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APPLICATION FOR COMMODITY CLEARANCE OF

DAS-68416-4

IN THE REPUBLIC OF SOUTH AFRICA

Data protection.
This application contains scientific data and other information which are protected in accordance with Section 18 of the Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997), including all amendments.

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digestibility of aryloxyalkanoate dioxygenase-12 (abbreviation AAD-12). Study ID: 080064.

Attachment N — Stagg, N.J. (2010) 1D-IgE immunoblot and ELISA inhibition analyses of DAS-68416-4. Study ID: 101900.

Attachment O — Harpham, N.V.J., Stagg, N.J. (2010) 2D-Immunoblot assessment of DAS-68416-4 Soybean. Dow AgroSciences Study ID: 101740.

Attachment P — Phillips A.M., Lepping M.D. (2010b) Nutrient composition of a transformed soybean cultivar containing aryloxyalkanoate dioxygenase-12 (AAD-12) - event DAS-68416-4. Dow AgroSciences Study ID: 09105.03.

Attachment Q — Fletcher, D.W. (2010) DAS-68416-4 (AAD-12) Soybean feeding study in the broiler chicken. Dow AgroSciences Study ID: 10188.

Attachment R — Cleveland, C. (2010) Human and livestock exposure and dietary assessment for AAD-12 protein in DAS-68416-4 soybean for the European Union. Dow AgroSciences Study ID: 102036.

PART I
1. BRIEF DESCRIPTION OF THE GENETICALLY MODIFIED ORGANISM

1.1 Include specific and common names of the organism, the country of origin of the plant and a description of the genetically modified trait.

Soybean is an erect, bushy herbaceous annual that can reach a height of 1.5 metres. Cultivated soybean, *Glycine max* (L.) Merr., is a diploidized tetraploid (2n=40), in the family *Leguminosae*, subfamily *Papilionoideae*, tribe *Phaseoleae*, genus *Glycine* Willd. and subgenus *Soja* (Moench). The subgenus *Soja* also contains *G. soja* and *G. gracilis*. *G. soja*, a wild species of soybean, grows in fields, hedgerows, roadsides and riverbanks in many Asian countries. Cytological, morphological and molecular evidence suggest that *G. soja* is the ancestor of *G. max*. *G. gracilis* is considered to be a weedy or semi-wild form of *G. max*, with some phenotypic characteristics intermediate to those of *G. max* and *G. soja*. *G. gracilis* may be an intermediate in the speciation of *G. max* from *G. soja* or a hybrid between *G. soja* and *G. max* (OECD, 2009).

Soybean is native to North and Central China and is commonly considered one of the oldest cultivated crops. The first recording of soybeans was in a series of books known as Pen Ts'ao Kong Mu written by the emperor Sheng Nung in the year 2838 B.C., in which the various plants of China are described. Historical and geographical evidence suggest that soybeans were first domesticated in the eastern half of China between the 17th and 11th century B.C. (OECD, 2009).

This is an application for commodity clearance approval of the soybean event, DAS-68416-4 in the Republic of South Africa (South Africa). DAS-68416-4 soybean was developed using *Agrobacterium*-mediated transformation to stably incorporate the *aad*-12 gene, from *Delftia acidovorans*, and the *pat* gene, from *Streptomyces viridochromogenes*, into soybean. The *aad*-12 gene encodes the aryloxyalkanoate dioxygenase-12 (AAD-12) enzyme which, when expressed in plants, degrades 2,4-D into herbicidally-inactive 2,4-dichlorophenol (DCP). DAS-68416-4 soybeans also provide tolerance to glufosinate-ammonium herbicide; driven by the expression of the *pat* gene. Glufosinate is a non-selective, contact herbicide that controls a broad spectrum of annual and perennial grasses and broadleaf weeds. The tolerance to glufosinate allows use of an additional mode of action as part of effective herbicide resistance management strategies. Glufosinate herbicides can also be used as selection agents in breeding nurseries to select herbicide-tolerant plants to maintain seed trait purity.

This application is submitted by Dow AgroSciences Southern Africa (Pty) Ltd. (on behalf of Mycogen Seeds c/o Dow AgroSciences LLC).

2. COMMODITY CLEARANCE

2.1 Please indicate the type of clearance requested.

This is an application for commodity clearance approval of DAS-68416-4 soybean. The scope of the application is for all uses of DAS-68416-4 soybean grain as for any other soybean grain, excluding cultivation, *i.e*. in food, feed and for processing.

The product described in this application consists of soybean products from progeny, containing the genetic modification, as derived from conventional breeding between DAS-68416-4 soybean and traditionally bred soybean.

2.2 Detail specific instructions for the storage and handling of the plant or plant parts.

Information and data provided in this application support the conclusion that, except for the specifically introduced herbicide tolerance trait, DAS-68416-4 is substantially equivalent to conventional soybean. Consequently, no specific storage or handling measures are required for the placing on the market of DAS-68416-4 soybean grain, for all uses as any other soybean grain. Furthermore, South Africa is not the centre of origin for *G.max* and there are no wild relatives in South Africa with which soybean can outcross (Singh, 2010). DAS-68416-4 grain will therefore be stored and handled in the same manner as current commercial soybean grain.

2.3 When will commodity import take place?

Grain imports are made by international grain traders, with the time of importation dependent on the local or regional need for grain. The grain traders would, as per the requirements in terms of the Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997), obtain the necessary permits from the Registrar prior to importation.

2.4 Where will commodity import take place?

As indicated in section 2.3, grain imports are made by international grain traders and could potentially enter South Africa through any of the ports of entry.

2.5 Detail the type of environment and the geographical areas for which the plant is suited.

Except for the introduced herbicide tolerance trait, DAS-68416-4 soybean is substantially equivalent to conventional soybean and would therefore, in countries where this product is approved for commercial use, be grown in all the soybean producing areas.

However, as indicated in section 2.1, this is an application for commodity clearance approval, and not cultivation, of DAS-68416-4 soybean in South Africa.

2.6 Who will undertake the commodity import?

As indicated in section 2.3, grain imports are made by international grain traders.

2.7 Estimate the amount of production of the genetically modified plant within South Africa per annum, or the amount that will be imported into South Africa per annum.

This is an application for commodity clearance approval, and not cultivation, of DAS-68416-4 soybean in South Africa.

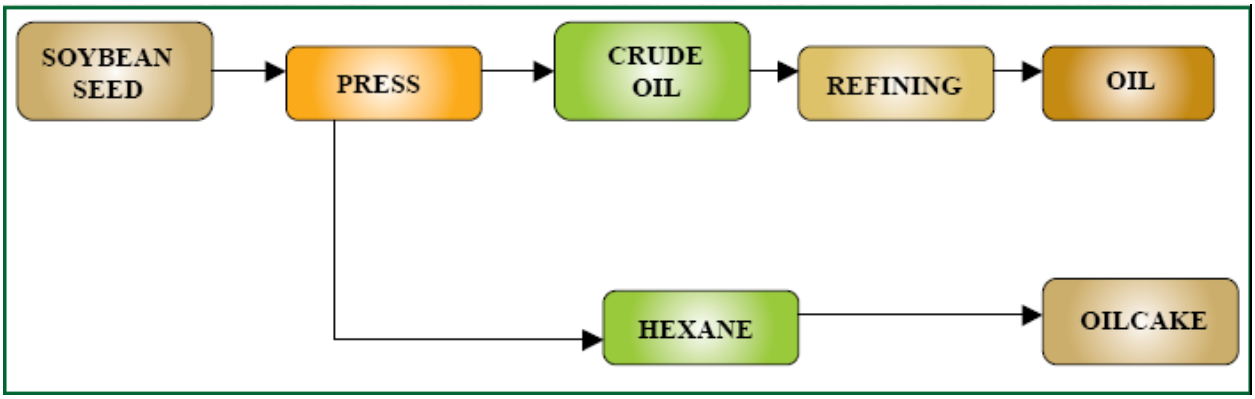
Statistics indicate that 143 873 tons of soybean were imported into South Africa in 2007, 17 986 tons in 2008 and 1 495 in 2009 (Department of Agriculture, Forestry and Fisheries [DAFF], (2011).

3. DESCRIPTION OF ANY PRODUCT DERIVED FROM THE PLANT

3.1 Identify the part of the plant to be used for the product, the type of product, and the use of the product, the market sector in which the product will be marketed and the tradename of the product.

The principal product of soybean is the seed which is contained inside a pod. Domestically, soybeans are sold to expressers who produce oil, oilcake and animal feed (DAFF, 2011). A smaller percentage of soybeans are sold directly to consumers for the edible market. Soybean seeds can be cooked and eaten as a vegetable and the dried seeds can be eaten whole, split or sprouted. They can also be processed to give soy milk which is a valuable protein supplement in infant feeding and can also be used to produce curds and cheese. When the seed is pressed during the processing of soybeans crude oil is released. Soybean oilcake is also derived from the process. The crude oil is then refined to produce soybean oil (Figure 1).

Figure 1. The processing of soybeans

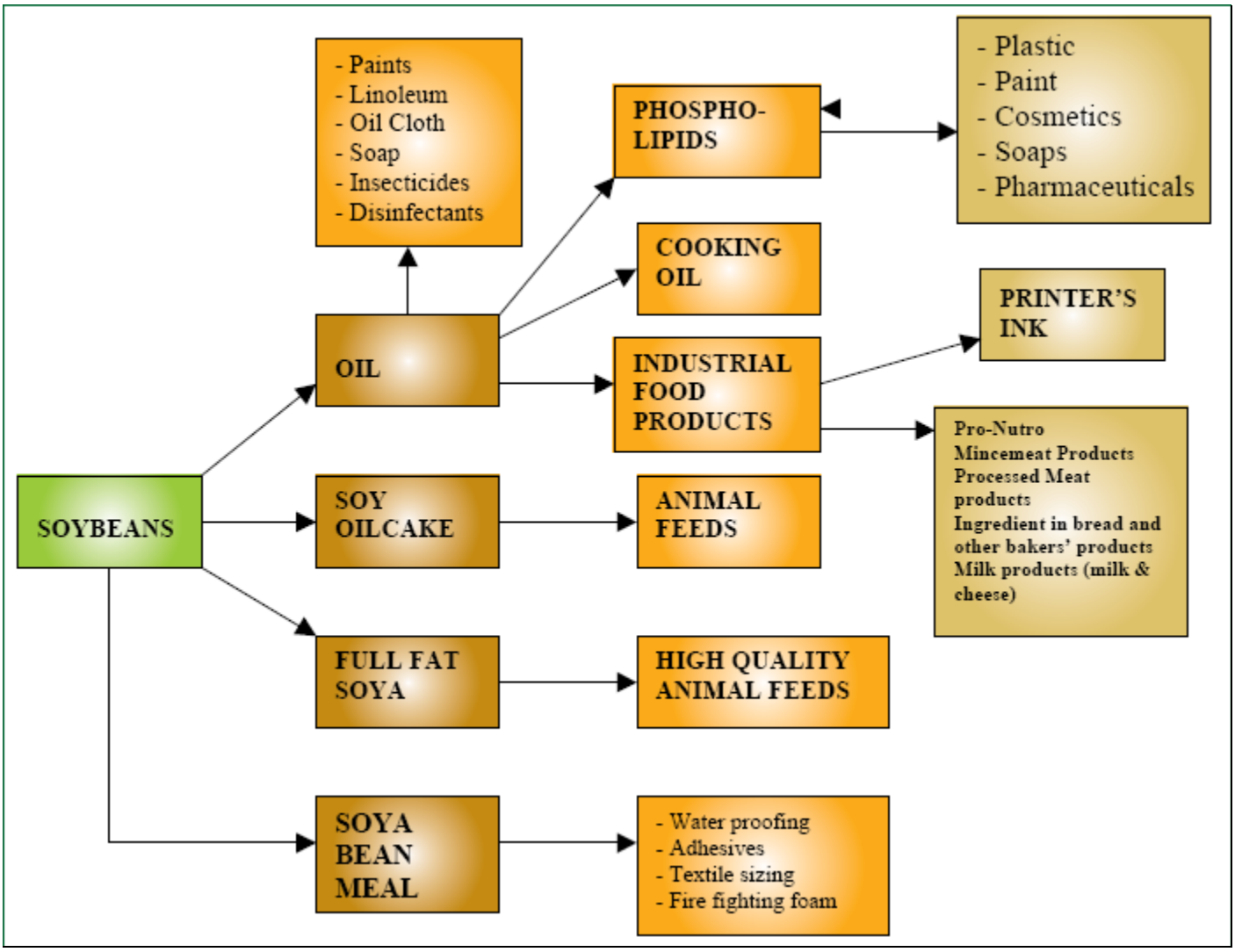


Source: Grain SA (http://www.grainsa.co.za/)

Soy sauce can be made from mature fermented beans while roasted seeds can be used as a coffee substitute. Soy flour can be prepared from beans while producing full fat flour with about 20% oil. The flour is used in bakeries and other food products, as additives and extenders to cereal flour and meat products and in health foods. Other industrial uses of the oil include, manufacture of paints, linoleum, oilcloth, printing inks, soap, insecticides and disinfectants. Lecithin phospholipids that are obtained as a by-product of the oil industry are used as wetting and stabilizing agents in food, cosmetics, pharmaceuticals, leather, paint, plastic, soaps and detergent industries. Soybean meal and soybean protein are used in the manufacture of synthetic fibre, adhesives, textile sizing, waterproofing and firefighting foam. The straw can be used to make paper that is stiffer than that made from wheat straw. Soybean meal, for which there is an increasing demand, is a very rich protein feedstuff for livestock while the vegetative portions of the plant can be used as silage, hay, pasture or ploughed in as green manure (DAFF, 2011). The various uses of soybeans are illustrated in Figure 2.

Since this is an application for commodity clearance approval of DAS-68416-4, the intended categories of users belong to the soybean crushing and packaging industry and their customers, as well as the consumers of soybean and soybean products.

Figure 2. The soybean value chain tree: Explaining its various uses



Source (National Agricultural Marketing Council [NAMC], 2011)

3.2 Specify the exact conditions of use of the product.

There is only limited animal feed use, and no food use for unprocessed soybeans, since they contain anti-nutrient compounds, such as trypsin inhibitors and lectins. Soybeans must therefore be heated prior to consumption to reduce the levels of these compounds. Whole soybeans are used to produce soy sprouts, baked soybeans, roasted soybeans, full fat soy flour and the traditional soy foods (miso, soy milk, soy sauce, and tofu). In addition to the use of whole oil for human consumption, refined soybean oil has many other technical and industrial applications. Glycerol, fatty acids, sterols, and lecithin are all derived from soybean oil (OECD, 2001). Soybean hulls are generally removed from the beans before oil extraction and used in animal feeds as carriers, i.e., to provide bulk in animal feed supplements. Soybean foliage can also be used as forage or hay. For use as forage the plants are harvested between the time when the plants reach the sixth node stage to the beginning of pod formation while soybean hay is usually harvested at mid-to-full bloom, before the bottom leaves begin to fall and when pods are approximately 50%

developed (OECD, 2001). These however would not apply to the product in this application, DAS-68416-4, since this application is for commodity clearance approval, and not cultivation, of DAS-68416-4 soybean in South Africa. In addition, no forage or hay from DAS-68416-4 would be imported.

The domestic uses of soybeans consist mostly of soybeans processed for animal feed-soybean meal and soybean oil (South African Bureau for Food and Agricultural Policy [BFAP], 2011). For instance, during 2009/10, 79.9 % (701 055 tons) of the total available soybean meal was used by the Animal Feed Manufacturers Association (AFMA) members. Processing capacity for high protein soybean meal for animal feed is currently 127 000 tons per annum and it is expected to increase to 327 000 tons per annum in the near future while processing capacity for high protein soybean meal for human consumption is currently 104 000 tons per annum. Soybeans processed for meal and oil increased by 20 % annually from 2005 to 2010.

3.3 Provide information on the proposed labelling of the product for marketing.

This is an application for commodity clearance approval, and not cultivation, of DAS-68416-4 soybean in the environment of South Africa.

The commercially cultivated soybean, including any GM events in the country of export, would determine which GM events would be in a consignment of grain destined for South Africa. As per the requirements of the Cartagena Protocol on Biosafety, if the consignment contains any GM soybean events, such as DAS-68416-4, the consignment would need to be accompanied by documentation stating that the consignment may contain GM soybean.

3.4 State whether the benefits of the product are available in any other non-genetically modified form. If so, state why the genetically modified form should be approved for general release when other, non-modified products are available.

There are no commercial soybean varieties that have been developed through conventional breeding practices in South Africa, which exhibit the herbicide tolerance traits present in DAS-68416-4 soybean.

3.5 Detail specific instructions for the storage and handling of GMO's that will avoid misuse or escape of the genetically modified plant into an environment for which it was not intended.

This is an application for commodity clearance approval, and not cultivation, of DAS DAS-68416-4 in the environment of South Africa.

As indicated previously, grain imports are made by international grain traders, with the time of importation dependent on the local or regional need for grain. The grain traders would, as per the requirements in terms of the Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997), obtain the necessary permits from the Registrar prior to

importation. Importation activities would thus be subjected to the conditions prescribed in the relevant import permits.

3.6 Detail the likelihood of the GMO being exported from South Africa, particularly if such export could result in the introduction of the plant into its centre of origin.

South Africa imports and exports soybean (NAMC, 2011). However, the South African soybean industry is not competitive when it comes to exports. Exports of soybeans from South Africa over the last few years have been very insignificant (DAFF, 2011) and it is therefore unlikely that grain imported into South Africa would be exported.

4. FOREIGN GENES AND GENE PRODUCTS

4.1 Identify all foreign genes in the genetically modified plant.

DAS-68416-4 was generated by *Agrobacterium*-mediated transformation using the plasmid pDAB4468 (Figure 3). The T-DNA insert in the plasmid contains a synthetic, plant-optimized sequence of the *aad*-12 gene from *Delftia acidovorans* and the *pat* gene from *Streptomyces viridochromogenes* (Figure 4). A summary of the genetic elements is given in Table 1.

Two gene expression cassettes were present in the pDAB4468 vector for insertion into soybeans. The *aad*-12 expression cassette, contained in the T-DNA insert of pDAB4468, is designed to express the plant-optimized aryloxyalkanoate dioxygenase (*aad*-12) gene that encodes the AAD-12 protein. The *aad*-12 gene was isolated from *Delftia acidovorans* and the synthetic version of the gene was optimized to modify the G+C codon bias to a level more typical for plant expression. The native and plant-optimized DNA sequences of *aad*-12 are 79.7% identical. The *aad*-12 gene encodes a protein of 293 amino acids that has a molecular weight of approximately 32 kDa. The insertion of *aad*-12 into soybean plants confers tolerance to herbicides such as 2,4-D by production of the aryloxyalkanoate dioxygenase-12 enzyme (AAD-12).

Delftia acidovorans, which has previously been identified as *Pseudomonas acidovorans* and *Comamonas acidovorans*, is a non glucose-fermenting, gram-negative, non spore-forming rod present in soil, fresh water, activated sludge, and clinical specimens (von Gravenitz, 1985; Tamaoka *et al*., 1987; Wen *et al*., 1999). *D. acidovorans* can be used to transform ferulic acid into vanillin and related flavor metabolites (Toms and Wood, 1970; Ramachandra and Ravishankar, 2000; Shetty *et al.*, 2006). This utility has led to a history of safe use for *D. acidovorans* in the food processing industry.

Expression of *aad*-12 in the T-DNA insert of pDAB4468 is controlled by the AtUbi10 promoter from *Arabidopsis thaliana* and AtuORF23 3' UTR sequence from *Agrobacterium tumefaciens* plasmid pTi15955. The AtUbi10 promoter is known to drive constitutive expression of the genes it controls (Norris *et al*., 1993).

A matrix attachment region (MAR) of RB7 from *Nicotiana tabacum* was included at the 5' end of the *aad*-12 PTU (plant transcriptional unit; includes promoter, gene, and terminator sequences) to potentially facilitate expression of the *aad*-12 gene in the plant.

Matrix attachments regions (MARs) are natural and abundant regions found in genomic DNA that are thought to attach to the matrix or scaffold of the nucleus. When positioned on the flanking ends of gene cassettes, some MARs have been shown to increase expression of transgenes and to reduce the incidence of gene silencing (Han *et al*., 1997; Abranches *et al*., 2005; Verma *et al.*, 2005). It is hypothesized that MARs may act to buffer effects from neighbouring chromosomal sequences that could destabilize the expression of genes (Allen *et al*., 2000). A MAR was included at the 5' end of *aad*-12 PTU to potentially facilitate the expression of AAD-12 in transgenic plants.

