (Delaney et al.,



**Fig. 7.** Heat stability of various forms of DMO. Purified DMO proteins were heated over a temperature range of 25–95 °C for 15 min and then subjected to functional activity analysis. Mean specific activity was determined from  $n = 3$ . The DMO activity of non heat treated samples was assigned 100% active. Relative activity = [specific activity of heat treated sample/specific activity of non heat treatment sample] x 100.

2008). Other international food safety organizations indicate that if a history of safe use is apparent and weight of evidence results suggest no concern regarding safety, oral toxicity studies should not be necessary (Codex Alimentarius, 2009). Despite these recommendations, a precautionary approach leading to oral toxicity studies of proteins incorporated into crops has become a part of safety assessment. Although DMO is homologous to proteins expressed in many plants commonly consumed as food and feed; and the weight of evidence from the safety studies with the sDMO, cDMO, and mDMO proteins support a conclusion that these proteins are safe, acute toxicity studies in mice were also conducted in an additional attempt at hazard identification. The experimental design for each of the studies conducted with sDMO, cDMO or mDMO was similar in that the test group of animals (male and female) received their doses by oral gavage and a concurrent control group received a similar dose level of bovine serum albumin to control for potential formulation effects (i.e., the administration of a relatively large volume of a concentrated protein solution could induce satiety and reduce food consumption) and thereby better distinguish test substance-related effects. As summarized in Table 3, the test groups of mice were dosed at 140, 283 or 1000 mg DMO/kg body weight for sDMO, cDMO or mDMO, respectively. Following the day of dosing, the animals were observed for 14 days, humanely euthanized, and subsequently subjected to a macroscopic examination of their gross anatomy. At completion of the 14 days of observations, no mortality occurred and there were no adverse test substance-related clinical findings observed following treatment with sDMO, cDMO and mDMO. Occasional findings were noted during the clinical observations (stained fur, unkempt appearance, etc.); but these findings did not correlate with signs of toxicity, are common clinical findings in the CD-1 mouse (e.g., unkempt appearance), occurred at a similar rate in concurrent control, and/or were of an isolated and transient nature. Consequently, they were determined to be unrelated to treatment. There were no adverse test substance-related effects of sDMO, cDMO or mDMO on body weight, body weight change, or food consumption. Occasional statistically significant differences were noted in these endpoints, but the differences were determined to be spurious because the difference was detected in a single gender, the direction of the change was not commonly associated with toxicity (e.g., higher weight gain at one study interval), and/or were within the

**Table 3**  
Summary of acute toxicity study results for various forms of DMO.

DMO (dose level mg/kg)	Mean BW Day 14 (grams)		Mean BW gain Day 0–14 (grams)		Mean food consumption Day 0–7, 7–14 (grams/Animal/Day)		Macroscopic observations at necropsy (test)
	Test	Control	Test	Control	Test	Control	
	sDMO (140)						
Male	33.2 [+2.2] <sup>a</sup>	32.5	2.6 [+23.8]	2.1	6.4 [−3.0], 6.8 [−2.9]	6.6, 7.0	NTF <sup>b</sup>
Female	28.1 [−0.7]	28.3	2.5 [−19.4]	3.1	6.5 [+6.6], 7.0 [+7.7]	6.1, 6.5	
cDMO (283)							
Male	35.0 [+0.9]	34.7	6.0 [+5.3]	5.7	7.3 [−11.0], 7.1 [−10.1]	8.2, 7.9	NTF
Female	26.9 [+1.5]	26.5	3.2 [+3.2]	3.1	6.1 [−1.6], 6.1 [0]	6.2, 6.1	
mDMO (1000)							
Male	35.1 [+0.6]	34.9	4.0 [+17.6]	3.4	7.6 [+4.1], 7.7 [+5.5]	7.3, 7.3	NTF
Female	27.8 [+0.7]	27.6	3.3 [−2.9]	3.4	6.7 [+4.7], 7.0 [+2.9]	6.4, 6.8	

<sup>a</sup> Number in [] represents percent ± compared to control.

