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Preliminary safety assessment of a membrane-bound delta 9 desaturase candidate protein for transgenic oilseed crops

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ABSTRACT

A gene encoding delta 9 desaturase (D9DS), an integral membrane protein, is being considered for incorporation into oilseed crops to reduce saturated fatty acids and thus improve human nutritional value. Typically, a safety assessment for transgenic crops involves purifying heterologously produced transgenic proteins in an active form for use in safety studies. Membrane-bound proteins have been very difficult to isolate in an active form due to their inherent physicochemical properties. Described here are methods used to derive enriched preparations of the active D9DS protein for use in early stage safety studies. Results of these studies, in combination with bioinformatic results and knowledge of the mode of action of the protein, along with a history of safe consumption of related proteins, provides a weight of evidence supporting the safety of the D9DS protein in food and feed.

1. Introduction

Development of oils with reduced saturated fatty acid content is desirable for the improvement of nutrition and the overall health of consumers. One way to accomplish this is by genetically modifying oil seed crops (*e.g.* canola and soybean) with a gene encoding acyl-CoA delta 9 desaturase (D9DS), an integral membrane protein that catalyzes desaturation of saturated fatty acids at the C9-C10

and cytochrome b5 reductase for activity. The substrate specificity of D9DS could be broad, and in one very well studied case, the rat liver microsomal stearoly-CoA deaturase was active on acyl-CoA substrates containing 12 to 19 carbon fatty acyl chains (Enoch et al., 1976). The active site of the related, soluble acyl-(ACP) delta 9 desaturase consists of eight conserved histidine residues that chelate two molecules of iron. The function and sequence comparison of the membrane-bound acyl-CoA delta 9 desaturase indicates this enzyme also uses a histidine coordinated diiron center. (Shanklin and Cahoon, 1998). The acyl-CoA delta 9 desaturase enzyme is expressed exclusively in the smooth endoplasmic reticulum (ER), and no apparent N-terminal signal sequences, ER retention motifs, or consensus glycosylation sites are present in the enzyme (Stukey et al., 1990). The D9DS gene from *Aspergillus nidulans* (Folkerts and Merlo, 2002) was chosen as a gene candidate for genetic modification of canola. This protein shares 40% identity with the D9DS isolated from the yeast *Saccharomyces cerevisiae* (Stukey et al., 1990) that is used commonly in baking and brewing and the Fe-binding histidine residues appear to be conserved. The D9DS protein is 93% identical to a corn (*Zea mays*) protein (Genbank accession NP_001169125.1), although it would be desirable to verify the presence of this protein in maize grain to rule out

ely include those that contain genes coding for membrane-bound and other intractable proteins (i.e. those difficult to purify in active form). The well documented difficulty in purifying membrane-bound proteins in an active form makes it challenging to conduct safety studies that have routinely been conducted with purified, active protein to support the registration of GM crops. Functional expression of membrane-associated proteins (*e.g.* the G-protein coupled receptors) is most commonly pursued in yeast (*Pichia*), mammalian, *Escherichia coli* and insect cell systems (Tate, 2001). Published research on membrane-bound protein expression suggests that, in general, homologous expression is far better at producing functional membrane-bound proteins compared with heterologous expression (Tate, 2001). Even with the recent developments in membrane protein expression, such as specialized expression systems and cell free expression systems, it is still challenging to obtain expression at a level comparable to what can be achieved for soluble proteins (Bill et al., 2011). There are no examples in the literature for

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producing multi-gram quantity of membrane-bound proteins in active form, and therefore the traditional safety assessment with proteins made in heterologous system is not feasible (e.g. mouse acute oral).

In addition to protein expression levels, use of routine safety assessment routes for D9DS is further complicated in two additional areas. (1) Demonstration of purified D9DS activity requires the reconstitution of the desaturase and cytochrome b5 reductase into a membrane environment. This has traditionally been accomplished by generating liposomes or by incubating the isolated desaturase with microsomes which are deficient of desaturase activity on their own (Joshi et al., 1977). This is further complicated by the need to achieve the appropriate component ratios. (Enoch et al., 1976) (2) Purified D9DS tends to aggregate and would need to be mixed with lipids or other proteins to reduce aggregation as prolonged detergent exposure is detrimental to activity. (Strittmatter and Enoch, 1978). Hence "stabilization" of pure D9DS could potentially confound toxicology results. Nevertheless, as a logical first step in developing a strategy for safety assessment with membrane-bound proteins, we expressed D9DS in multiple expression hosts and carried out a preliminary safety assessment using a novel strategy. Here we describe multiple expression systems, isolation procedures, and report the relative success of each system. We also describe the safety studies conducted with the resulting enriched extracts from insect cells. Finally, we describe the mode-of-action and bioinformatic investigations that bear on the safety assessment of the D9DS protein.

2. Methods and materials

2.1. Construction of expression vectors

For expression in *E. coli*, the gene coding for acyl-CoA delta 9 desaturase was cloned under the control of the T7 promoter in vector pET30a (Promega, Madison, WI). The recombinant plasmid was transformed into BL21 (DE3) host strain and the protein was expressed using the manufacturer's recommended conditions (Promega, Madison, WI). For mammalian expression, the *d9ds* coding sequence was cloned into a pCDNA4/TO vector according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Human kidney cells were co-transfected with *d9ds*/pCDNA4/TO and the tetracycline repressor pCDNA6/TR using the Profection mammalian transfection kit (Promega, Madison, WI). Expression was induced 48 h following transfection, with 1 μg/mL tetracycline for an additional 24–48 h. Cells were harvested and D9DS protein was assessed by Western blot using anti-D9DS polyclonal antibodies raised against N- and C-terminal polypeptides of the D9DS protein.

For expression in yeast, *d9ds* was cloned into pPICZ α expression vector according to the manufacturer's (Invitrogen, Carlsbad, CA) protocol. For transformation, linearized vector was introduced into the X-33 *Pichia* strain using electroporation and transformants were selected on Zeocin. Selected colonies were grown in 100 mL of Buffered Complex Glycerol medium (complete medium) or minimal media at 28–30 °C to an OD_{600} of ~2–6, and expression was induced by resuspending cells in medium containing methanol (0.5% final concentration). Aliquots at 0, 1, 2, and 3 days were analyzed for expression by immunoblotting with anti-D9DS antibodies. For expression in insect cells, the *d9ds* gene was cloned into the baculovirus vector pAcSG2 containing a Kozak translation initiation signal at the 5' end (continued below).