The *pat* expression cassette contained in the T-DNA insert of pDAB4468 is designed to express the plant-optimized phosphinothricin *N*-acetyl transferase (*pat*) gene that encodes the PAT protein. The *pat* gene was isolated from *Streptomyces viridochromogenes* and the synthetic version of the gene was optimized to modify the G+C codon bias to a level more typical for plant expression. The insertion of the *pat* gene into soybean genome confers tolerance to glufosinate and was used as a selectable marker during the soybean transformation. The *pat* gene encodes a protein of 183 amino acids that has a molecular weight of approximately 21 kDa. The *pat* gene has been widely used both as a selectable marker and to confer herbicide tolerance traits in previously deregulated products (e.g., Canadian Food Inspection Agency [CFIA], 1998; CFIA 1999; United States Department of Agriculture [USDA], 2005; European Food Safety Authority [EFSA], 2007).

Expression of the *pat* gene in the T-DNA insert of pDAB4468 is controlled by the CsVMV promoter from cassava vein mosaic virus and AtuORF1 3' UTR sequence from *Agrobacterium tumefaciens* plasmid pTi15955. The cassava vein mosaic virus is a double stranded DNA virus which infects cassava plants (*Manihot esculenta* Crantz) and has been characterized as a plant pararetrovirus belonging to the caulimovirus subgroup. The CsVMV promoter is known to drive constitutive expression of the genes it controls (Verdaguer *et al*., 1996).

Figure 3. Plasmid map of pDAB4468

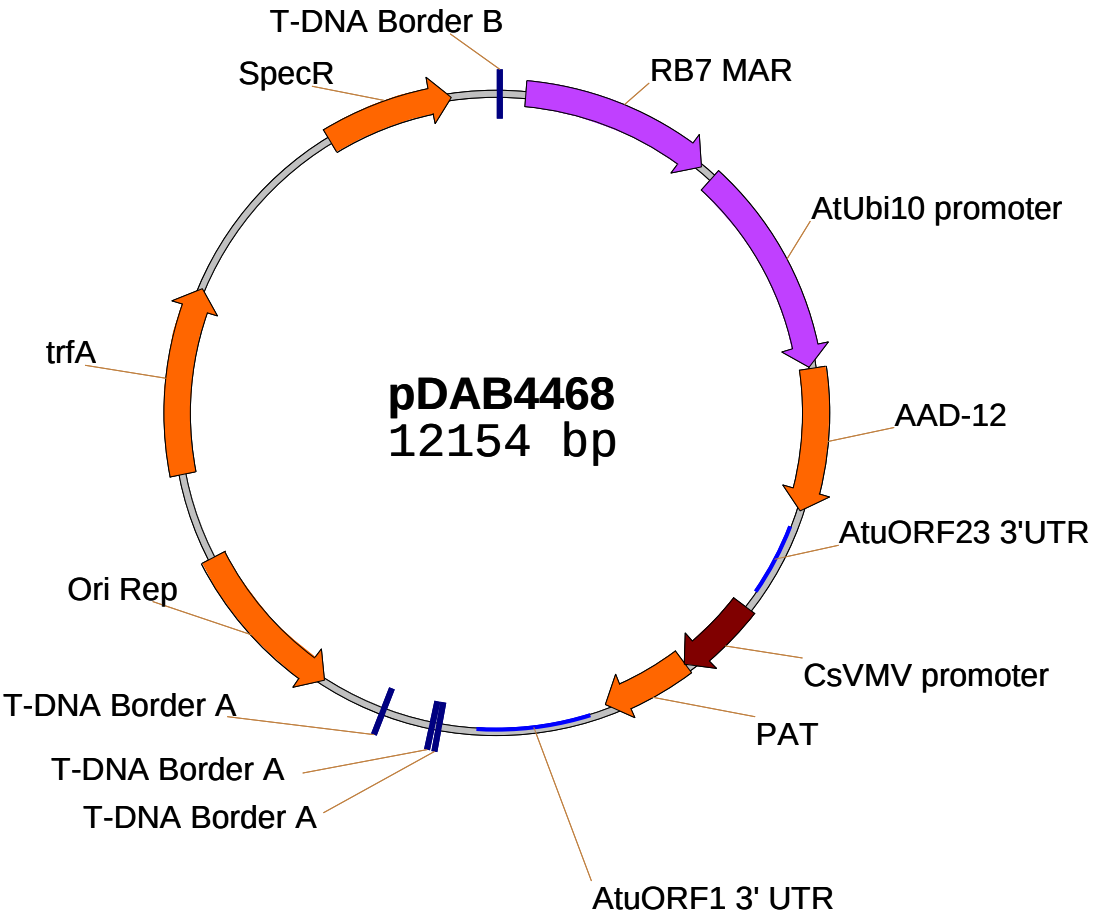
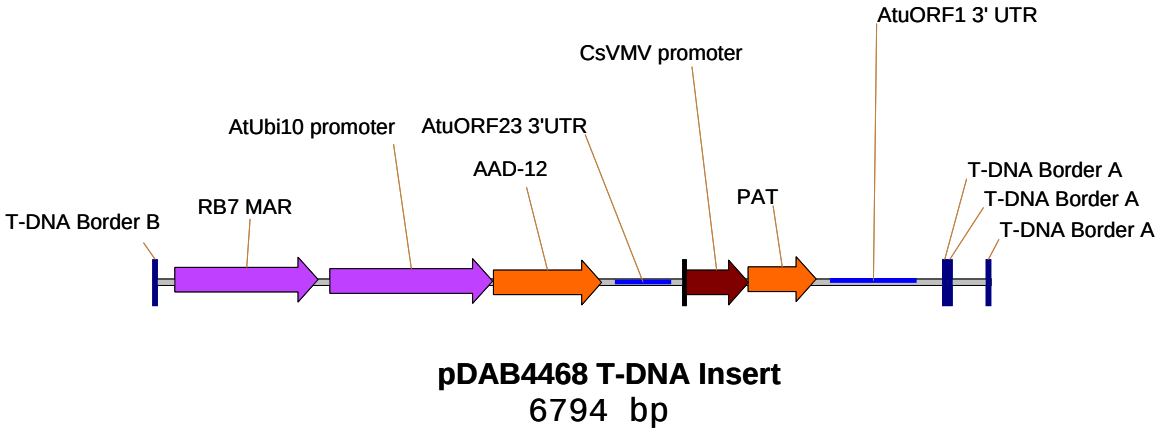


Figure 4. Diagram of T-DNA insert in plasmid pDAB4468



Molecular characterization of event DAS-68416-4 was conducted by southern blot analysis. The results demonstrate that:

(i) the transgene insert in soybean event DAS-68416-4 occurred as a simple integration of the T-DNA insert from plasmid pDAB4468, including a single, intact copy of the *aad*-12 and *pat* expression cassettes,

(ii) the event is stably integrated, as shown by its stable inheritance pattern across several breeding generations, and

(iii) no plasmid backbone sequences are present in DAS-68416-4 soybean (Song *et al.*, 2009a-Attachment A [**CBI-DELETED: Section 68(a),(b) and (c)ii of the Promotion of Access to Information Act**].

Table 1. Genetic elements of the plasmid pDAB4468

Location on pDAB4468[1]	Genetic Element	Size (base pairs)	Description
1−24	T-DNA Border B	24	Transferring DNA sequences
25−160	Intervening sequence	136	Sequence from Ti plasmid pTi15955 (Barker *et al*., 1983)
161−1326	RB7-MAR	1166	Matrix attachment region (MAR) from *Nicotiana tabacum* (Hall *et al*., 1991)
1327−1421	Intervening sequence	95	Sequence from plasmid pENTR/D-TOPO (Invitrogen Cat. No. A10465) and multiple cloning sites
1422−2743	AtUbi10	1322	*Arabidopsis thaliana* polyubiquitin UBQ10 comprising the promoter, 5' untranslated region and intron (Norris *et al*., 1993)
2744−2751	Intervening sequence	8	Sequence used for DNA cloning
2752−3633	*aad*-12	882	Synthetic, plant-optimized version of an aryloxyalkanoate dioxygenase gene from *Delftia acidovorans* (Wright *et al*., 2007)
3634−3735	Intervening sequence	102	Sequence used for DNA cloning
3736−4192	AtuORF23	457	3' untranslated region (UTR) comprising the transcriptional terminator and polyadenylation site of open reading frame 23 (ORF23) of *Agrobacterium tumefaciens* pTi15955 (Barker *et al*., 1983)
4193−4306	Intervening sequence	114	Sequence from plasmid pENTR/D-TOPO (Invitrogen Cat. No. A10465) and multiple cloning sites
4307−4823	CsVMV	517	Promoter and 5’ untranslated region derived from the cassava vein mosaic virus (Verdaguer *et al*., 1996)
4824−4830	Intervening sequence	7	Sequence used for DNA cloning
4831−5382	*pat*	552	Synthetic, plant-optimized version of phosphinothricin N-acetyl transferase (PAT) gene, isolated from *Streptomyces viridochromogenes* (Wohlleben *et al*., 1988)
5383−5484	Intervening sequence	102	Sequence from plasmid pCRI2.1(Invitrogen Cat. No. K205001) and multiple cloning sites

Table 1 (cont.). Genetic elements of the plasmid pDAB4468

Location on pDAB4468[1]	Genetic Element	Size (base pairs)	Description
5485−6188	AtuORF1	704	3' untranslated region (UTR) comprising the transcriptional terminator and polyadenylation site of open reading frame 1 (ORF1) of *Agrobacterium tumefaciens* pTi15955 (Baker *et al*., 1983)
6189−6416	intervening sequence	228	Sequence from Ti plasmid C58 (Zambryski *et al*., 1982)
6417−6440	T-DNA border A	24	Transferring DNA sequences
6441−6459	intervening sequence	19	Sequence from Ti plasmid C58 (Zambryski *et al*., 1982)
6460−6483	T-DNA border A	24	Transferring DNA sequences
6484−6770	intervening sequence	287	Sequence from Ti plasmid pTi15955 (Baker *et al*., 1983)
6771−6794	T-DNA border A	24	Transferring DNA sequences
6795-7173	Plasmid backbone sequences	379	Plasmid backbone sequences from RK2 plasmid (Stalker *et al*., 1981)
7174-8193	Ori Rep	1020	Replication origin sequences from RK2 plasmid (Stalker *et al*., 1981)
8194-8738	Plasmid backbone sequences	545	Plasmid backbone sequences from RK2 plasmid (Stalker *et al*., 1981)
8739-9887	Trf A	1149	Plasmid replication sequences for Trf A protein from RK2 plasmid (Stalker *et al*., 1981)
9888-11091	Plasmid backbone sequences	1204	Plasmid backbone sequences from RK2 plasmid (Stalker *et al*., 1981)
11092-11880	SpecR	789	Sequences for Spectinomycin resistance gene (Fling *et al*., 1985)
11881-12154	Plasmid backbone sequences	274	Plasmid backbone sequences for cloning

[1] Base pair position.

Detailed southern blot analysis was conducted using probes specific to the gene coding sequences, promoters, terminators, and other regulatory elements contained in the pDAB4468 transformation plasmid. The locations of each probe on the pDAB4468 plasmid are described in Table 2 and shown in Figure 5. The expected and observed fragment sizes with specific digest and probe combinations, based on the known restriction enzyme sites of the pDAB4468 plasmid are shown in Table 3, Figure 6 and Figure 7, respectively. The southern blot analyses described here made use of two types of restriction fragments: a) internal fragments in which known enzyme restriction sites are completely contained within the T-DNA insert of pDAB4468 and b) border fragments in which a known enzyme site is located within the T-DNA insert and a second site is located in the soybean genome flanking the insert. Border fragment sizes vary by event because they rely on the DNA sequence of flanking genomic regions. Since integration sites are unique for each event, border fragments provide a means to determine the number of DNA insertions and to specifically identify the event.

Genomic DNA for southern blot analysis was prepared from leaf material of individual DAS-68416-4 soybean plants from three distinct breeding generations (Note: there were two populations in the T5 generation). Genomic DNA from leaves of non-transgenic variety Maverick was used as the control material. Plasmid DNA of pDAB4468 added to genomic DNA from the conventional control served as the positive control for the southern blot analysis.

The expected restriction fragments of the inserted DNA are shown in Figure 7. Southern blot analysis showed that event DAS-68416-4 contains a single intact copy of the *aad*-12 and *pat* expression cassettes integrated at a single locus. No vector backbone sequences were detected in event DAS-68416-4 (Song *et al*., 2009a).

Table 2. List of probes and their positions in plasmid pDAB4468

Probe Name	Size (bp)	Location in pDAB4468
aad-12	882	2752 – 3633
pat	552	4831 – 5382
RB7	1010	306 – 1315
AtUbi10	760	1422 – 2181
CsVMV	478	4332 – 4809
AtuORF1 3’ UTR	684	5474 – 6157
AtuORF23 3’ UTR	413	3762 – 4174
T-DNA Flanking A	339	6793 – 7131
T-DNA Flanking B	303	11894 – 42
*Spec*R	789	11092 – 11880
Backbone 1	1310	9854 – 11163
Backbone 2	1728	8157 – 9884
Ori-Rep	1068	7111 – 8178

Figure 5. Location of probes on pDAB4468 used in southern blot analysis of DAS-68416-4 soybean (Song *et al.*, 2009a)

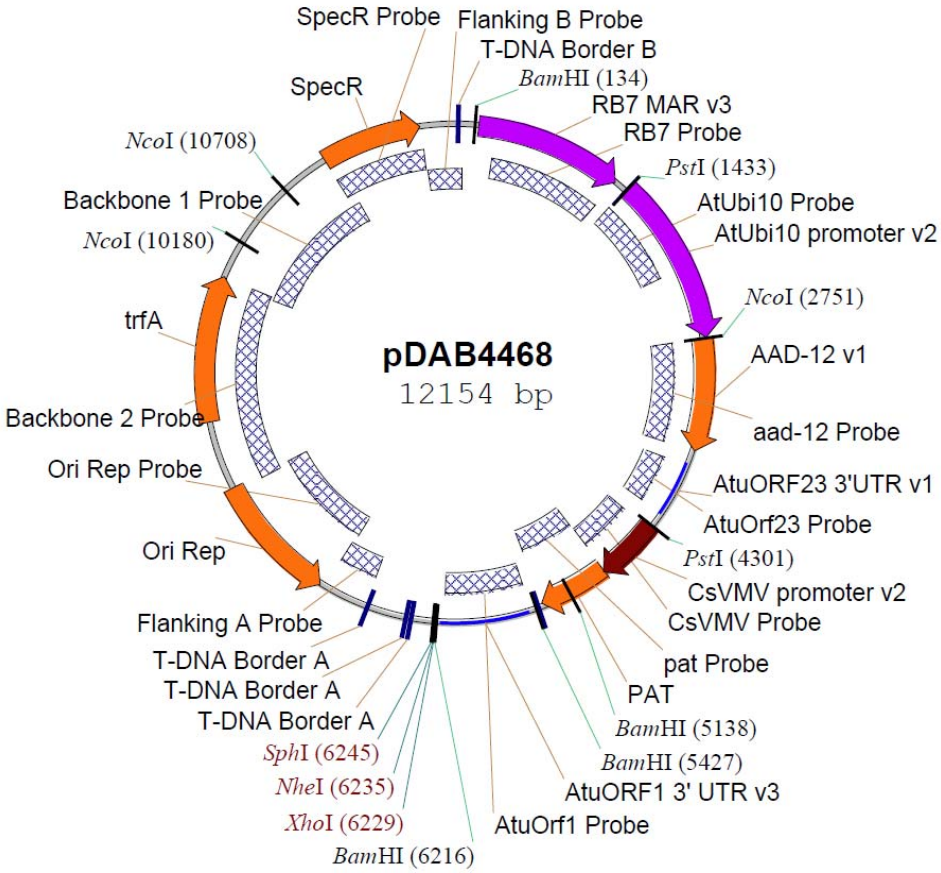


Table 3. Predicted and observed sizes of hybridizing fragments in southern blot analyses of DAS-68416-4 soybean

Probe	Restriction Enzyme	Sample	Southern Blot Figure	Fragment Size (bp)	
				Expected	Observed
aad-12	*Nco* I	Plasmid pDAB4468	8, 11	7429	~7400
		DAS-68416-4	8,11	> 4043*	~5500*
		Maverick	8, 11	None	None
	Sph I/*Xho* I	Plasmid pDAB4468	9, 11	12138	~12100
		DAS-68416-4	9, 11	> 6229*	~8500*
		Maverick	9, 11	None	None
	Nhe I/*Xho* I	Plasmid pDAB4468	10, 11	12148	~12100
		DAS-68416-4	10, 11	> 6229*	~7200*
		Maverick	10, 11	None	None
pat	*Nco* I	Plasmid pDAB4468	16, 19	7429	~7400
		DAS-68416-4	16, 19	> 4043*	~5500*
		Maverick	16, 19	None	None
	Sph I/*Xho* I	Plasmid pDAB4468	17, 19	12138	~12100
		DAS-68416-4	17, 19	> 6229*	~8500*
		Maverick	17, 19	None	None
	Nhe I/*Xho* I	Plasmid pDAB4468	18, 19	12148	~12100
		DAS-68416-4	18, 19	> 6229*	~7200*
		Maverick	18, 19	None	None
aad-12	*Pst* I (Release PTU)	Plasmid pDAB4468	12, 13A	2868	~2900
		DAS-68416-4	12, 13A	2868	~2900
		Maverick	12, 13A	None	None
AtUbi10		Plasmid pDAB4468	14, 13B	2868	~2900
		DAS-68416-4	14, 13B	2868	~2900
		Maverick	14, 13B	None	None
AtuORF23		Plasmid pDAB4468	15, 13C	2868	~2900
		DAS-68416-4	15, 13C	2868	~2900
		Maverick	15, 13C	None	None
pat	*Pst* I/*Xho* I (Release PTU)	Plasmid pDAB4468	20, 21A	1928	~1900
		DAS-68416-4	20, 21A	1928	~1900
		Maverick	20, 21A	None	None
CsVMV		Plasmid pDAB4468	22, 21B	1928	~1900
		DAS-68416-4	22, 21B	1928	~1900
		Maverick	22, 21B	None	None
AtuORF1		Plasmid pDAB4468	23, 21C	1928	~1900
		DAS-68416-4	23, 21C	1928	~1900
		Maverick	23, 21C	None	None
RB7	*Bam*H I/*Nco* I	Plasmid pDAB4468	24, 26A	2617	~2600
		DAS-68416-4	24, 26A	2617	~2600
		Maverick	24, 26A	None	None
AtUbi10		Plasmid pDAB4468	25, 26B	2617	~2600
		DAS-68416-4	25, 26B	2617	~2600
		Maverick	25, 26B	None	None

Table 3 (cont.). Predicted and observed sizes of hybridizing fragments in southern blot analyses of DAS- 68416-4 soybean

Probe	Restriction Enzyme	Sample	Southern Blot Figure	Fragment Size (bp)	
				Expected	Observed
Flanking A	*Nco* I	Plasmid pDAB4468	27, 31	7429	~7400
		DAS-68416-4	27, 31	None	None
		Maverick	27, 31	None	None
	Sph I/*Xho* I	Plasmid pDAB4468	29, 31	12138	~12100
		DAS-68416-4	29, 31	None	None
		Maverick	29, 31	None	None
Backbone1	*Nco*I	Plasmid pDAB4468	27, 31	7429, 4197, 528	~7400, ~4200, ~500
		DAS-68416-4	27, 31	None	None
		Maverick	27, 31	None	None
	Sph I/*Xho* I	Plasmid pDAB4468	29, 31	12138	~12100
		DAS-68416-4	29, 31	None	None
		Maverick	29, 31	None	None
SpecR	*Nco* I	Plasmid pDAB4468	27, 31	4197	~4200
		DAS-68416-4	27, 31	None	None
		Maverick	27, 31	None	None
	Sph I/*Xho* I	Plasmid pDAB4468	29, 31	12138	~12100
		DAS-68416-4	29, 31	None	None
		Maverick	29, 31	None	None
Flanking B	*Nco* I	Plasmid pDAB4468	28, 32	4197	~4200
		DAS-68416-4	28, 32	None	None
		Maverick	28, 32	None	None
	Sph I/*Xho* I	Plasmid pDAB4468	30, 32	12138	~12100
		DAS-68416-4	30, 32	None	None
		Maverick	30, 32	None	None
Backbone 2	*Nco* I	Plasmid pDAB4468	28, 32	7429	~7400
		DAS-68416-4	28, 32	None	None
		Maverick	28, 32	None	None
	Sph I/*Xho* I	Plasmid pDAB4468	30, 32	12138	~12100
		DAS-68416-4	30, 32	None	None
		Maverick	30, 32	None	None
Ori-Rep	*Nco* I	Plasmid pDAB4468	28, 32	7429	~7400
		DAS-68416-4	28, 32	None	None
		Maverick	28, 32	None	None
	Sph I/*Xho* I	Plasmid pDAB4468	30, 32	12138	~12100
		DAS-68416-4	30, 32	None	None
		Maverick	30, 32	None	None

Note: * These bands include border region of soybean genome;

1. Expected fragment sizes are based on the plasmid map of the pDAB4468 and its T-DNA insert as shown in Figure 6 and Figure 7.
2. Observed fragment sizes are considered approximately from these analyses and are based on the indicated sizes of the DIG-labeled DNA Molecular Weight Marker II fragments. Due to the incorporation of DIG molecules for visualization, the marker fragments typically run approximately 5-10% larger than their actual indicated molecular weight.