<sup>b</sup> No treatment-related findings; BW: body weight.

normal range defined by historical control data. No treatment-related findings were noted at necropsy following treatment with sDMO, cDMO or mDMO. The only findings noted at necropsy in the three studies were periovarian cysts in females in the study where mDMO was administered. These findings were not considered treatment-related because they are common findings in CD-1 mice and occurred at the same rate in the control (1 animal out of 10) and test groups (1 animal out of 10). Thus, the No Observable Adverse Effect Levels (NOAEL) for sDMO, cDMO and mDMO were the highest dose levels administered in each case.

#### 4. Discussion

DT soybean, cotton and maize were developed through constitutive expression of DMO targeted into chloroplasts. The DMO proteins expressed in these DT crops exhibit 91.6%–97.1% amino acid sequence identity with the DMO cloned from *S. maltophilia*, strain DI-6 that was found at the site of a dicamba manufacturing plant (Krueger et al., 1989). The focus of the present study was to assess if small amino acid sequence differences outside the active site of DMO in these three DT crops would raise any safety concerns.

Given the importance of functional characterization of introduced proteins for protein safety assessment (Codex Alimentarius, 2009; Delaney et al., 2008; Hammond et al., 2013; Herouet et al., 2005; Kier and Petrick, 2008), several functional and biochemical properties of the various forms of DMO in these three DT crops were evaluated. Because glycosylation can alter the physicochemical properties of a protein (e.g., function, half-life), assessing whether the plant-produced protein is glycosylated is one component of protein safety assessment (Raybould et al., 2013; Wang et al., 2015). Glycosylation assays showed that all forms of DMO, like the bacterial DMO on which they were based, were not glycosylated (data not shown). This result is consistent with the fact that a search for potential glycosylation sites of DMO found only a few possible O-linked glycosylation sites and no likely N-linked glycosylation sites. Another characteristic of the plant-produced DMO that was assessed was whether the immunoreactive properties of plant-produced DMO from DT cotton and DT maize were comparable to their respective *E. coli*-produced DMO forms. Immunoreactive analysis verifies the protein intactness and distinguishes potential differences in immunoreactive properties, which is an essential component of biotech protein characterization (Raybould et al., 2013). In both cases, immunoreactive properties were shown to be similar. Similarly, the plant-produced sDMO were also immunoreactive to the polyclonal antibody raised against the *E. coli*-produced wild type DMO protein and not glycosylated. Therefore, these results support DT crop-produced DMO do not have other

modification except small sequence differences illustrated in Fig 1B.

Crystal structure analysis of DMO indicates that the catalytic domain of DMO contains no contribution from an N-terminal extension region (D'Ordine et al., 2009; Dumitru et al., 2009). The fact that all of the forms of DMO in these DT crops were active both *in vitro* by enzymatic assay and *in vivo* by conferring dicamba tolerance (Behrens et al., 2007; Cao et al., 2011), and yet some of these DMO forms have varying degrees of an extended N-terminal sequence from incomplete processing of the CTP, demonstrates that these modifications at the N-terminus of DMO do not interfere with its functional activity, as previously concluded (D'Ordine et al., 2009; Dumitru et al., 2009; Herman et al., 2005). A W112C substitution in sDMO, structurally located outside the Rieske cluster and catalytic domain (D'Ordine et al., 2009; Dumitru et al., 2009; Herman et al., 2005), also does not interfere with its functional activity. All residues involved in the coordination of the Rieske cluster and catalytic domain (D'Ordine et al., 2009; Herman et al., 2005) are completely conserved in all DMO forms in these three DT crops (Fig. 1 B). In addition, the substrate specificity of DMO for dicamba is due to the specific interactions between residues in the active site and the chlorine atoms, carboxylate moiety and ring structure of dicamba, which are primarily involved in orienting the substrate in the catalytic pocket (D'Ordine et al., 2009; Dumitru et al., 2009). Previously, DMO activity was tested by two independent laboratories using O-anisic acid as a pseudo substrate, which is structurally similar to dicamba except for the absence of chlorines (D'Ordine et al., 2009; Dumitru et al., 2009). No significant DMO activity was detected with O-anisic acid under standard assay conditions. 4-Hydroxy-3-methoxybenzoic acid (vanillic acid), also structurally similar to dicamba but lacking the chlorine moieties, was tested and similarly showed no DMO activity, supporting the conclusion that the chlorine atoms of dicamba are required for the proper positioning of the substrate for DMO catalytic activity. The DMO crystal structure, combined with the functional and physicochemical characterization presented in this study, confirm that the alternative processing of the N-terminal CTP, single amino acid insertion at N-terminal and an amino acid substitution among various forms of DMO do not result in alterations in the DMO functional structure, substrate specificity, enzymatic mode of action and phenotypic trait of dicamba tolerance (Behrens et al., 2007; Cao et al., 2011; D'Ordine et al., 2009; Feng and Brinker, 2014). Therefore, the data generated on these various forms of DMO proteins are representative of the DMO family.