2.2. Generation of recombinant baculovirus

*Spodoptera frugiperda Sf*9 cells were transfected with the plasmid pAcSG2 containing the *d9ds* gene using BacPAK6 viral DNA and Bacfectin transfection reagent following the manufacturer recommended protocols (Clontech, Mountain View, CA). To amplify a high titer stock of the recombinant plasmid, a 50-mL culture of *Sf*9 cells was seeded at 2×10^6 cells/mL and infected with 1 mL of a viral plaque suspension. The culture was incubated 140 rpm at 27 °C for 72 h. After 72 h, the culture was clarified and the supernatant was used as a high titer virus stock.

2.3. Scale-up of acyl-CoA delta 9 desaturase in expresSF+ cells

Once a high titer virus stock was generated, sufficient virus was available for large scale production of the D9DS protein using expresSF+ cells. To scale up expression, an 800-mL culture of expresSF+ cells was seeded at 1×10^6 cells/mL in SF900 II medium in a 2 L non-baffled Fernbach flask. The culture was incubated at 27 °C at 130 rpm for 72 h. The culture was subsequently split into 6×800 mL cultures which were incubated at 27 °C at 130 rpm overnight. The following day, a sample of the cultures was counted with a hemacytometer, the total number of cells in each flask was calculated, and the recombinant virus (pAcSG2/*d9ds*) was added to a multiplicity of infection of 0.5. Incubation was continued at 27 °C at 130 rpm for 48 h. After 48 h, a sample of the culture was taken for Western blot analysis, and the remaining 4.8 L was used for purification of the D9DS protein.

2.4. Preparation of microsomes

Microsomes were prepared from eukaryotic expression hosts as follows. In the case of *Pichia*, cells were resuspended 1 g/10 mL of buffer containing 50 mM KPO_4, pH 7.9, 1 mM EDTA, 5% glycerol, 2 mM DTT, 1 mL of protease inhibitor cocktail for yeast from Sigma per 100 mL of cell lysate and disrupted in a bead beater (SPEX SamplePrep, Metuchen, NJ) using 0.5 mm beads for 8 cycles of 1 min each with 1 min intervals between each cycle. After disruption, the supernatant was centrifuged at 12,000*g* for 10 min at 4 °C followed by a second centrifugation at 100,000*g* for 1 h at 4 °C to recover a microsomal pellet. In the case of insect cells, baculovirus infected *S. frugiperda* (Sf9) cells were resuspended in sterile PBS. The cells were lysed on ice by mild sonication. The cell debris was removed by centrifugation at 10,000*g* for 20 min and the supernatant was further centrifuged at 100,000*g* for 90 min to obtain the microsome pellet.

2.5. Acyl-CoA delta 9 desaturase assay

Once the microsomes were isolated, the D9DS enzyme activity was measured by a modified assay based on the procedure described by Young (2003). To eliminate the need for radioactivity, heptadecanoyl-CoA was used in place of ^{14}C-palmitoyl-CoA and product formation was measured via GC-flame ionization detector (FID). In detail, microsomes were incubated in a 30 °C shaking water bath (150 rpm) for 30 min under the following conditions: 90 μM heptadecanoyl-CoA, 70 mM potassium phosphate buffer pH 7.2, and 1 mM NADH. The reaction was stopped by adding an equal volume of 10% KOH in methanol and samples were saponified by incubating on an orbital shaker at 80 °C for 30 min. The product and substrate of the oxidation reaction were measured after saponification of the microsomes. The resulting free fatty acids were extracted by acidifying the reaction with 0.5 mL of 6 N HCl. The protonated acids were extracted with hexane and the resulting organic fraction was evaporated to dryness. The dry residue was resuspended in 0.5 mL MeOHCl 3 N and reacted at 80 °C for 30 min. The resulting fatty acid methyl esters (FAMEs) were extracted in hexane and evaporated to dryness prior to reconstitution in 0.1 mL hexane containing 100 ppm tridecanoate-methyl ester as an internal standard (IS). The quantitation of the FAMEs was achieved by direct comparison of the peak area with a commercial calibration standard (Matreya, rape seed #1083) containing the IS. The stearate methyl ester response was used for quantification of heptadecanoate and heptadecenoyl methyl esters. The separation of resulting FAMEs was achieved using Agilent GC6890 equipped with a capillary column BPX-70 (SGE, 15 m × 0.25 mm × 0.25 μm). The oven was programmed as follows: splitless injection at 60 °C for 1.3 min followed by 41.3 °C/min ramping to 150 °C then to 180 °C at 9.1 °C/min and finishing at 220 °C at 41.3 °C/min to be held for 1.8 min. The injection port was maintained at 230 °C and the detector at 240 °C. The make-up gas was nitrogen at 25 mL/min and the carrier gas was hydrogen at 40 mL/min. Reported FAMEs concentrations were expressed in ppm.

2.6. Membrane-bound protein extraction and purification

The microsomes were thawed and kept on ice. Detergent or combinations of detergents, ranging from 0.1% to 2% (v/v) were added and the reaction was incubated at 4 °C overnight with stirring. Samples were then subjected to ultracentrifugation, as described above, and the supernatant was collected for column separation. In most cases, sample was further diluted in the extraction buffer to reduce the detergent concentration to less than 0.5% final concentration. Detergent Fos-choline-12 was selected for production and purification after scouting experiments eliminated ~ 50 other detergents from various classes. The solution was applied onto a 5 mL or a tandem 5 mL nickel-HP column (GE Healthcare, Piscataway, NJ), and washed in buffer A (50 mM Tris–HCl, pH 7.5, 0.3 M KCl, 5% glycerol, 0.2% FC-12, and 20 mM imidazole). Bound protein samples were eluted from 20–500 mM imidazole gradient in buffer A and collected in individual fractions. Samples containing D9DS were pooled, either concentrated on a 5 mL Heparin affinity column (GE Healthcare) or further separated on a Mono S column (GE Healthcare) using 0–1 M NaCl salt gradient. SDS–PAGE was used to monitor separation and Western blot confirmed sample identity using polyclonal antibodies.