Figure 6. Plasmid map of pDAB4468 with restriction enzyme sites used for southern blot analysis

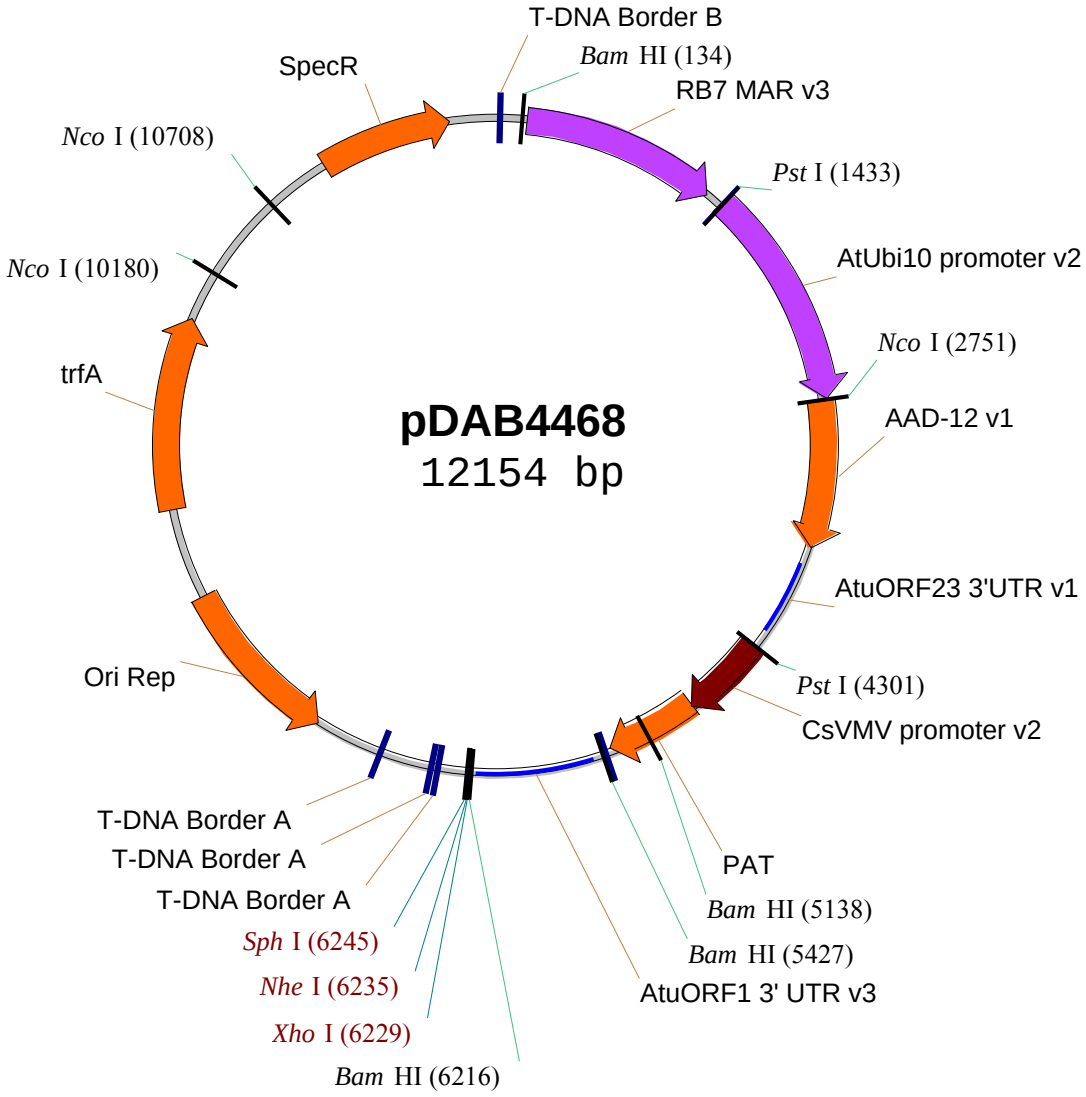
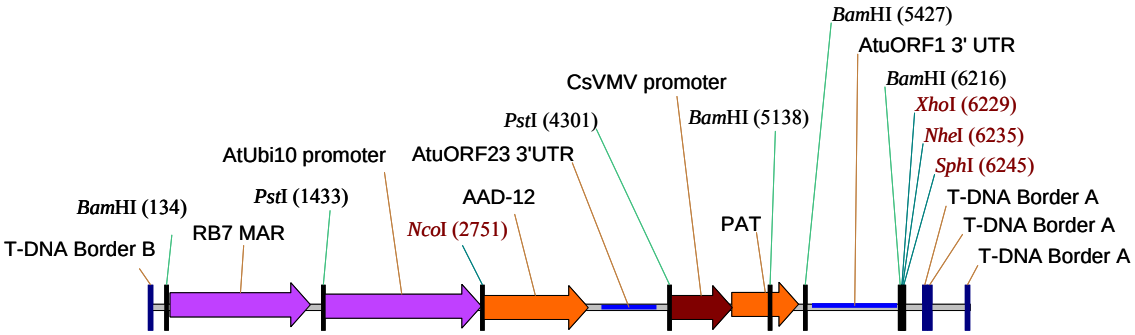


Figure 7. pDAB4468 T-DNA insert[1], restriction enzymes used in DNA digestion and expected hybridization bands



pDAB4468 T-DNA Insert
6794 bp

Nco I	> 2751 bp	> 4043 bp				
Nhe I/*Xho* I	> 6229 bp					> 559 bp
Sph I/*Xho* I	> 6229 bp					> 549bp
Pst I	> 1433 bp	2868 bp	> 2493 bp			
Pst I/*Xho* I	> 1433 bp	2868 bp	1928 bp			> 565 bp
*Bam*H I/*Nco* I	2617 bp	2387 bp	289 bp	789 bp	> 578 bp	

[1] *"Representation of insertion site before sequencing data was available -6794 bp corresponds to the expected size of pDAB4468 T-DNA (source Song et al. 2009)."*

Analysis of the *aad*-12 gene

To characterize the *aad*-12 gene insert in event DAS-68416-4, restriction enzymes *Nco* I, *Sph* I/*Xho* I and *Nhe* I/*Xho* I were used (Song *et al*., 2009a; Song *et al*., 2009b-Attachment B [**CBI-DELETED: Section 68(a),(b) and (c)ii of the Promotion of Access to Information Act**]). These enzymes possess unique restriction sites in the pDAB4468 T-DNA insert. Border fragments of >4043 bp, >6229 bp, >6229 bp were predicted to hybridize with the *aad*-12 gene probe following digestion with *Nco* I, *Sph* I/*Xho* I and *Nhe* I/*Xho* I enzymes respectively (Table 3). The results showed single hybridization bands of ~5500 bp, ~8500 bp and ~7200 bp respectively when *Nco* I, *Sph* I/*Xho* I and *Nhe* I/*Xho* I enzymes were used, indicating a single insertion site of *aad*-12 in the soybean genome of event DAS-68416-4 (Figure 8, Figure 9, Figure 10, Figure 11). An enzyme digestion with *Pst* I was conducted to release a PTU fragment of 2868 bp which contains the AtUbi10 promoter, *aad*-12 gene, and AtuORF23 terminator sequences. The predicted ~2900 bp fragment was observed following the *Pst* I digestion and hybridization with *aad*-12 probe (Figure 12, Figure 13A). Results obtained from the individual and double enzyme digestions indicated that a single copy of an intact *aad*-12 expression cassette from pDAB4468 was inserted into the soybean genome of event DAS-68416-4 as shown in the restriction map in Figure 7 (Song *et al*., 2009a).

Analysis of the AtUbi10 promoter

Restriction enzyme *Pst* I was used to characterize the AtUbi10 promoter region for *aad*-12 in event DAS-68416-4 (Song *et al*., 2009a). *Pst* I digestion was expected to release a PTU fragment of 2868 bp which contains the AtUbi10 promoter, *aad*-12 gene, and AtuORF23 terminator sequences. The predicted ~2900 bp fragment was observed following the *Pst* I digestion and hybridization with AtUbi10 promoter probe (Figure 13B, Figure 14). The AtUbi10 promoter was further characterized with a double digestion of *Bam*H I and *Nco* I which releases a fragment of 2617bp containing AtUbi10 promoter and RB7 MAR element. The predicted ~2600 bp fragment was detected following the enzyme digestion and hybridization with AtUbi10 promoter probe (Figure 25, Figure 26B). Results obtained with *Pst* I or *Bam*H I/*Nco* I digestion of the DAS-68416-4 sample followed by AtUbi10 promoter probe hybridization further confirmed that a single copy of an intact *aad*-12 PTU from plasmid pDAB4468, along with a RB7 MAR element at its 5' end, was inserted into the soybean genome of event DAS-68416-4 (Song *et al*., 2009a).

Analysis of the AtuORF23 3'UTR

The terminator sequence, AtuORF23, for *aad*-12 in event DAS-68416-4 was characterized using *Pst* I digestion, followed by hybridization of AtuORF23 probe (Song *et al*., 2009a). *Pst* I was expected to release a PTU fragment of 2868 bp which contains the AtUbi10 promoter, *aad*-12 gene, and AtuORF23 terminator sequences. The predicted ~2900 bp fragment was observed following the enzyme digestion and hybridization with AtuORF23 probe (Figure 13C, Figure 15). Results obtained with *Pst* I digestion of the DAS-68416-4 sample followed by AtuORF23 probe hybridization further confirmed that a single copy of an intact *aad*-12 PTU from plasmid pDAB4468 was inserted into the soybean genome of event DAS-68416-4 (Song *et al*., 2009a).

Analysis of the *pat* gene

To characterize the *pat* gene insert in event DAS-68416-4, restriction enzymes *Nco* I, *Sph* I/*Xho* I and *Nhe* I/*Xho* I were used (Song *et al*., 2009a). These enzymes possessed unique restriction sites in the pDAB4468 T-DNA insert. Border fragments of >4043 bp, >6229 bp, >6229 bp were predicted to hybridize with the *pat* gene probe following digestion with *Nco* I, *Sph* I/*Xho* I and *Nhe* I/*Xho* I enzymes respectively (Table 3). The results showed single hybridization bands of ~5500 bp, ~8500 bp and ~7200 bp respectively when *Nco* I, *Sph* I/*Xho* I and *Nhe* I/*Xho* I enzymes were used, indicating a single site of *pat* gene insertion in the soybean genome of event DAS-68416-4 (Figure 16, Figure 17, Figure 18, Figure 19). An enzyme digestion with *Pst* I/*Xho* I was conducted to release a PTU fragment of 1928 bp which contained the CsVMV promoter, *pat* gene, and AtuORF1 terminator sequences. The predicted ~1900 bp fragment was observed following the enzyme digestion and hybridization with *pat* probe (Figure 20, Figure 21A). Results obtained from the individual and double enzyme digestions indicated that a single copy of an intact *pat* expression cassette from pDAB4468 was inserted into the soybean genome of event DAS-68416-4 as shown in the restriction map in Figure 7 (Song *et al*., 2009a).

Analysis of the CsVMV promoter

Restriction enzyme combination of *Pst* I/*Xho* I was used to characterize the CsVMV promoter region for *pat* in event DAS-68416-4 (Song *et al*., 2009a). *Pst* I/*Xho* I digestion was expected to release a PTU fragment of 1928 bp which contains the CsVMV promoter, *pat* gene, and AtuORF1 terminator sequences. The predicted ~1900 bp fragment was observed following the enzyme digestion and hybridization with CsVMV promoter probe (Figure 21B, Figure 22). Results obtained with *Pst* I/*Xho* I digestion of the DAS-68416-4 sample followed by CsVMV promoter probe hybridization further confirmed that a single copy of an intact *pat* PTU from plasmid pDAB4468 was inserted into the soybean genome of event DAS-68416-4.

Analysis of the AtuORF1 3'UTR

The terminator sequence, AtuORF1, for *pat* in event DAS-68416-4 was characterized using *Pst* I/*Xho* I double digestion, followed by hybridization of AtuORF1 probe (Song *et al*., 2009a). The double digestion of *Pst* I/*Xho* I was expected to release a PTU fragment of 1928 bp which contained the CsVMV promoter, *pat* gene, and AtuORF1 terminator sequences. The predicted ~1900 bp fragment was observed following the enzyme digestion and hybridization with AtuORF1 probe (Figure 21C, Figure 23). Results obtained with *Pst* I/*Xho* I double digestion of the DAS-68416-4 sample followed by AtuORF1 probe hybridization further confirmed that a single copy of an intact *pat* PTU from plasmid pDAB4468 was inserted into the soybean genome of event DAS-68416-4 (Song *et al*., 2009a).

Analysis of the RB7 MAR

Restriction enzyme combination of *Bam*H I and *Nco* I was selected to characterize the RB7 MAR elements from the T-DNA insert in pDAB4468 (Table 3) (Song *et al*., 2009a). A double digestion with *Bam*H I and *Nco* I was expected to release a fragment of 2617 bp containing the RB7 MAR and AtUbi10 promoter. The predicted ~2600 bp fragment was observed following the double enzyme digestion and hybridization with RB7 MAR and Atubi10 probe, respectively (Figure 24, Figure 25, Figure 26A, Figure 26B). Results

obtained with *Bam*H I/*Nco* I double digestion of the DAS-68416-4 sample followed by hybridization with RB7 MAR (Figure 24, Figure 26 A) and AtUbi10 (Figure 25, Figure 26B) further confirmed that a single copy of an intact RB7 MAR, along with an intact *aad*-12 PTU from plasmid pDAB4468, was inserted into the soybean genome of event DAS-68416-4 (Song *et al*., 2009a).

Confirmation of absence of vector backbone DNA

To verify that no plasmid vector backbone sequences exist in event DAS-68416-4, six probes covering the whole backbone region of pDAB4468 were used to hybridize the blots from digestions with *Nco* I and *Sph* I/*Xho* I (Table 2, Figure 5) (Song *et al*., 2009a). For the T5-B generation, a blot from digestion with *Nhe* I/*Xho* I was also hybridized with backbone probes. The probes were grouped into 2 sets by mixing them with equal ratio for hybridization purposes. Probe Set 1 included backbone1, flanking A, and SpecR, and Probe Set 2 included backbone 2, flanking B, and Ori-Rep (Figure 5, Table 3). The blots were hybridized with Probe Set 1, and then followed by Probe Set 2 after complete removal of previously deployed probes. No hybridization signals were detected in any sample across the T3 to T5 generations (Table 3, Figure 27, Figure 28, Figure 29, Figure 30, Figure 31, Figure 32[1]) except for the positive controls, indicating no backbone sequences from pDAB4468 were incorporated into event DAS-68416-4.

Stability of the insert across generations

Southern blot hybridizations were conducted with three distinct generations, T3, T4, and 2 populations of T5 (T5-A and T5-B), of event DAS-468416-4. Prior to initiation of southern blot analysis, all plants were tested for AAD-12 protein expression using a lateral flow strip test kit to allow confirmation of AAD-12 expression positive plants. All of the genetic element probes: *aad*-12 gene, AtUbi10 promoter, AtuORF23 terminator, CsVMV promoter, *pat* gene, AtuORF1 terminator, and RB7 MAR, and the backbone of plasmid pDAB4468, were hybridized with the three generations of DAS-68416-4 soybean. Results across all DAS-68416-4 samples in three generations were as expected (Table 3, Figures 8 – 32[1]), indicating stable integration and inheritance of the intact, single copy insert across multiple generations of DAS-68416-4 soybean (Song *et al*., 2009a).

[1] According to the Figure 5, three bands are expected (7429 bp , 4197 bp, 528 bp) when the plasmid control DNA is mixed with non-transgenic Maverick DNA and digested with *Nco* I followed by hybridization with Probe Set 1 - including probes of Backbone 1, Flanking A, and *Spec*R -, whilst two bands are expected (7429 bp and 4197 bp) when hybridized with Probe Set 2 -including probes of Backbone 2, Flanking B, and *Ori*Rip.

We note that in Fig 32, 3 bands were present (7429 bp, 4197 bp, 528 bp) in Lane 2, instead of the predicted two (7429 bp and 4197 bp) where plasmid control DNA is mixed with non-transgenic Maverick DNA. We understand that this is possibly due to an error during the hybridization of the blot since a subsequent confirmatory southern blot (see Figure 1 in Song, 2009a), where plasmid control DNA is mixed with non-transgenic Maverick DNA, digested with *Nco* I and hybridised with individual probes - Backbone 1, Backbone 2, *Spec*R, and *Ori*Rip - matched the predicted pattern of plasmid pDAB4468 digested with *Nco* I, as shown in Figure 5 of current application.

Figure 8. Southern blot analysis of *Nco* I digest with *aad*-12 probe

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3 (Panel A), T4, T5-A (Panel B) and the non-transgenic control were digested with *Nco* I and hybridized with *aad*-12 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signal in Lane 15 was due to a larger amount of DNA recovered after digestion. Panel A and B were from the same blot and hybridized in the same container).

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #2	11	T4 #9
3	control (Maverick) #3	12	T5 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

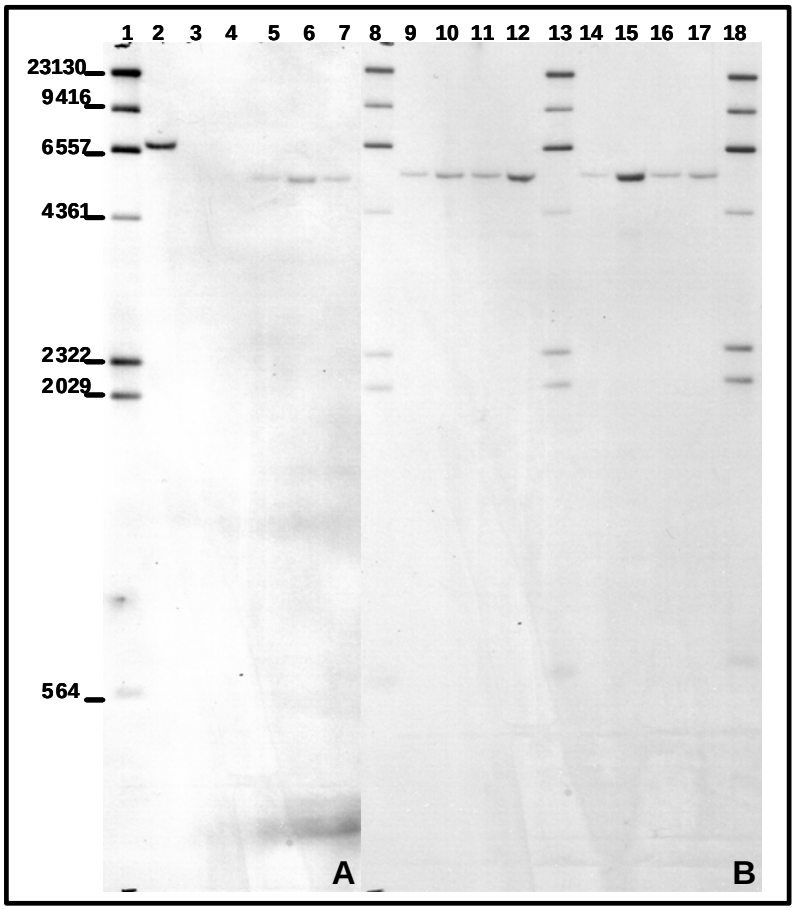


Figure 9. Southern blot analysis of *Sph* I/*Xho* I digest with *aad*-12 probe

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3 (Panel A), T4, T5-A (Panel B) and the non-transgenic control were digested with *Sph* I/*Xho* I and hybridized with *aad-12* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signals in Lane 10 and 15 were due to a larger amount of DNA recovered after digestion. Panel A and B were from the same blot and hybridized in the same container).

