FOOD STANDARDS
Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

SUPPORTING DOCUMENT 1

APPLICATION A1046 – FOOD DERIVED FROM HERBICIDE-TOLERANT SOYBEAN LINE DAS-68416-4

SAFETY ASSESSMENT REPORT

SUMMARY AND CONCLUSIONS

Background

Dow AgroSciences Australia Ltd has developed a genetically modified (GM) soybean line known as DAS-68416-4 that is tolerant to two herbicides, glufosinate ammonium and 2,4-dichlorophenoxyacetic acid (2,4-D). Tolerance to glufosinate ammonium is achieved through expression of the enzyme phosphinothricin acetyl transferase (PAT) encoded by the *pat* gene derived from the soil bacterium *Streptomyces viridochromogenes*. The *pat* and closely related *bar* genes have been widely used in the genetic modification of a number of crop species. Tolerance to 2,4-D has been achieved through the introduction of the *aad*-12 gene, from another soil bacterium *Delftia acidovorans*, expressing the enzyme aryloxyalkanoate dioxygenase-12 (AAD-12).

In conducting a safety assessment of food derived from herbicide-tolerant soybean line DAS-68416-4, a number of criteria have been addressed including: a characterisation of the transferred genes, their origin, function and stability in the corn genome; the changes at the level of DNA, protein and in the whole food; compositional analyses; evaluation of intended and unintended changes; and the potential for the newly expressed proteins to be either allergenic or toxic in humans.

This safety assessment report addresses only food safety and nutritional issues. It therefore does not address: environmental risks related to the environmental release of GM plants used in food production; the safety of animal feed or animals fed with feed derived from GM plants; or the safety of food derived from the non-GM (conventional) plant.

History of Use

Soybean (*Glycine max*), the host organism is grown as a commercial crop in over 35 countries worldwide. Soybean-derived products have a range of food and feed as well as industrial uses and have a long history of safe use for both humans and livestock. Oil accounts for 94% of the soybean products consumed by humans and is used mainly as a salad and cooking oil, bakery shortening, and frying fat as well as being incorporated into processed products such as margarine.

Molecular Characterisation

Soybean line DAS-68416-4 contains two novel gene cassettes, one containing the *pat* gene and the other containing the *aad-12* gene. There are no antibiotic resistance marker genes present.

Comprehensive molecular analyses of soybean line 68416 indicate there is a single insertion site containing one complete copy of each of the two gene cassettes. The introduced genetic elements are stably inherited from one generation to the next. No known endogenous genes have been interrupted by insertion of the new genetic material.

Characterisation of Novel Protein

Soybean line DAS-68416-4 expresses two novel proteins, AAD-12 and PAT. Expression analyses of the two proteins showed that, in the plant parts tested, the AAD-12 is lowest in the roots and grain (approximately 16 µg/g dry weight) and highest in leaves (approximately 55 µg/g dry weight). PAT protein concentrations are much lower than those for AAD-12 but similarly, the leaves contain the highest levels (approximately 11 µg/g dry weight) and the roots contain the lowest levels (approximately 2 µg/g dry weight).

Several studies were done to confirm the identity and physicochemical properties of the plant-derived AAD-12 and PAT proteins, and demonstrated they both conform in size and amino acid sequence to that expected, and do not exhibit any post-translational modification including glycosylation.

In relation to potential toxicity and allergenicity, previous assessments of the PAT protein have shown it is inherently non-toxic to mammals and does not exhibit any potential to be allergenic to humans.

For the AAD-12 protein, bioinformatic studies confirmed the lack of any significant amino acid sequence similarity to known protein toxins or allergens; a digestibility study suggests the protein would be rapidly degraded in the stomach following ingestion; and a thermolability study showed that the protein is inactivated by heating. An acute oral toxicity study in mice also confirmed the absence of toxicity of AAD-12. Taken together, the evidence indicates that AAD-12 is unlikely to be toxic or allergenic to humans.

Herbicide Metabolites

Use of PAT to confer tolerance to glufosinate ammonium has been previously considered in a wide range of food crops, including soybean, and therefore glufosinate ammonium residues were not considered in this Safety Assessment. The major residue generated on soybean line DAS-68416-4 as a result of spraying with 2,4-D is dichlorophenol. This residue is the same as that found on conventional crops sprayed with 2,4-D and would be present at very minor levels; there are no safety concerns.

Compositional Analyses

Detailed compositional analyses were done to establish the nutritional adequacy of seed-derived products from soybean line DAS-68416-4 under four herbicide-spraying regimes. Analyses were done of 84 analytes encompassing proximates (crude fat/protein, carbohydrate and ash), acid detergent fibre, neutral detergent fibre, fatty acids, amino acids, isoflavones, anti-nutrients, minerals, and vitamins. The levels were compared to levels in the seeds of the non-GM parent ‘Maverick’.

These analyses indicated that the seeds of soybean line DAS-68416-4 are compositionally equivalent to those of the parental line. Out of all the analytes tested, there were significant differences between the non-GM control and soybean 68416 in only 19 analytes. In 17 of these, the mean levels observed in seeds of soybean line DAS-68416-4 were within the range of natural variation reported in the literature. There were no consistent trends in the effect that herbicide spraying of soybean line DAS-68416-4 had on mean analyte levels.

In addition, no difference between seeds of soybean line DAS-68416-4 and 'Maverick' were found in IgE-binding studies using sera from soybean-allergic individuals.

The compositional data are consistent with the conclusion that there are no biologically significant differences in the levels of key components in seed from soybean line DAS-68416-4 when compared with the non-GM control or with the range of levels found in commercial soybean cultivars.

Nutritional Impact

Soybean line DAS-68416-4 is the result of a simple genetic modification to confer herbicide tolerance with no intention to significantly alter nutritional parameters in the food. In addition, the extensive compositional analyses of seed that have been undertaken to demonstrate the nutritional adequacy of soybean line DAS-68416-4 indicate it is equivalent in composition to conventional soybean cultivars. The introduction of soybean line DAS-68416-4 into the food supply is therefore expected to have little nutritional impact.

Conclusion

No potential public health and safety concerns have been identified in the assessment of soybean line DAS-68416-4. On the basis of the data provided in the present Application, and other available information, food derived from soybean line DAS-68416-4 is considered to be as safe for human consumption as food derived from conventional soybean cultivars

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List of abbreviations

AAD	aryloxyalkanoatedioxygenase
ADF	acid detergent fibre
ADI	acceptable daily intake
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BSA	Bovine serum albumin
bw	Body weight
CCI	Confidential Commercial Information
2,4-D	2,4-dichlorophenoxyacetic acid
DCP	2,4-dichlorophenol
DIG	digoxigenin
DNA	deoxyribonucleic acid
T-DNA	transferred DNA
dw	dry weight
EFSA	European Food Safety Authority
ELISA	enzyme linked immunosorbent assay
ESI-LC/MS	electrospray ionisation-liquid chromatography mass spectrometry
FARRP	Food Allergy Research and Resource Program
FSANZ	Food Standards Australia New Zealand
GM	genetically modified
ILSI	International Life Sciences Institute
kDa	kilo Dalton
LSM	least squares mean
MAR	matrix attachment region
MALDI-TOF MS	matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry
MCPA	2-methyl-4-chlorophenoxyacetic acid
ND	Not detected
NDF	neutral detergent fibre
NS	not significant
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame
PATt	phosphinothricin acetyltransferase gene
PCR	polymerase chain reaction
PHI	pre-harvest interval
L-PPT	L-phosphinothricin
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGF	simulated gastric fluid
U.S.	United States of America
USDA	United States Department of Agriculture

1. Introduction

A genetically modified (GM) soybean line, DAS-68416-4 (hereafter also referred to as soybean 68416), has been developed that is tolerant to herbicides of the aryloxyalkanoate family including the phenoxy auxins, and to herbicides containing glufosinate ammonium.

Tolerance to phenoxy auxins is achieved through expression of the enzyme aryloxyalkanoatedioxygenase-12 (AAD-12) encoded by the *aad-12* gene derived from *Delftia acidovorans,* a gram-negative soil bacterium. Tolerance to glufosinate ammonium is achieved through expression of the enzyme phosphinothricin acetyltransferase (PAT) encoded by the *pat* gene derived from another soil bacterium *Streptomyces viridochromogenes*. The *pat* gene has been widely used for genetic modification of a number of crop species.

It is anticipated that soybean 68416 will be grown in at least the United States (U.S.), Canada, Argentina and Brazil, subject to approval. There is currently no intention to grow the plant line in Australia or New Zealand.

2. History of use

2.1 Host organism

The host organism is a conventional soybean (*Glycine max* (L.) Merr.), belonging to the family Leguminosae. The commercial soybean cultivar 'Maverick' was used as the parental variety for the genetic modification described in this application, and thus is regarded as the near-isogenic line for the purposes of comparative assessment with soybean 68416. It was developed by the Missouri and Illinois Agricultural Experiment Stations and released in 1996 (Sleper *et al*., 1998).

Soybean is grown as a commercial food and feed crop in over 35 countries worldwide (OECD, 2000) and has a long history of safe use for both humans and livestock. The major producers of soybeans, accounting for 90% of world production, are the U.S., Argentina, Brazil and China. Australia, while a net importer of soybean, grows crops in latitudes extending from the tropics (16^{o} S) to temperate regions (37^{o} S), mainly in the eastern states and as a rotational crop (James and Rose, 2004). The seed is used mainly to produce meal for use in animal feed (Grey, 2006).

In many soybean producing countries, GM soybean (mainly with a herbicide tolerant trait) accounts for a significant proportion of the total soybean grown e.g. U.S. (91%); Argentina (99%); Brazil (63%); South Africa (87%); Uruguay (99%) (Brookes and Barfoot, 2009). Australia does not currently grow any commercial GM soybean lines[1].

Soybean food products are derived either from whole or cracked soybeans as follows:

- whole soybeans are used to produce soy sprouts, baked soybeans, roasted soybeans and traditional soy foods such as miso, tofu, soy milk and soy sauce.
- cracked soybeans have the hull (seed coat) removed and are then rolled into flakes which undergo solvent extraction to remove the oil. This crude oil is further refined to produce cooking oil, shortening and lecithins as well as being incorporated into a variety of edible and technical/industrial products. The flakes are dried and undergo further processing to form products such as meal (for use in e.g. livestock, pet and

[1] See information on approved commercial; releases of GM crops in Australia on the website of the Office of the Gene Technology Regulator - http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/ir-1

poultry food), protein concentrate and isolate (for use in both edible and technical/industrial products), and textured flour (for edible uses). The hulls are used in mill feed.

Unprocessed (raw) soybeans are not suitable for food use, and have only limited feed uses, as they contain toxicants and anti-nutritional factors, such as lectins and trypsin inhibitors (OECD, 2001a). Appropriate heat processing inactivates these compounds and may involve one of a number of techniques such as extrusion cooking, roasting, or micronisation (World Food Program, 2004).

Soybean oil constitutes approximately 71% of global consumption of edible fats and oils (The American Soybean Association, 2008), and is currently the second largest source of vegetable oil worldwide (USDA, 2009).

Another possible food product that can be derived from the soybean plant is bee pollen. This substance is produced by bees during foraging and is taken back to the hive to be fed to larvae and young adult bees (Krell, 1996). It comprises pollen grains that are pelleted by the bee in the corbiculae ('pollen baskets') located on the posterior pair of legs. Beekeepers can collect the pellets by placing a screen at the entrance to a hive; as the bees pass through the screen, the pellets are dislodged and fall into a collection tray. The pellets are frozen or dried for storage and are then packaged for sale as bee pollen, which is generally consumed as the raw product without any further processing. It is highly unlikely that this product would be imported to Australia or New Zealand as domestic supply would satisfy market requirements.

2.2 Donor organisms

2.2.1 Delftia acidovorans

The *aad-12* gene was sourced from the bacterial species *Delftia acidovorans* strain MC1, a strain isolated from herbicide-contaminated building rubble (Müller *et al*., 1999). This bacterium is a member of the Pseudomonads, a ubiquitous group of environmental gram negative bacteria. It was originally classified in the genus P*seudomonas*, then renamed in the genus *Comamonas* (Tamaoka *et al*., 1987) and finally renamed again to *Delftia* (Wen *et al*., 1999).

Delftia spp. are aggressive colonisers of the rhizosphere of various crop plants and have a broad spectrum of antagonistic activity against plant pathogens (see e.g. El-Banna, 2007; Han *et al*., 2005). They have also been found to possess a variety of biodegradation mechanisms that could be exploited in the development of systems for the removal of chemicals that may be released into the environment (Müller *et al*., 1999; Patel *et al*., 1998; Urata *et al*., 2004). On rare occasions, *Delftia* spp. have been implicated in human infections (see e.g. Chun *et al*., 2009 and references therein).

D. acidovorans is one of several microorganisms that has been proposed as a bioconverter for use in the food industry to transform ferulic acid into vanillin and related flavour metabolites (Labuda *et al*., 1992). However, commercial application has not been realised (see e.g. Yoon *et al*., 2005). The polyhydroxyalkanoates produced by *D. acidovorans* and other bacteria have been proposed for use as biomaterial for use in tissue engineering and other medical applications (Sudesh, 2004).

2.2.2 Streptomyces viridochromogenes

The source of the *pat* gene is the bacterial species *Streptomyces viridochromogenes*, strain Tü494 (Wohlleben *et al*., 1988). The *Streptomycetae* bacteria were first described in the early 1900's. These organisms are generally soil-borne, although they may also be isolated

from water. They are not typically pathogenic to animals including humans, and few species have been shown to be phytopathogenic (Bradbury, 1986; Kützner, 1981).

Although these organisms are not used in the food industry, the *pat* gene from *S. viridochromogenes*, has been used to confer glufosinate ammonium-tolerance in a range of food producing crops. The *bar* gene from the closely related *S. hygroscopicus* produces a protein that is structurally and functionally equivalent to the protein encoded by the *pat* gene (Wehrmann *et al*., 1996) and has similarly been used widely for genetic modification of crop species.

2.2.3 Other organisms

Genetic elements from several other organisms have been used in the genetic modification of soybean 68416 (refer to Table 1). These non-coding sequences are used to drive, enhance or terminate expression of the two novel genes. None of the sources of these genetic elements is associated with toxic or allergenic responses in humans. The genetic elements derived from plant pathogens are not pathogenic in themselves and do not cause pathogenic symptoms in soybean 68416.

3. Molecular characterisation

Molecular characterisation is necessary to provide an understanding of the genetic material introduced into the host genome and helps to frame the subsequent parts of the safety assessment. The molecular characterisation addresses three main aspects:

- the transformation method together with a detailed description of the DNA sequences introduced to the host genome
- a characterisation of the inserted DNA including any rearrangements that may have occurred as a consequence of the transformation
- the genetic stability of the inserted DNA and any accompanying expressed traits.

Studies submitted:

Poorbaugh, J.; Zhou, N.; Mo, J. (2009). Cloning and characterization of the DNA sequence for the insert and flanking border regions of AAD-12 soybean event DAS-68416-4. Study ID 091048. Dow AgroSciences LLC (unpublished).

Song, P.; Cruse, J.; Thomas, A. (2009). Molecular characterisation of AAD-12 soybean event DAS-68416-4. Study ID 081087. Dow AgroSciences LLC (unpublished).