2.7. Bioinformatic assessment of protein allergenicity

For the allergenicity assessment, the amino acid sequence of the D9DS was compared with a peer-reviewed database containing 1603 known and putative allergens as well as celiac-induction proteins residing in the FARRP dataset (Version 12, Released in February 2012, University of Nebraska, http://www.allergenonline.org). Potential identities between the D9DS and proteins in the allergen

database were evaluated with the FASTA program (v35) using the default algorithm parameters (Matrix = BLOSUM50; Gap Penalties = −12/-2; ktup = 2; except Expectation = 100). The FASTA search was run by an in-house Perl script on an internal UNIX computer with Linux operating system. If a query sequence is longer than 80 amino acids, the script parses the query sequence into a complete (overlapping) set of 80 amino acid long fragments and each fragment is subjected to a FASTA search. A greater than 35% identity threshold over any 80 or more amino acid sequences between a query sequence and an allergen was used to indicate the potential for cross-reactivity. To ensure that high identity over a short stretch (for example, 80% over 60 amino acids) will not be overlooked, a calculation, (Identity% × number of overlapped amino acids)/80, was implemented as a conversion to check the criteria of >35% over 80 amino acids when the FASTA alignment (overlapped amino acids) is less than 80 amino acids. The D9DS protein sequence was also screened for any matches of 8 contiguous amino acids to the allergens contained in the database noted above. This was done using an in-house Perl script that generates all sequentially possible (overlapping) 8-residue peptides from a query protein, followed by Fuzzpro program (Emboss Package v2.10.0) search that compares each query sequence with all allergen sequences in the database for perfect matches.

2.8. Bioinformatics assessment of protein toxicity

To assess potential toxicity of the D9DS protein, a search for similarity of protein sequences was conducted using the BLASTp program (Altschul et al., 1990). The amino acid sequence of the D9DS was queried using the BLASTp (Version 2.2.21+) against GenBank non-redundant protein sequences (update to February 10, 2012), which incorporates non-redundant entries from all GenBank and RefSeq nucleotide translations (Genpept "nr") along with protein sequences from SWISSPROT (http://www.expasy.org/sprot/), PIR (http://pir.georgetown.edu/), PRF (http://www.prf.or.jp/aboutdb-e.html), and PDB (http://www.wwpdb.org/). The search was done through the BLAST program installed on an internal UNIX computer with default settings (Matrix = BLOSUM62, Gap Costs = Existence: 11, Extension: 1) except that a cutoff expectation *E*-value of 1.0 was used to generate biologically meaningful similarity between the query sequences and proteins in the database, the low complexity filtering was turned off, and the sequence description and alignment display were set to 5000 alignments. Although a statistically significant sequence similarity generally requires a match with an expectation value less than 0.01, a cutoff of E()<1.0 insures that proteins with even limited similarity will not be overlooked in the search (Pearson, 2000).

2.9. Heat treatment of acyl-CoA delta 9 desaturase

The D9DS microsomal preparation (prepared as described earlier) was thawed on ice, vortexed, and dispensed into 5 microfuge tubes in 150 μL aliquots. The control sample was held on ice and the others were heated at 30, 50, 70, or 95 °C for 30 min. After heat treatment, all samples were immediately placed on ice. Aliquots were analyzed by Western blot (probed with a D9DS specific polyclonal rabbit antibody) and evaluated for enzymatic activity by monitoring the formation of 17:1 as described earlier.

2.10. Digestibility of partially purified and microsomes expressing acyl-CoA delta 9 desaturase

Equimolar (~0.074 mM) solutions of the control substances were prepared as follows: BSA (pepsin labile) was solubilized by weighing 24.7 mg of powder in a 15-mL centrifuge tube and adding 5 mL of Milli-Q water. β-lactoglobulin A (pepsin resistant) was solubilized by weighing 7.5 mg of powder in a 15-mL centrifuge tube and adding 5 mL of Milli-Q water. The varying amounts of the test and control substances reflect differences in purity and molecular weight. Simulated gastric fluid (SGF, pH ~1.2) containing a final concentration of approximately 0.32% (w/v) pepsin (Sigma Aldrich, St. Louis, MO, catalog #P6887, 89% w/w pure, 3,600 units of activity/mg protein) was prepared as recommended in The United States Pharmacopeia, 1995. Pepsin was solubilized by weighing 0.1904 g of powder and dissolving into 50 mL of 34 mM NaCl, pH 1.2. Methods generally followed those of (Herman et al., 2005; Herman et al., 2007)

The digestions for microsomal-derived D9DS, BSA, and β-lactoglobulin A were performed for time intervals of approximately 15 s, 30 s, 1, 2, 4, and 8 min in a water bath set to 37 °C. The three samples; D9DS microsomes (that were thawed on ice), BSA, and β-lactoglobulin A were digested as follows: Three 2.75-mL aliquots of SGF were placed in the 37 °C water bath. After 5 min, 250 μL of the D9DS microsomal solution (as prepared above), 0.74 mM BSA, and 0.74 mM β-lactoglobulin A were each added to a separate vial of the SGF and a timer was set. After each specified incubation interval, 100 μL of the reaction mixture was removed and added to tubes containing stop solution (40 μL of 200 mM sodium carbonate, pH ~11.0). The stopped reactions were then placed on ice until all of the time points were sampled for the three proteins. An SGF control was prepared by substituting water for the sample protein and incubating for the duration of the experiment at 37 °C. The SGF control was prepared as follows: A 2.75-mL aliquot of SGF was heated in a 37 °C water bath for 5 min, 250 μL of Milli-Q water was added and a timer was set. A 100 μL aliquot was immediately removed as the zero time point and placed into a tube containing the stop reaction (40 μL of 200 mM sodium carbonate, pH ~11.0), when all digestion reactions for all proteins were complete, one final aliquot was taken at the duration of the experiment. For each of the proteins above, a zero time point (neutralized control) was prepared as follows: First, a 91.7 μL aliquot of SGF was heated for 5 min at 37 °C and stopped with 40 μL 200 mM sodium carbonate, then 8.3 μL of the respective protein was added to the solution.