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #2	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

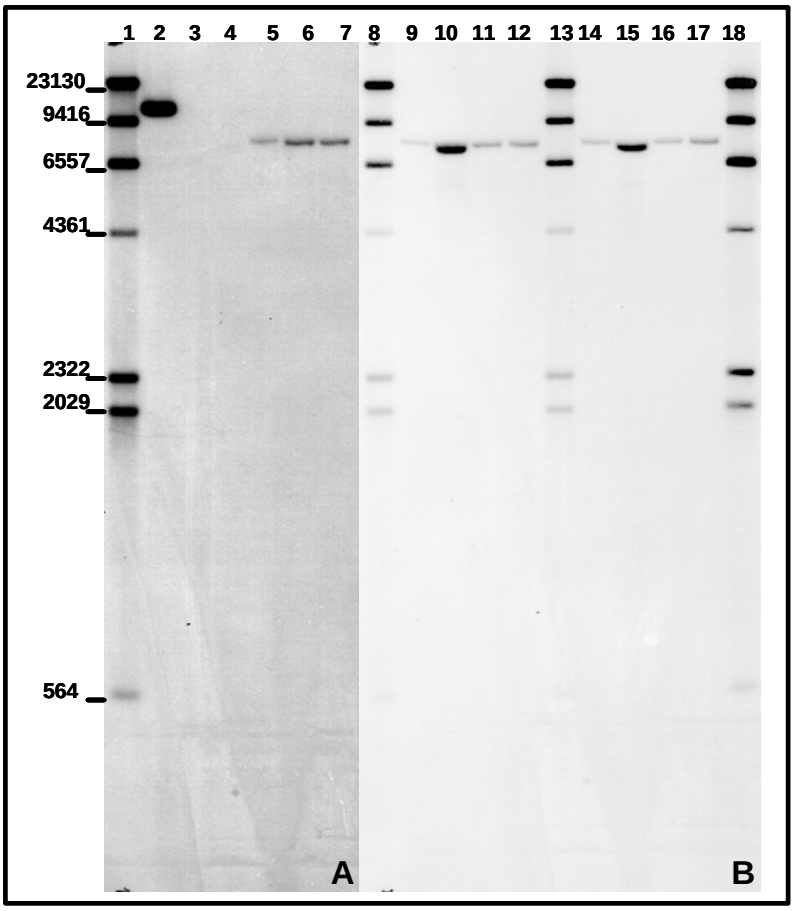


Figure 10. Southern blot analysis of *Nhe* I/*Xho* I digest with *aad*-12 probe

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3, T4, T5-A and the non-transgenic control were digested with *Nhe* I/*Xho* I and hybridized with *aad*-12 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signal in Lane 9 was due to a larger amount of DNA recovered after digestion).

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #2	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

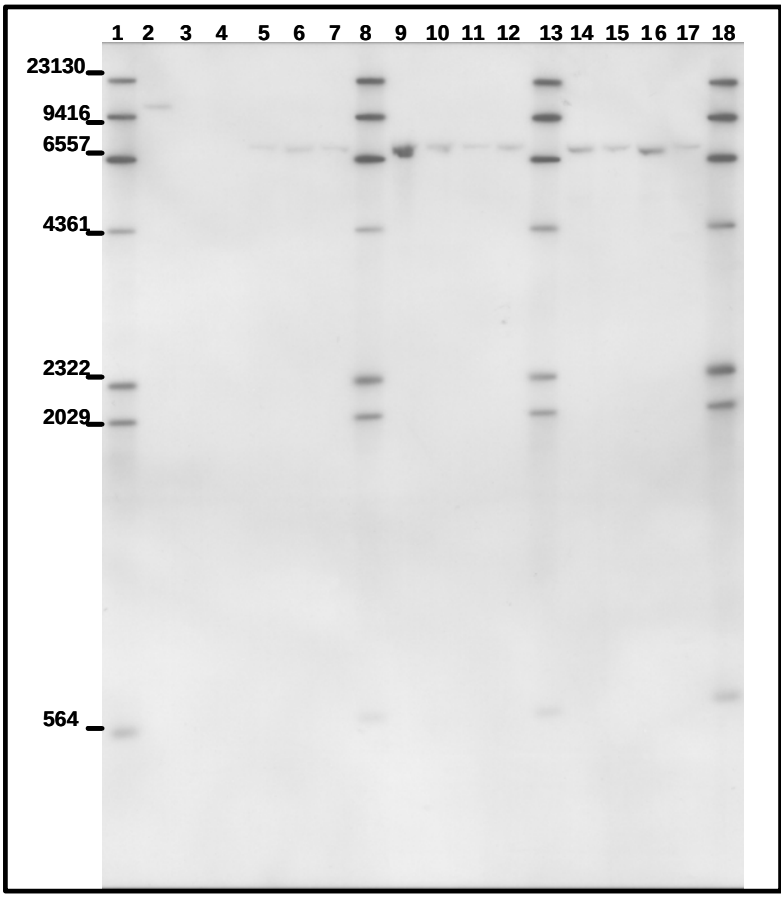


Figure 11. Southern blot analysis of *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I digests of T5-B generation with *aad*-12 probe

DNA isolated from individual plants of soybean event DAS-68416-4 generation T5-B and the non-transgenic control was digested with *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I and hybridized with *aad*-12 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signal in Lane 19 was due to a larger amount of DNA recovered after digestion).

Lane	Description	Enzyme	Lane	Description	Enzyme
1	DNA molecular marker (bp)		12	T5-B #1	*Sph* I/ *Xho* I
2	pDAB4468 + control (Maverick) #2	*Nco* I	13	T5-B #4	
3	control (Maverick) #2		14	T5-B #6	
4	control (Maverick) #3		15	T5-B #8	
5	T5-B #1		16	pDAB4468 + control (Maverick) #2	*Nhe* I/ *Xho* I
6	T5-B #4		17	control (Maverick) #2	
7	T5-B #6		18	control (Maverick) #3	
8	T5-B #8		19	T5-B #1	
9	pDAB4468 + control (Maverick) #4	*Sph* I/ *Xho* I	20	T5-B #4	
10	control (Maverick) #4		21	T5-B #6	
11	control (Maverick) #5		22	T5-B #8	

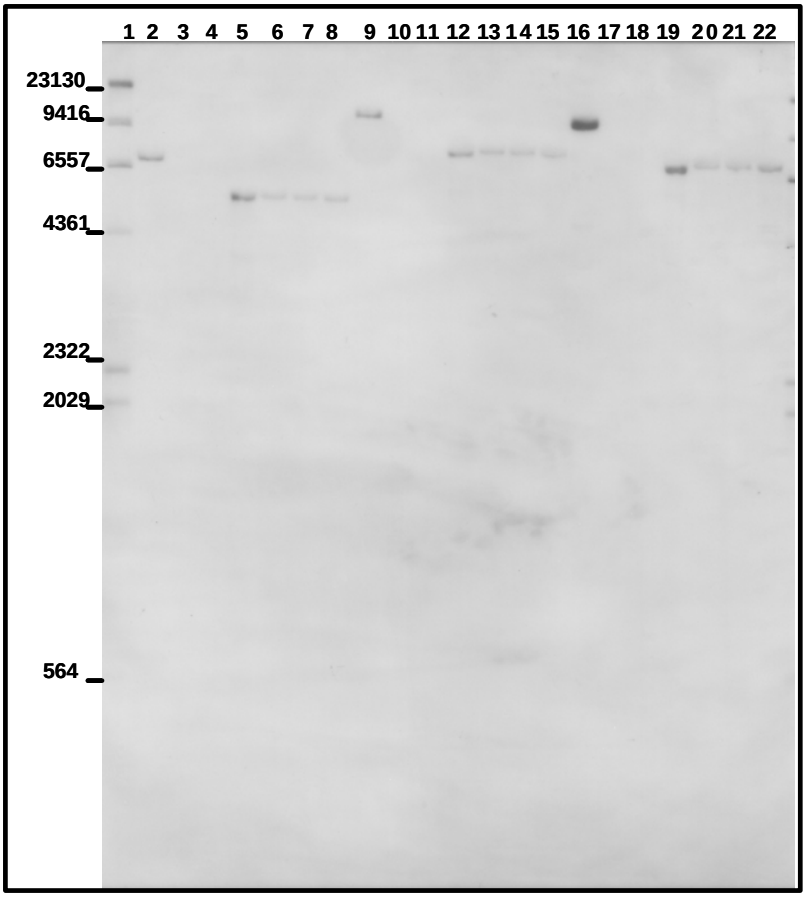


Figure 12. Southern blot analysis of *Pst* I digest with *aad*-12 probe

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3, T4, T5-A and the non-transgenic control were digested with *Pst* I and hybridized with *aad*-12 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively weak signal in Lane 7 was due to a lesser amount of DNA recovered after digestion).

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #2	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

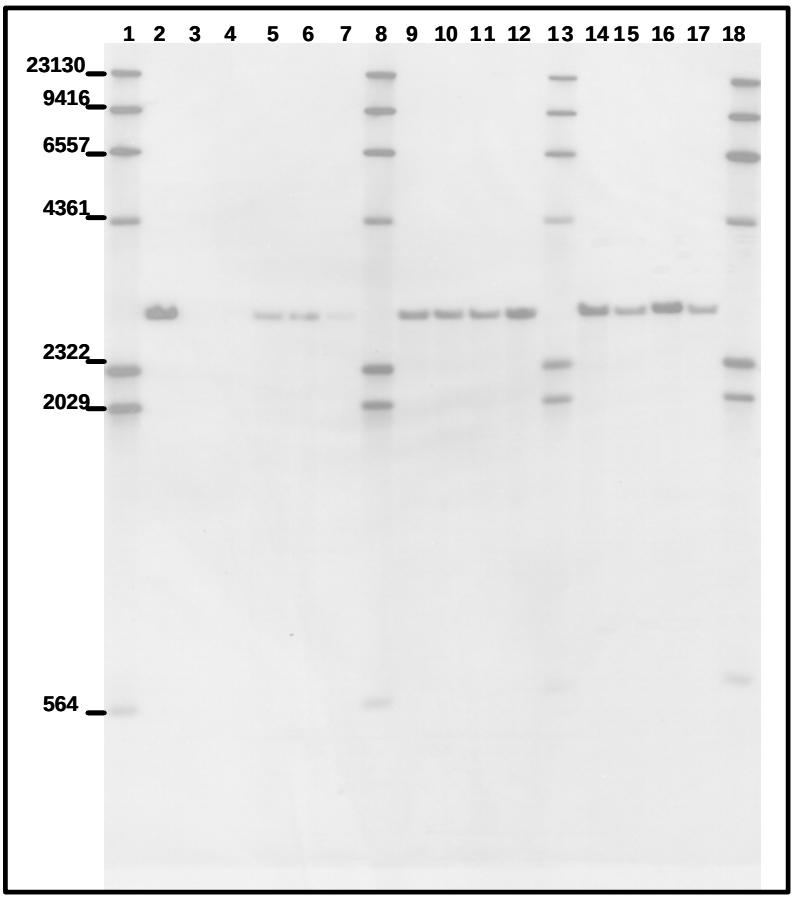


Figure 13. Southern blot analysis of *Pst* I digest of T5-B generation with *aad*-12, AtUbi10, and AtuORF23 probes

DNA isolated from individual plants of soybean event DAS-68416-4 generation T5-B and the non-transgenic control were digested with *Pst* I and hybridized with *aad*-12 (Panel A), AtUbi10 (Panel B), and ORF23 probes (Panel C). Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.

Lane	Description
1	DNA molecular marker (bp)
2	pDAB4468 + control (Maverick) #2
3	control (Maverick) #2
4	control (Maverick) #3
5	T5-B #1
6	T5-B #4
7	T5-B #6
8	T5-B #8

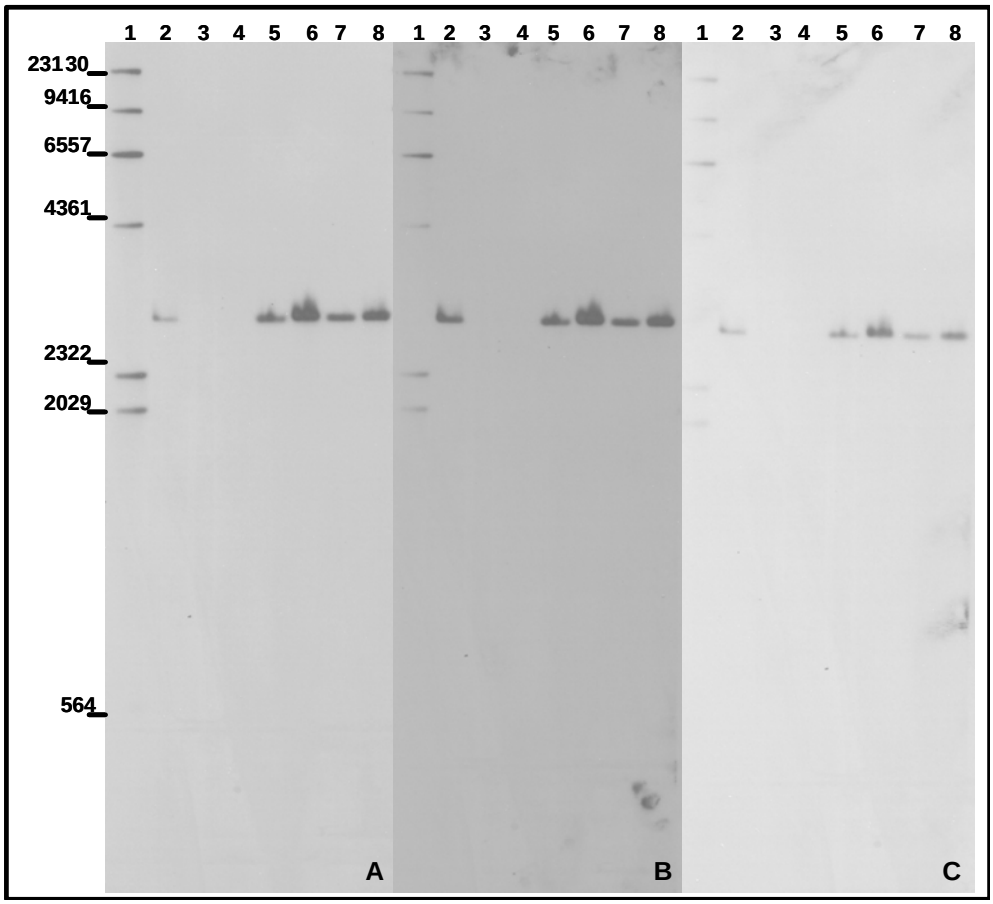


Figure 14. Southern blot analysis of *Pst* I digest with AtUbi10 probe

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3, T4, T5-A and the non-transgenic control were digested with *Pst* I and hybridized with AtUbi10 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively weak signal in Lane 7 was due to a lesser amount of DNA recovered after digestion).

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #2	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

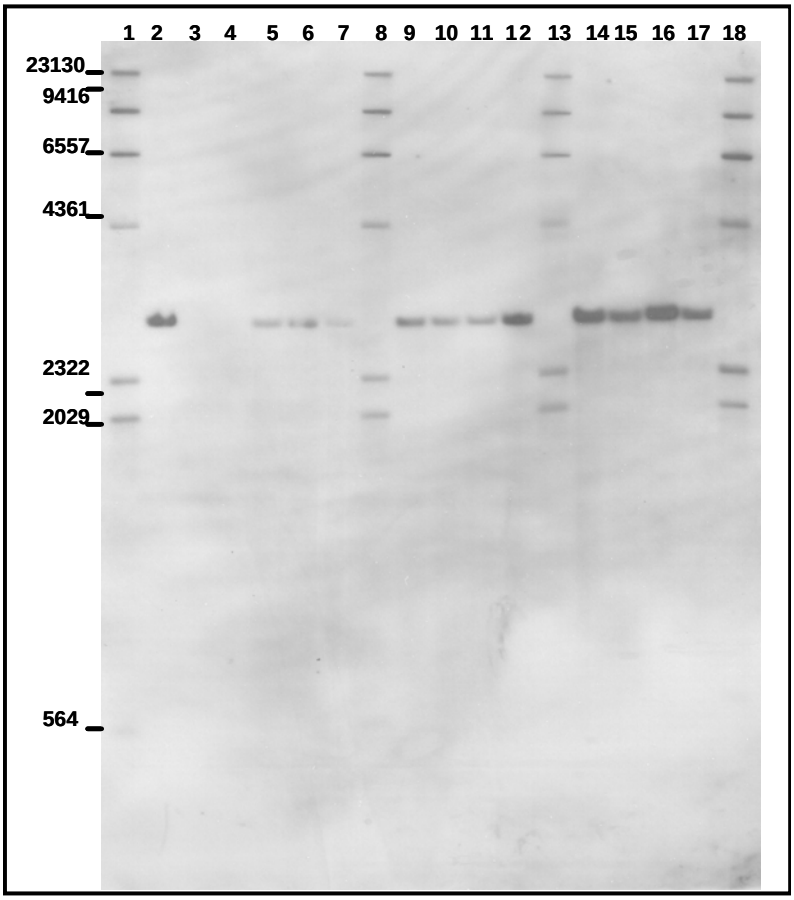


Figure 15. Southern blot analysis of *Pst* I digest with AtuORF23 probe

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3, T4, T5-A and the non-transgenic control were digested with *Pst* I and hybridized with AtuORF23 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively weak signal in Lane 7 was due to a lesser amount of DNA recovered after digestion).

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #2	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

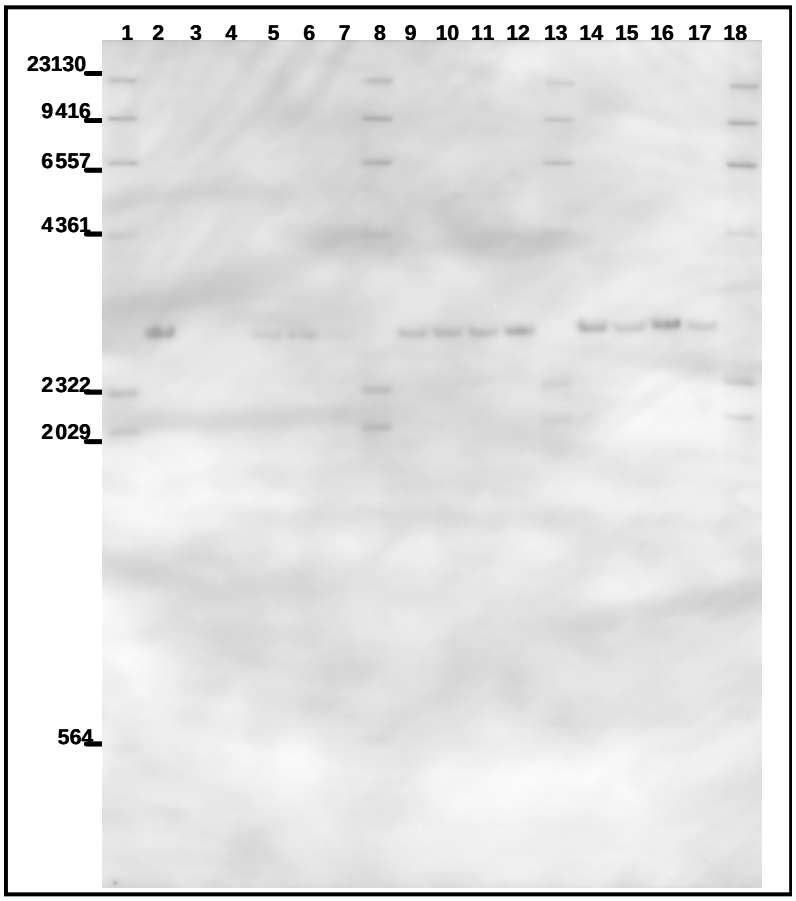


Figure 16. Southern blot analysis of *Nco* I digest with *pat* probe

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3 (Panel A), T4, T5-A (Panel B) and the non-transgenic control were digested with *Nco* I and hybridized with *pat* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signals in Lane 12 and 15 were due to the greater amount of DNA recovered after digestion. Panel A and B were from the same blot and hybridized in the same container).