For proteins, as is also true for much of biology, structure governs function (Hammond et al., 2013). Hence, bioinformatic analysis is a key dataset in the weight of evidence assessment of protein safety since it will readily identify if an introduced protein is

structurally and therefore functionally, similar to toxins or allergens. The analysis presented in this study for the various forms of DMO in these three DT crops demonstrated that no structurally relevant similarity exists with any known toxic or allergenic proteins that would be harmful to human or animal health. An absence of homology between various forms of DMO and known allergens and protein toxins supports the conclusion that there is no safety concern with DMO expressed in these three DT crops and that the sequence differences described have no impact on this conclusion.

While bioinformatic analysis determined that all forms of DMO in these three DT crops are not structurally and functionally similar to any toxins or allergens, it can also provide information about whether proteins known to be safe in the diet have significant similarity with DMO. Because high sequence and/or structural similarity often correlates with a conserved biological role (Hammond et al., 2013), bioinformatic analysis to evaluate evolutionary relationships of proteins are valuable in safety evaluations. DMO belongs to the family of Rieske non-heme iron oxygenases that perform an exocyclic monooxygenation. Rieske domain proteins are ubiquitous in numerous bacterial and plant species; more than 130 Rieske non-heme iron oxygenases have been reported from different species (Chen et al., 2014). Structures for a number of these proteins have been determined. As presented in this study, a sequence and structure analysis of 18 representative proteins revealed oxygenases with highly structural (domain/tertiary structure) and functional (oxygenation) similarity to the DMO protein are present in crops with a history of safe consumption, such as sugar beet (*Betula vulgaris*), maize (*Zea mays*), tomato (*Solanum lycopersicum*) and rice (*Oryza sativa*) (Fig. 2). Therefore, DMO proteins expressed in DT crops are structurally similar to Rieske oxygenases present in the human diet and directly consumed in common foods, including tomato which is frequently eaten raw, supporting a conclusion that DMO has a comparable history of safe use. It should be noted that the various N-terminal sequence extensions do not have additional safety concerns because Rubisco protein and CTPs in the three DT crops are natively expressed in plants with a history of safe use (Hammond et al., 2013).