Aliquots of the neutralized and digested proteins were mixed with equal volumes of Laemmli sample buffer (Bio-Rad), containing 5% freshly added 2-mercaptoethanol (Bio-Rad) and heated for 5 min at ~95 °C. Single 4–12% Bis–Tris polyacrylamide gels (Bio-Rad) of BSA β-lactoglobulin A, and duplicate gels of D9DS were prepared. One gel was stained with Gel-Code Blue (Thermo-Pierce) and the other was transferred to a nitrocellulose membrane and probed with anti-D9DS polyclonal antibodies and detected with anti-rabbit HRP-labeled antibodies and chemiluminescent detection. The optical densities (ODs) of three digestion fragments of the microsomal-derived D9DS observed on the film were determined by scanning the Western blot film with a densitometer similar to the procedure described by Brussock and Currier (1990). The densities of each fragment were regressed against the digestion time between 15 s and 8 min (time of appearance till last time point) using a three-parameter exponential model ($OD = ae^{-kT} + b$, where $a = y -$ intercept; b = asymptote and $-k$ = 1st-order rate constant) using SAS version 9.1.

3. Results and discussion

The safety assessment of proteins expressed in genetically modified plants routinely requires multi-gram quantities of highly purified, active recombinant proteins for toxicological and allergenicity studies. Membrane-bound proteins such as D9DS are difficult to produce at such levels in a highly purified form. The ubiquitous nature of this protein and the important function it performs in all living organisms suggest that it is unlikely to be detrimental to human health if consumed, however, weight of evidence studies are needed to address this assumption. D9DS presents two additional challenges: (1) besides being a membrane-bound protein; it is not functional when extracted from the membrane and (2) it requires another membrane-bound accessory protein, cytochrome B5 reductase, for activity. Because of these challenges, D9DS was expressed in multiple expression systems, and several strategies were explored for safety assessment.

3.1. Expression of acyl-CoA delta 9 desaturase

A broad range of expression systems such as *E. coli*, mammalian, Pichia, and insect cells were explored to ensure successful production of D9DS in quantities that would enable a thorough safety assessment. Expression levels in *E. coli* and mammalian cell culture systems were very low; only *P. pastoris* and insect cells provided a sufficient level of expression (milligrams per liter) to warrant moving forward with enrichment and purification. Most of the purification work was performed with D9DS expressed in these two systems. Fatty acid analysis of insect and Pichia cells expressing D9DS demonstrated a decrease in C16:1 and an increase in C18:1 fatty acids compared with control cells suggesting that the D9DS was expressed and functional (data not shown). Membrane localization in insect and Pichia cells was confirmed by evaluating microsomes by Western blot analysis for the presence of D9DS. D9DS was present in the microsomal fraction and not in the soluble fraction confirming membrane localization (Fig. 1).

When a non-natural substrate heptadecanoic acid (C 17:0) was used to verify that the expressed proteins were functional, microsomes from insect cells and *P. pastoris* expressing D9DS were able to convert 12% and 35%, respectively, of the added substrate to heptadecenoic acid (C 17:1) indicating that the expressed D9DS was active in both host systems. Subsequent experiments targeted a conversion of <10% to ensure that subrate was not limiting and supported a linear reaction rate. We also examined the fatty acid profile of whole cells expressing D9DS. In *P. pastoris* and insect cells

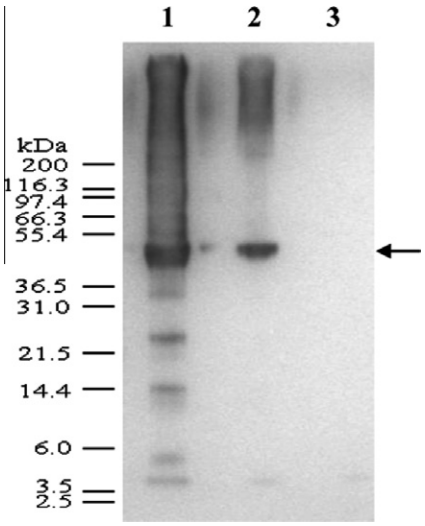

Fig. 1. Western hybridization analysis of ExpressSF cells expressing acyl-CoA delta 9 desaturase after processing for the isolation of microsomes. Lane 1 –ExpresSF+ cells expressing the desaturase; Lane 2 – microsome pellet after 100,000g centrifugation; Lane 3 –supernatant after centrifugation at 100,000g. Arrow indicates the full-length acyl-CoA delta 9 desaturase protein.

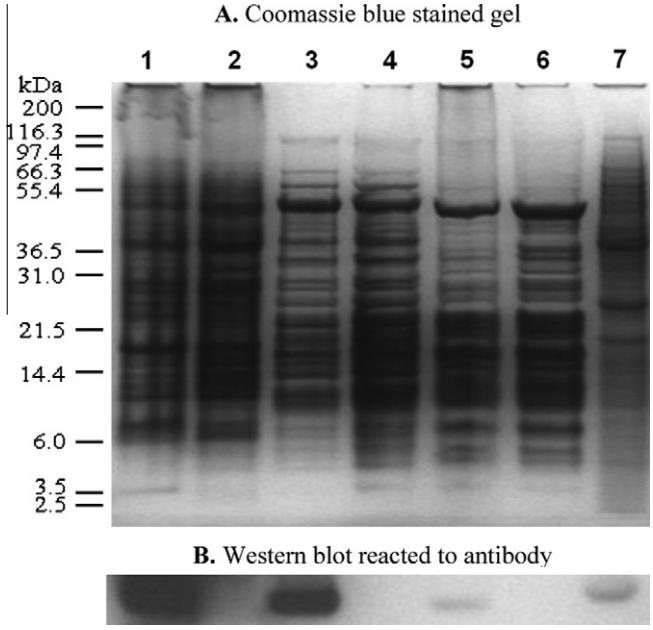

Fig. 2. Comparison of acyl-CoA delta 9 desaturase expression in multiple hosts. Lane 1 - Sf9 cells expressing desaturase; Lane 2 - Sf9 control cells; Lane 3 – *Pichia* expressing desaturase; Lane 4 - *Pichia* host only control; Lane 5 – *S. cerevisiae* expressing desaturase; Lane 6 – *S. cerevisiae* host only control; Lane 7 – *A. nidulans* untransformed (source of the desaturase gene). Total proteins from different hosts were separated by SDS–PAGE (panel A) and probed by western hybridization analysis with the polyclonal antibody generated against the C-terminal domain of desaturase expressed in an insoluble form in *E. coli.*

expressing D9DS, a clear shift in fatty acid profile was generally seen, especially for the pool of C18:1.