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #2	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

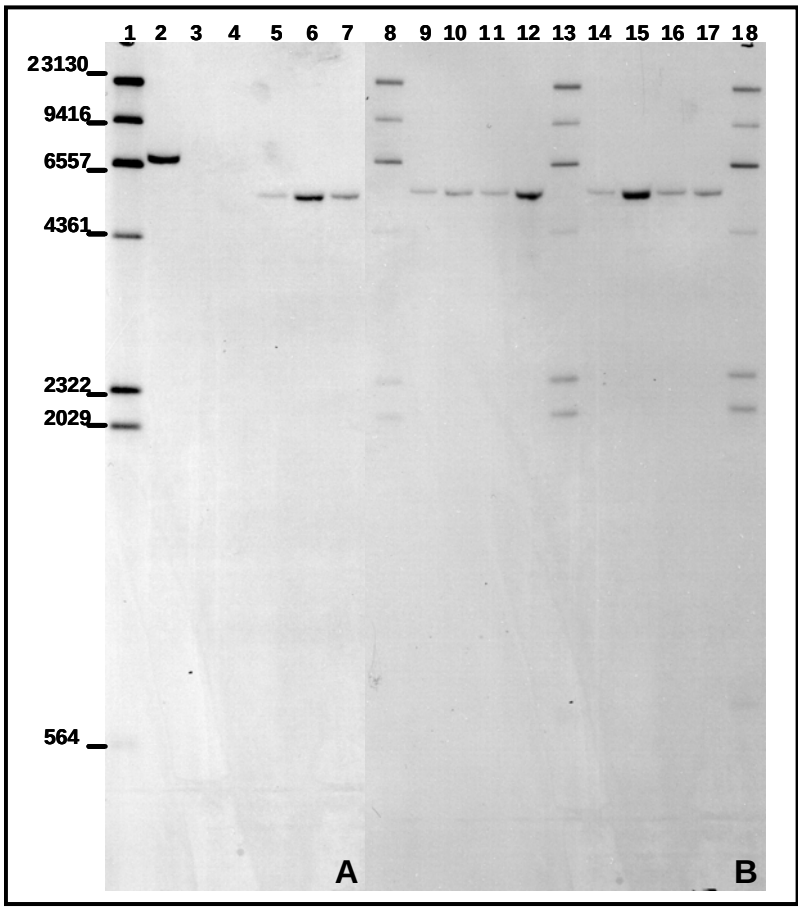


Figure 17. Southern blot analysis of *Sph* I/*Xho* I digest with *pat* probe

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3 (Panel A), T4, T5-A (Panel B) and the non-transgenic control were digested with *Sph* I/*Xho* I and hybridized with *pat* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signals in Lane 10 and 15 were due to the greater amount of DNA recovered after digestion. Panel A and B were from the same blot and hybridized in the same container).

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #2	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

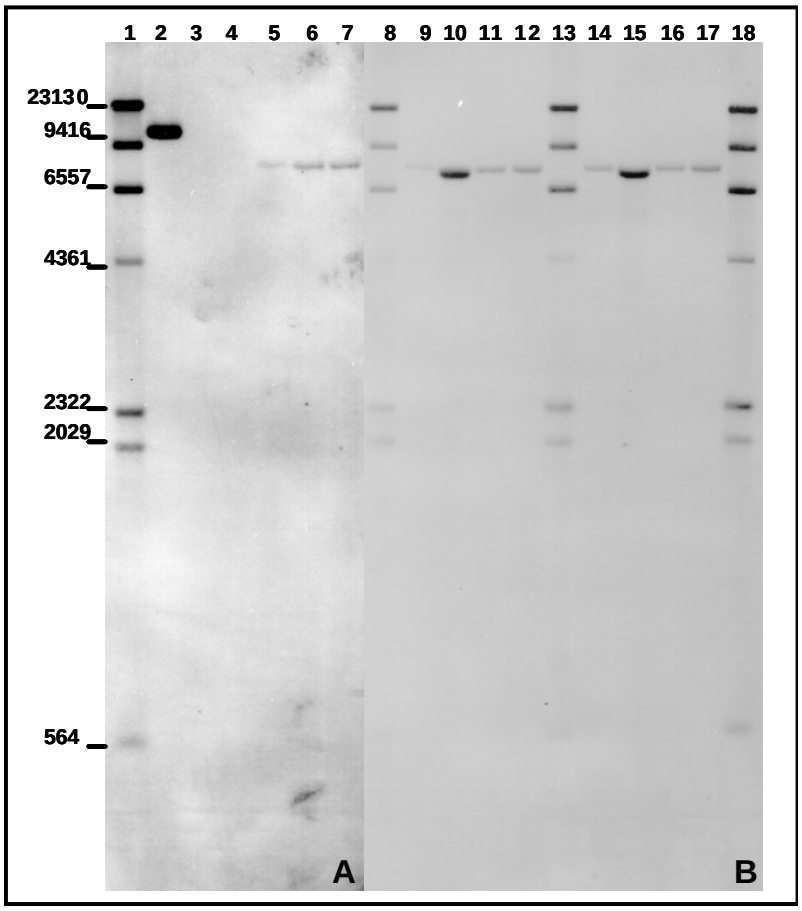


Figure 18. Southern blot analysis of *Nhe* I/*Xho* I digest with *pat* probe

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3, T4, T5-A and the non-transgenic control were digested with *Nhe* I/*Xho* I and hybridized with *pat* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signal in Lane 9 was due to the greater amount of DNA recovered after digestion).

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #2	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

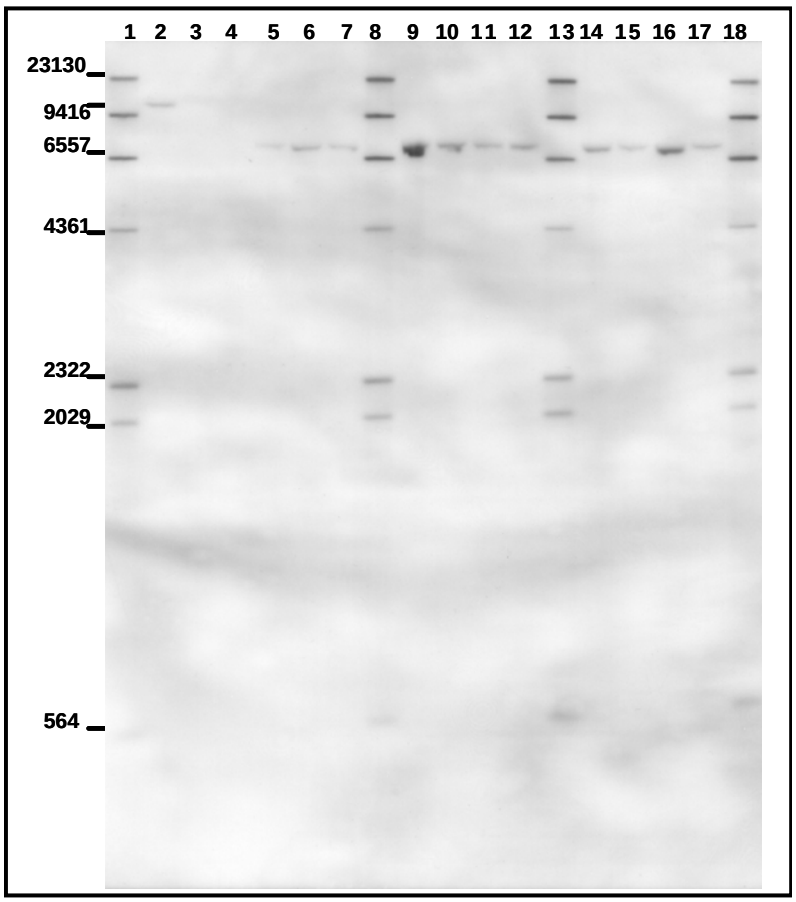


Figure 19. Southern blot analysis of *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I digests of T5-B generation with *pat* probe

DNA isolated from individual plants of soybean event DAS-68416-4 generation T5-B and the non-transgenic control was digested with *Nco* I, *Sph* I/*Xho* I and *Nhe* I/*Xho* I and hybridized with *pat* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signal in Lane 19 was due to the greater amount of DNA recovered after digestion. The faint band in Lane 16 is probably degraded plasmid DNA).

Lane	Description	Enzyme	Lane	Description	Enzyme
1, 23	DNA molecular marker (bp)		12	T5-B #1	*Sph* I/ *Xho* I
2	pDAB4468 + control (Maverick)l #2	*Nco* I	13	T5-B #4	
3	control (Maverick) #2		14	T5-B #6	
4	control (Maverick) #3		15	T5-B #8	
5	T5-B #1		16	pDAB4468 + control (Maverick) #2	*Nhe* I/ *Xho* I
6	T5-B #4		17	control (Maverick) #2	
7	T5-B #6		18	control (Maverick) #3	
8	T5-B #8		19	T5-B #1	
9	pDAB4468 + control (Maverick) #4	*Sph* I/ *Xho* I	20	T5-B #4	
10	control (Maverick) #4		21	T5-B #6	
11	control (Maverick) #5		22	T5-B #8	

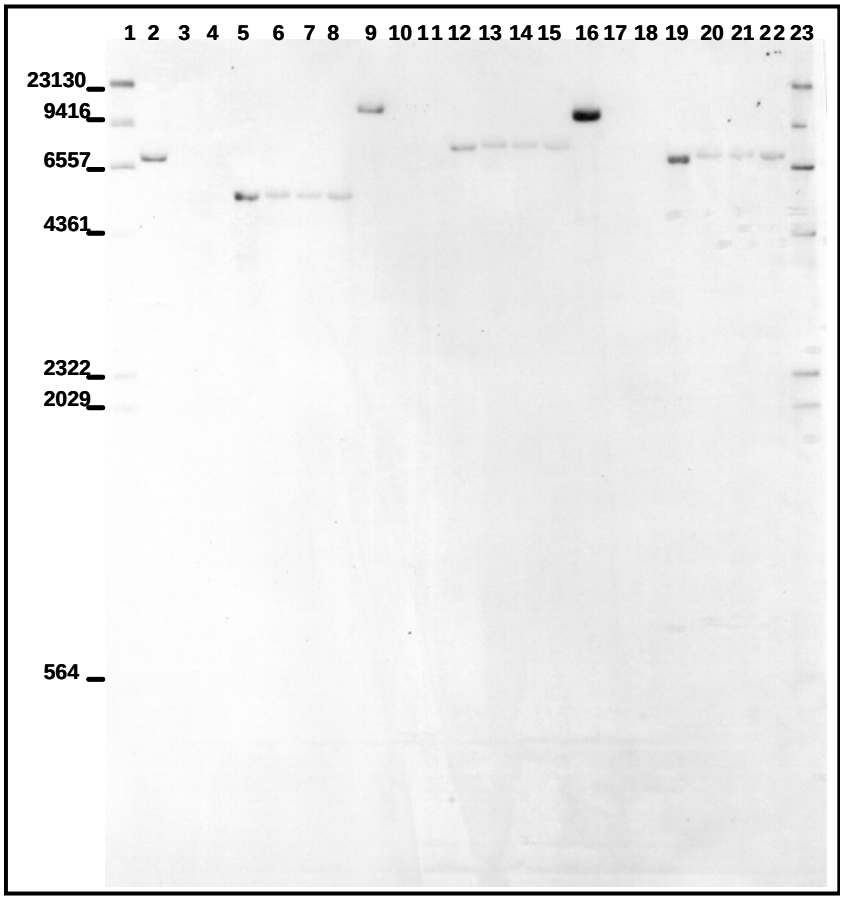


Figure 20. Southern blot analysis of *Pst* I/*Xho* I digest with *pat* probe

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3, T4, T5-A and the non-transgenic control were digested with *Pst* I/*Xho* I and hybridized with *pat* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #3	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

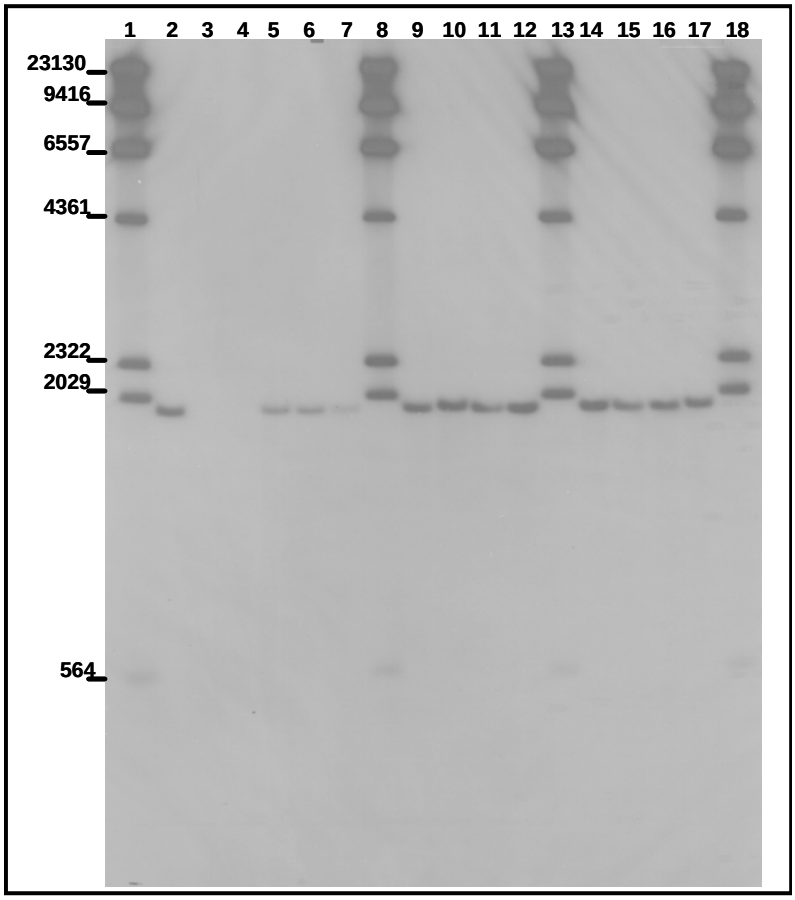


Figure 21. Southern blot analysis of *Pst* I/*Xho* I digest of T5-B generation with *pat,* CsVMV, and AtuORF1 probes

DNA isolated from individual plants of soybean event DAS-68416-4 generation T5-B and the non-transgenic control were digested with *Pst* I/*Xho* I and hybridized with *pat* (Panel A), CsVMV (Panel B), and AtuORF1 probes (Panel C). Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.

Lane	Description
1	DNA molecular marker (bp)
2	pDAB4468 + control (Maverick) #2
3	control (Maverick) #2
4	control (Maverick) #3
5	T5-B #1
6	T5-B #4
7	T5-B #6
8	T5-B #8

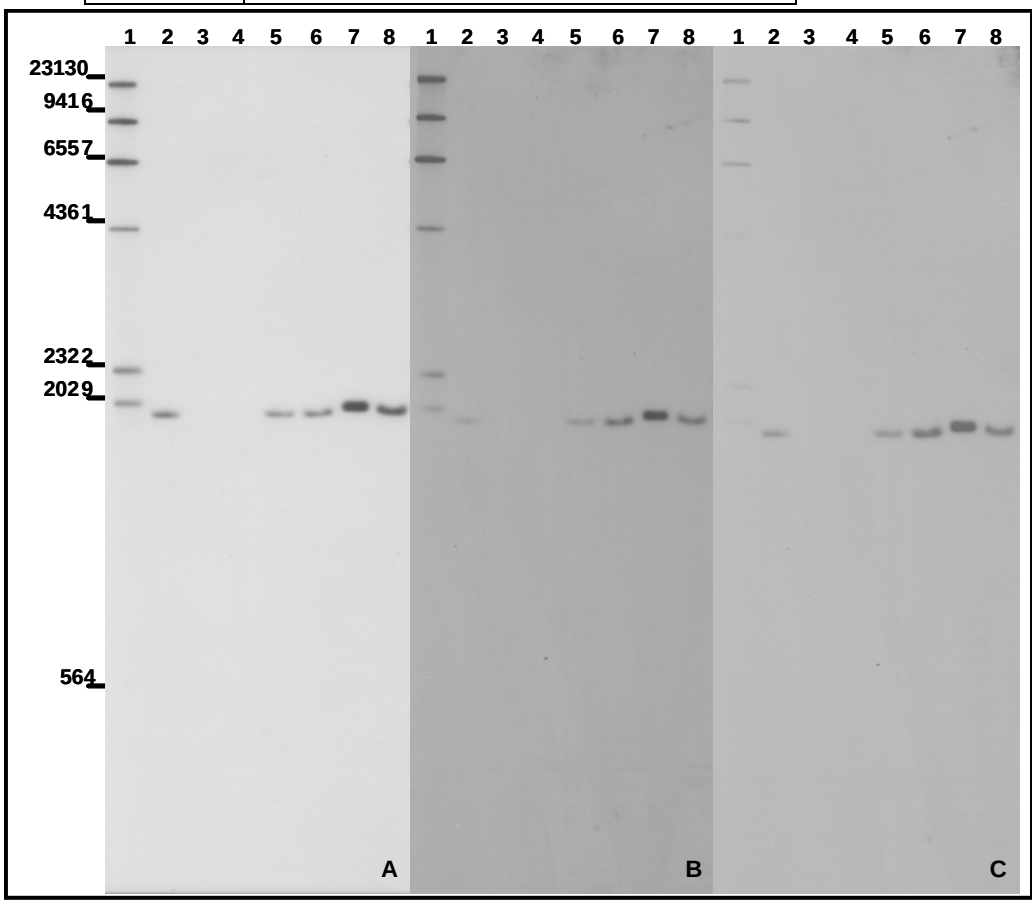


Figure 22. Southern blot analysis of *Pst* I/*Xho* I digest with CsVMV probe

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3, T4, T5-A and the non-transgenic control were digested with *Pst* I/*Xho* I and hybridized with CsVMV probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #3	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

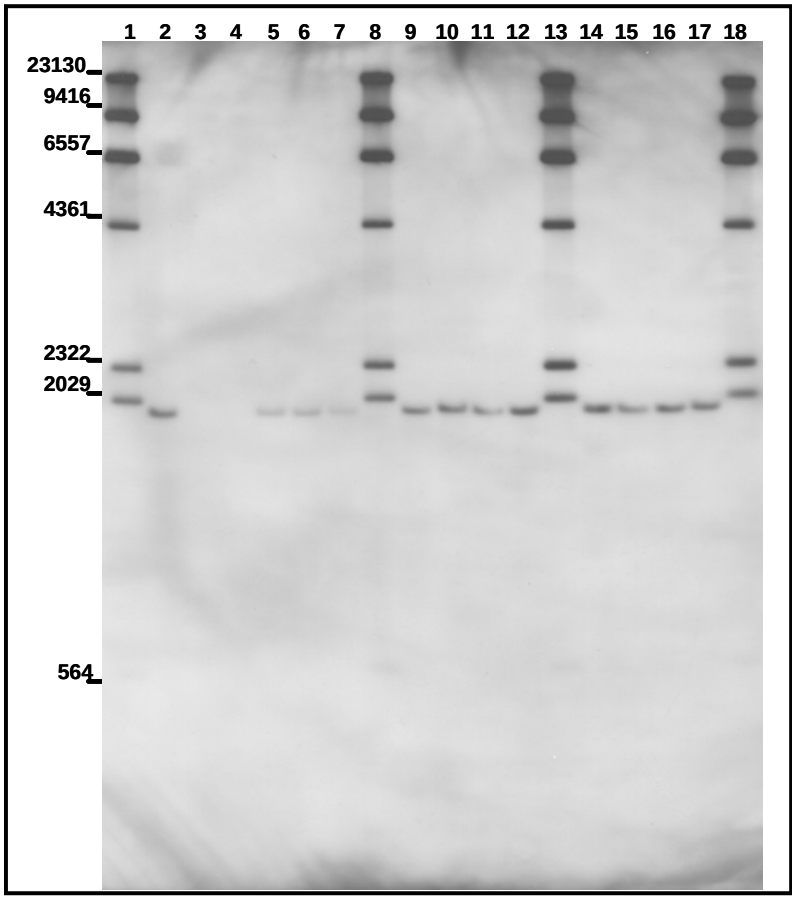


Figure 23. Southern blot analysis of *Pst* I/*Xho* I digest with AtuORF1 probe

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3, T4, T5-A and the non-transgenic control were digested with *Pst* I/*Xho* I and hybridized with AtuORF1 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #3	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

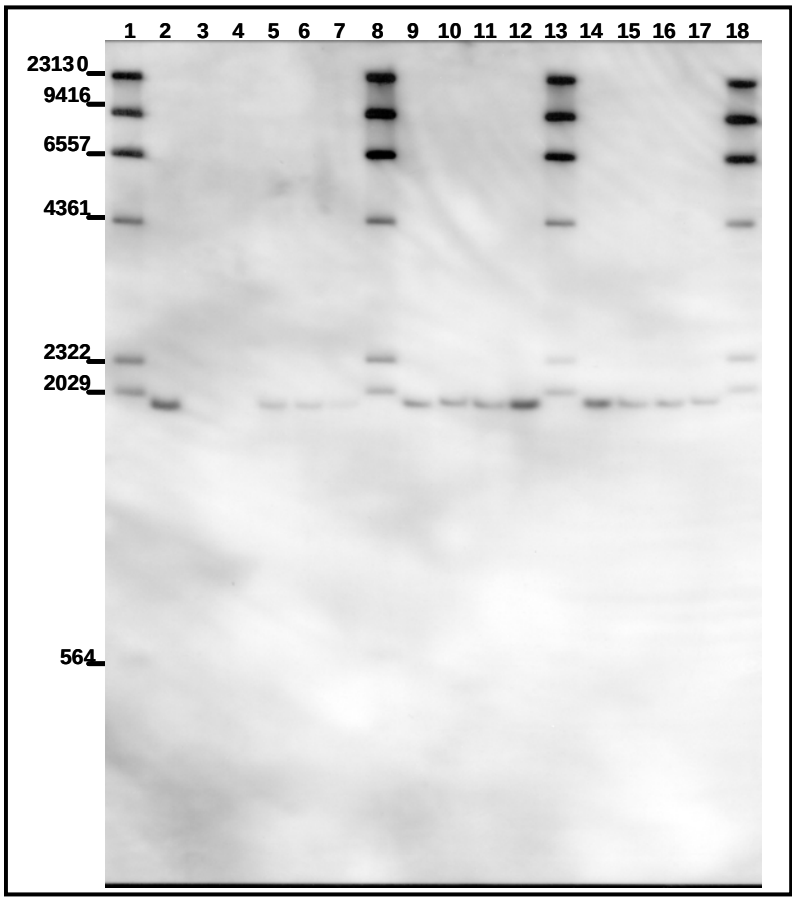


Figure 24. Southern blot analysis of *Bam*H I/*Nco* I digest with RB7 probe

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3, T4, T5-A and the non-transgenic control were digested with *Bam*H I/*Nco* I and hybridized with RB7 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively weak signals in Lane 6 and 7 were due to the less amount of DNA recovered after digestion).