Song, P. (2010). Bioinformatics analysis of soybean event DAS-68416-4 insert and its flanking border sequences. Study 101710. Dow AgroSciences LLC (unpublished).

Song, P. (2010). Bioinformatics evaluation of the putative reading frames across the junctions in soybean event DAS-68416-4 for potential allergenicity and toxicity. Study ID 101711. Dow AgroSciences LLC (unpublished).

Shan, G. (2010). Seed segregation of soybean event DAS-68416-4. Study ID 102059. Dow AgroSciences LLC (unpublished).

3.1 Method used in the genetic modification

Soybean cultivar 'Maverick' was transformed via *Agrobacterium*-mediated transformation (Deblaere *et al*., 1987) basically following the method of Zeng *et al*.(2004). The genes of interest were inserted into plasmid pDAB4468 (refer to Figure 1) between DNA sequences known as the Left and Right Borders (Border A and Border B in Figure 1). These border sequences were isolated from the tumour-inducing (Ti plasmid of *Agrobacterium tumefaciens* and normally delimit the DNA sequence (T-DNA) transferred into the plant (Zambryski, 1988).

Basically, the cotyledonary nodes of *in vitro* germinated seedlings were co-cultivated with the *Agrobacterium tumefaciens* strain EHA101 (Hood *et al*., 1986) containing the binary vector pDAB4468. Following shoot development, putative transformed shoots were selected on a

medium containing glufosinate ammonium as the selection agent. The selected shoots were then rooted, and the terminal leaflets of the resulting plantlets were leaf painted with glufosinate ammonium as a further screen. Selected plantlets (T_0) were screened for absence of vector backbone, copy number, tolerance to 2,4-D and then transferred to a glasshouse for further characterisation and selection.

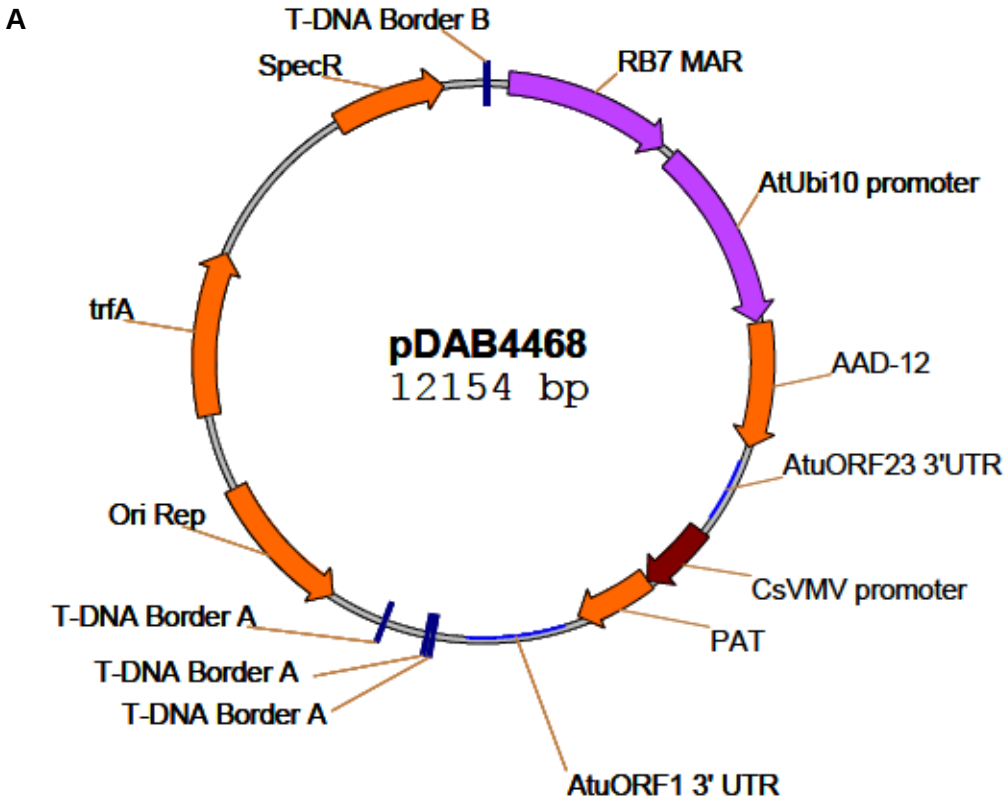

Figure 1: Vector map of plasmid pDAB4468

3.2 Function and regulation of introduced genes

A diagram of the T-DNA insert in plasmid pDAB4468 is given in Figure 2. Information on the genetic elements in the T-DNA insert is summarised in Table 1.

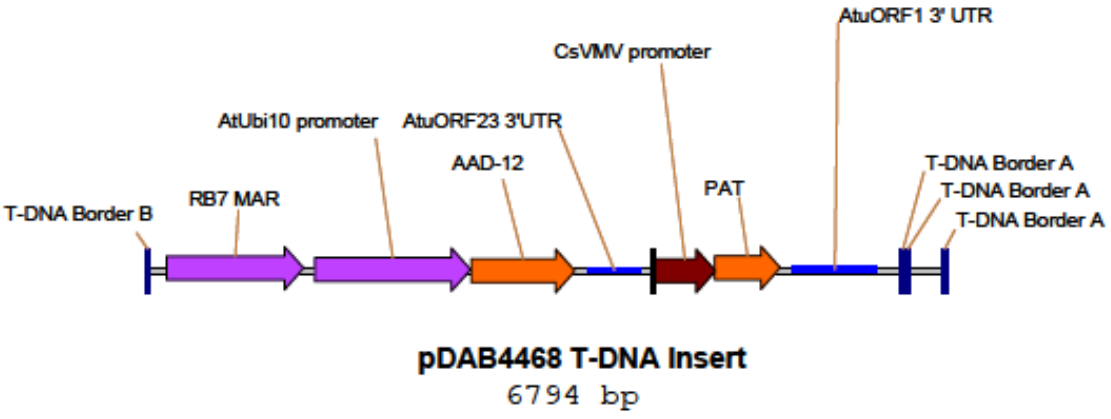

Figure 2: Representation of the genetic elements in the T-DNA insert of plasmid pDAB4468

Table 1: Details of the genetic elements contained in the T-DNA insert in plasmid pDAB4468

Genetic element	bp location on pDAB4468	Size (bp)	Source	Orient.	Description & Function	References
Border B	1 - 24	24	*Agrobacterium tumefaciens*		• Border repeat • Required for the transfer of the T-DNA into the plant cell	Zambryski (1988)
Intervening sequence	25 - 160	136	*Agrobacterium tumefaciens*		• Sequence from Ti plasmid pTi15955	Barker et al.(1983)
RB7-MAR	161 - 1326	1166	*Nicotiana tabacum*	Clockwise	• Matrix attachment region • Facilitates expression of the *aad-12* gene	Hall et al (1991)
Intervening sequence	1327 - 1421	95	Plasmid pENTR/D-TOPO		• The plasmid had been used as a cloning vector	Invitrogen Cat. # A10465
AtUbi10	1422 - 2743	1322	*Arabidopsis thaliana*	Clockwise	• Polyubiquiton promoter, 5'UTR and intron • Drives constitutive expression of the *aad-12* gene	Norris et al (1993)
Intervening sequence	2744 - 2751	8			• Cloning sequence	
aad-12	2752 - 3633	882	*Delftia acidovorans*	Clockwise	• Coding sequence of the aryloxyalkanoate dioxygenase gene (adapted to plant codon usage)	Wright et al (2007)
Intervening sequence	3634 - 3735	102			• Cloning sequence	
AtuORF23	3736 - 4192	457	*Agrobacterium tumefaciens*		• Transcriptional terminator and polyadenylation site of ORF23 from Ti plasmid pTi15955	Barker et al.(1983)
Intervening sequence	4193 - 4306	114			• The plasmid had been used as a cloning vector	Invitrogen Cat. # A10465
CsVMV	4307 - 4819	513	Cassava vein mosaic virus	Clockwise	• Promoter and 5'UTR • Drives constitutive expression of the *pat* gene	Verdaguer et al (1996)
pat	4820 - 5371	552	*S.treptomyces viridochromogenes*	Clockwise	• Coding sequence of the phosphinothricin acetyl transferase gene (adapted to plant codon usage)	Wohlleben et al (1988)
Intervening sequence	5372 - 5484	113	Plasmid pCR2.1		• Cloning sequence	Invitrogen Cat. # K2050-01 cloning kit
ATuORF1	5485 - 6188	704	*Agrobacterium tumefaciens*		• Transcriptional terminator and polyadenylation site of ORF1 from Ti plasmid pTi15955	Barker et al.(1983)
Intervening sequence	6189 - 6416	228	*Agrobacterium tumefaciens*		• Sequence from Ti plasmid C58	Zambryski et al (1982); Wood et al (2001)
Border A	6417 - 6440	24	*Agrobacterium tumefaciens*		• Border repeat • Required for the transfer of the T-DNA into the plant cell	Zambryski (1988)
Intervening sequence	6441 - 6459	24	*Agrobacterium tumefaciens*		• Sequence from Ti plasmid C58	Zambryski et al (1982); Wood et al (2001)
Border A	6460 - 6483	24	*Agrobacterium tumefaciens*		• Border repeat • Required for the transfer of the T-DNA into the plant cell	Zambryski (1988)
Intervening sequence	6484 - 6770	287	*Agrobacterium tumefaciens*		• Sequence from Ti plasmid pTi15955	Barker et al.(1983)
Border A	6771 - 6794	24	*Agrobacterium tumefaciens*		• Border repeat • Required for the transfer of the T-DNA into the plant cell	Zambryski (1988)

3.2.1 aad-12 *expression cassette*

The *aad-12* gene of *D. acidovorans*, also referred to as *sdpA* (Schleinizt *et al*., 2004; Wright *et al.*, 2007; Wright *et al*., 2010) has low homology (approximately 37% sequence identity) with the *tfdA* gene first isolated from *Ralstonia eutropha* (Streber *et al*., 1987) but found in phylogentically diverse bacteria (Baelum *et al*., 2008). The *tfdA* gene codes for an α-ketoglutarate-dependent dioxygenase which converts chlorinated phenoxyalkanoate herbicides such as 2,4-dichlorophenoxyacetc acid (2,4-D) into a harmless phenol and glyoxylate (refer to Section 4.2.1). Expression of the *aad-12* gene confers tolerance to both phenoxyalkanoate herbicides as well as to pyridyloxyacetic acids such as trichlopyr and fluroxypyr (Wright *et al.*, 2007). *Sdpa* genes have also been reported to occur in *Sphingomonas herbicidovorans* and *Rhodoferax* sp. as well as in *D. acidovorans* but show considerable sequence diversity (Lee and Matheson, 1984).

The DNA sequence of the *aad-12* gene has been optimised for expression in plants and is approximately 80% identical to the DNA sequence of the native *aad-12* gene. The *aad-12* coding region in plasmid pDAB4468 is 882 bp in length and is driven by the constitutive polyubiquiton promoter from *Arabidopsis thaliana*. A matrix attachment region (MAR) from the root-specific *Rb7* gene (Hall, Jr. *et al*., 1991; Verma *et al*., 2005) of *Nicotiana tabacum* (tobacco) was included at the 5' end of cassette to potentially increase the consistency of *aad-12* expression (Abranches *et al*., 2005). At the 3' untranslated region of the coding region is a transcript termination and polyadenylation region from *Agrobacterium tumefaciens.*

3.2.2 pat gene *expression cassette*

The *pat* gene from *Streptomyces viridochromogenes* and the *bar* gene from *S. hygroscopicus* confer tolerance to herbicides containing glufosinate ammonium (phosphinothricin) – see Section 4.2.2. Both genes code for polypeptides of 183 amino acids and share 87% homology at the nucleotide sequence level (Wehrmann *et al*., 1996). Both genes have been widely used for genetic modification of food species.

The *pat* gene coding region (Strauch *et al*., 1988; Wohlleben *et al*., 1988) used in plasmid pDAB4468 is 552 bp in length and has been modified for expression in plants. It is driven constitutively by a promoter region of the Cassava vein mosaic virus and terminated by a sequence of the 3'untranslated region of an open reading frame originating from plasmid pTi5955 of *Agrobacterium tumefaciens*.

3.3 Breeding of soybean plants containing transformation event DAS-68416-4

A breeding programme was undertaken for the purposes of:

- obtaining generations suitable for analysing the molecular and genetic characteristics of soybean 68416
- ensuring that the DAS-68416-4 event is incorporated into elite breeding line(s) for commercialisation of 2,4-D- and glufosinate ammonium-tolerant soybean.
-

The breeding pedigree for the various generations is given in Figure 3.

Following selection of T_0 plants (see Section 3.1) a series of selfing and seed bulking proceeded up to generation T_7. At the T_4 generation, plants were crossed with a number of elite lines to produce an F1 generation which was either selfed to produce F2 and F3 generations, or backcrossed to the appropriate parental elite cultivar.

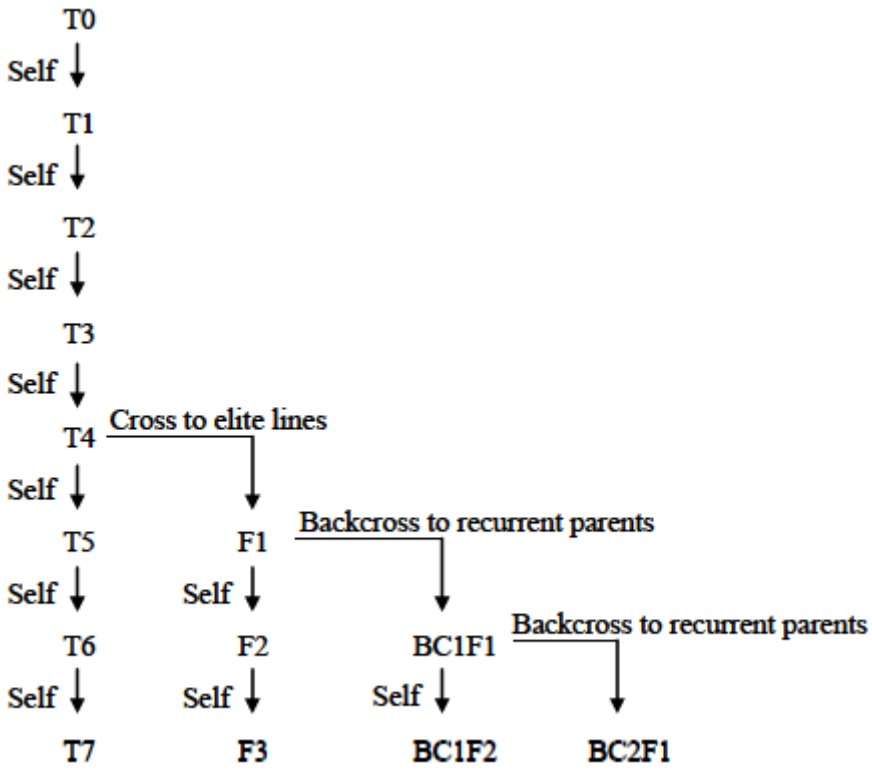

Figure 3: Breeding strategy for plants containing event DAS-68416-4

3.4 Characterisation of the genes in the plant

A range of analyses were undertaken in order to characterise the genetic modification in soybean 68416. These analyses focussed on the nature of the insertion of the introduced genetic elements and whether any unintended genetic re-arrangements may have occurred as a consequence of the transformation procedure.