Western blot analysis of microsomes from the two separate hosts expressing D9DS indicated that the volumetric productivity (productivity per unit volume of culture) was comparable (Fig. 2). Expression of D9DS was examined in Pichia using a quantitative Western blot analysis method using the polyclonal antibodies to the C-terminal domain of D9DS. In Pichia D9DS expressed at 0.8–1 mg/l and in insect cells the expression was higher than Pichia (Fig. 2) indicating that both systems were suitable for an early tier safety assessment (Delaney et al., 2008). Specifically, mode of action, *in vitro* digestibility and heat lability, protein expression level, and dietary exposure of the transgenic protein could all be assessed for the *A. nidulans*-derived D9DS protein. However, it was clear that none of the hosts expressing D9DS are capable of generating sufficient protein for an *in vivo* toxicology assessment which would be required if an early tier assessment indicated risk (Delaney et al., 2008). Such testing would require several grams of active purified protein. To develop suitable D9DS enrichment methods, the insect cell system was chosen as a source of D9DS as the native D9DS protein in insect cells is significantly different both in sequence and size compared with *A. nidulans*-D9DS facilitating differentiation between native and transgenic versions. Insect D9DS proteins have been cloned and characterized (Knipple et al., 1998) and do not contain the cytochrome B5 domain that is present in the *A. nidulans*-D9DS whereas yeast D9DS (including *P. pastoris*) also contains a cytochrome B5 domain. Therefore, the native insect D9DS was less likely to interfere with chromatographic purification as it is unlikely to co-elute with *A. nidulans*-D9DS.

3.2. Purification of *A. nidulans* acyl-CoA delta 9 desaturase

Isolation of sufficient D9DS in an active form posed many challenges. The ideal detergent needed to be very efficient at extracting the D9DS from membrane preparations, and at the same time should not impact stability or interfere with activity. Reconstitution of the purified protein in an artificial membrane is essential for validating the function of the purified protein. This is a major bottleneck in developing purification methods to monitor progress as it is not trivial to reconstitute active protein given the requirement for the accessory protein cytochrome b_5 reductase (cytb5). Therefore, a number of detergents were evaluated for their efficiency in extracting D9DS from microsomes and for impact on activity of D9DS.

Approximately 50 detergents from all classes (ionic, non-ionic and zwittterionic) were examined for extraction efficiency (Table 1) and 36 were evaluated for interference with the activity of D9DS. In the latter case, the enzyme's tolerance for residual detergent was examined at 0.05% detergent concentration because higher detergent concentrations were detrimental to activity for all detergents tested (unpublished data). Initial purification work focused on the detergents which demonstrated low impact on activity, octyl glucoside and zwittergent 3–10.

As mentioned earlier, activity of the purified protein could not be demonstrated without reconstitution of the membrane environment as cytb5 reductase is required in the artificial membrane along with D9DS to enable activity. Our efforts to express cytb5 reductase resulted in very limited success. Therefore, reconstitution of D9DS activity was attempted with SF9 microsomes from cells which had not been transfected for D9DS production based on the method of Joshi et al., 1977. Convincing reconstitution was not observed. Given these results and that the protein recovery levels with these detergents were not optimal, follow up extraction efforts focused soley on obtaining high levels of pure protein. Among the detergents tested, Fos-choline-12 and ASB-14 were found to perform very well in extracting D9DS from microsome membranes. As expected, the activity of D9DS was abolished after extraction with high concentration of detergents as these enzymes function only when present in membranes (Chabaud et al., 1998; Strittmatter and Enoch, 1978). As the level of recombinant expressed D9DS was only a small fraction of the microsomal preparation, conventional methods (combination of ion exchange, isoelectric focusing, hydroxyapatite chromatographies) to isolate the

Table 1
Detergents tested for *An* acyl-CoA delta 9 desaturase (D9DS) extraction efficacy. The types of detergents: A, anionic; C, cationic; N, nonionic; Z, zwitterionic. Abbreviation: NA: data not available; Purity of detergents from Anatrace was measured by the manufacturer using HPLC analysis. Detergents from other manufacturers were of ultrapure grade reagent. Extraction efficacy was rated by SDS–PAGE analysis followed by Western blot using a polyclonal antibody for the C-terminal fragment of D9DS, "–" indicates no extraction and recovery; "+/–" as non-reproducibly positive extraction; " + " as positive extraction; "++" as strong positive extraction; while "x" indicates extraction resulting in significant destruction, where only protein smears were obtained with no distinct protein bands after gel separating. Majority of the detergent concentration examined were from 0.5–2% (v/v) and only a few were used in either <0.5% or >5% depending on their critical micelle concentration (CMC).