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #2	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

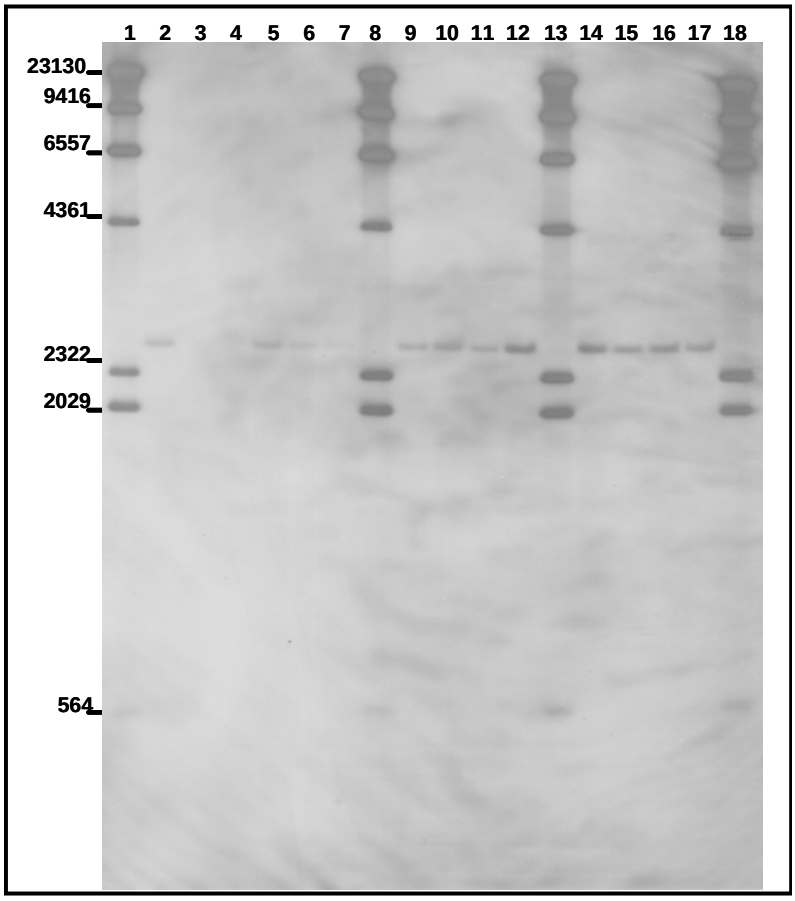


Figure 25. Southern blot analysis of *Bam*H I/*Nco* I digest with AtUbi10 probe

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3, T4, T5-A and the non-transgenic control were digested with *Bam*H I/*Nco* I and hybridized with AtUbi10 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #2	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

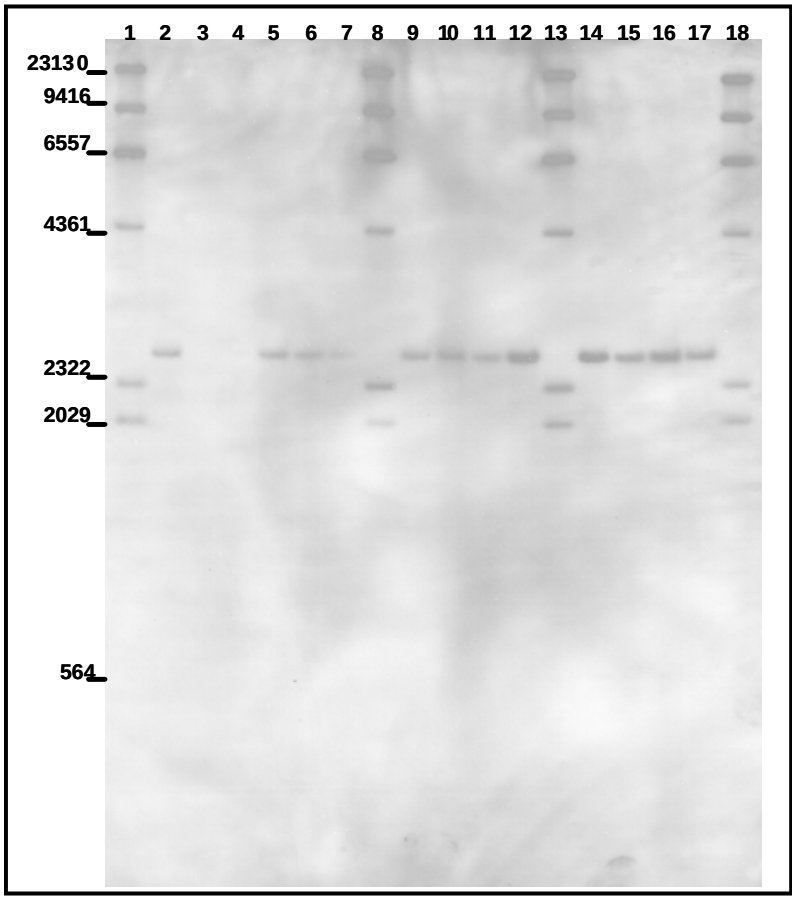


Figure 26. Southern blot analysis of *Bam*H I/*Nco* I digest of T5-B generation with RB7 and AtUbi10 probes

DNA isolated from individual plants of soybean event DAS-68416-4 generation T5-B and the non-transgenic control were digested with *Bam*H I/*Nco* I and hybridized with RB7 (Panel A), AtUbi10 (Panel B). Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.

Lane	Description
1	DNA molecular marker (bp)
2	pDAB4468 + control (Maverick) #2
3	control (Maverick) #2
4	control (Maverick) #3
5	T5-B #1
6	T5-B #4
7	T5-B #6
8	T5-B #8

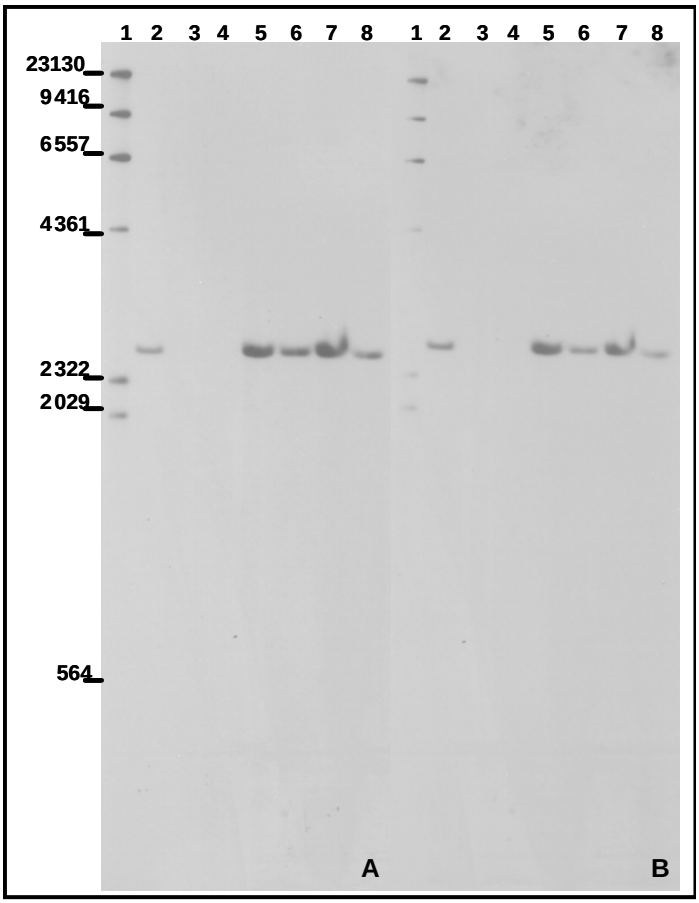


Figure 27. Southern blot analysis of *Nco* I digest with backbone probe set 1 from plasmid pDAB4468 vector backbone

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3 (Panel A), T4, T5-A (Panel B) and the non-transgenic control were digested with *Nco* I and hybridized with Backbone Probe Set 1 (Backbone1, Flanking A, and *SpecR*). Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. No specific hybridization signals were detected in the lanes containing the DAS-68416-4 samples or in the negative control. Expected hybridized bands were observed in the positive control lane, which contained the pDAB4468 plasmid + Maverick. (Note: Panel A and B were from the same blot and hybridized in the same container).

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #2	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

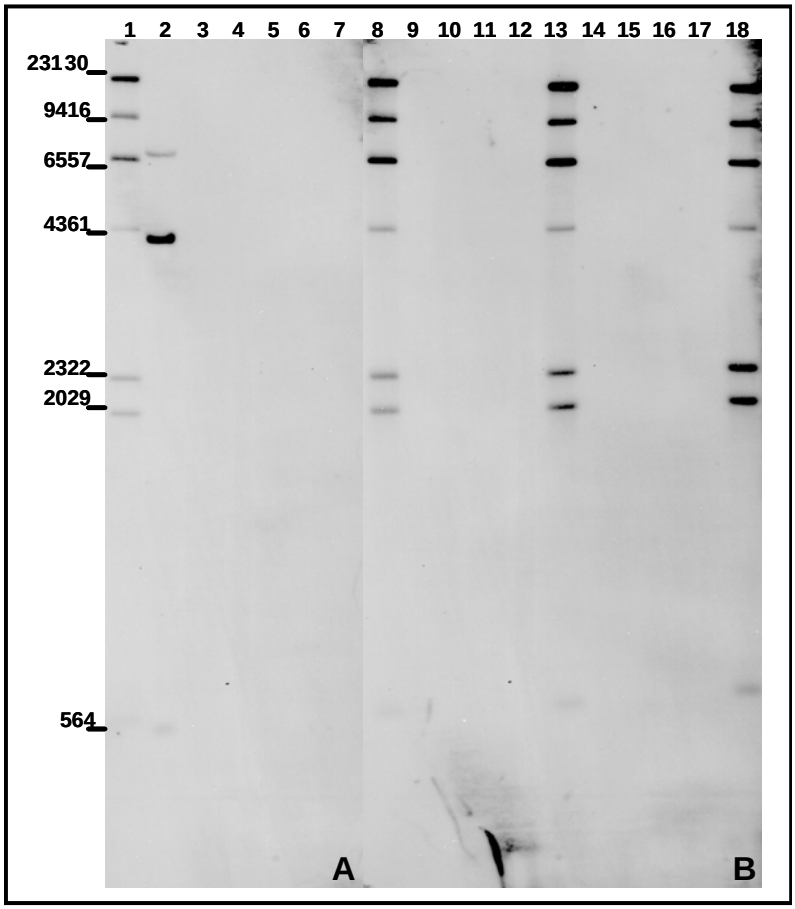


Figure 28. Southern blot analysis of *Nco* I digest with backbone probe set 2 from plasmid pDAB4468 vector backbone

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3 (Panel A), T4, T5-A (Panel B) and the non-transgenic control were digested with *Nco* I and hybridized with Backbone Probe Set 2 (Backbone2, Flanking B, and *Ori-Rep*) probes. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. No specific hybridization signals were detected in the lanes containing the DAS-68416-4 samples or in the negative control. Expected hybridized bands were observed in the positive control lane, which contained the pDAB4468 plasmid + Maverick. (Note: Panel A and B were from the same blot and hybridized in the same container).

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #2	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

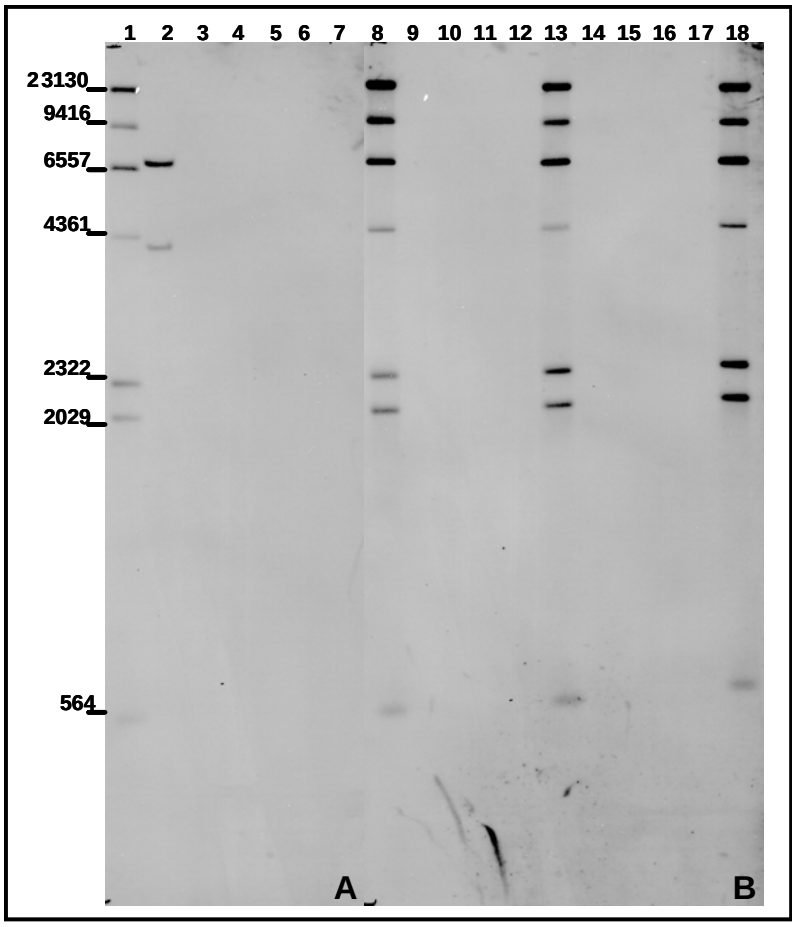


Figure 29. Southern blot analysis of *Sph* I/*Xho* I digest with backbone probe set 1 from plasmid pDAB4468 vector backbone

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3 (Panel A), T4, T5-A (Panel B) and the non-transgenic control were digested with *Sph* I/*Xho* I and hybridized with Backbone Probe Set 1 (Backbone1, Flanking A, and *SpecR*). Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. No specific hybridization signals were detected in the lanes containing the DAS-68416-4 samples or the negative control. An expected hybridized band was observed in the positive control lane, which contained the pDAB4468 plasmid + Maverick. (Note: Panel A and B were from the same blot and hybridized in the same container).

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #2	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

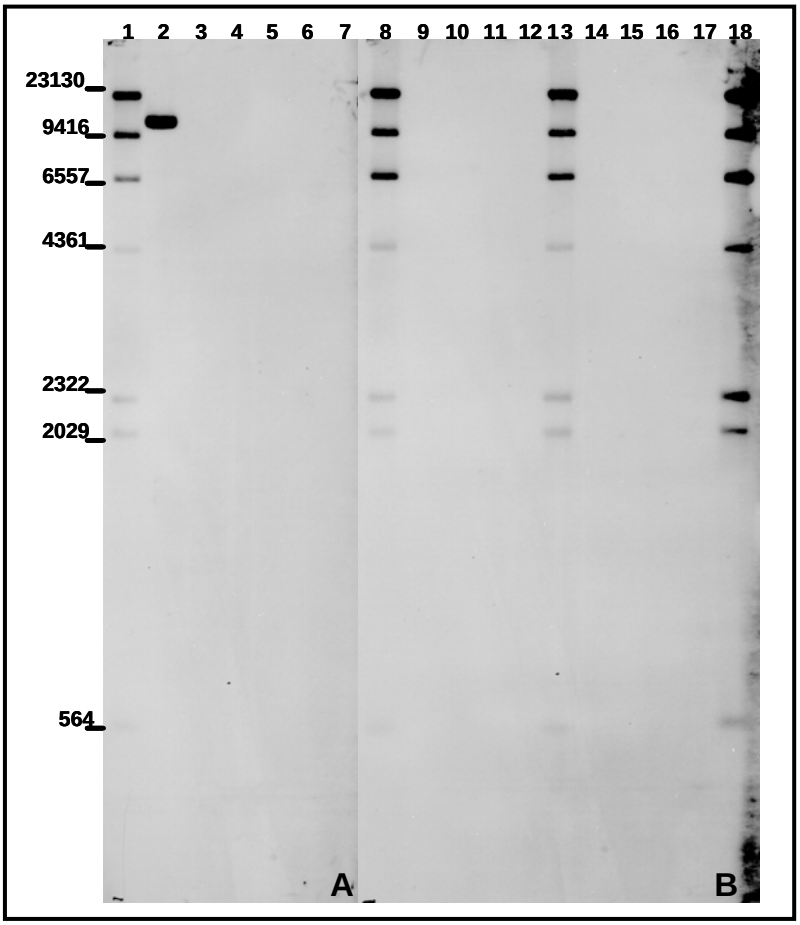


Figure 30. Southern blot analysis of *Sph* I/*Xho* I digest with backbone probe set 2 plasmid pDAB4468 vector backbone

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3 (Panel A), T4, T5-A (Panel B) and the non-transgenic control were digested with *Sph* I/*Xho* I and hybridized with Backbone Probe Set 2 (Backbone2, Flanking B, and *Ori-Rep*). Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. No specific hybridization signals were detected in the lanes containing the DAS-68416-4 samples or the negative control. An expected hybridized band was observed in the positive control lane, which contained the pDAB4468 plasmid + Maverick. (Note: Panel A and B were from the same blot and hybridized in the same container).

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #2	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

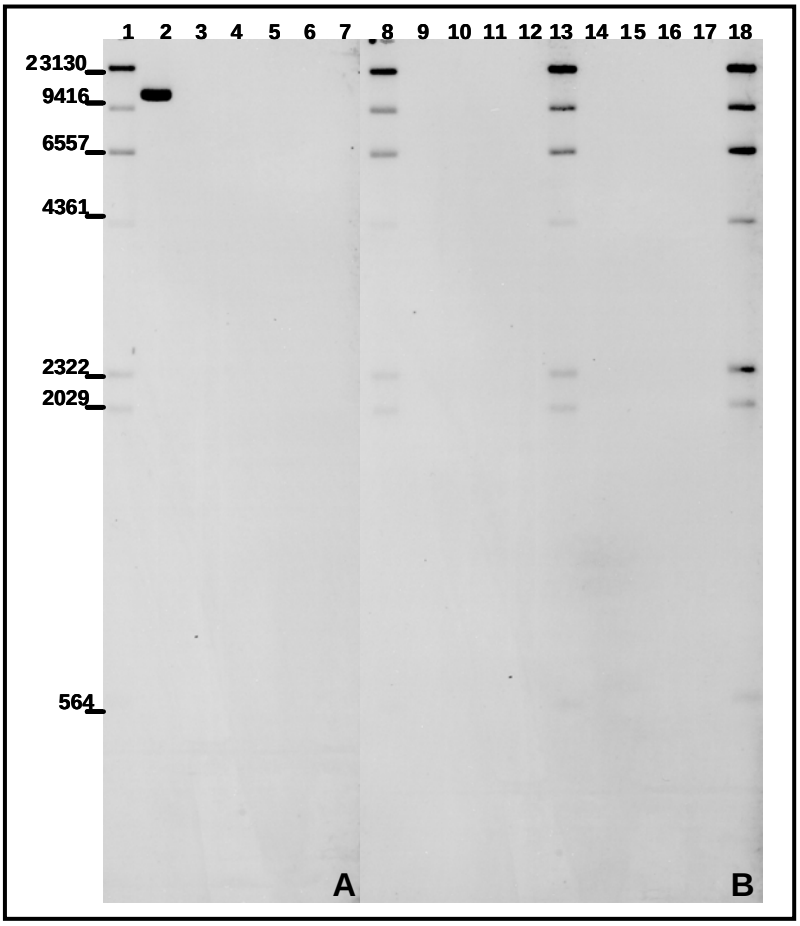


Figure 31. Southern blot analysis of *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I digests of T5-B generation with the backbone probe set 1 from pDAB4468 vector backbone

DNA isolated from individual plants of soybean event DAS-68416-4 generation T5-B and the non-transgenic control was digested with *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I and hybridized with Backbone Probe Set 1 (Backbone1, Flanking A, and *SpecR*). Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. No specific hybridization signals were detected in the lanes containing the DAS-68416-4 samples or the negative control. Expected hybridized bands were observed in the positive control lanes, which contained the pDAB4468 plasmid + Maverick. (Note: The faint bands in lane 16 may be a result of hybridization to the degraded plasmid DNA; no visible band of ~500bp in the plasmid control was probably caused by incomplete digestion of plasmid DNA.).