3.4.1 Transgene copy number, insertion integrity and plasmid backbone analysis

Total genomic DNA from leaf tissue of individual soybean 68416 seedlings from each of the T_3 to T_5 generations and a negative control (non-GM cultivar 'Maverick') was used for Southern blot analyses. A positive control (DNA from 'Maverick' spiked with T-DNA from plasmid pDAB4468) was also included in the Southern blot analyses. Lateral Flow Strip testing was done of the soybean 68416 seedlings to confirm the presence of the AAD-12 protein. The DNA from soybean 68416 seedlings, the negative control and the positive control was digested with one, or a combination, of the following enzymes *Nco*I, *Bam*HI, *Pst*I, *Sph*I, *Nhe*I, *Xho*I (refer to Figure 4 for the location of the restriction sites). The resulting DNA fragments were separated and transferred to a membrane for sequential hybridisation with thirteen different digoxigenin (DIG)-labelled probes that represented various sections of the T-DNA and vector backbone (refer to Figure 4). The lengths of all hybridisation fragments were estimated by comparison with DIG-labelled DNA Molecular Weight Marker II (Roche Diagnostics).

The Southern blot analyses indicated that there is a single insert in event DAS-68416-4 and that the arrangement of the T-DNA genetic material is the same as that in the pDAB4468 plasmid (refer to Figure 4). Further, since no hybridisation fragments were observed with

either DAS-68416-4 or 'Maverick' DNA while the positive control samples showed the expected hybridisation fragments after hybridisations with the vector backbone probes, it is concluded that there are no vector backbone sequences present in soybean 68416.

Additional to these data, the analyses also demonstrated the stability of the insert across three generations.

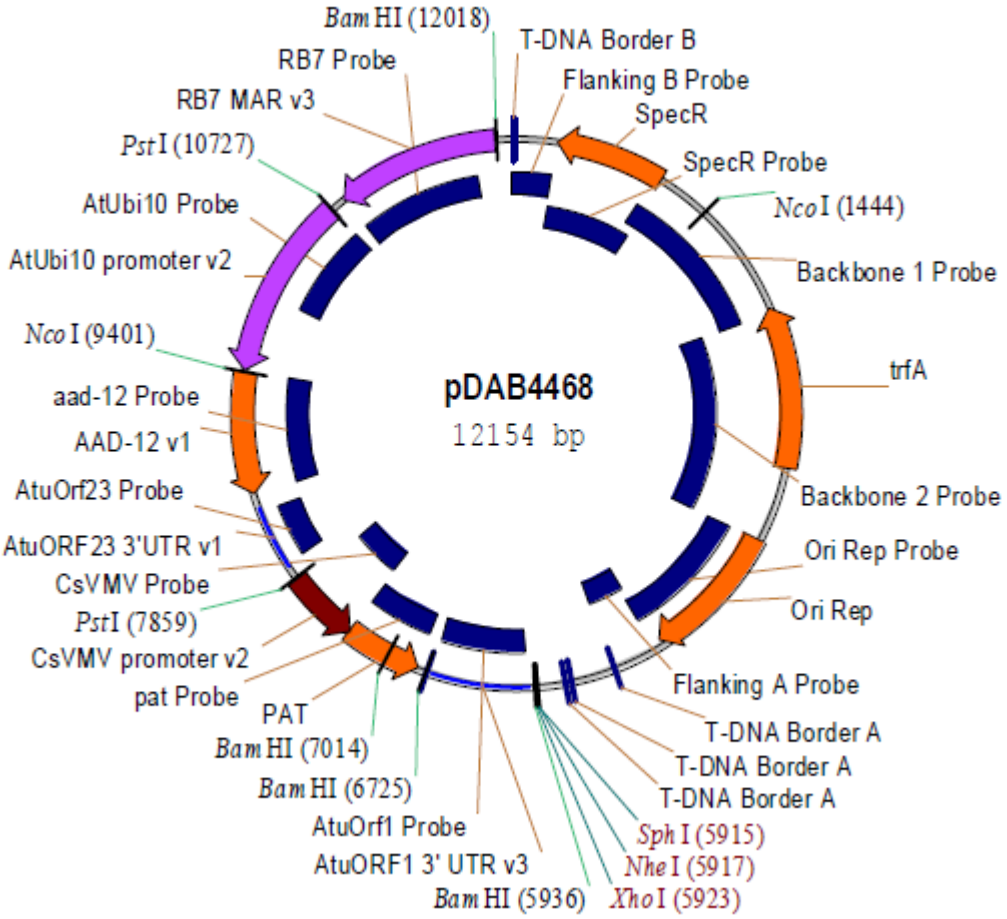

Figure 4: Plasmid map of pDAB4468 showing restriction enzyme sites and probe positions

3.4.2 Full DNA sequence of insert

Genomic DNA was obtained from leaf tissue of T_4 or T_5 generation soybean 68416 plants and a negative control (cultivar 'Maverick'). These samples were used to characterise the DNA sequence in the transgene insertion and its flanking border regions. A commercially available kit (Genome Walker™) was used to clone the 5' and 3' flanking border regions of event DAS-68416-4 and then obtain the sequences by primer walking. Standard polymerase chain reaction (PCR) was used to clone the pDAB4468 insert and confirm the flanking sequences as well as the insertion site in the host genome. DNA sequencing analysis was done using commercially available software (Sequencher®).

Border sequence analysis showed that the 5' end junction of T-DNA border B and the 3' end junction of T-DNA border A are both present in the transgenic locus which, itself comprises 6400 bp and is identical in sequence to that of the T-DNA of the pDAB4468 plasmid.

A comparison of the sequences of the 5' and 3' flanking sequences in the transgenic event with sequences of amplified fragments from the non-GM 'Maverick' showed that the 5' and 3' flanking sequences are identical to sequences in the pre-insertion locus except that there is a 9 bp insertion in the 3' integration junction. Two junctions were identified between the T-DNA insert and the host genomic DNA. A 55 bp sequence (known as a target site deletion) was found to have been deleted during the transformation process and is not present in the transgenic locus. A diagram of the insert is given in Figure 5.

As additional negative controls, genomic DNA was also isolated from leaves of plants derived from four other independent soybean transgenic events containing functional *aad-12* and *pat* cassettes. The results showed that the primers used for amplifying the 5' and 3' flanking regions as well; as those used for amplifying the entire transgene DNA region were specific only to event DAS-68416-4.

The sequence analysis confirms that the flanking sequences in event DAS-68416-4 are of *Glycine max* origin and that all inserted sequences originate from the T-DNA of the transforming pDAB4468 plasmid.

3.4.3 Novel open reading frame analysis

Since the transgenic insert has the identical sequence to the T-DNA of the pDAB4468 plasmid (see Section 3.4.2), it follows that no unexpected open reading frames have been created within the insert.

Using sequence data from the 5' (2730 bp) and 3' (1082 bp) flanking regions an *in silico* analysis was performed to determine whether any novel open reading frames had been created in either of the two junctions between the T-DNA insert and the host genomic DNA. The analysis comprised a search of six-frame translations between stop codons across the two junction regions, regardless of the presence of a start codon or the number of amino acid residues coded by the nucleotide sequence.

Twelve open reading frames were identified (see Table 2). Four of these coded for only four amino acids and were therefore excluded from further consideration. Further analysis of the remaining eight open reading frames is detailed in Section 4.1.

Table 2: Location and characterisation of novel open reading frames

Location of flanking region	Nucleotide location of reading frame	Number of amino acids coded	Deduced amino acid sequence
5' border	2719 -2766	16	IIQAPVSIITPKVRPE
	2705 – 2818	38	KFIFKSFKHQSASSHQKLGPNSLKLESSQLRSTGQIRS
	2700 – 2756	19	IKNLFLNHSSTSQHHHTKS
	2817 – 2704	38	ERIQPVDLNCELSNFKLFGPNFWCDDADWCLNDLKINF
	2798 – 2727	24	PSLYSASRYGAVDCNSMVRAGRTREG
	2734 - 2723	4	LVLE
3' border	9082 - 9132	17	KRPQCVIKLSKRQYFNS
	9068 - 9154	29	LQYIKNVRNVLLSCLSVNILILNNQYFNS
	9114 - 9125	4	ASIF
	9138 - 9073	22	LLRIKILTLRQLNNTLRTFLMY
	9131 - 9120	4	ELKY
	9124 - 9113	4	NIDA

3.4.4 Analysis of possible disruption to endogenous genes at the insertion locus

An analysis was done in order to ascertain whether endogenous soybean genes and/or open reading frames may have been disrupted by the insertion of the transgenic sequences.

The nucleotide sequences of the flanking regions and parental locus were searched for sequence similarities against the GenBank nucleotide database using the BLASTN[2] and BLASTX[3] algorithms (for a general discussion of this type of analysis refer to Section 4.5.2).

No genes, interrupted as a result of the transformation procedure, were identified. The searches indicated that the insert of DAS-68416-4 has probably integrated into a locus close to the 3' end (i.e. downstream) of a putative peroxidase gene in the soybean genome (refer to Figure 5). The identification as a peroxidase gene is putative because the similarity search indicated a 78% identity with a *Medicago truncatula* (lucerne) peroxidase. The function of the gene is unlikely to be affected by the insertion as there is a sequence of 550 bp between the stop codon of the putative gene and the site of insertion of the T-DNA.

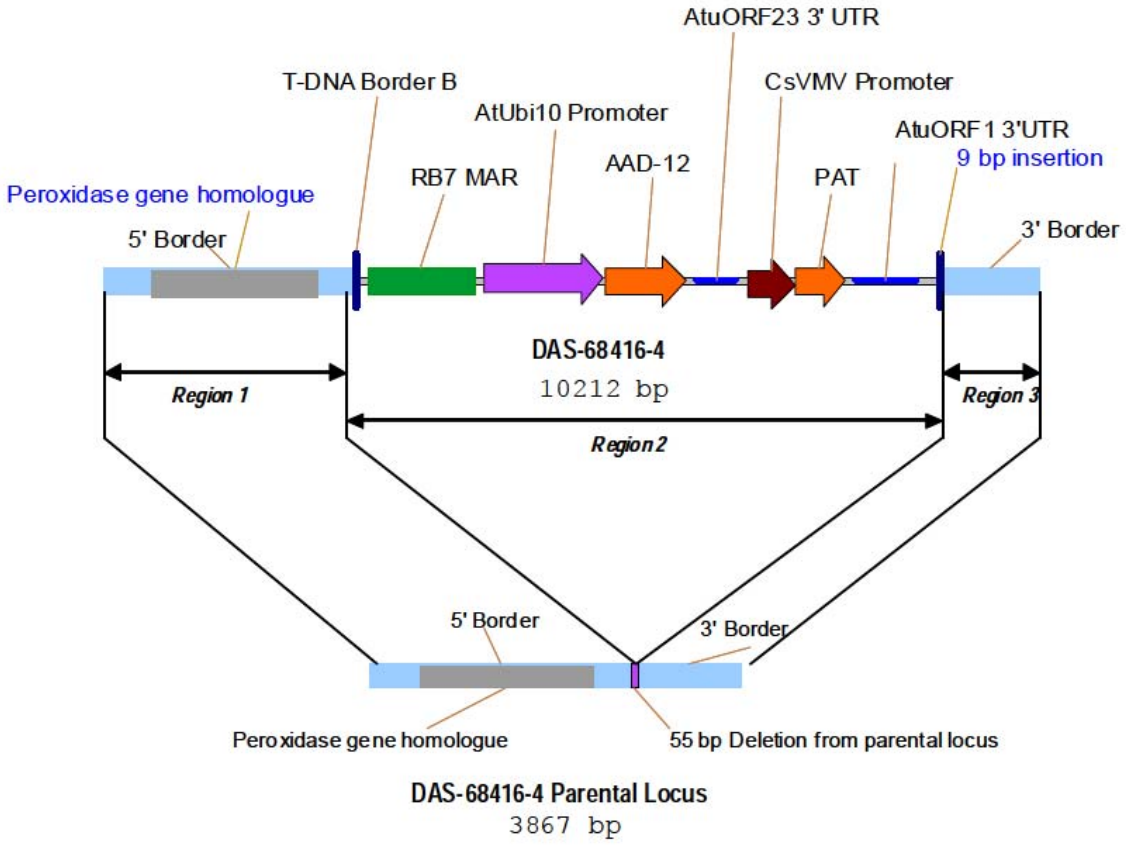

Figure 5: Diagram of the insert, flanking borders and parental locus in soybean event DAS-68416-4

[2] BLASTN is used to compare a nucleotide sequence with a nucleotide database.
[3] BLASTX takes a nucleotide sequence, translates it, and then queries it against a protein sequence (Gish and States, 1993)

3.5 The inheritance pattern of soybean 68416

Studies submitted:

Song, P.; Cruse, J.; Poorbaugh, J.; Thomas, A. (2009). Molecular characterisation of AAD-12 soybean event DAS-68416-4 within a single segregating generation. Study ID 091071. Dow AgroSciences LLC (unpublished).

Shan, G. (2010). Seed segregation of soybean event DAS-68416-4. Study ID 102059. Dow AgroSciences LLC (unpublished).

Both phenotypic and genetic approaches were used to assess greenhouse-grown plants of a segregating F_2 generation of soybean 68416 generated by crossing T_4 plants with an elite non-GM line taken from the Applicant's soybean germplasm development programme. The F_1 plants were self-pollinated to obtain the F_2 generation (refer to Figure 3).

3.5.1 Phenotypic analysis

Prior to sampling for molecular analysis (see below), leaves of 147 F_2 soybean 68416 plants were analysed by Lateral Flow Strip testing for expression of the AAD-12 protein. A Chi squared (X^2) analysis of the results was done for the 3:1 segregation ratio of AAD-12 positive versus negative plants. A total of 102 plants were positive for AAD-12 while 45 were negative. The X^2 value of 2.469 (P>0.05) indicated that the segregation ratio was consistent with the Mendelian inheritance pattern of a single dominant trait.

An additional study was undertaken using plants grown from F_2 seed in a field trial in Puerto Rico. Leaf punches (8/plant) were taken from 6,774 plants that had reached the V4-6 stage[4]. The samples were assayed for the presence or absence of the *aad-12* gene using a gene-specific fluorescence-based detection method specific for *aad-12*. A total of 5056 plants were positive, while 1,718 were negative. The X^2 value of 0.4726 (P>0.05) indicated that the segregation ratio was consistent with a Mendelian inheritance pattern.

3.5.2 Molecular analysis

The Mendelian inheritance of event DAS-68416-4 was evaluated in 143 of the 147 F_2 plants sampled for phenotypic analysis. The non-GM cultivar 'Maverick' (10 plants) was used as a negative control and cultivar 'Maverick' spiked with DNA from plasmid pDAB4468 was used as a positive control.

Genomic DNA, isolated from leaf tissue of soybean 68416 and the negative control, was digested with *Nco1, Pst1* and *Pst1/Xho1* restriction enzymes (refer to Figure 4 for sites in the transgenic locus). The positive control mixture was similarly digested. The resulting DNA fragments were separated and transferred to a membrane for hybridisation with DIG-labelled probes to *aad-12* and *pat* in pDAB4468.