Detergent	Type	Purity (%)	Manufacturer	Extraction efficacy (recovery)
None, no detergent control				0
ANAPOE-20	N	NA	Anatrace, Maumee, OH, USA	+/–
ANAPOE-35	N	NA	Anatrace	+
ANAPOE-58	N	NA	Anatrace	++
ANAPOE-80	N	NA	Anatrace	+/–
ANAPOE- $C_{12}E_{10}$	N	NA	Anatrace	+
ANAPOE-X-100	N	NA	Anatrace	+
ANAPOE-X-114	N	NA	Anatrace	+
ANZERGENT 3–8	Z	99.6	Anatrace	+
ANZERGENT 3–10	Z	99.5	Anatrace	+
Apo-10	N	NA	Calbiochem, Billerica, MA, USA	–
ASB-14	Z	>98	Calbiochem	++
ASB-14–4	Z	NA	Calbiochem	+
ASB-16	Z	NA	Calbiochem	+
ASB-C_8	Z	NA	Calbiochem	–
BRIJ 72	N	NA	Calbiochem	–
$C_{10}E_8$	N	NA	Calbiochem	+/–
$C_{12}E_8$	N	NA	Calbiochem	+/–
C–DODECAFOS	Z	99.5	Anatrace	–
CHAPS	Z	99.5	Anatrace	+
CYMAL-2	N	99.5	Anatrace	+
CYMAL-6	N	99.5	Anatrace	+/–
CYCLOFOS-2	Z	99.5	Anatrace	+
CYCLOFOS-5	Z	>98	Anatrace	+
CYPFOS-3	Z	99.5	Anatrace	–
Decyl-D-thiomaltopyranoside	N	NA	Anatrace	–
Deriphat 160	N	NA	Cognis Corp., Cincinnati, OH, USA	+
Dodecyl-D-maltoside	N	99.5	Anatrace	+
Dodecyltrimethylammonium chloride	C	98.9	Anatrace	–
FOS-choline-10	Z	99.5	Anatrace	+
FOS-choline-12	Z	99.5	Anatrace	++
FOS-choline-13	Z	>99	Anatrace	++
FOS-choline-16	Z	99.2	Anatrace	++
FOS-choline-ISO-9	Z	99.5	Anatrace	–
FOS-choline-UNSAT-11–10	Z	>99	Anatrace	+
FOS-MEA-8	A	99.5	Anatrace	–
Heptyl-D-glucopyranoside	N	99.5	Anatrace	–
Hexadecyltrimethylammonium chloride	C	98.9	Anatrace	+
IGEPAL CA-630 (NP-40)	N	NA	Sigma	–
LDAO	Z	99.5	Anatrace	+
LDS	A	>99	Anatrace	x
Na Cholate	A	NA	Anatrace	–
Na deoxycholate	A	99.5	Anatrace	–
NDSB 201	N	~100	Calbiochem	–
Octaethylene glycol monododecyl ether ($C_{12}E_8$)	N	99.5	Anatrace	+
Octyl-D-glucopyranoside	N	>99	Anatrace	+
Pentaethylene glycol monodecyl ether	N	99.5	Anatrace	–
PMAL-C10	Z	NA	Anatrace	–
SDS	A		Sigma	x
Sucrose monodedecanoate	N	98.9	Anatrace	+
Tridecyl-D-maltopyranoside	N	99.5	Anatrace	+
Triton X-100	N	NA	Sigma, St. Louis, MO, USA	+
Triton X-114	N	NA	Sigma	+
Tween 20	N	NA	Sigma	+/–
Tween 80	N	NA	Sigma	+/–
Tergitol NP-9	N	NA	Sigma	–
Undecyl-D-maltopyranoside	N	99	Anatrace	+
Zwittergent 3–10	Z	NA	Calbiochem	+
Zwittergen 3–14	Z	NA	Calbiochem	+

protein were not feasible. Therefore a more robust procedure was explored.

Polyclonal antibodies were raised against the C-terminal soluble domain immediately following the second TM domain corresponding to amino acids 235–454 expressed in an insoluble form in *E. coli* (unpublished results) and were used to monitor the purification process by Western blot. The same polyclonal antibody was used to purify D9DS, initially by immunoaffinity chromatography, although it failed to capture D9DS efficiently. Among the various chromatographic options evaluated, a metal affinity column followed by a heparin affinity column was found to be most effective in enriching D9DS. This method yielded about 500-fold enrichment with an overall purity of 30–50% in standard PBS buffer. Under these conditions, the protein maintained good stability though it did not maintain activity. Further purification to remove other host proteins and/or phosphorylated proteins/lipids resulted in

Table 2
Sequence homology between acyl-CoA delta 9 desaturase and several human and plant fatty acid desaturase and cytochrome b5 proteins.

GenBank accession	Description	Identity (%)/number of amino acids	*E*-value
NP_005054.3	Stearoyl-CoA desaturase (delta-9-desaturase) [*Homo sapiens*]	40/267	1×10^{-48}
AAD29870.1	Stearoyl-CoA desaturase (delta-9-desaturase) [*Homo sapiens*]	40/267	1×10^{-48}
CAA73998.1	Stearoyl CoA desaturase [*Homo sapiens*]	40/267	9×10^{-48}
NP_001169125.1	Uncharacterized protein LOC100382970 [*Zea mays*]	93/456	0
BAC43716.1	Putative delta 9 desaturase [*Arabidopsis thaliana*]	32/234	2×10^{-21}
AAK92773.1	Putative delta 9 desaturase [*Arabidopsis thaliana*]	30/244	2×10^{-20}
BAJ91418.1	Predicted protein [*Hordeum vulgare* subsp. vulgare]	56/394	1×10^{-129}
XP_003525924.1	PREDICTED: cytochrome b5-like [*Glycine max*]	41/75	2×10^{-5}
XP_003523994.1	PREDICTED: cytochrome b5-like [*Glycine max*]	41/71	3×10^{-5}
XP_003636809.1	Cytochrome B5 [Medicago truncatula]	43/67	8×10^{-5}
XP_003638456.1	Cytochrome B5 [*Medicago truncatula*]	43/67	2×10^{-4}

significant D9DS precipitation likely caused by previously noted aggregation issues. From 10 L of insect cell cultures expressing D9DS, about 1 mg of D9DS at ~30–50% purity was obtained. Given the low recovery and level of purity achieved prior to protein precipitation, we concluded that preparation and reconstitution of a functional, purified D9DS on the scale needed for *in vivo* toxicology studies was not feasible.

3.3. Safety assessment

Expression and purification studies demonstrated that it is not feasible to obtain a sufficient amount of high purity D9DS in a functionally active form to enable *in vivo* toxicology studies. While the enzyme is ubiquitous and is part of the human diet (including yeast and corn which have homologous D9DS), assessment of initial safety was deemed beneficial, but the strategy employed had to be modified. Strategies that did not require purification of protein to homogeneity were considered. Feeding studies with microsomes from insect cell expressing D9DS were considered for toxicity assessment. However, the traditional high-dose acute oral mouse toxicity study is not possible with microsomes as the oral ingestion of microsomes needed to meet the common dosage of 2000–5000 mg/kg. In addition, ingestion of microsomes might be deleterious to the health of the animals. For these reasons, animal toxicity studies with enriched D9DS were not considered feasible as a part of overall safety assessment and a modified safety assessment regime was used.