Lane	Description	Enzyme	Lane	Description	Enzyme
1, 23	DNA molecular marker (bp)		12	T5-B #1	*Sph* I/*Xho* I
2	pDAB4468 + control (Maverick) #2	*Nco* I	13	T5-B #4	
3	control (Maverick) #2		14	T5-B #6	
4	control (Maverick) #3		15	T5-B #8	
5	T5-B #1		16	pDAB4468 + control (Maverick) #2	*Nhe* I/*Xho* I
6	T5-B #4		17	control (Maverick) #2	
7	T5-B #6		18	control (Maverick) #3	
8	T5-B #8		19	T5-B #1	
9	pDAB4468 + control (Maverick) #4	*Sph* I/*Xho* I	20	T5-B #4	
10	control (Maverick) #4		21	T5-B #6	
11	control (Maverick) #5		22	T5-B #8	

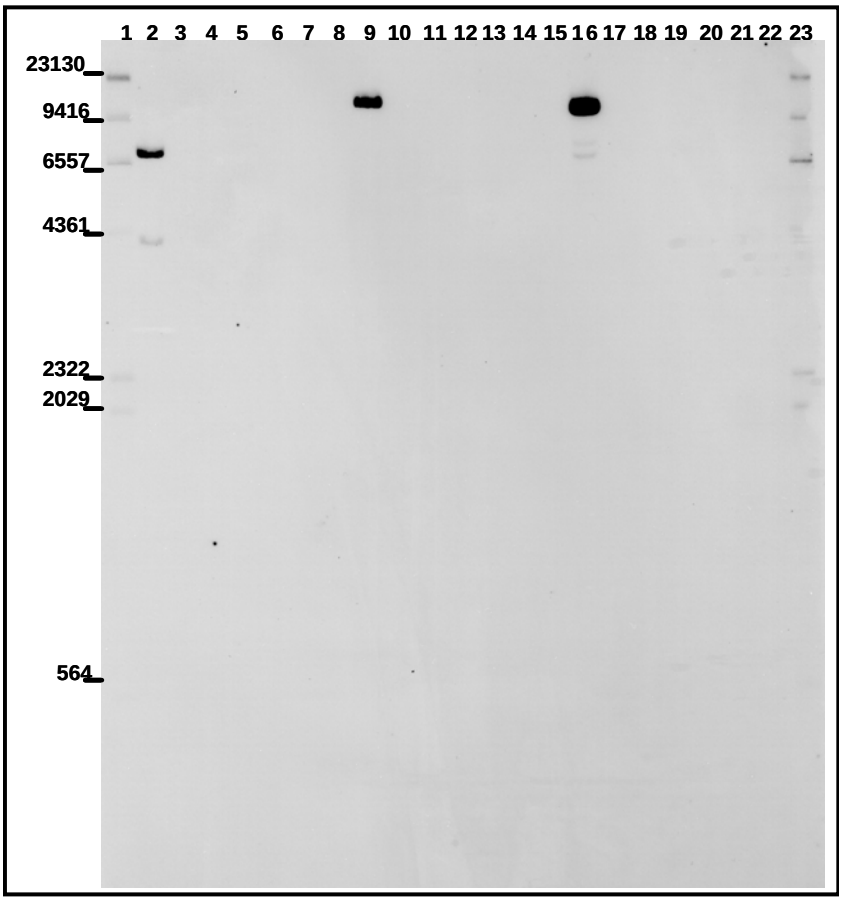
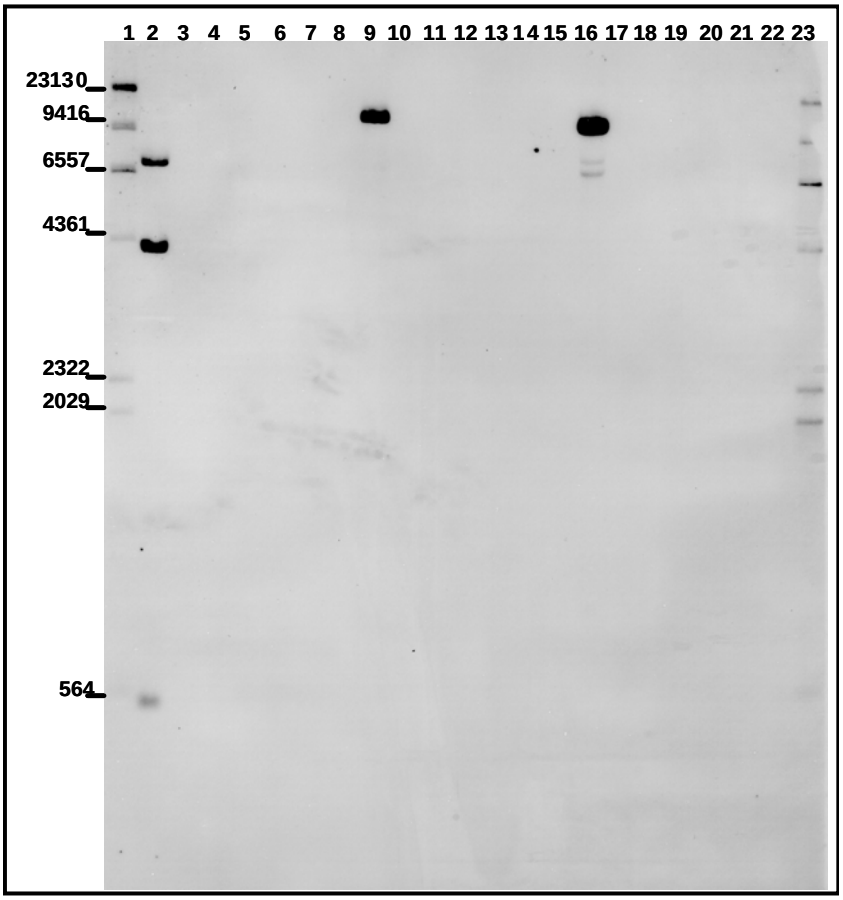


Figure 32. Southern blot analysis of *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I digests of T5-B generation with the backbone probe set 2 from pDAB4468 vector backbone

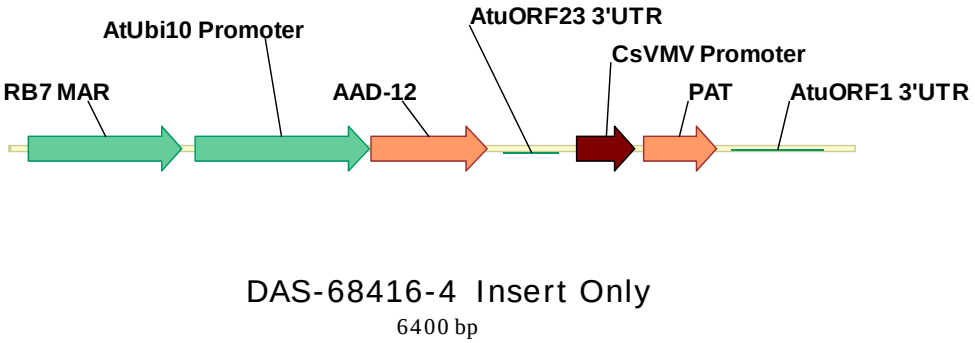
DNA isolated from individual plants of soybean event DAS-68416-4 generation T5 and the non-transgenic control was digested with *Nco* I, *Sph* I/*Xho* I and *Nhe* I/*Xho* I and hybridized with Backbone Probe Set 2 (Backbone2, Flanking B, and *Ori-Rep*). Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. No specific hybridization signals were detected in the lanes containing the DAS-68416-4 samples or the negative control. Expected hybridized bands were observed in the positive control lanes, which contained the pDAB4468 plasmid + Maverick. (Note: The faint bands in lane 16 may be a result of hybridization to the degraded plasmid DNA).

Lane	Description	Enzyme	Lane	Description	Enzyme
1, 23	DNA molecular marker (bp)		12	T5-B #1	*Sph* I/*Xho* I
2	pDAB4468 + control (Maverick) #2	*Nco* I	13	T5-B #4	
3	control (Maverick) #2		14	T5-B #6	
4	control (Maverick) #3		15	T5-B #8	
5	T5-B #1		16	pDAB4468 + control (Maverick) #2	*Nhe* I/*Xho* I
6	T5-B #4		17	control (Maverick) #2	
7	T5-B #6		18	control (Maverick) #3	
8	T5-B #8		19	T5-B #1	
9	pDAB4468 + control (Maverick) #4	*Sph* I/*Xho* I	20	T5-B #4	
10	control (Maverick) #4		21	T5-B #6	
11	control (Maverick) #5		22	T5-B #8	



The organisation of the DAS-68416-4 soybean insertion site can therefore be divided into three separate major sections i)the 5' border sequence, comprising the flanking region of soybean genomic DNA; ii)the full-length, single copy pDAB4468 T-DNA insert; and iii) the 3' border sequence, comprising the flanking region of soybean genomic DNA. A schematic representation of the DAS-68416-4 soybean insertion site is given in Figure 33.

Figure 33. Schematic representation of DAS-68416-4 insert



4.2 Describe the gene products that are derived from the foreign genes.

DAS-68416-4 soybean was developed using *Agrobacterium*-mediated transformation to stably incorporate the *aad*-12 gene from *Delftia acidovorans* and *pat* gene from *Streptomyces viridochromogenes* into soybean. The *aad*-12 gene encodes the aryloxyalkanoate dioxygenase-12 (AAD-12) enzyme which, when expressed in plants, degrades 2,4-D into herbicidally-inactive 2,4-dichlorophenol (DCP). The *pat* gene encodes the enzyme phosphinothricin acetyl transferase that inactivates glufosinate.

4.3 Describe the biological activity associated with the foreign gene products.

The AAD-12 protein degrades 2,4-D into herbicidally-inactive 2,4-dichlorophenol (DCP) while the PAT protein inactivates glufosinate. The availability of DAS-68416-4 soybean is expected to have a beneficial impact on weed control practices by providing growers with another tool to address their weed control needs.

The development of 2,4-D-tolerant soybeans provides an excellent option for controlling broadleaf, glyphosate-resistant (or highly tolerant) weed species for in-crop applications, allowing the grower to focus applications at the critical weed control stages and extending the application window without the need for specialized sprayer equipment.

DAS-68416-4 soybeans also provide tolerance to glufosinate herbicides. Glufosinate is a non-selective, contact herbicide that controls a broad spectrum of annual and perennial grasses and broadleaf weeds. The tolerance to glufosinate allows use of an additional mode of action as part of effective herbicide resistance management strategies.

Glufosinate herbicides can also be used as selection agents in breeding nurseries to select herbicide-tolerant plants to maintain seed trait purity.

4.4 Provide information on the rate and level of expression of the foreign genes and the sensitivity of the measurement of the rate and level. State whether expression is constitutive or inducible. Are foreign genes expressed throughout the plant or only in certain organs or tissues?

The AAD-12 and PAT proteins are expressed in several tissues, including leaf (stages V5 and V10-12), forage, root R3 and grain (R8-Maturity), samples from DAS-68416-4 soybean throughout the growing season (Phillips and Lepping, 2010a-Attachment C [**CBI-DELETED: Section 68(a),(b) and (c)ii of the Promotion of Access to Information Act**]). However, grain is the most relevant tissue for the food and feed safety assessment of DAS-68416-4 and therefore only the results for grain are summarised in this application.

Field expression, nutrient composition, and agronomic trials of a non-transgenic control and a soybean line containing Aryloxyalkanoate Dioxygenase-12 (DAS-68416-4 soybean), were conducted in 2009 at eight sites located in Arkansas, Iowa, Illinois (2 sites), Indiana, Missouri (2 sites), and Nebraska (2 sites planted in Ontario, Canada did not reach harvest maturity due to climatological conditions) (Phillips and Lepping, 2010a).

Expression of AAD-12 and PAT proteins in grain from DAS-68416-4 soybean was characterized using a specific Enzyme Linked Immunosorbent Assay (ELISA) developed specifically for each protein. The results obtained from the expression analysis have been summarized in Table 4.

Mean expression values of AAD-12 protein in grain ranged from 20.19-22.92 ng/mg whilst the overall mean expression of AAD-12 was 21.49 ng/mg for grain (Table 4).

Conversely, mean expression values of PAT protein in grain ranged from 2.57-2.66 ng/mg and the overall mean expression of PAT was 2.63 ng/mg for grain (Table 4).

Table 4. Summary of mean concentration levels of AAD-12 and PAT proteins measured in the DAS-68416-4 soybean unsprayed, sprayed with glufosinate, sprayed with 2,4-D and sprayed with glufosinate and 2,4-D (Tissue: soybean grain)

	PAT ng/mg Tissue Dry Weight			AAD-12 ng/mg Tissue Dry Weight		
Description	**Mean**	**Std. Dev. (n=7)**	**Min/Max Range**	**Mean**	**Std. Dev. (n=7)**	**Min/Max Range**
Control	ND	NA	ND-ND	ND	NA	ND-ND
DAS-68416-4	2.66	0.46	1.80-3.71	22.92	4.17	16.29-32.18
DAS-68416-4Gluf	2.66	0.37	1.81-3.52	21.67	4.47	14.21-31.59
DAS-68416-4 2,4-D	2.57	0.4	1.91-3.34	20.19	4.16	12.14-29.77
DAS-68416-4 Both	2.62	0.44	1.55-3.41	21.16	4.63	11.51-31.97
Overall Mean	2.63			21.49		

4.5 Provide protocols for the detection of the foreign genes in the environment including sensitivity, reliability and specificity of the techniques.

PCR detection methods to confirm the molecular identity of DAS-68416-4 soybean, as well as certified reference materials, have been developed (see http://www.sumobrain.com/patents/wipo/Detection-aad-12-soybean-event/WO2011066360A1.pdf).

5. RESISTANCE

5.1 Detail whether the genetically engineered plant is able to initiate resistance, in any biotic component of the environment, to any biologically active foreign gene product.

As this is an application for commodity clearance of DAS-68416-4, *i.e.* use as food, feed or for processing, and not an application for release of DAS-68416-4 into the environment of South Africa, this question is not applicable.

5.2 Detail what methods are available to minimise the risk of resistance developing in the environment.

Considering that this is not an application for environmental release, this question is not applicable.

5.3 Detail how resistance will be managed during release of the genetically modified plant.

Considering that this is not an application for environmental release, this question is not applicable.

6. HUMAN AND ANIMAL HEALTH

6.1 State whether the genetically modified plant or its products will enter human or animal food chains.

This is an application for commodity clearance of DAS-68416-4. DAS-68416-4 will therefore enter human and animal food chains.

6.2 Detail the results of experiments undertaken to determine the toxicity of the foreign gene products (including marker genes) to humans and animals.

The safety assessment of biotechnology-derived crops addresses two major areas *viz*. (i) the safety of the introduced trait (most often a protein) and (ii) the safety of the derived food and feeds.

This section is divided into three major parts:
- Toxicological assessment
- Allergenicity assessment
- Nutritional and agronomic assessment

Toxicological assessment

Safety assessment of newly expressed proteins

The human and animal safety of the AAD-12 protein as expressed in DAS-68416-4 was established first via a biochemical characterisation followed by toxicity assessment as indicated below:

Biochemical characterisation
- Characterisation of mode of action;
- Heat lability;
- Equivalence of microbially-derived AAD-12 and PAT and DAS-68416-4 soybean expressed protein used in toxicological studies.

Toxicity assessment
- History of safe use of donor organism;
- Amino acid sequence comparison to known toxins;
- Assessment of gene disruption on the parental genomic locus and structural similarity of potential novel reading frames of the insert and its flanking regions to known toxins or other biologically active proteins that could cause adverse effects in humans or animals;
- Assessment of toxicity to mammals.

Biochemical characterisation

The amino acid sequence is identical to the native enzyme sequence, except for the addition of an alanine at position number 2. The additional alanine codon encodes part of an *Nco* I restriction enzyme recognition site (CCATGG) spanning the ATG translational start codon. This additional codon serves the dual purpose of facilitating subsequent cloning operations and improving the sequence context surrounding the ATG start codon to optimize translation initiation. The proteins encoded by the native and plant-optimized coding regions are 99.3% identical, differing only at amino acid number 2. The AAD-12 protein is comprised of 293 amino acids and has a molecular weight of ~32 kDa (Figure 34).

Figure 34. Amino acid sequence of the AAD-12 protein

```
001 MAQTTLQITPTGATLGATVTGVHLATLDDAGFAALHAAWLQHALLIFPGQ
051 HLSNDQQITFAKRFGAIERIGGGDIVAISNVKADGTVRQHSPAEWDDMMK
101 VIVGNMAWHADSTYMPVMAQGAVFSAEVVPAVGGRTCFADMRAAYDALDE
151 ATRALVHQRSARHSLVYSQSKLGHVQQAGSAYIGYGMDTTATPLRPLVKV
201 HPETGRPSLLIGRHAHAIPGMDAAESERFLEGLVDWACQAPRVHAHQWAA
251 GDVVVWDNRCLLHRAEPWDFKLPRVMWHSRLAGRPETEGAALV
```

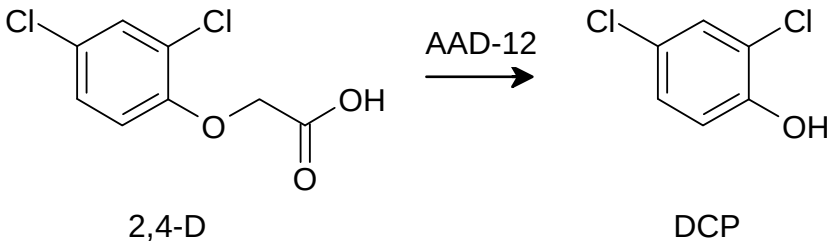
Mode of action

Expression of the AAD-12 protein in transgenic crops provides tolerance to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) by catalyzing the conversion of 2,4-D to 2,4-dichlorophenol (DCP) (Müller *et al*., 1999; Westendorf *et al*., 2002; Wright *et al*., 2007; Wright *et al*., 2010), a herbicidally inactive compound (Figure 35).

AAD-12 is also able to degrade related achiral phenoxyacetate herbicides such as MCPA ((4-chloro-2-methylphenoxy) acetic acid) and pyridyloxyacetate herbicides such as triclopyr and fluroxypyr to their corresponding inactive phenols and pyridinols, respectively.

AAD-12 has enantiomeric selectivity for the (S)-enantiomers of the chiral phenoxy acid herbicides (*e.g.*, dichlorprop and mecoprop), but does not catalyze degradation of the (R)-enantiomers. It is the R-enantiomers in this class of chemistry that are herbicidally active, therefore AAD-12 does not provide tolerance to commercially-available chiral phenoxy acid herbicides.

Figure 35. Degradation reaction of 2,4-D catalyzed by AAD-12



2,4-D DCP

Enzyme specificity

AAD-12 has enantiomeric selectivity for the (S)-enantiomers of the chiral phenoxy acid herbicides (*e.g.*, dichlorprop and mecoprop), but does not catalyze degradation of the (R)-enantiomers. It is the R-enantiomers in this class of chemistry that are herbicidally active, therefore AAD-12 does not provide tolerance to commercially-available chiral phenoxy acid herbicides.

The AAD-12 enzyme was screened for the ability to utilize endogenous plant substrates using a sensitive coupled *in vitro* enzyme assay. Substrates were screened based on chemical structure, similar physiological function to known xenobiotic substrates, and abundance within primary/secondary metabolic pathways of plants as a means to determine if AAD-12 may be anticipated to perturb important pathways by utilizing any of a number of likely endogenous substrates when expressed in genetically modified plants (Cicchillo *et al*., 2010-

Attachment D [**CBI-DELETED: Section 68(a),(b) and (c)ii of the Promotion of Access to Information Act**]).