All samples from AAD-12 positive plants and the spiked positive controls showed the expected bands when digested and then hybridized with either the *aad-12* or *pat* probes. None of the DNA samples from AAD-12 negative plants or 'Maverick' negative control showed any hybridisation bands. The Southern blot analysis thus confirmed the Mendelian inheritance pattern of event DAS68416-4.

[4] For information on soybean growth stages see e.g. NDSU (2004).

3.6 Stability of the genetic changes

The concept of stability encompasses both the genetic and phenotypic stability of the introduced trait over a number of generations. Genetic stability refers to maintenance of the modification over successive generations, as produced in the initial transformation event. It is best assessed by molecular techniques, such as Southern analysis or PCR, using probes and primers that cover the entire insert and flanking regions. Phenotypic stability refers to the expressed trait remaining unchanged over successive generations. It is often quantified by a trait inheritance analysis to determine Mendelian heritability via assay techniques (chemical, molecular, visual).

The stability of event DAS-68416-4 in the soybean genome was established by the experimental work described in Section 3.4.1 in which the hybridisation pattern of the event was shown to be identical across the T_3, T_4 and T_5 generations.

3.7 Antibiotic resistance marker genes

No antibiotic marker genes are present in soybean 68416. Plasmid backbone analysis shows that no plasmid backbone has been integrated into the soybean genome during transformation, i.e. the *Spec* gene, which was used as a bacterial selectable marker gene, is not present in soybean 68416.

3.8 Conclusion

Soybean line DAS-68416-4 contains two novel gene cassettes. One contains an *aad-12* gene that encodes a protein conferring tolerance to phenoxy herbicides such as 2,4-D and the other contains a *pat* gene that encodes a protein conferring tolerance to herbicides containing glufosinate ammonium (phosphinothricin). There are no antibiotic resistance markers present in soybean line 68416.

Comprehensive molecular analyses of soybean line 68416 indicate there is a single insertion site containing one complete copy of each of the two cassettes comprising the T-DNA from plasmid pDAB4468. The introduced genetic elements are stably inherited from one generation to the next. Plasmid backbone analysis shows that no plasmid backbone has been incorporated into the transgenic locus. A number of small open reading frames were created at the junctions regions with the inserted DNA. No known endogenous genes have been interrupted by insertion of the new genetic material.

4. Characterisation of novel proteins

In considering the safety of novel proteins it is important to consider that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects, although a small number have the potential to impair health, e.g., because they are allergens or anti-nutrients (Delaney *et al*., 2008). As proteins perform a wide variety of functions, different possible effects have to be considered during the safety assessment including potential toxic, anti-nutritional and allergenic effects. To effectively identify any potential hazards requires knowledge of the characteristics, concentration and localisation of all novel proteins expressed in the organism as well as a detailed understanding of their biochemical function and phenotypic effects. It is also important to determine if the novel protein is expressed as expected, including whether any post-translational modifications have occurred.

Two types of novel proteins were considered:

- those that may be potentially generated as a result of the creation of novel open reading frames during the introduction of the T-DNA of plasmid pDAB4468 (see Section 3.4.4)
- those that were expected to be produced as a result of the expression of the introduced genes. Soybean 68416 expresses two novel proteins, an AAD-12 protein and a PAT protein. A number of different analyses were done to determine the identity, physiochemical properties, and *in planta* expression of both proteins. Potential toxicity and allergenicity was considered only for the AAD-12 protein. *Streptomyces hygroscopicus* and *S. viridochromogenes* are common soil bacteria, and therefore humans have a long history of exposure to the PAT protein through the consumption of roots and vegetables. In addition, since 1995, humans have been directly exposed to the PAT protein through the consumption of foods derived from glufosinate ammonium tolerant canola, soybean, cotton and corn, without any evidence of toxicity (Delaney et al., 2008; Hérouet et al., 2005). No safety concerns are raised from its inclusion in human food and animal feed.

  Because the expression of proteins *in planta* is usually too low to allow purification of sufficient quantities for safety assessment studies, a bacterial expression system was used to generate large quantities of both proteins. The equivalence of the bacterial-produced proteins to the plant-produced proteins was determined as part of the protein characterisation.

4.1 Potential allergenicity/toxicity of sequences encoded by novel open reading frames

Study submitted:

Song, P. (2010). Bioinformatics evaluation of the putative reading frames across the junctions in soybean event DAS-68416-4 for potential allergenicity and toxicity. Study ID 101711. Dow AgroSciences LLC (unpublished).

Eight novel open reading frames were identified in the junction regions of the insert in event DAS-68416-4 (refer to Section 3.4.3). The amino acid sequences corresponding to these eight open reading frames were analysed for potential allergenicity and toxicity using an *in silico* approach. These analyses are entirely theoretical; since there is no reason to expect that any of the identified ORFs would, in fact, be expressed.

4.1.1 Allergenicity assessment

The amino acid sequence of each identified open reading frame was compared with a peer-reviewed database containing 1,471 known and putative allergens, as well as celiac-induction sequences, residing in the FARRP(Food Allergy Research and Resource Program) dataset (Version 10) within AllergenOnline (University of Nebraska; http:www.allergenonline.org/).

Only three of the eight novel open reading frames were longer than 29 amino acids. These three underwent a similarity search using the FASTA algorithm and the BLOSUM50 scoring matrix (for general information of this type of analysis see Section 4.5.2). The criterion indicating potential allergenicity was a 35% identity, over at least 80 consecutive amino acids, with a known allergen. No similarities with known allergens were found. The five open reading frames comprising less than 29 amino acids were screened for any matches of eight contiguous amino acids to known allergens. No matches of eight or more contiguous amino acids were found in any of the sequences.

4.1.2 Toxicity assessment

The sequences corresponding to the eight identified open reading frames were compared with protein sequences present in a number of large public reference databases including Uniprot_Swissprot, PIR (Protein Information Resource), PRF (Protein Research Foundation) and PDB (Protein Data Bank). The similarity searches used the BLASTP (Basic Local Alignment Search Tool Protein) algorithm (refer to Section 4.5.2 for an explanation). No significant similarities of the eight open reading frames to any sequences (including those of known toxins) in the databases were found.

4.1.3 Conclusion

It is concluded that, in the unlikely event that any of the identified open reading frames were expressed, there is no significant similarity between the encoded sequences and any known protein toxins or allergens..

4.2 Function and phenotypic effects of the AAD-12 and PAT proteins

4.2.1 AAD-12 protein

The native AAD-12 (GenBank Accession AAP88277) is an α-ketoglutarate dependent dioxygenase that catalyses the breakdown of pyridyloxyacetate auxins and achiral[5] phenoxy auxins to an intermediate that, itself, is then spontaneously broken down to a herbicidally inactive phenol and glyoxylate (refer to Figure 6). In the case of soybean 68416, the active herbicide that would be applied is 2,4-D and the inactive phenol produced would be 2,4-dichlorophenol (DCP).

Native AAD-12 is also known as (S)-phenoxypropionate/α-ketoglutarate-dioxygenase (SdpA) and is one of two enantiomers[6] that occurs in *Delftia acidovorans*. The term AAD-12 was first used by Wright *et al.* (2007).

The (S) and (R) enzymes show less than 35% amino acid identity with the TfdA protein but share the consensus sequence H*X*(D/E)X138-207H*X*10R/K with TdfA and other (referred to as group II) α-ketoglutarate dependent dioxygenases (Schleinizt *et al*., 2004). While RdpA is highly specific to the R enantiomer of 2-phenoxypropionates and shows weak activity towards phenoxyacetates, SdpA is enantioselective to the S enantiomers but can also convert certain phenoxyacetates such as 2,4-D and 4-chloro-2-methylphenoxyacetate (MCPA) (Paulin *et al*., 2010).

AAD-12 is closely related to AAD-1, a protein conferring tolerance to 2,4-D and quizalofop-P-ethyl, that is considered in Application A1042 (FSANZ, 2010). AAD-12 has significantly greater *in vitro* activity on 2,4-D than AAD-1 (Wright *et al*., 2010).

[5] a term used to describe a molecule which, in a given configuration, is superimposible on its mirror image

[6] either one of a pair of compounds (crystals or molecules) that are mirror images on each other but are not identical

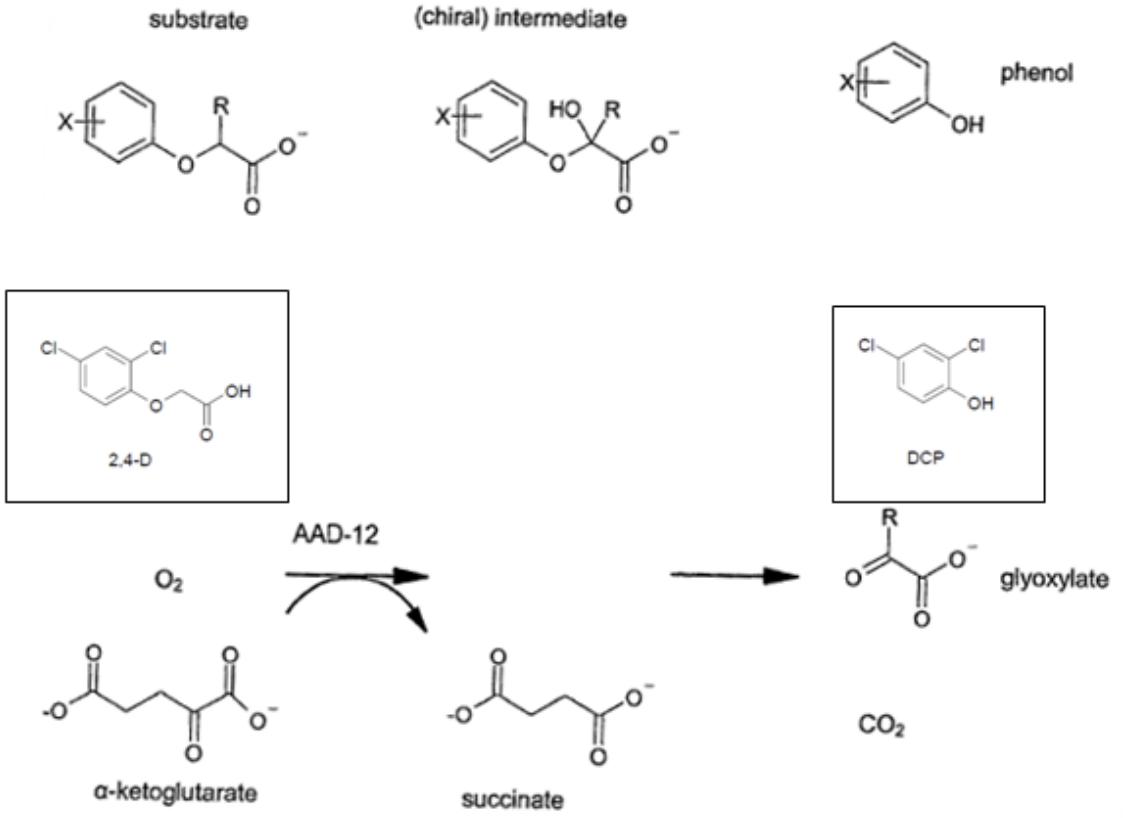

Figure 6: General representation of the conversion of pyridyloxyacetate and phenoxyacetate herbicides to an inactive phenol in the presence of AAD-12 (Diagram modified from Wright et al. *(2007)). The structures of 2,4-D and its inactive phenol, DCP, are given in the boxes.*

4.2.2 PAT protein

Members of the genus *Streptomyces* produce antibiotics, one of which is bialaphos. These bacteria have evolved a mechanism to avoid the toxicity of their own products. Thus the *pat* gene from *Streptomyces viridochromogenes* and the *bar* gene from *S. hygroscopicus* both confer tolerance to bialaphos (Wehrmann *et al*., 1996). Bialaphos, now also used as a non-selective herbicide, is a tripeptide composed of two L-alanine residues and an analogue of glutamate known as L-phosphinothricin (L-PPT) (see Thompson *et al*., 1987) more recently known also as glufosinate ammonium. Free L-PPT released from bialaphos by peptidases (or applied directly as a synthetic herbicide) inhibits glutamine synthetase which in turn leads to rapid accumulation of ammonia and subsequent cell death.

The homologous polypeptide produced by the *bar* and *pat* genes (see Section3.2.2) is known as phosphinothricin acetyltransferase (PAT); it is an acetyl transferase with enzyme specificity for both L-PPT and demethylphosphinothricin (DMPT) in the acetylation reaction (Thompson et al., 1987). In the presence of acetyl-CoA, PAT catalyses the acetylation of the free amino group of L-PPT to N-acetyl-L-PPT, a herbicidally-inactive compound. The kinetics and substrate specificity of the PAT enzyme are well characterised; it has a high specificity for L-PPT and has been shown to have a very low affinity to related compounds and amino acids; even excess glutamate is unable to block the L-PPT-acetyltransferase reaction (Thompson *et al*., 1987).

The acetyltransferase activity is heat- and pH-dependent (Wehrmann *et al*., 1996). PAT is active between temperatures of 25-55^{o}C, with maximum activity occurring between 40 and 45°C. Complete thermoinactivation occurs after 10 minutes at 60^{o}C and above. The optimum pH for PAT activity is 8.5, but it is active over a broad pH range of 6 to 11. The protein is expressed in a wide range of GM crop plants and is regarded as safe (see e.g. Hérouet *et al*., 2005).

4.3 Protein expression analysis

Studies submitted:

Lira, J.M. (2010). Strain review for *Delftia acidovorans*, the source organism for *aad-12*. Study ID JML 100001, Dow AgroSciences LLC (unpublished).

Schafer, B.W. (2008). Effect of heat treatment on a recombinant aryloxyalkanoate dioxygenase-12 (AAD-12). Study ID 080140, Dow AgroSciences LLC (unpublished).

Schafer, B.W.;Embrey, S.K. (2009). Characterization of aryloxyalkanoate dioxygenase-12 (AAD-12) protein derived from transgenic soybean event DAS-68416-4. Study ID 081113, Dow AgroSciences LLC (unpublished).

Schafer, B.W.; Embrey, S.K. (2009). Characterization of phosphinothricin acetyltransferase (PAT) protein derived from transgenic soybean event DAS-68416-4. Study ID 081132, Dow AgroSciences LLC (unpublished).

Smith-Drake, J.K.;Thomas, A.S.;Sosa, M.J. (2009). Field expression of a transformed soybean cultivar (DAS-68416-4) containing arylyoxyalkanoate dioxygenase-12 (AAD-12) and phosphinothricin acetyltransferase (PAT). Study ID 080003, Dow AgroSciences LLC (unpublished).

4.3.1 Novel protein expression in plant tissues

The AAD-12 and PAT proteins are expected to be expressed in all plant tissues since the *aad-12* and *pat* genes are driven by constitutive promoters (refer to Table 1). Six sites in North America (in Iowa, Illinois, Indiana, Nebraska and Ontario) representing regions of diverse agronomic practices and environmental conditions for soybean were planted with soybean 68416 (generation T_4) and 'Maverick'. Four spraying treatments were applied to soybean 68416 namely, unsprayed, sprayed with 2,4-D, sprayed with glufosinate, and sprayed with 2,4-D + glufosinate. Samples for analysis of expression of AAD-12 and PAT were taken from a number of plant parts at specific growth stages (refer to Table 3).