3.4. Potential protein allergenicity and toxicity

When a new transgenic protein is produced in crops, one assessment considers allergenic potential. Thus far, no single property of a protein is known to predict allergenic potential. For this reason, a weight-of-evidence approach to predicting allergenic risk has been adopted which considers multiple factors. To assess potential allergenicity of a protein, two criteria for evaluating structural similarities between query proteins and known allergens are currently used based on amino acid sequence alignments Codex, 2009; Ladics, 2008 FAO/WHO, 2001). The first criterion is a search over 80-amino-acid stretches (sliding window search) to detect >35% identity between a query protein and known allergens. The window size of 80 amino acids was selected to correspond with a typical domain size in a protein, and recognizes that single protein domains may contain epitopes that mediate antibody binding. The second criterion involves evaluating short amino-acid stretches for identity between the query protein and known allergens although this approach has been criticized for high rate of false positive and lack of scientific value (Silvanovich et al. 2006; Stadler and Stadler, 2003; Goodman et al., 2008; Thomas et al., 2008; Cressman and Ladics, 2009; Herman et al., 2009). When the amino acid sequence of the D9DS protein was compared with the FARRP allergen dataset (Version 12), no over threshold identities (greater than 35% identity over 80 or more amino acid residues) were detected in the FASTA search outputs. No matches of eight or greater contiguous identical amino acids with known allergens in the database were observed in the entire D9DS sequence. These results show that D9DS does not share any significant amino acid sequence similarity with known protein allergens.

Another safety assessment of proteins expressed in transgenic crops is the evaluation of relatedness to known toxins. It has been reported that assessing the potential toxicity of a protein may include comparison of the protein sequence to known protein toxin sequences Codex, 2009. Since there is no commonly recognized definition of a protein toxin based on its sequence, a comparison should be made to a database of all available protein sequences as a conservative approach. Proteins identified with statistically significant similarity using a local alignment algorithm are then evaluated for their relevance as potential toxins. The D9DS protein is a stearoyl CoA desaturase isolated from the fungus *A. nidulans*. This protein has a fatty acid deasaturase domain. Unlike many enzymes of this class, it also contains a cytochrome b5 (cytb5) domain. Therefore, it was expected to have homology with two classes of proteins from various species when comparing to other proteins in a database.

The search of D9DS against the Genbank non-redundant protein sequences identified 3007 hits with *E*-value < 1. As expected, the majority of the identified proteins were fatty acid-desaturase and/or contained a cytb5 domain. Those proteins can be grouped into 4 categories: fatty acid-desaturase (fatty acid hydrolyase), cytochromes b5, hypothetically predicted proteins, and nitrate reductase with very weak similarity with D9DS (*E*-value >0.001). Fatty acid-desaturase is a common enzyme found in a broad range of organisms including human, animal, plants, and fungus, and bacterium. Cytochromes b5 are ubiquitous electron transport hemoproteins found in animals, plants, fungi and purple phototrophic bacteria. None of these proteins returned by the BLASTp search is associated with toxicity. Furthermore, D9DS

Table 3
Measurement of the effect of heat treatment on acyl-CoA delta 9 desaturase enzymatic activity. Activity was measured based on the conversion of a non-natural C17:0 substrate (to differentiate from background fatty acids) to a C17:1 product. The initial specific activity of the microsomal preparation was 0.13 nmol/min/mg of total protein.

Temperature (°C)	% Activity
4	100.0
30	49.1
50	5.2
70	0.0
95	0.0

showed some significant sequence similarity with fatty acid-desaturases from human and plants, and cytochromes b5 from food crops (Table 2). An uncharacterized maize protein (NP_001169125.1) is almost identical to D9DS, indicating proteins similar to D9DS exist in food crops, although it would be desirable to verify that this protein is present in maize grain to eliminate the possibility of an erroneous entry into the Genebank database. Therefore D9DS protein contains no significant sequence similarity with any known toxic protein that is harmful to humans or animals