Evolutionary changes within protein families used as food processing enzymes have not resulted in the enzymes becoming toxic to humans (Pariza and Cook, 2010). Similarly, it is highly unlikely that genetic modification of a 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 (Hammond et al., 2013). Analysis of the source organism from which DMO was cloned is also part of the weight of evidence approach for safety assessment. It was reported over forty years ago that microbes were able to convert dicamba to 3,6-dichlorosalicylic acid (Smith, 1974). Krueger et al. (1989) established that dicamba can be degraded by pure cultures of bacteria under aerobic conditions. They isolated eight species of bacteria from soil and water samples with long histories of dicamba exposure that were capable of using dicamba as the sole carbon source. The *dmo* gene is derived from the bacterium *S. maltophilia* (Herman et al., 2005), which is an aerobic, ubiquitous environmental gram negative bacterium commonly found in aquatic environments, soil, and plants. The *dmo* gene is present on a megaplasmid in cells of *S. maltophilia* (Herman et al., 2005). Recently, homologous DMO proteins with 97–99% amino acid sequence identity to the wild type DMO were also identified from *Sphingobium fuliginis* (accession number: AJS1906.1) and *Sphingomonas* sp. Y57 (accession number: WP\_047168602.1) bacteria species, indicating that many soil bacteria are capable of degrading dicamba through the DMO activity. *S. maltophilia* can be found in a variety of foods and feeds (Echemendia, 2010; Qureshi et al., 2005), and is widespread in the home environment (Denton and Kerr, 1998; Denton et al., 1998; Ryan et al., 2009).

*S. maltophilia* was also detected in raw and pasteurized milks (Juffs, 1973). Infections caused by *S. maltophilia* are extremely uncommon (Cunha, 2009). Additionally, *S. maltophilia* has not been reported to be a source of allergens (FARRP, 2016). The ubiquitous presence of *S. maltophilia* in the environment and the incidental contamination of foods without any adverse safety reports support a conclusion that the source organism for the *dmo* gene is not associated with any toxic or allergenic biological processes.

While functional characterization, bioinformatics and history of safe use analyses show that the consumption of the various forms of DMO from these three DT crops is safe, additional safety studies were also conducted. Most dietary proteins are digested to constituent amino acids and small peptides in the mammalian gastrointestinal system and absorbed for nutritive purposes. Although the correlation between allergenicity and pepsin resistance is imperfect (Herman et al., 2006), some proteins that are food allergens are relatively resistant to pepsin digestion (Asero et al., 2000; Astwood et al., 1996; Yagami et al., 2000). In addition, rapid degradation by pepsin provides evidence that exposure to the introduced protein will be minimized following consumption (Hammond et al., 2013; Kier and Petrick, 2008). Test results show that all forms of DMO from these three DT crops were completely digested within the first measured time point by pepsin and, similarly in a separate assay by pancreatin, supporting a conclusion that exposure to any form of structurally and functionally intact DMO is unlikely. All forms of DMO also showed complete loss of functional activity by heating at temperatures 55 °C and above. Given virtually all consumed foods from soybeans and maize are exposed to heating during processing or cooking (Hammond and Jez, 2011), the heat labile property of DMO is consistent with the conclusion that dietary exposure to functionally intact DMO is unlikely.

Although bioinformatic and history of safe use assessments identified no hazards with respect to a potential for toxicity, acute toxicity studies were conducted with the various DMO proteins. An acute toxicity study is a supplemental method to assess if a protein is toxic because most known protein toxins exert toxicity through acute mechanisms (Pariza and Johnson, 2001; Sjoblad et al., 1992). The forms of DMO introduced into soybean, cotton and maize were orally administered to mice independently. These three independent toxicity studies together included a total of 50 mice (25 males and 25 females), with doses of up to 1000 mg DMO/kg body weight, and no adverse effects were observed on the three studies.

To put the maize DMO acute toxicity study dose level into context, consider the following extreme example: if 100% of all cultivated maize used for food production had the DT trait (with an average expression level of 0.19 µg DMO protein/g maize grain), a 60 kg human would have to eat approximately  $3.2 \times 10^5$  kg (704,000 pounds) of DT maize in a single day to achieve a DMO intake level similar to the NOAEL of the acute toxicity study with mDMO (1000 mg/kg).