The AAD-12 and PAT protein levels were determined by enzyme linked immunosorbent assay (ELISA) using commercial ELISA kits specific for each protein. A commercially available software programme (SoftMax Pro™) was used to calculate the concentrations of immunoreactive AAD-12 and PAT proteins from optical density values.

No AAD-12 or PAT proteins were detected in samples taken from 'Maverick' plants. For soybean 68416 plants, AAD-12 and PAT proteins were detected in all parts (Table 3). AAD-12 was lowest in the roots and seed (approximately 16 µg/g dry weight) and highest in leaves (approximately 55 µg/g dry weight). PAT protein concentrations were much lower than those for AAD-12 but similarly, the leaves contained the highest levels (approximately 11 µg/g dry weight) and the roots contained the lowest levels (approximately 2 µg/g dry weight).

Table 3: Average concentration of AAD-12 and PAT proteins in various plant parts from soybean 68416

Sample source	Growth Stage	Treatment	Average protein content in µg/g dry weight ±SD	
			AAD-12	PAT
Leaf (8 trifoliate leaves)	V5	unsprayed	51.42 ± 25.22	9.17 ± 2.99
		+ glufosinate	50.63 ± 23.69	9.83 ± 2.66
		+ 2,4-D	51.68 ±25.41	9.01 ± 3.03
		+ glufosinate + 2,4-D	66.08 ± 37.82	10.05 ± 3.76
Leaf (8 trifoliate leaves)	V10	unsprayed	53.95 ± 20.85	10.94 ±1.31
		+ glufosinate	56.06 ± 21.95	11.51 ± 1.69
		+ 2,4-D	55.24 ± 20.62	11.76 ± 2.02
		+ glufosinate + 2,4-D	57.07 ± 22.97	11.58 ± 1.45
Root (3 plants)	R3	unsprayed	17.10 ±5.68	1.73 ± 0.51
		+ glufosinate	15.48 ± 4.58	1.92 ± 0.45
		+ 2,4-D	16.01 ± 6.64	1.73 ± 0.68
		+ glufosinate + 2,4-D	16.66 ± 6.81	1.93 ± 0.55
Forage (3 plants)	R3	unsprayed	41.11 ± 25.72	3.63 ± 2.88
		+ glufosinate	39.35 ± 24.47	4.81 ± 3.75
		+ 2,4-D	40.56 ± 25.58	5.28 ± 4.20
		+ glufosinate + 2,4-D	39.65 ± 22.41	4.73 ± 3.63
Seed (500 g)	R8 - maturity	unsprayed	16.47 ± 3.55	2.73 ± 0.34
		+ glufosinate	16.94 ± 3.15	2.74 ± 0.28
		+ 2,4-D	16.47 ± 3.78	2.79 ± 0.26
		+ glufosinate + 2,4-D	16.21 ± 3.62	2.82 ± 0.23

4.4 Protein characterisation and equivalence

The AAD-12 protein produced by soybean 68416 has an amino acid sequence that is 99% homologous with the native AAD-12, differing only in that an alanine has been added at position 2. The Applicant claims that this addition serves the dual purpose of facilitating cloning operations and optimising translation initiation. The AAD-12 protein comprises 293 amino acids and has an approximate molecular weight of 32 kDa.

The PAT protein produced by soybean 68416 is identical to the native protein (Uniprot Accession No. Q57146). It comprises 183 amino acids and has an approximate molecular weight of 21 kDa.

The AAD-12 and PAT proteins are not produced in sufficient quantity in soybean 68416 to isolate enough for the toxicological and biochemical studies required for a safety assessment. A standard procedure to overcome this type of problem is to produce the protein in a bacterial system and, if this protein shows equivalence to the *in planta*-produced protein, to then use the bacterially-produced protein for the toxicological and biochemical studies. The AAD-12 and PAT proteins were therefore expressed in recombinant *Pseudomonas fluorescens* and characterisation tests were done to confirm the identity and equivalence of these bacterially-produced proteins to those produced in soybean 68416.

4.4.1 Microbially expressed proteins

Microbial AAD-12 protein and PAT protein were obtained from *P. fluorescens* bacterial expression systems using standard methods. In order to characterise the bacterially-produced proteins, two parameters were measured for both proteins:

- molecular weight (sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE))
- immunoreactivity (western blotting)

The molecular weights of the proteins were calculated from visualisation of Coomassie-stained SDS-PAGE gels. A single band at approximately 20 kDa was obtained for the microbially-derived PAT protein and a major band at 30 - 36 kDa (over separate analyses) was obtained for the AAD-12 protein. This is considered to be good agreement, within the limitation of analysis, with the actual molecular weights of 32 kDa for the AAD-12 protein and 21 kDa for the PAT protein.

Immunoreactivity was tested using

- a rabbit polyclonal antibody raised against the appropriate (i.e. AAD-12 or PAT) protein followed by incubation with an enzyme (horseradish peroxidase) linked goat-anti-rabbit secondary antibody.
- A mouse monoclonal antibody raised against the appropriate (i.e. AAD-12 or PAT) protein followed by incubation with an enzyme (horseradish peroxidase) linked goat-anti-mouse secondary antibody.

For both proteins a single immunoreactive band at the expected molecular weight was observed using the polyclonal antibody. For the monoclonal antibody, a single band (at the expected molecular weight) was also observed for the microbially-derived AAD-12 protein whereas two bands were observed for the PAT protein; one at the expected molecular weight and a faint band at 40 kDa. The 40 kDa band most likely represents a dimer.

Taken together, these two analyses confirmed the identity of the proteins obtained from the bacterial expression system as AAD-12 and PAT. The characteristics of the PAT protein are already well established. In order to further characterise the AAD-12 protein, three further analyses were undertaken:

- peptide analysis (electrospray ionisation-liquid chromatography mass spectrometry (ESI-LC/MS))
- glycosylation status (glycoprotein detection).
- protein activity (enzymatic assay)

4.4.1.1 ESI-LC/MS peptide mass fingerprinting and N-terminal and C-terminal sequencing of microbially-derived AAD-12

Lyophilized AAD-12 protein isolated from *P. fluorescens* was resuspended in buffer before ESI-LC/MS analysis of the intact protein to obtain intact mass spectral characteristics. Micromass-supplied electrospray maximum entropy algorithm (MAXENT 1) was used to transform the spectra to a mass axis and to allow resolution enhancement. One major peak corresponding to a mass of 31.59 kDa was obtained; this was consistent with the molecular weight estimated from SDS-PAGE gels. Additionally there were two minor satellite peaks, the larger of which contained oxidised Met residue(s).

ESI-LC/MS analysis was also used to generate peptide coverage maps, N-terminal and C-terminal sequences and to determine post-translational processing sites from trypsin, chymotrypsin, Arg-C, Asp-N and Glu-C digests of AAD-12. The combined sequence coverage was essentially 100% and most of the peptides observed showed patterns consistent with their expected amino acid sequences.

N- and C-terminal peptides were then further analysed by tandem mass spectrometry of tryptic and chymotryptic digests to confirm their amino acid sequences. The fragments observed for the N-terminal peptides for both digests were consistent with the expected sequence of amino acids from positions 2 – 62 inclusive (the N-terminal Met at position 1 is expected to be removed post-translationally). The fragments observed for the C-terminal peptides for both digests were consistent with the expected sequence of amino acids from positions 281 - 293 inclusive.

4.4.1.2 Glycosylation analysis

N-glycosylated proteins are glycosylated on an asparagine residue and commonly contain an asparagine-X-serine/threonine sequence (N-X-S/T), where X is any amino acid except proline (Orlando and Yang, 1998). Although rare, the sequence asparagine-X-Cysteine (N-X-C) can also be an N-glycosylation site (Miletich and Broze Jr., 1990). The occurrence of these motifs does not, however, indicate that the protein will necessarily be glycosylated and since *P. fluorescens*, like most prokaryotes, lacks the capacity for protein glycosylation (Abu-Qarn *et al*., 2008; Wacker *et al*., 2002) it would be unusual to find that N-glycosylation had occurred in the bacterially-derived proteins.

To assess whether post-translational glycosylation had occurred, glycosylation analysis of purified AAD-12 protein sample from *P. fluorescens* was undertaken using a commercially available glycoprotein detection kit. No glycoprotein staining was detected.

4.4.1.3 Protein activity

The activity of the bacterially-derived AAD-12 protein was confirmed by a spectrophotochemical method (Fukumori and Hausinger, 1993) based on the ability of AAD-12 to convert 2,4-D to DCP (refer to Figure 6) which can then react with 4-aminoantipyrine to form a stable dye.

4.4.2 Protein equivalence

Having established the authenticity and characteristics of the bacterially-derived AAD-12 and PAT proteins, it was then necessary to confirm whether they were equivalent to the plant-derived proteins. For the AAD-12 analyses, both SDS-PAGE and western blotting were used to compare the bacterially-derived proteins with immunopurified plant AAD-12 protein and with crude aqueous extract from lyophilised tissue, both obtained from leaves of plants grown from T_5 seed (refer to Figure 3). For the PAT protein analyses, the bacterially-derived protein was compared only with crude extract. Extract from leaves of 'Maverick' was used as a negative control. The presence of AAD-12 and PAT proteins in the soybean 68416 aqueous extracts, and absence in the 'Maverick' extracts, was confirmed by lateral flow test strip assay.

Immunoreactivity was detected on the Western blots using both:

- an anti-AAD-12 or anti-PAT polyclonal rabbit primary antibody with a goat anti-rabbit horseradish peroxidise-linked secondary antibody
- an anti-AAD-12 or anti-PAT monoclonal mouse primary antibody with a goat anti-mouse horseradish peroxidise-linked secondary antibody.

The SDS-PAGE analysis of crude plant extracts, as expected, showed a wide range of bands for both soybean 68416 and 'Maverick' and was not informative. The SDS-PAGE gel containing the purified plant and bacterial AAD-12 proteins showed a major band at approximately 32 kDa.

Western blots of both polyclonal and monoclonal antibodies to PAT showed an immunoreactive band at approximately 20 kDa in both crude extract of soybean 68416 and bacterially-derived PAT. Similarly, the blots of both polyclonal and monoclonal antibodies to AAD-12 showed an immunoreactive band at approximately 35 kDa in both crude extract and bacterially-derived AAD-12.

The AAD-12 protein from soybean 68416 was run on an SDS-PAGE system and the AAD-12 protein band was excised and subjected to in-gel digestion by trypsin and endoproteinase Asp-N followed by matrix-assisted laser desorption/ionisation time-of-flight mass

spectrometry (MALDI-TOF MS) and ESI-LC/MS analyses. The masses of the detected peptides were compared to those deduced from potential trypsin or Asp-N cleavage sites. Sequence coverage was approximately 73% of the theoretical peptide sequences and included both the N- and C-termini. The analysis confirmed that the sequence of the soybean 68416-derived protein matched that of the microbially-derived protein. ESI-LC/MS analysis showed that, as for the microbially-derived protein, the N-terminal methionine of the soybean 68416-derived protein had been removed. Unlike the microbially-derived protein, the alanine at position 2 of the soybean 68416-derived protein had been acetylated. Amino-terminal acetylation is common in eukaryotes (Polevoda and Sherman, 2002). The C-terminal sequences of the soybean 68416- and microbially-derived AAD-12 were indistinguishable.

Glycosylation analysis of the AAD-12 protein derived from soybean 68416 indicated that no covalently-linked carbohydrates were detectable. This result was the same as for the microbially-derived AAD-12. The Applicant did not undertake a glycoprotein staining analysis of the plant-derived PAT protein. However, an *in silico* approach can be used to search for the occurrence of the two motifs discussed in the opening paragraph of Section 4.4.1.2., and no potential N-glycosylation sites have been identified for the PAT protein (Hérouet *et al*., 2005). Further evidence of the non-glycosylated status of both proteins from both sources is provided in the molecular weight analyses in which the proteins ran at the expected molecular weight in the SDS-PAGE/western blot analyses; glycosylation would add to the overall protein molecular weight.

4.4.3 Conclusion

The studies described above allowed the determination of the identity and physicochemical properties of the AAD-12 and PAT proteins. A range of characterisation methods confirmed the identity and equivalence of the two proteins produced in both a bacterial expression system and in leaves from soybean 68416. Based on weight of evidence, it is concluded that microbially-derived AAD-12 and PAT proteins are suitable surrogates for use in safety assessment studies.

4.5 Potential toxicity of the AAD-12 protein

While the vast majority of proteins ingested as part of the diet are not typically associated with toxic effects, a small number may be harmful to health. Therefore, if a GM food differs from its conventional counterpart by the presence of one or more novel proteins, these proteins should be assessed for their potential toxicity. The main purpose of an assessment of potential toxicity is to establish, using a weight of evidence approach, that the novel protein will behave like any other dietary protein.

The assessment focuses on: whether the novel protein has a prior history of safe human consumption, or is sufficiently similar to proteins that have been safely consumed in food; amino acid sequence similarity with known protein toxins and anti-nutrients; structural properties of the novel protein including whether it is resistant to heat or processing and/or digestion. Appropriate oral toxicity studies in animals may also be considered, particularly where results from the biochemical, bioinformatic, digestibility or stability studies indicate a concern.

4.5.1 History of human consumption

As stated in Section 4.2.1, the AAD-12 protein is also known as SdpA. While the TfdA enzyme, to which the SdpA and RdpA enantiomers are related, is widespread in soil bacteria, few bacterial strains have been isolated that contain the *sdpA* and *rdpA* genes (refer to Section 3.2.1). There have also been few studies on the presence, natural

abundance and activation of the *rdpA* and *sdpA* genes. However, it is known that the *sdpA* gene is present in unsprayed agricultural soil (Lee and Matheson, 1984). In crops that are sprayed with phenoxyalkanoic herbicides, a significant amount of spray reaches the ground and can lead to an increase in the abundance of phenoxyalkanoic herbicide degraders (Gazitúa *et al*., 2010). Therefore, humans would be naturally exposed to the AAD-12 (SdpA) protein, particularly in those areas where phenoxyalkanoic herbicides are sprayed.

4.5.2 Similarities with known protein toxins

Study submitted:

Larrinua, I.M.; Herman, R.A. (2007). AAD-12 amino-acid homology search for similarity to toxins. Study ID 071035, Dow AgroSciences LLC (unpublished).

Song, P. (2010). Toxicity similarity assessment of AAD-12 protein expressed in soybean event DAS-68416-4 by bioinformatic analysis (Update, March, 2010). Study ID 101573, Dow AgroSciences LLC (unpublished).

Song, P. (2010). Supplemental information for toxicity similarity assessment of AAD-12 protein expressed in soybean event DAS-68416-4 by bioinformatic analysis (Update, March 2010). Study ID 101573S. Dow AgroSciences LLC (unpublished).

Bioinformatic analyses are useful for assessing whether introduced proteins share any amino acid sequence similarity with known protein toxins. The AAD-12 sequence was compared with protein sequences present in the GenBank non-redundant protein dataset.