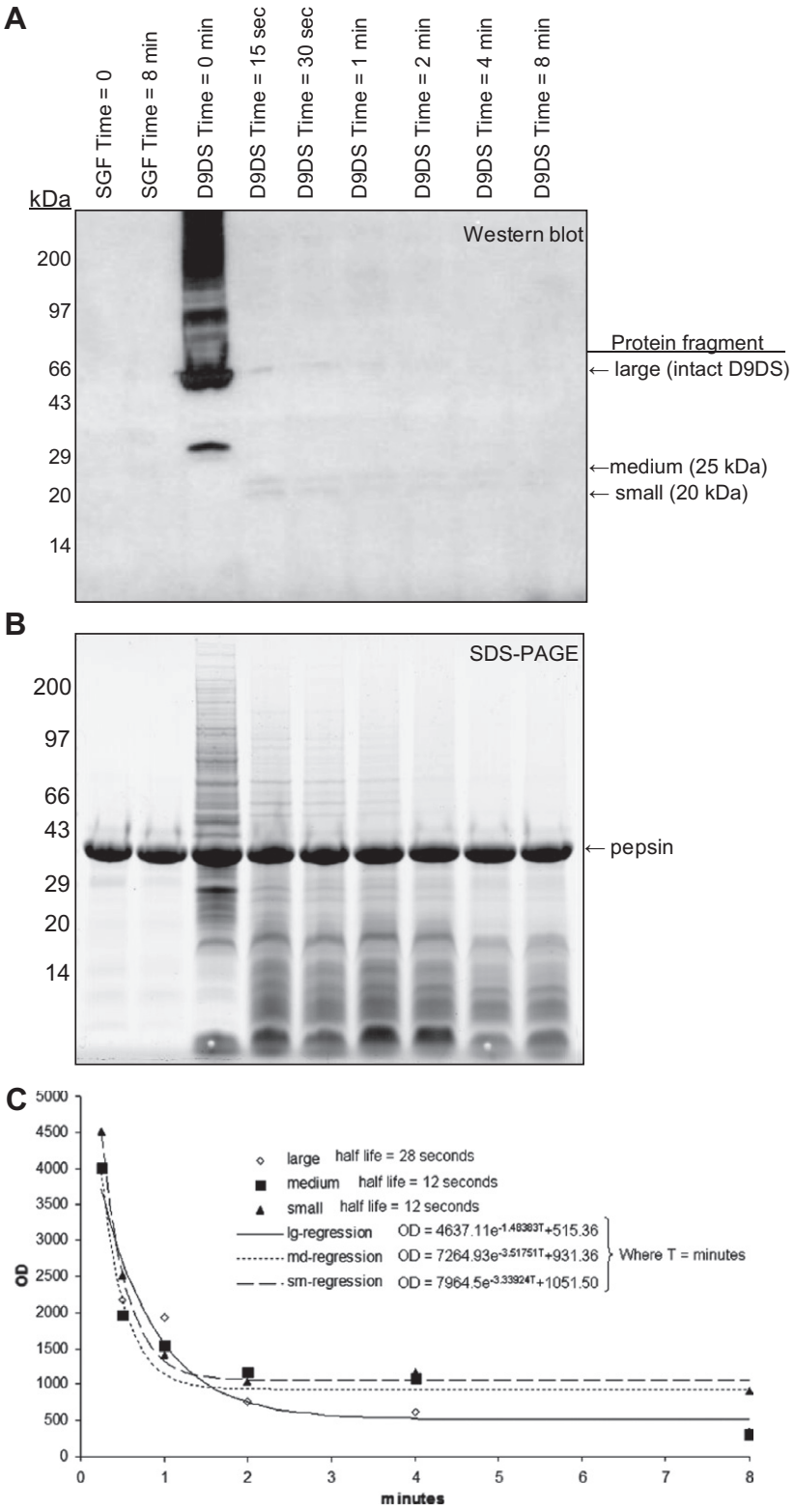

Fig. 3. Western blot analysis of microsomal-derived acyl-CoA delta 9 desaturase (M.W. ~52 kDa) protein subjected to digestion in simulated gastric fluid. Western blot (panel A), SDS PAGE (panel B). Plot of digestion of three digestion fragments of *An*acyl-CoA delta 9 desaturase showing the fit of a three-parameter exponential decay model (panel C).

3.5. Early stage safety assessment

3.5.1. Biochemical properties

While no significant amino-acid sequence homology was seen to known allergens and protein toxins by sequence analysis, two additional weight of evidence studies were examined for the active microsomes bound D9DS or partially purified soluble protein. Simulated gastric fluid studies and heat lability were considered as a part of overall safety assessment to determine the stability of the D9DS.

3.5.2. Heat treatment of acyl-CoA delta 9 desaturase

Proteins are usually denatured and inactivated by high temperatures and the degree of inactivation is dependent upon the temperature and duration of the heat treatment. All heat treatments conducted as part of this investigation involved temperatures that occur at or below the temperatures experienced during canola seed processing (Gunstone, 2004). In canola industrial processing, multiple steps of heat treatment are usually involved in the production of the desolventized meal and extraction of oil. For example, during pre-pressing of the seed, temperatures of ~82 to 100 °C are used in the production of crude canola oil and pressed cake. During the production of the refined, bleached, and deodorized canola oil (RBD oil), the oil extracted from canola meal is further processed by exposing the oil to acid, caustic soaps, bleaching reagents, and temperatures up to 240 °C. This harsh treatment ensures the final RBD oil is completely free from odor and off flavors allowing use in many aspects of food manufacture.

The thermal stability of D9DS protein was evaluated by heating microsomal solutions for 30 min at 30, 50, 70, 95 °C. The D9DS protein activity was measured by a modified enzyme assay based on the procedure described by Young (2003). The study demonstrated that the D9DS protein has significantly reduced activity when heated. In the activity assay (the measurement of the conversion of C17:0 to 17:1), the protein lost greater than 50% of its enzymatic activity at 30 °C and virtually all of the activity was eliminated at the higher temperatures tested (Table 3). These data indicate that industrial canola processing would largely inactivate the D9DS protein.

3.5.3. SGF digestibility of microsomal-derived acyl-CoA delta 9 desaturase

The digestibility of D9DS was assessed according to published methods that are universally accepted by global regulatory agencies. The digestible and non-digestible control proteins, BSA and β-lactoglobulin A, respectively, responded as expected as BSA was not detected at the 30-s time point and β-lactoglobulin A remained readily detectable for the duration of the experiment (data not shown). The D9DS protein, with an apparent molecular weight of approximately 52 kDa, was barely detectable at 15 s on the Western blot for the microsomal-preparation Fig. 3. Three minor fragments of the protein were produced during the digestion, and the half lives of the three fragments ranged from 12 to 28 s as demonstrated by quantitative densitometric analysis (Fig. 3). Multiple time points were were used to calculate the digestion rate and the rapid digestion of the D9DS protein and fragments leaves little uncertainty about its digestibility, even in this complex matrix. To further validate this finding, a similar experiment was performed using partially-purified D9DS and the protein was not detectable after 30 s and no fragments were observed in any of the time points (data not shown). This was probably due to protein denaturation that occurred during purification resulting in more accessible protease sites or the digestion of the microsomal-derived D9DS was slowed by the additional proteins contained in the microsomal preparation. The undigested D9DS portion of both preparations also showed dimeric and/or oligomerized forms in addition to the monomeric 52 kDa band on the Western blot, probably owing to the complex mixture of the protein sample and aggregation upon heating.

4. Conclusions

The ability to purify active proteins produced from new transgenic traits may not be feasible where such proteins are membrane-bound. The membrane-bound protein, D9DS, is an example of a protein which could not be isolated in an adequate quantity for traditional toxicology studies using mouse models. Current weight of evidence information does not indicate any known safety concerns: (1) D9DS –type proteins are present in food. (2) The mode of action of the D9DS protein is not associated with any known safety issues. (3) Bioinformatic studies indicated that the D9DS protein does not have any biologically relevant similarity to proteins known to pose a safety risk. (4) Enriched preparations of active D9DS protein were denatured by heat and readily digested in simulated gastric fluid indicating no remarkable stability that might increase exposure or persistence relative to normal dietary proteins. This information, in combination with the history of safe consumption of highly related proteins, provides a weight-of-evidence supporting the human health safety of D9DS as expressed in oil-seed crops. When transgenic events of interest are selected animal-feeding studies could also be carried out to confirm safety.

5. Conflict of Interest

The authors declare that there are no conflicts of interest.

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