In the acute toxicity study with sDMO, the plant-produced DMO was used because it consists of a mixture of two forms of the DMO protein. Therefore, the plant-derived DMO retains the natural ratio and active trimeric form expected to be present in DT-soybean. sDMO is expressed at a relatively low level in seed tissue that contains high levels of oils and carbohydrates, which complicate protein purification in general and limited the ability to produce large amounts of the DMO protein. Considering the challenges associated with the isolation and purification of DMO from soybean grain, a margin of exposure-based dosing strategy for the mammalian toxicity study was used. This is a toxicologically sound approach because dose levels that provide a large safety factor (i.e., Margin of Exposure  $\geq 100$ ) are typically considered an appropriate demonstration of a substance's safety (Faustman and Omenn,

2008). In comparison to the mDMO dose level discussed in the previous paragraph, the sDMO dose level (140 mg/kg) may appear moderate. However, it is worth noting that other substances routinely ingested by humans are lethal at lower dose levels (e.g., caffeine has an oral LD<sub>50</sub> of <140 mg/kg in mice) (Windholz, 1983). The significance of a 140 mg/kg NOAEL can also be demonstrated re-applying the grain consumption scenario from the preceding paragraph with soybean in place of maize. A 60 kg human would have to eat more than three times their body weight (approximately 195 kg) of DT soybean (with an average expression level of 43 µg DMO protein/g soybean seed) in a single day to achieve an sDMO intake level similar to the NOAEL of the acute toxicity study with sDMO (140 mg/kg). This theoretical soybean consumption scenario dwarfs actual soybean consumption rates, even in world areas with high levels of soybean consumption. For example, the WHO GEMS program indicates Japanese children ages 6 years and under have a soybean consumption value of 5.55 g/kg/day (the highest 97.5th percentile “Eater-Only” worldwide). In the theoretical scenario above, the person would have a soybean consumption value of 3250 g/kg/day (195 kg soybean by a 60 kg human).

Dietary exposure to DMO from DT cotton is negligible because the processed fractions of cotton consumed by humans (refined, bleached and deodorized oil and linters) contain negligible amounts of protein (Reeves and Weihrauch, 1979). In spite of this fact, an acute mouse study was conducted to comply with global requirements that do not entirely consider these differences in cotton. In that case a dose level of 283 mg/kg was used and no adverse health effects were observed. Therefore, the apparent absence of hazard and lack of exposure result in negligible risk.

Furthermore, these are very conservative estimates of human exposure to DMO because it assumes no loss of the DMO protein during storage, processing and/or cooking of the grain or food or during digestion. As described above, all forms of DMO from three DT crops were shown to be heat labile and readily susceptible to proteolytic degradation, therefore resulting in no meaningful dietary exposure to intact DMO from any of these DT crops.

As presented in this report, the three forms of DMO introduced in DT crops (soy, cotton, maize): (1) is sourced from bacteria that have no association with allergenicity; (2) are related to proteins that have a history of safe use; (3) are not structurally or functionally related to known toxins or allergens; (4) have a substrate-specific activity that confers tolerance to dicamba in crops; (5) are readily cleaved by mammalian digestive enzymes; and (6) are heat labile. Furthermore, acute toxicity studies with each of these three forms of DMO demonstrated no adverse effects following treatment. Thus, these weights of evidence align well with conclusions that the various forms of DMO in these three DT crops, while having small amino acid sequence differences have similar structure, function and safety properties and that there is no risk for humans to consume foods containing DMO.

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## Abbreviations used

bw	body weight
CTP	chloroplast transit peptide
DCSA	3,6-dichlorosalicylic acid
DMO	dicamba mono-oxygenase from <i>Stenotrophomonas maltophilia</i>
DT	dicamba tolerance
<i>E. coli</i>	<i>Escherichia coli</i>
EPSPS	5-Enolpyruvylshikimate-3-phosphate Synthase
LOD	limit of detection
MALDI-TOF MS	Matrix assisted laser desorption ionization - time of flight mass spectrometry
MOE	margin of exposure
NOAEL	no observable adverse effect level
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sDMO	the DT soybean-expressed DMO
cDMO	the DT cotton-expressed DMO
mDMO	the DT maize-expressed DMO

## Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.yrtph.2016.08.014>.

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