Over time, two similarity searches were done (in 2007 and again in 2010), both using the BLASTP[7] (Basic Local Alignment Search Tool Protein) algorithm (Altschul *et al*., 1997). BLASTP is now frequently applied for searching for similarities in protein sequences by performing local alignments. This detects more similarities that would be found using the entire query sequence length. A parameter known as the *E* value (see eg Baxevanis, 2005) represents the probability that a particular alignment is due to random chance. Comparisons between highly homologous proteins yield *E*-values approaching zero, indicating the very low probability that such matches would occur by chance. A larger *E*-value indicates a lower degree of similarity. All database sequences with an E-value of 1 or lower were identified by default by the BLASTP program. Although a statistically significant sequence similarity generally requires a match with an *E*-value of less than 0.01 (Pearson, 2000), setting a threshold *E*-value of 1.0 ensures that proteins with even limited similarity will not be excluded. Commonly, for protein-based searches, hits with *E*-values of 10^{-3} or less and sequence identity of 25% or more are considered significant although any conclusions reached need to be tempered by an investigation of the biology behind the putative homology (Baxevanis, 2005).

The first AAD-12 similarity search identified 618 proteins. Of these, 474 proteins were identified as taurine dioxygenases (TauD) which are related to TfdA and to which, it has been documented, that AAD-12 shares homology (Westendorf *et al*., 2002). A total of 138 proteins were clavaminic acid synthetases (alpha-ketoglutarate-dependent oxygenases central to the biosynthesis of clavulanic acid). Neither of these classes of proteins nor any of the other six proteins identified in the search is a known toxin.

The second search returned 1,577 alignments, the most predominant classes of which were taurine dioxygenases (468), dioxygenases (295) α-ketoglutarate dioxygenases (254), taurine catabolism dioxygenases (155) and 2,4-D/ α-ketoglutarate dioxygenases (91). Again, none of the proteins is associated with toxicity.

[7] BLASTP is used to compare a protein sequence with a database of protein sequences.

4.5.3 In vitro *digestibility*

See Section 4.6.3

4.5.4 Thermolability

The thermolability of a protein provides an indication of the stability of the protein under cooking/processing conditions.

Study submitted:

Schafer, B.W. (2008). Effect of heat treatment on a recombinant aryloxyalkanoate dioxygenase-12 (AAD-12). Study ID 080140, Dow AgroSciences LLC (unpublished).

Authenticated AAD-12 protein purified from a bacterial expression system (refer to Section 4.4) was dissolved in buffer and then treated in a number of temperature regimes, namely, held on ice, incubated for 30 minutes at 50^{o}, 70^{o} or 95^{o} C, and autoclaved at 120^{o} C for 20 minutes (to mimic commercial processing). After treatment, total protein samples were detected by SDS-PAGE coupled with Coomassie blue staining, and a protein activity assay (refer to Section 4.4.1.3) was also done.

The results (refer to Table 4) demonstrate that while no significant changes to protein structure were indicated by SDS-PAGE after any of the treatments except autoclaving, the enzymatic activity of the protein was eliminated by any heating.

Table 4: Summary of results testing the thermolability of the AAD-12 protein

Temperature/ Time	SDS-PAGE (total protein)	% activity
4^{o} C/indefinite	band @ 32 kDa	100
50^{o} C/30 min	band @ 32 kDa	0
70^{o} C/30 min	band @ 32 kDa	0
95^{o} C/30 min	band @ 32 kDa	0
120^{o} C/20 min	no band	0

It is concluded that cooking/processing of soybean 68416 would eliminate its enzyme activity.

4.5.5 Acute oral toxicity study

An acute oral toxicity study using mice was conducted in order to examine the potential toxicity of authenticated AAD-12 protein obtained from a bacterial expression system (refer to Section 4.4

Studies submitted:

Wiescinski, C.M.; Golden, R.M. (2008). AAD-12: acute oral toxicity study in CRL_CD1 (ICR) mice. Study ID 081037, Dow AgroSciences LLC (unpublished).

Cleveland, C.B.; Herman, R.A.; Krieger, M.S. (2009). Human and livestock exposure assessment for AAD-12 protein in DAS 68416-4 soybeans. Study ID 091141, Dow AgroSciences LLC (unpublished).

The study design is summarised in Table 5

Table 5: Study design for acute oral toxicity testing of AAD-12

Test material	AAD-12 derived from *Pseudomonas fluorescens*
Vehicle	0.5% aqueous methylcellulose
Test Species	Crl:CD1 (ICR) mice (five females and five males) – 8 weeks old on day of treatment
Dose	2 x separate doses of test substance by oral gavage, within 1 h. Actual total dose was 2,000 mg/kg body weight AAD-12**
Control	None

** The dose of 2,000 mg/kg body weight is the maximum unexceptional dose recommended by the OECD for the testing of acute oral toxicity using the fixed dose procedure (OECD, 2001b).

Mice were observed for mortality, body weight gain and clinical signs over 14 days. At the end of the study all animals were killed and examined for organ or tissue damage or dysfunction. All mice survived for the duration of the study. No clinical signs of systemic toxicity were observed. No macroscopic abnormalities were present in the mice at necropsy on day 14. A cyst in the cortex of the kidney of one female mouse was observed; this was not considered to be associated with the administration of AAD-12. Under the conditions of this study, administration of AAD-12 protein to female and male mice at a dose of 2,000 mg /kg bw produced no test substance-related clinical signs of toxicity, body weight losses, macroscopic abnormalities or mortality. These results support the conclusion that the AAD-12 protein is not acutely toxic.

4.6 Potential allergenicity of the AAD-12 protein

The potential allergenicity of novel proteins was evaluated using an integrated, step-wise, case-by-case approach relying on various criteria used in combination. This is because no single criterion is sufficiently predictive of either allergenicity or non-allergenicity (see e.g. Thomas *et al*., 2009). The assessment focuses on:

- the source of the novel protein;
- any significant amino acid sequence similarity between the novel protein and known allergens;
- the structural properties of the novel protein, including susceptibility to digestion, heat stability and/or enzymatic treatment; and
- specific serum screening if the novel protein is derived from a source known to be allergenic or has amino acid sequence similarity to a known allergen, additional *in vitro* and *in vivo* immunological testing may be warranted.

Applying this approach systematically provides reasonable evidence about the potential of the novel protein to act as an allergen.

The allergenic potential of the AAD-12 protein was assessed by:

- consideration of the source of the gene encoding the protein and history of use or exposure
- bioinformatic comparison of the amino acid sequence of the AAD-12 protein with known protein allergen sequences
- evaluation of the lability of the microbially produced and purified AAD-12 protein using *in vitro* gastric digestion models; and thermolability

4.6.1 Source of protein

The AAD-12 protein is derived from a common soil bacterium to which humans have been naturally exposed and which may have been inadvertently ingested on fresh produce without

eliciting adverse effects. *Delftia acidovorans* has not been reported to be a source of allergenic proteins.

4.6.2 Similarity to known allergens

Studies submitted:

Herman, R.A. (2007). AAD-12 amino-acid homology search for similarity to allergens. Study ID 071036, Dow AgroSciences LLC (unpublished).

Song, P. (2010). Potential allergenicity assessment of AAD-12 protein expressed in soybean event DAS-68416-4 by bioinformatic analysis (Update, March, 2010). Study ID 101572, Dow AgroSciences LLC (unpublished).

Song, P. (2010). Supplemental information for potential allergenicity assessment of AAD-12 protein expressed in soybean event DAS-68416-4 by bioinformatic analysis (Update, March 2010). Study ID 101572S. Dow AgroSciences LLC (unpublished).

Bioinformatic analysis provides part of a 'weight of evidence' approach for assessing potential allergenicity of novel proteins introduced to GM plants (Goodman, 2006; Thomas *et al*., 2005). It is a method for comparing the amino acid sequence of the introduced protein with sequences of known allergens in order to indicate potential cross-reactivity between allergenic proteins and the introduced protein. As with the bioinformatic analysis that looked at similarities of the AAD -12 protein with known protein toxins (refer to Section 4.5.2), the generation of an *E* value provides an important indicator of significance of matches (Baxevanis, 2005; Pearson, 2000).

The AAD-12 (293 amino acids) sequence was compared with all known allergen sequences contained in a reference allergen database (Food Allergy Research and Resource Program - FARRP, version 7.0) using the FindPatterns and FASTA algorithms. The sequence search was repeated in 2010 (FARRP version 10.0) using the FASTA algorithm and BLOSUM50 scoring matrix. The criteria used to indicate potential allergenicity in both searches were a minimum of 8-contiguous amino acid identity or 35% identity on a window of 80 amino acids within the sequence of an allergenic protein.

No matches were found in either search with known allergenic proteins or with known allergenic epitopes. It was concluded that it is unlikely the AAD-12 protein is allergenic.

4.6.3 In vitro digestibility

Typically, food proteins that are allergenic tend to be stable to enzymes such as pepsin and the acidic conditions of the digestive system, exposing them to the intestinal mucosa and leading to an allergic response (Astwood and Fuchs, 1996; Kimber *et al*., 1999; Metcalfe *et al*., 1996). Therefore a correlation exists between resistance to digestion by pepsin and potential allergenicity although this may be inconsistent (Herman *et al*., 2007; Thomas *et al*., 2004). As a consequence, one of the criteria for assessing potential allergenicity is to examine the stability of novel proteins in conditions mimicking human digestion. Proteins that are rapidly degraded in such conditions are considered less likely to be involved in eliciting an allergic response. However, evidence of slow or limited protein digestibility does not necessarily indicate that the protein is allergenic.

A pepsin digestibility assay (Thomas *et al*., 2004) was conducted to determine the digestive stability of the AAD-12 protein using simulated gastric fluid (SGF).

Study submitted:

Embrey, S.K.; Schafer, B.W. (2008). In vitro simulated gastric fluid ndigestibility of aryloxyalkanoate dioxygenase-12 (abbreviation AAD-12). Study ID 080064, Dow AgroSciences LLC (unpublished).

The *in vitro* digestibility of the *P. fluorescens*-derived, authenticated AAD-12 protein in SGF (U.S.Pharmacopeia, 1990) containing pepsin was evaluated by incubating samples at 37º for selected times (1, 2, 4, 6, 8, 16 minutes). The equivalent of a zero time point was prepared by neutralizing the SGF with sodium carbonate prior to adding the AAD-12. All samples were then run on SDS-PAGE. Proteins were visualised by GelCode Blue staining of the resulting gel. Two control proteins were treated in parallel: bovine serum albumin (BSA) is known to hydrolyse readily in pepsin and served as a positive control; β-lactoglobulin is known to persist in pepsin and was used as a negative control. Western blotting of the SDS gels was also performed using an anti-AAD-12 rabbit polyclonal antibody.

Both the SDS gels and western blotting indicated that the AAD-12 protein was rapidly hydrolysed in SGF, with no detectable presence at >=30 seconds. The BSA positive control was rapidly hydrolysed (>=30 seconds) while the β-lactoglobulin negative control had not been digested after 16 minutes.

4.6.4 Thermolability

See Section 4.5.4

4.7 Conclusion

Soybean line DAS-68416-4 expresses two novel proteins, AAD-12 and PAT. Expression analyses of the two proteins showed that, in the plant parts tested, the AAD-12 is lowest in the roots and seed (approximately 16 µg/g dry weight) and highest in leaves (approximately 55 µg/g dry weight). PAT protein concentrations are much lower than those for AAD-12 but similarly, the leaves contain the highest levels (approximately 11 µg/g dry weight) and the roots contain the lowest levels (approximately 2 µg/g dry weight). The level in the seed is approximately 2.8 µg/g dry weight.

Several studies were done to confirm the identity and physicochemical properties of the plant-derived AAD-12 and PAT proteins, and demonstrated they both conform in size and amino acid sequence to that expected, and do not exhibit any post-translational modification including glycosylation.

In relation to potential toxicity and allergenicity, the PAT protein has previously been demonstrated to be non-toxic to mammals and also does not exhibit any potential to be allergenic to humans.

For the AAD-12 protein, bioinformatic studies confirmed the lack of any significant amino acid sequence similarity to known protein toxins or allergens; a digestibility study suggests the protein would be rapidly degraded in the stomach following ingestion; and a thermolability study showed that the protein is inactivated by heating. An acute oral toxicity study in mice also confirmed the absence of toxicity of AAD-12. Taken together, the evidence indicates that AAD-12 is unlikely to be toxic or allergenic to humans.

Analysis of the sequences encoded by eight identified novel open reading frames in the junction regions of the inserted DNA did not identify any similarity with known protein toxins or allergens.

5. Herbicide metabolites

In the case of herbicide-tolerant GM lines there is the possibility that novel metabolites may be produced following application of the herbicide and these metabolites may be present in the final food. It is therefore necessary for those lines incorporating a herbicide/gene combination not previously assessed, to establish whether such metabolites occur. If they do, their toxicity needs to be determined in order to enable the establishment of an appropriate health-based guidance value (e.g. Acute Reference Dose – ARfD; Acceptable Daily Intake – ADI). Residue data also need to be considered to confirm the concentration of the novel GM trait-specific metabolites relative to the parent herbicide in the final food.

Use of the *pat* gene to confer tolerance to glufosinate ammonium has been considered in a wide range of food crops, including soybean, and therefore glufosinate ammonium residues are not considered in this Assessment.

As a result of the activity of AAD-12 (refer to Section 4.2.1), the herbicide metabolite generated by soybean line DAS-68416-4 from spraying with 2,4-D is expected to be identical to that detected in conventional crops (i.e. 2,4-dichlorophenol (DCP)). That is, no herbicide metabolites would be produced in soybean line DAS-68416-4 as a result of spraying with 2,4-D that are not also produced in conventional crops treated with the herbicide.

Studies submitted:

Graper, L.K.; Balcer, J.L.; Smith, K.P.; Hogan, P.S. (2011) A nature of the residue study with [^{14}C]-2,4-D DMA applied to AAD-12 soybeans

Culligan, J.F. (2010). Magnitude of the residue of 2,4-D in/on herbicide tolerant soybeans containing the aryloxyalkanoate dioxygenase-12 (AAD-12) gene

5.1 Residue chemistry studies

Field trials were conducted in 2009 at 24 sites in North America that included two sites in Canada. The trials were located in areas where soybean is commonly grown commercially. Each site had an untreated control plot and a treated plot that were planted with soybean 68416. The treated soybean was sprayed with three applications of 2,4-D (Weedar 64) with each application targeted at a rate of 1.12 kg acid equivalents/ha (ae/ha). These rates are equivalent to the maximum seasonal rates for the herbicide. The three applications of 2,4-D were applied at each of the pre-emergent stage, approximately 12 days before R2 was reached, and the R2 stage[8]. All applications, except for the pre-emergent treatment, were via a boom sprayer.

The residue data in soybean seed harvested at commercial maturity are summarised in Table 6.

Table 6: Residue levels (µg/g) in seed of soybean line DAS-68416-4 sprayed with 2,4-D

Residue	PHI[1]	N[2]	Minimum[3]	Maximum	Median	Mean
2,4-D	51 - 103	48	ND	ND	ND	ND
DCP	51 - 103	48	ND	0.054	0.015	0.017

[1]PHI = Pre-harvest interval; days between last application of herbicide and collection of field sample
[2]Two independently composite samples were collected from each treated plot i.e. 2 x 24 = 48
[3]ND = not detected (less than the limit of detection of 0.003 µg/g)

[8] For information on soybean growth stages see e.g. NDSU (2004).

Decline samples of seeds were also collected from two trial sites at approximately 7 and 14 days before the harvested samples and 7 and 14 days after the harvested samples. A residue decline profile was established for DCP and indicated that the residue remained relatively unchanged over the 28-day collection interval. No decline profile could be established for 2,4-D because of the non-detectable levels.

In addition bulk samples were also collected from two trial sites in Georgia and Nebraska sprayed with twice the rate of 2,4-D (i.e. 2.24 kg ae/ha) and residue levels were determined in several processed commodities (refer to Table 7). The results indicate that there may be some concentration of DCP in meal but no concentration in hulls. No residue was detected in oil.

Table 7: Residue levels in processed commodities of soybean line DAS-68416-4 sprayed with 2x2,4-D

Commodity	Mean Residue Levels (µg/g)			
	Georgia Trial Site		Nebraska Trial Site	
	2,4-D	DCP	2,4-D	DCP
Seed	ND	0.109	ND	0.012
Hulls	ND	0.050	ND	(0.004)[1]
Meal	ND	0.155	ND	0.015
Oil	ND	ND	ND	ND

[1]the value is greater than the limit of detection but less than the limit of quantitation (0.01 µg/g)

Based on the proposed use of 2,4-D in soybean line DAS-68416-4 there would be no detectable residue of 2,4-D in either seed or processed commodities. The metabolite DCP may be present at levels that are just above the limit of quantitation in raw seed and processed commodities except oil, in which it is unlikely the metabolite is detectable.

5.2 ADI for 2,4-D

As no novel herbicide metabolites are present in treated soybean line DAS-68416-4 as a result of spraying with 2,4-D, the existing health-based guidance value (i.e. Acceptable Daily Intake - ADI) for 2,4-D is appropriate and relevant for assessing dietary risk with soybean line DAS-68416-4. In Australia the ADI for 2,4-D is 0.01 mg/kg bw/day[9].

5.3 Conclusion

The major residue generated on soybean line DAS-68416-4 as a result of spraying with 2,4-D is dichlorophenol. This residue is the same as that found on conventional crops sprayed with 2,4-D and would be present at very minor levels.

6. Compositional analysis

The main purpose of compositional analysis is to determine if any unexpected changes in composition have occurred to the food and to establish its nutritional adequacy. Compositional analysis can also be important for evaluating the intended effect where there has been a deliberate change to the composition of food.

[9]ADIs are established by the Office of Chemical Safety within the Department of Health and Ageing http://www.health.gov.au/internet/main/publishing.nsf/Content/E8F4D2F95D616584CA2573D700770C2A/$File/ADI-report-may10.pdf

The classic approach to the compositional analysis of GM food is a targeted one; rather than analysing every single constituent, which would be impractical. The aim is to analyse only those constituents most relevant to the safety of the food or that may have an impact on the whole diet. Important analytes therefore include the key nutrients, toxicants and anti-nutrients for the food in question. The key nutrients and anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates or enzyme inhibitors as anti-nutrients) or minor constituents (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in an organism, such as compounds whose toxic potency and level may be significant to health (eg solanine in potatoes).

6.1 Key components

For soybean there are a number of components that are considered to be important for compositional analysis (EuropaBio, 2003; OECD, 2001a). As a minimum, the key nutrients of soybean seed appropriate for a comparative study include the proximates (crude protein, fat, ash, acid detergent fibre and neutral detergent fibre), amino acids and fatty acids. In addition, mineral and vitamin levels may be considered and international guidance also suggests that levels of the key anti-nutrients phytic acid, trypsin inhibitors, lectins, isoflavones and stachyose and raffinose should be determined for new varieties of soybean. The reasons for focussing on these particular anti-nutrients are:

- phytic acid causes chelation of mineral nutrients (including calcium, magnesium, potassium, iron and zinc) thereby making them unavailable to monogastric animals, including humans
- trypsin inhibitors interfere with digestion of protein; lectins are proteins that bind to carbohydrate-containing molecules. Both trypsin inhibitors and lectins can inhibit animal growth. The activity of trypsin inhibitors and lectins is heat-labile and they are inactivated during processing of soybean protein products and soybean meal so that the final edible soybean product should contain minimal levels of these anti-nutrients.
- isoflavones are reported to possess biochemical activity including estrogenic, anti-estrogenic and hypocholesterolaemic effects that have been implicated in adversely affecting animal reproduction. Reports in the literature suggest that 6”-O-malonylgenistin, genistin, 6”-O-malonyldaidzin and daidzin together represent at least 80% of total isoflavone content in soybean seeds (see discussion in Wang and Murphy, 1994)
- stachyose and raffinose are low molecular weight carbohydrates (oligosaccharides) that are associated with production of intestinal gas and resulting flatulence when they are consumed.

6.2 Study design and conduct for key components

Study submitted:

Smith-Drake, J.K.;Dunville, C.M.; Phillips, A.M.; Herman, R.A. (2009). Field expression, nutrient composition analysis and agronomic characterisation of a transformed soybean cultivar (DAS-68416-4) containing aryloxyalkanoate dioxygenase (AAD-12) and phosphinothricin acetyltransferase (PAT). Study ID 080003, Dow AgroSciences LLC (unpublished)

The test (DAS-68416-4, seed of T4 lineage), and control (‘Maverick’) lines were grown under similar conditions at six field sites across North America[10] during the 2008 growing season. The Applicant noted that temperature and rainfall during this season were well outside (lower temperature and higher rainfall) the 20th century means. ‘Maverick’ is the original

[10] The six sites were: Richland, IA ; Carlyle, IL; Rockville, IN, York, NE; Thorndale, Ontario (Canada); Branchton, Ontario (Canada)

transformed line and therefore represents the isogenic control line for the purposes of the comparative analyses. The identity of the test and control lines was verified by event-specific PCR analysis.

Four herbicide treatments were applied to soybean 68416, namely: unsprayed; sprayed with glufosinate; sprayed with 2,4-D; and sprayed with 2,4-D + glufosinate. Seeds of the test and control soybean lines were planted in a randomised complete block design, with three replicated plots at each of the six sites.

Seed and forage from soybean 68416 and 'Maverick' were harvested from all replicated plots and analysed for composition. Forage was collected at the R3 plant growth stage, and seed was harvested at physiological maturity (refer to Table 3 for reference to growth stages). Methods of composition analysis were based on internationally recognised procedures (e.g. those of the Association of Official Analytical Chemists), methods specified by the manufacturer of the equipment used for analysis, or other published methods.

Data were transformed into Statistical Analysis Software[11] (SAS) data sets and analysed using SAS version 8. A least squares mean (LSM) value was generated and used for each analyte comparison, and standard deviation and minimum and maximum values were also calculated for each analyte. Analysis of Variance was used for over-all analysis. The significance of an overall treatment effect was estimated using an F-test, while paired contrasts were made between the unsprayed and each of the 3 sprayed treatments using t-tests. Probability values were adjusted using False Discovery Rate procedures to improve discrimination of true differences (Benjamini and Hochberg, 1995). In assessing the significance of any difference between the mean analyte value for soybean 68416 and 'Maverick' a P-value of 0.05 was used. This means that approximately 5% of statistically significant differences are expected to occur due to chance alone.

The results for the treatments were compared to a combined literature range for each analyte, compiled from published literature for commercial cultivars that included Codex (Codex, 2001); Douglas (1996); ILSI (2007); Kakade et al. (1972); Liener (1994); Novak & Haslberger (2000); OECD (2001a); Vaidehi & Kadam (1989). Any mean value for a soybean 68416 analyte that fell within the combined literature range was considered to be within the normal variability of commercial soybean cultivars even if the mean value was statistically different from the 'Maverick' control. It is noted, however, that information in the published literature is limited and is unlikely to provide a broad reflection of the natural diversity that occurs within soybean. Therefore, even if means fall outside the published range, this is not necessarily a concern.

6.3 Analyses of key components

Although the Applicant provided results for the compositional analyses of forage, the focus of this assessment is necessarily on the food uses of soybean and therefore the forage data are not presented in this report. However, it should be noted that there were no significant differences between the control and the treated plants for any of the analytes measured in forage except fat. In this instance the results were equivocal, with plants from unsprayed DAS-68416-4 having a higher mean than the control while DAS-68416-4 plants sprayed with glufosinate or sprayed with both glufosinate and 2,4-D had a lower mean than control plants. All means, were, however, within the literature range.

Compositional analyses of the soybean seed included proximates (protein, fat, and ash), acid detergent fibre (ADF), neutral detergent fibre (NDF), fatty acids, amino acids,

[11] SAS website - http://www.sas.com/technologies/analytics/statistics/stat/index.html

isoflavones, anti-nutrients (stachyose, raffinose, lectins, phytic acid and trypsin inhibitor), minerals and vitamins. The results presented in the following tables show least square means pooled from all sites and an adjusted probability value. Probability values >0.05 are written as NS (Not Significant).

6.3.1 Proximates and fibre

Results of the proximate and fibre analysis are shown in Table 8. The mean level of protein was significantly lower in soybean 68416 sprayed with 2,4-D compared to the level in 'Maverick' and the mean level of carbohydrate was significantly higher in soybean 68416 sprayed with 2,4-D compared to the level in 'Maverick' but for both analytes the means for all soybean 68416 spray treatments were within the ranges reported in the literature. While certain means for soybean 68416 spray treatments (shaded in Table 8) were just outside the literature range, they did not represent a significant difference from the control.

Table 8: Mean Percentage dry weight (dw) of proximates and fibre in seed from 'Maverick' and DAS-68416-4.

Analyte	'Maverick'	68416 no spray	68416 + glufos.	68416 + 2,4-D	68416 + glufos. + 2,4-D	Overall treat effect (P-value)	Combined literature range
Protein (%dw)	39.2	38.3	38.8	37.8	38.5	<0.01	32.0 – 47.4
Fat (%dw)	17.1	17.1	16.6	16.7	17.2	NS	8.10 – 24.7
Carbohydrate (%dw)[1]	38.8	39.6	39.6	40.3	39.3	0.01	29.6 – 50.2
ADF (%dw)	17.8	17.6	18.0	18.8	18.1	NS	7.81 – 18.6
NDF (%dw)	20.1	20.8	21.9	21.6	20.3	NS	4.50 – 21.3
Ash (%dw)	4.92	5.04	5.04	5.10	5.07	NS	3.89 – 6.99

[1] Carbohydrate calculated as 100% - (protein %dw + fat %dw + ash %dw)

6.3.2 Fatty Acids

The levels of 22 fatty acids were measured. Of these, the following were below the limits of quantitation - C8:0 caprylic, C10:0 capric, C12:0 lauric, C14:0 myristic, C14:1 myristoleic, C15:0 pentadecanoic, C15:1 pentadecenoic, C17:1 heptadecenoic, C18:3 gamma linolenic, C20:2 eicosadienoic, C20:3 eicosatrienoic and C20:4 arachidonic acids. Results for the remaining 10 fatty acids are given in Table 9 and can be summarised as follows:

- There was no significant difference between the control and soybean 68416 in terms of the levels of palmitic, heptadecanoic, stearic, linoleic and eicosenoic and behenic acids. While means for eicosenoic acid from two soybean 68416 sprayed treatments (shaded in Table 9) were just outside the literature range, they did not represent a significant difference from the control.
- The mean level of palmitoleic acid was significantly lower in the unsprayed soybean 68416 than in 'Maverick' and this level (shaded in Table 9) was also slightly outside the literature range
- The mean levels of oleic and arachidic acids were significantly lower in plants from all soybean 68416 treatments compared to the level in control plants. The levels in soybean 68416 (all herbicide treatments) were, however, within the literature range.

- The mean level of linolenic acid was significantly higher in plants from all soybean 68416 treatments compared to the level in control plants. The levels in soybean 68416 (all herbicide treatments) were, however, within the literature range.

Table 9: Mean percentage composition, relative to total fat, of major fatty acids in seed from 'Maverick' and DAS-68416-4.

Analyte	Maverick (% total)	68416 no spray (% total)	68416 + glufos. (% total)	68416 + 2,4-D (% total)	68416 + glufos. + 2,4-D (% total)	Overall treat effect (P-value)	Combined literature range (% total)
Palmitic acid (C16:0)	10.1	10.0	9.78	9.94	9.85	NS	7.00 - 15.8
Palmitoleic acid (C16:1)	0.097	0.085	0.088	0.087	0.089	0.029	0.0860 - 0.194
Heptadecanoic acid (C17:0)	0.111	0.114	0.113	0.114	0.113	NS	0.0850 - 0.146
Stearic acid (C18:0)	4.28	4.03	3.98	4.05	4.06	NS	2.00 - 5.88
Oleic acid (C18:1)	21.8	19.8	19.5	19.9	19.9	0.01	14.3 – 34.0
Linoleic acid (C18:2)	50.3	52.5	51.9	52.6	52.0	NS	42.3 - 60.0
Linolenic acid (C18:3)	7.83	8.23	8.15	8.10	8.21	0.022	2.00 - 12.5
Arachidic acid (C20:0)	0.307	0.284	0.282	0.285	0.287	0.023	0 - 1.00
Eicosenoic acid (C20:1)	0.143	0.140	0.136	0.141	0.138	NS	0.140 - 0.350
Behenic acid (C22:0)	0.305	0.288	0.285	0.288	0.288	NS	0.277- 0.595

6.3.3 Amino acids

Levels of 18 amino acids were measured. Since asparagine and glutamine are converted to aspartate and glutamate respectively during the analysis, levels for aspartate include both aspartate and asparagine, while glutamate levels include both glutamate and glutamine. Results of the analysis are given in Table 10 and can be summarised as follows:

- There was no significant difference between the control and soybean 68416 in terms of the levels of cystine, isoleucine, methionine, proline, serine, tryptophan, tyrosine and valine.
- For each of the remaining amino acids, the mean levels in two or more of the soybean 68416 treatments were significantly lower than the levels in the control, but all means were within the literature range.

Table 10: Mean percentage dry weight (dw), relative to total dry weight, of amino acids in seed from 'Maverick' and DAS-68416-4.

Analyte	Maverick (%dw)	68416 no spray (%dw)	68416 + glufos.(%dw)	68416 + 2,4-D (%dw)	68416 + glufos. + 2,4-D (%dw)	Overall treat effect (P-value)	Combined literature range (%dw)
Alanine	1.74	1.70	1.70	1.69	1.71	<0.01	1.49 - 2.10
Arginine	3.15	2.97	3.00	2.94	2.96	<0.01	2.29 - 3.49
Aspartate	4.52	4.41	4.44	4.38	4.43	<0.01	3.81 - 5.12
Cystine	0.604	0.602	0.602	0.605	0.614	NS	0.370 - 0.808
Glutamate	6.98	6.76	6.83	6.70	6.80	<0.01	5.84 - 8.72
Glycine	1.74	1.69	1.70	1.69	1.70	<0.01	1.46 - 2.02
Histidine	1.09	1.06	1.07	1.05	1.07	<0.01	0.878 - 1.22
Isoleucine	1.87	1.83	1.85	1.82	1.85	NS	1.46 - 2.12
Leucine	3.06	3.00	3.02	2.98	3.01	0.01	2.20 - 4.00
Lysine	2.56	2.51	2.52	2.49	2.52	<0.01	2.29 - 2.86
Methionine	0.556	0.551	0.549	0.546	0.554	NS	0.431 - 0.681
Phenylalanine	2.02	1.97	1.98	1.94	1.97	<0.01	1.60 - 2.35
Proline	1.91	1.85	1.88	1.87	1.87	NS	1.69 - 2.61
Serine	1.99	1.95	1.95	1.91	1.93	NS	1.11 - 2.48
Threonine	1.62	1.57	1.58	1.55	1.57	<0.01	1.14 - 1.89
Tryptophan	0.433	0.429	0.433	0.434	0.421	NS	0.356 - 0.670
Tyrosine	1.36	1.34	1.35	1.33	1.33	NS	1.02 - 1.62
Valine	1.97	1.92	1.94	1.92	1.95	NS	1.50 - 2.44

6.3.4 Isoflavones

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In total, there are 12 different soybean isoflavone isomers, namely three parent isoflavones (genistein, daidzein and glycitein), their respective β-glucosides (genistin, daidzin, and glycitin), and three β-glucosides each esterified with either malonic or acetic acid (Messina, 2005). The parent isoflavones are also referred to as free or aglycon isoflavones, while the glucosides and their esters are also referred to as conjugated isoflavones.

The Applicant used an AOAC International method (AOAC, 2005), to measure the levels of the three parent isoflavones and the conjugates in seed from soybean 68416 and the control. Levels of the three parent isoflavones were below the limit of quantitation and no data are presented. Levels for the conjugates (comprising glucoside esters and β-glucosides and reported as aglycon equivalents) are given in Table 11 and show the following:

- The mean levels of total genistein and total daidzein in soybean 68416 were not significantly different from those in 'Maverick'.
- The level of total glycitein in soybean 68416 seeds from plants sprayed with both herbicides was significantly higher (shaded in Table 11) than the level in control seeds.

The mean levels of all the measured isoflavones in both the GM and non-GM soybeans fall within the literature range.

Table 11: Mean weight (µg/g dry weight expressed as aglycon equivalents) of isoflavones in soybean 68416 and 'Maverick' seed

Analyte[1]	Maverick (µg/g dw)	68416 no spray (µg/g dw)	68416 + glufos. (µg/g dw)	68416 + 2,4-D (µg/g dw)	68416 + glufos. + 2,4-D (µg/g dw)	Overall treat effect (P-value)	Combined literature range[2.] (µg/g dw)
Total Genistein (aglycon equivalents)	1282	1321	1327	1357	1389	NS	60 - 2453
Total Daidzein (aglycon equivalents)	1085	1103	1112	1128	1179	NS	144 - 2837
Total Glycitein (aglycon equivalents)	253	267	270	268	285	0.032	15.3 - 310

[1.] Although the Total levels normally represent the total of all 12 isomers, the fact that there were unquantifiable levels of the aglycons means that the conjugates essentially represent the Total levels.
[2] This literature range has been compiled from ILSI (2010) figures but encompasses a number of references e.g. – Murphy & Wang (1993), USDA-ISU (2002), Harrigan *et al*. (2010). The USDA-ISU and ILSI figures were converted from fresh weight to dry weight assuming an approximate moisture content of 11% in harvested seed (Kumar *et al*., 2009).

6.3.5 Anti-nutrients

Levels of key anti-nutrients are given in Table 12. No significant differences between means were obtained and all means fell within the literature range.

Table 12: Mean levels of anti-nutrients in soybean 68416 and 'Maverick' seed.

Analyte	Maverick (%dw)	68416 no spray (%dw)	68416 + glufos.(%dw)	68416 + 2,4-D (%dw)	68416 + glufos. + 2,4-D (%dw)	Overall treat effect (P-value)	Combined literature range (%dw)
Lectin (hemagglutinat. units/mg)	2.18	2.74	2.84	2.98	3.09	NS	0.105 - 9.04
Phytic acid (%dw)	1.20	1.20	1.22	1.21	1.25	NS	0.634 - 2.74
Raffinose (%dw)	0.344	0.339	0.310	0.317	0.315	NS	0.634 - 1.96
Stachyose (%dw)	2.42	2.34	2.23	2.28	2.32	NS	1.21 - 3.50
Trypsin inhibitor (trypsin inhibitor units/mg)	25.3	27.2	24.7	24.9	25.3	NS	19.6 - 119

6.3.6 Minerals

Levels of 13 minerals were measured. Sodium was below the level of quantitation. The means for the remaining 12 minerals are given in Table 13 and show that the only significant treatment effect was for potassium in which the mean levels for plants in all of the soybean 68416 treatments were higher than the level in the control. The potassium level in the control was outside (lower than) the literature range and the means for the magnesium levels in all of the treatments were also outside (lower than) the literature range (shaded in Table 13). These results do not raise any safety concerns.

Table 13: Mean values for mineral levels in seed from 'Maverick' and DAS-68416-4.

Analyte	Maverick	68416 no spray	68416 + glufos.	68416 + 2,4-D	68416 + glufos. + 2,4-D	Overall treat effect (P-value)	Combined literature range
Calcium (mg/100g dw)	256	265	264	274	269	NS	117 – 307
Chromium (ppb)	145	149	175	126	137	NS	Not reported
Copper (mg/100g dw)	1.31	1.28	1.30	1.27	1.28	NS	Not reported
Iodine (mg/100g dw)	0.027	0.023	0.021	0.032	0.023	NS	Not reported
Iron (mg/100g dw)	8.15	8.46	8.95	8.53	8.59	NS	5.54 – 11.0
Magnesium (mg/100g dw)	210	212	215	213	215	NS	219 –313
Manganese (mg/100g dw)	2.56	2.60	2.60	2.58	2.59	NS	Not reported
Molybdenum (ppb)	2165	2557	2462	2563	2284	NS	Not reported
Phosphorus (mg/100g dw)	583	589	599	596	594	NS	507 - 935
Potassium (mg/100g dw)	1801	1876	1882	1883	1864	<0.01	1868 - 2316
Selenium (ppb)	490	523	520	511	418	NS	Not reported
Zinc (mg/100g dw)	5.06	5.07	5.19	5.21	5.25	NS	Not reported

6.3.7 Vitamins

Levels of 14 vitamins were measured. Levels for β-tocopherol, β-carotene (vitamin B1), vitamin B12 and vitamin D were below the level of quantitation. Means for the remaining 10 vitamins are given in Table 14. Folic acid levels were significantly lower in seeds from all of the soybean 68416 treatments than in the control, however these levels were within the literature range. The levels of γ-tocopherol in seeds of all the soybean 68416 treatments were significantly higher than in seeds of 'Maverick'. There are a number of papers reporting values for soybean γ-tocopherol (Carrão-Panizzi and Erhan, 2007; Scherder *et al*., 2006; Seguin *et al*., 2010; Seguin *et al*., 2009).but the units of measurement vary or are not always clear. What is clear, however, is that genotype, environment and growing conditions have a significant and inconsistent effect on all of the tocopherols. Given that there can be a 400% difference between lowest and highest values reported in the literature (Carrão-Panizzi and Erhan, 2007), the maximum of 11% difference between the means from 'Maverick' and soybean 68416 does not raise safety concerns.

Table 14: Mean weight (µg/g dry weight) of vitamins in seed from 'Maverick' and DAS-68416-4

Analyte	Maverick (µg/g dw)	68416 no spray (µg/g dw)	68416 + glufos. (µg/g dw)	68416 + 2,4-D (µg/g dw)	68416 + glufos. + 2,4-D (µg/g dw)	Overall treat effect (P-value)	Combined literature range[1] (µg/g dw)
Vitamin B1 (thiamin)	2.10	2.14	1.94	1.97	2.14	NS	1.01 – 2.54
Vitamin B2 (riboflavin)	4.49	4.52	4.60	4.52	4.55	NS	1.90 – 3.21
Vitamin B3 (niacin)	27.4	25.3	25.4	26.9	26.7	NS	Not reported
Vitamin B5 (pantothenate)	15.1	14.9	14.2	14.5	14.3	NS	Not reported
Vitamin B6 (pyridoxine)	5.50	5.51	5.40	5.40	5.39	NS	Not reported
Vitamin C	84.1	79.6	85.4	82.5	83.5	NS	Not reported
Vitamin E (α-tocopherol)	14.8	15.1	14.5	15.9	14.3	NS	1.9 – 61.7
δ-tocopherol	92.6	95.1	96.5	97.1	94.5	NS	31 – 186[1]
γ-tocopherol	153	164	158	169	157	<0.01	18 - 461[1]
Folic acid	3.70	3.49	3.56	3.38	3.48	<0.01	2.39 – 4.71

[1] values converted from µg/g oil to µg/g seed dry weight based on the oil content in seed being approximately 20% dry weight.

6.3.8 Summary of analysis of key components

Statistically significant differences in the analyte levels found in seed of soybean 68416 and 'Maverick' are summarised in Table 15. For all those analytes for which there is a range reported in the literature, the soybean 68416 means fall within that range, except for palmitoleic acid (unsprayed), which is 1% lower than the lowest value in the range and potassium (sprayed with 2,4-D and glufosinate) which is also 1% lower than the lowest value in the range. It is noted that even the 'Maverick' mean potassium level falls below the literature range.

These statistically significant differences do not raise safety concerns, given that there are no trends in the results, that for the majority of analytes there were no significant differences, and that where significant differences did occur, the means were within the normal biological range for soybean.

Table 15: Summary of analyte levels found in seed of soybean 68416 that are significantly (P < 0.05) different from those found in seed of 'Maverick'.

Analyte	Unit of measure.	Maverick	68416 no spray	68416 + glufos.	68416 + 2,4-D	68416 + glufos. + 2,4-D	Soybean 68416 within reported range
Protein	%dw	39.2	38.3	38.8	37.8	38.5	yes
Carbohydrate	%dw	38.8	39.6	39.6	40.3	39.3	yes
Palmitoleic acid (C16:1)	% total fat	0.097	0.085	0.088	0.087	0.089	Yes, except for no spray
Oleic acid (C18:1)	% total fat	21.8	19.8	19.5	19.9	19.9	yes
Linolenic acid (C18:3)	% total fat	7.83	8.23	8.15	8.10	8.21	yes
Arachidic acid (C20:0)	% total fat	0.307	0.284	0.282	0.285	0.287	yes
Alanine	%dw	1.74	1.70	1.70	1.69	1.71	yes
Arginine	%dw	3.15	2.97	3.00	2.94	2.96	yes
Aspartate	%dw	4.52	4.41	4.44	4.38	4.43	yes
Glutamate	%dw	6.98	6.76	6.83	6.70	6.80	yes
Glycine	%dw	1.74	1.69	1.70	1.69	1.70	yes
Histidine	%dw	1.09	1.06	1.07	1.05	1.07	yes
Leucine	%dw	3.06	3.00	3.02	2.98	3.01	yes
Lysine	%dw	2.56	2.51	2.52	2.49	2.52	yes
Phenylalanine	%dw	2.02	1.97	1.98	1.94	1.97	yes
Threonine	%dw	1.62	1.57	1.58	1.55	1.57	yes
Potassium	mg/100g dw	1801	1876	1882	1883	1864	Yes, except for + 2,4-D + glufos
γ-tocopherol	mg/kg dry weight	153	164	158	169	157	Not reported
Folic acid	mg/kg dry weight	3.70	3.49	3.56	3.38	3.48	yes

6.4 Assessment of endogenous allergenic potential

Soybean naturally contains allergenic proteins and is one of a group of known allergenic foods including milk, eggs, fish, shellfish, wheat, peanuts, tree nuts and sesame. This group of foods accounts for approximately 90% of all food allergies (Metcalfe *et al*., 1996). The presence of allergenic proteins in the diet of hypersensitive individuals can cause severe adverse reactions. The allergenic effect of soybeans is attributed to the globulin fraction of soybean proteins that comprise about 85% of total protein (OECD, 2001). Soybean-allergic individuals will also be allergic to soybean 68416.

Since soybean is associated with allergic effects in susceptible individuals, a study was done to assess whether seed from soybean 68416 may have an endogenous allergen content that was different from the non-GM parent line.

Study submitted:

Stagg, N.J. (2010). Endogenous allergenicity analysis of DAS-68416-4 soybean. Study ID 101001, Dow AgroSciences LLC (unpublished).

Extracts were prepared from ground seeds of soybean 68416 and the non-GM parent, 'Maverick', and run on SDS-PAGE. The protein profiles were compared after Coomassie blue staining. Proteins were transferred to nitrocellulose membranes which were then incubated with pooled serum from 20 soy-allergic subjects for ID-IgE one-dimensional

immunoblot, and ELISA inhibition studies using GraphPad Prism 4 software for the analysis. These immunoassays are used routinely to identify protein-specific IgE binding by sera of individuals allergic to a particular food (Goodman and Leach, 2004; Ogawa *et al*., 2000).

The protein profiles in the SDS-PAGE gels of samples from the two sources were similar, indicating no difference in protein content between seed protein from soybean 68416 and 'Maverick'. The ID immunoblot data indicated that protein in soybean 68416 seed is similar in IgE binding profile to protein from 'Maverick' seed. The ELISA inhibition data suggested close similarity in inhibition patterns between protein from soybean 68416 seed and that from 'Maverick', in concentrations ranging from 0.004 to 4,000 µg/mL of total soluble protein. The effective concentration for half-maximal inhibition (EC_{50}) was also similar for the protein from both sources.

Overall, these results suggest that protein from seed of soybean 68416 and 'Maverick' is similar in both protein profile and allergen content. Thus, soybean 68416 appears to be equivalent to the non-transgenic counterpart in terms of its IgE-binding capacity.

6.5 Conclusion

Detailed compositional analyses were done to establish the nutritional adequacy of seed-derived products from soybean 68416 under four herbicide-spraying regimes. Analyses were done of 84 analytes encompassing proximates (crude fat/protein, carbohydrate and ash), acid detergent fibre, neutral detergent fibre, fatty acids, amino acids, isoflavones, anti-nutrients, minerals, and vitamins. The levels were compared to levels in the seeds of the non-GM parent 'Maverick'.

These analyses indicated that the seeds of soybean 68416 are compositionally equivalent to those of the parental line. Out of all the analytes tested, there were significant differences between the non-GM control and soybean 68416 in only 19 analytes. In 17 of these, the mean levels observed in seeds of soybean 68416 were within the range of natural variation reported in the literature. There were no consistent trends in the effect that herbicide spraying of soybean 68416 had on mean analyte levels.

In addition, no difference between seeds of soybean 68416 and 'Maverick' were found in IgE-binding studies using sera from soybean-allergic individuals.

The compositional data are consistent with the conclusion that there are no biologically significant differences in the levels of key components in seed from soybean 68416 when compared with the non-GM control or with the range of levels found in commercial soybean cultivars.

7. Nutritional impact

In assessing the safety of a GM food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and well being. In most cases, this can be achieved through an understanding of the genetic modification and its consequences, together with an extensive compositional analysis of the food.

Where a GM food has been shown to be compositionally equivalent to conventional varieties, the evidence to date indicates that feeding studies using target livestock species will add little to the safety assessment and generally are not warranted (EFSA, 2008; OECD, 2003).

If the compositional analysis indicates biologically significant changes to the levels of certain nutrients in the GM food, additional nutritional assessment should be undertaken to assess the consequences of the changes and determine whether nutrient intakes are likely to be altered by the introduction of such foods into the food supply.

Soybean 68416 is the result of a simple genetic modification to confer herbicide tolerance with no intention to significantly alter nutritional parameters in the food. In addition, the extensive compositional analyses of seed that have been undertaken to demonstrate the nutritional adequacy of 68416, indicate it is equivalent in composition to conventional soybean cultivars. The introduction of soybean line DAS-68416-4 into the food supply is therefore expected to have little nutritional impact.

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