The metabolites tested were separated into three groups: i) the natural plant hormones indole-3-acetic acid (IAA), abscisic acid (ABA), gibberellin (GA), and aminocyclopropane-1-carboxylate (ACC); ii) phenylpropanoid intermediates cinnamate, coumarate, and sinapate and; iii) all twenty L-amino acids were evaluated. The putative substrates were initially screened by an *in vitro* enzyme-coupled system which detects succinate production. This system was used to guide detailed Fourier transform mass spectrometric (FT/MS) analyses of oxidation products (Cicchillo *et al*., 2010).

Together the data demonstrate that only oxidation of *trans*-cinnamic acid and IAA could be detected when exorbitant amounts of enzyme were used. AAD-12 kinetic parameters were determined by an enzyme-coupled assay using *trans*-cinnamic acid and IAA as substrates. The k_{cat} and K_m for AAD-12 using *trans*-cinnamic acid were found to be 0.1 s^{-1} and 645.2 µM, respectively. The K_m was significantly elevated using IAA at ~3.4 mM and the k_{cat} was 0.03 s^{-1}. Hence, although AAD-12 is capable of oxidizing both *trans*-cinnamic acid and IAA, the extremely poor kinetics confirms, that these transformations are unlikely to have a metabolic impact within transgenic plants (Cicchillo *et al*., 2010).

Heat lability

The thermal stability of the AAD-12 protein was evaluated by heating protein solutions for 30 min at 50, 70, and 95 °C in a phosphate-based buffer. All heating conditions eliminated the enzymatic activity of the AAD-12 protein. The study also demonstrated that the AAD-12 protein is immunochemically less reactive when heated. When the AAD-12 was exposed to the heat conditions (50 – 95 °C) the protein lost more than 99% of its immunoreactivity, as measured by a polyclonal antibody sandwich ELISA. Gel electrophoresis analysis indicated that the molecular mass of the AAD-12 protein (approximately 32 kDa) was unchanged. These data indicate that industrial processing of the soybean grain would significantly degrade the tertiary structure of the AAD-12 protein, reduce its immunoreactivity, and eliminate its enzymatic activity (Schafer 2010-Attachment E [**CBI-DELETED: Section 68(a),(b) and (c)ii of the Promotion of Access to Information Act**]).

Equivalence of microbially-derived AAD-12 and DAS-68416-4 soybean expressed protein

Large quantities of purified AAD-12 protein are required to perform safety assessment studies. Because it is technically infeasible to extract and purify sufficient amounts of recombinant protein from transgenic plants (Evans, 2004), the AAD-12 protein was microbially-produced using *Pseudomonas fluorescens* (*Pf*). Characterization studies were performed to confirm the equivalency of the AAD-12 protein expressed *in planta* in soybean line DAS-68416-4 with the *Pf* microbe-derived AAD-12 protein. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot, glycoprotein detection, and protein sequence analysis by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and electrospray ionization-liquid chromatography mass spectrometry (ESI-LC/MS) were used to characterize the biochemical properties of the protein. Using these methods, the AAD-12 protein from *Pf* and the transgenic soybean event, DAS-68416-4, were shown to be biochemically equivalent, thereby supporting the use of the microbially-produced protein in safety assessment studies (Schafer and Embrey, 2009a).

DAS-68416-4 transgenic soybean material

Greenhouse-grown DAS-68416-4 soybean plants (T4 generation) were used as the plant source of the AAD-12 protein. Prior to use, individual plants were leaf tested to confirm

expression of the AAD-12 protein using a rapid lateral flow test strip according to the manufacturer's instructions. Leaves (and some stems) from AAD-12 expressing plants were harvested, lyophilized, ground to a fine powder, and stored frozen until needed.

Control soybean material
Control soybean line Maverick had a genetic background representative of the DAS-68416-4 soybean plants, but did not contain the *aad*-12 gene. Absence of AAD-12 expression in the control plants was confirmed by immunoassay using an AAD-12 specific rapid lateral flow test strip. Leaves (and some stems) of control plants were harvested, lyophilized, ground and stored under the same conditions as the DAS-68416-4 soybean.

Reference material
Recombinant AAD-12 microbial protein was produced in *Pseudomonas fluorescens* (*Pf*) and purified to a lyophilized powder. The microbe-derived AAD-12 protein preparation was stored dry and resuspended in a buffer to maintain activity prior to use.

Protein purification from DAS-68416-4 soybean plant tissue
The AAD-12 protein was extracted from lyophilized leaf tissue in a PBST (Phosphate Buffered Saline with 0.05% Tween 20, pH 7.4) based buffer with added stabilizers, and the soluble proteins were collected by centrifugation. The supernatant was filtered and the soluble proteins were allowed to bind to Phenyl Sepharose (PS) beads (GE Healthcare). After an hour of incubation, the PS beads were washed with PBST and the bound proteins were eluted with Milli-Q water. Sodium chloride was added to increase the conductivity and the PS purified proteins were loaded onto an anti-AAD-12 immunoaffinity column which had been conjugated with an AAD-12 specific polyclonal antibody. The non-bound proteins were collected from the column and the column was washed extensively with pre-chilled PBS (phosphate buffered saline, pH 7.4). The bound proteins were eluted from the column with a 3.5 M NaSCN, 50 mM Tris, pH 8.0 buffer and examined by SDS-PAGE and western blotting.

SDS-PAGE and western blot analysis of crude extracts
Lyophilized leaf tissue from event DAS-68416-4 and Maverick was mixed with PBST buffer containing ~2.0% protease inhibitor cocktail (Sigma) and the protein was extracted by grinding with ball bearings in a Geno-Grinder. The samples were centrifuged and the supernatants were mixed with Laemmli sample buffer, heated, and briefly centrifuged. The samples were loaded directly on to a Bio-Rad Criterion SDS-PAGE gel. The positive reference standard, microbe-derived AAD-12, was also mixed with sample buffer and loaded on to the gel. Electrophoresis was conducted with Tris/glycine/SDS buffer (Bio-Rad). Following electrophoresis, the gel was cut in half, with one half stained with Pierce GelCode Blue protein stain and the other gel half was electro-blotted onto a nitrocellulose membrane. The nitrocellulose membrane was then probed with an AAD-12 specific polyclonal rabbit antibody. A chemiluminescent substrate was used to visualize the immunoreactive bands.

Detection of post-translational glycosylation
The immunoaffinity-purified, plant-derived AAD-12 protein was analyzed for evidence of glycosylation by electrophoresis with microbe-derived AAD-12 protein, soybean trypsin inhibitor, bovine serum albumin, and horseradish peroxidase as controls. The control protein samples were adjusted to concentrations approximately equal with the plant-derived AAD-12 protein and mixed with Laemmli buffer. The proteins were heated, centrifuged, and applied directly to a Bio-Rad Criterion SDS-PAGE gel. Following electrophoresis, the gel was cut in half. One gel half was stained with Pierce GelCode Blue stain for total protein. The remaining half of the gel was stained with GelCode Glycoprotein Stain to visualize the

glycoproteins. The glycoproteins present on the gel were visualized as magenta bands on a light pink background.

Mass spectrometry peptide mass fingerprinting and sequencing of plant- and microbe-derived AAD-12 protein

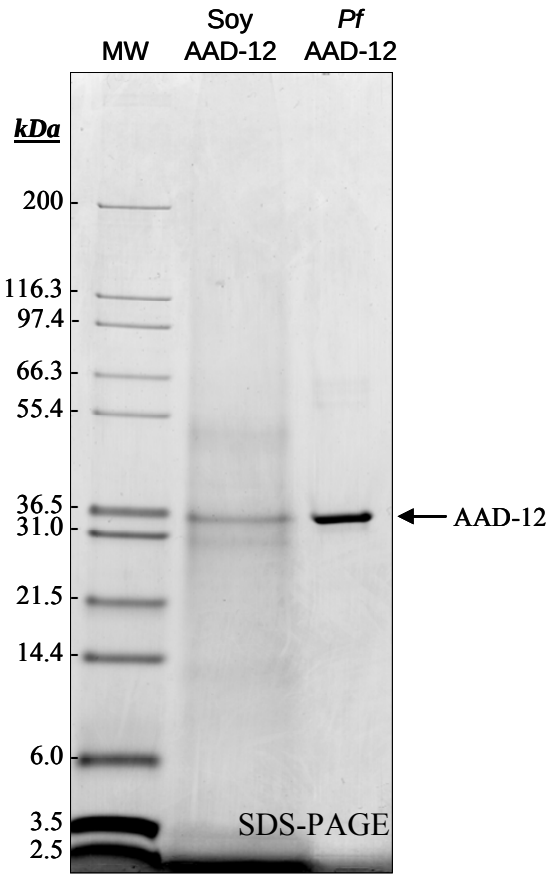
The immunoaffinity purified AAD-12 plant-derived protein was subjected to in-solution digestion by trypsin and Asp-N followed by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and electrospray-ionization liquid chromatography/mass spectrometry (ESI-LC/MS). The peptide fragments of the plant-derived AAD-12 protein (including the N- and C-termini) were analyzed and compared with the sequence of the microbe-derived protein.

Results of the SDS-PAGE and western blot analysis

In the microbe-derived AAD-12, the major protein band, as visualized on the Coomassie stained SDS-PAGE gel, was approximately 32 kDa (Figure 36). As expected, the corresponding plant-derived AAD-12 protein was identical in size to the microbe-derived protein. Predictably, the plant purified fractions contained a minor amount of non-immunoreactive impurities in addition to the AAD-12 protein. The co-purified proteins were likely retained on the column by weak interactions with the column matrix (Holroyde *et al*., 1976; Kennedy and Barnes, 1983; Williams *et al.*, 2006).

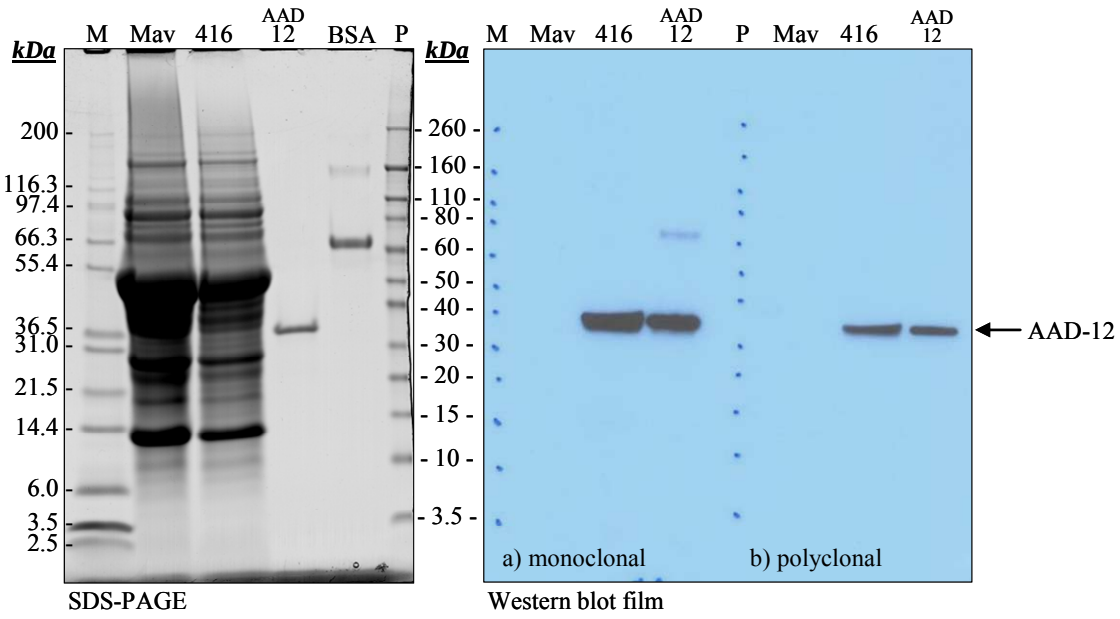
The microbe-derived AAD-12 and DAS-68416-4 plant tissue extract showed a positive signal of the expected size on the western blot using the anti-AAD-12 polyclonal antibody (Figure 37). In the AAD-12 western blot analysis, no immunoreactive proteins were observed in the control Maverick extract and no alternate size proteins (aggregates or degradation products) were seen in the samples from the transgenic plant. The monoclonal antibody did detect a small amount of the AAD-12 dimer in the microbe-derived protein. These results add to the evidence that the protein expressed in soybean is not glycosylated which would add to the overall protein molecular weight.

Figure 36. SDS-PAGE of soybean- and microbe-derived AAD-12



Lane	*Sample*	*Amount*
M	Invitrogen Mark12 MW markers	10 µL
Soy	Soybean-Derived AAD-12 (DAS-68416-4)	500 µL
Pf	Microbe-Derived AAD-12	1000 ng

Figure 37. SDS-PAGE and western blot of soybean- and microbe-derived AAD-12 protein extracts



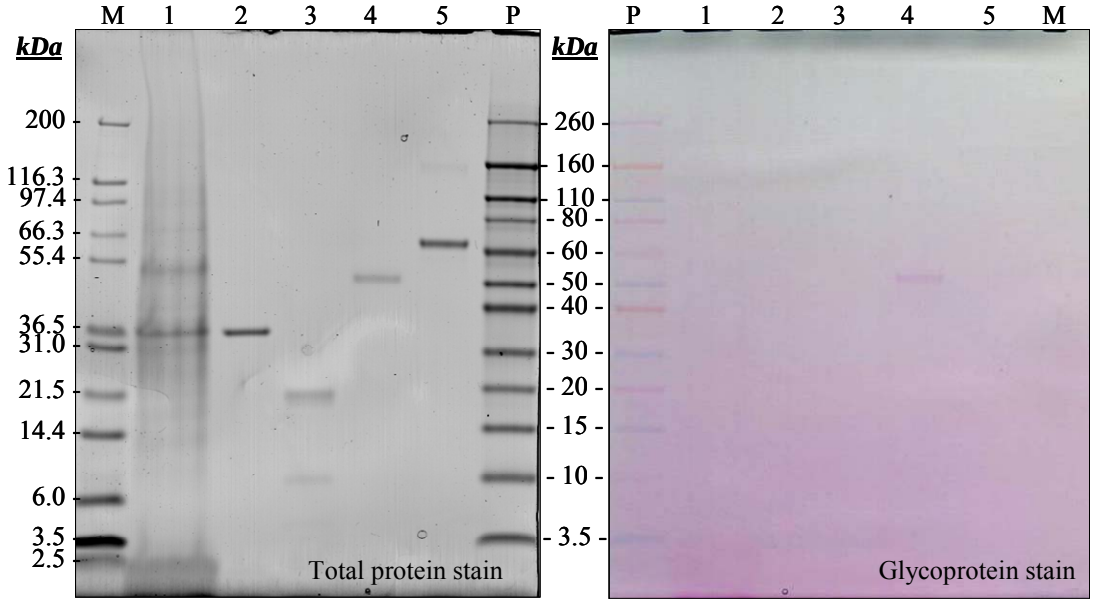
Lane	*Sample*	*Amount*
M	Invitrogen Mark12 MW markers	10 µL
Mav	Nontransgenic Soybean Extract	32 µL
416	Event DAS-68416-4 extract	32 µL
AAD-12	Microbe-Derived AAD-12	~785 ng
BSA	Bovine Serum Albumin (BSA)	~785 ng
P	Novex Prestained MW Markers	10 µL

Results of detection of glycosylation of AAD-12 protein

No covalently-linked carbohydrates were detectable on the plant- or microbe-derived AAD-12 proteins (Figure 38). Horseradish peroxidase, a glycoprotein, was used as a positive indicator for glycosylation. Soybean trypsin inhibitor and bovine serum albumin, both non-glycoproteins, served as negative controls.

Figure 38. Glycosylation analysis of soybean- and microbe-derived AAD-12 proteins

Note: The immunoaffinity-purified, soybean-derived AAD-12 protein, microbe-derived AAD-12, soybean trypsin inhibitor, bovine serum albumin, and horseradish peroxidase were diluted to a similar concentration prior to loading on the gel. After electrophoresis, the gel was cut in half and one half was stained with GelCode Blue stain for total protein, the other half of the gel was stained with a GelCode Glycoprotein Staining Kit to visualize the glycoproteins.



Lane	*Sample*	*Amount*
M	Invitrogen Mark12 MW markers	10 µL
1	Soybean-Derived AAD-12 (Frac 3)	500 µL
2	Microbe-Derived AAD-12	500 ng
3	Soybean Trypsin Inhibitor (STI)	500 ng
4	Horseradish Peroxidase (HRP)	500 ng
5	Bovine Serum Albumin (BSA)	500 ng
P	Novex Prestained MW markers	10 µL

Results of MALDI-TOF and ESI/LC-MS tryptic and Asp-N peptide mass fingerprints of AAD-12 proteins

Following digestion of the plant-derived AAD-12 protein by trypsin and Asp-N, the masses of the detected peptides were compared with those deduced based on potential cleavage sites in the sequence of the AAD-12 protein. Figure 39 illustrates the theoretical peptide cleavage which was generated *in silico* using PAWs software (Proteometrics LLC).

The trypsin and Asp-N digestion of soybean-derived AAD-12 protein yielded high detection of the expected peptides, resulting in 73.4% coverage of the AAD-12 protein sequence (Figure 40). The analysis confirmed the plant-derived protein amino acid sequence matched that of the microbe-derived AAD-12 protein and that of the predicted amino acid sequence. Results of these analyses indicated that the amino acid sequence of the soybean-derived AAD-12 protein was equivalent to the *P. fluorescens*-expressed protein.

Results of tryptic and Asp-N peptide N-and C-terminal sequence analysis of AAD-12

The N-terminal sequence of the first 27 residues of the plant-derived and all 292 residues of the microbe-derived AAD-12 protein was obtained by mass spectrometry. The amino acid sequences for N-terminus of both proteins was A^{2} H A A L S P L S Q I T P T G A T L G A T V T G V H L A T L^{27}, indicating the N-terminal methionine had been removed (Table 5 and Figure 40). These results suggest that during or after translation in the plant and *P. fluorescens*, the N-terminal methionine is cleaved by a methionine aminopeptidase. In addition to the methionine being removed, the N-terminal peptide of the AAD-12 protein was shown to be acetylated after the N-terminal methionine was cleaved. These two co-translational processes, cleavage of N-terminal methionine residue and N-terminal acetylation, are by far the most common modifications and occur on the vast majority (~85%) of eukaryotic proteins (Polevoda and Sherman, 2000; Polevoda and Sherman, 2002).

The C-terminal sequences of the plant- and microbe-derived AAD-12 proteins were determined to be identical to the expected sequences (Table 6 and Figure 40).

Figure 39. Theoretical trypsin (top panel) and Asp-N (bottom panel) cleavage of the AAD-12 protein

Note: Alternating blocks of upper (black) and lower (red) case letters within the amino acid sequence are used to differentiate the potential peptides after trypsin digestion. The numbers on the left and right sides indicate the amino acid residue numbers.

Digestion at K (lysine) and R (arginine)

1	M A Q T T L Q I T P T G A T L G A T V T G V H L A T L D D A	30
31	G F A A L H A A W L Q H A L L I F P G Q H L S N D Q Q I T F	60
61	A K r F G A I E R i g g g d i v a i s n v k A D G T V R q h	90
91	s p a e w d d m m k V I V G N M A W H A D S T Y M P V M A Q	120
121	G A V F S A E V V P A V G G R t c f a d m r A A Y D A L D E	150
151	A T R a l v h q r S A R h s l v y s q s k L G H V Q Q A G S	180
181	A Y I G Y G M D T T A T P L R P L V K v h p e t g r p s l l	210
211	i g r H A H A I P G M D A A E S E R f l e g l v d w a c q a	240
241	p r V H A H Q W A A G D V V V W D N R c l l h r A E P W D F	270
271	K l p r V M W H S R l a g r p e t e g a a l v	293

Digestion at D (aspartate)

1	M A Q T T L Q I T P T G A T L G A T V T G V H L A T L d D A	30
31	G F A A L H A A W L Q H A L L I F P G Q H L S N d q q i t f	60
61	a k r f g a i e r i g g g D I V A I S N V K A d g t v r q h	90
91	s p a e w D d m m k v i v g n m a w h a D S T Y M P V M A Q	120
121	G A V F S A E V V P A V G G R T C F A d m r a a y D A L d e	150
151	a t r a l v h q r s a r h s l v y s q s k l g h v q q a g s	180
181	a y i g y g m D T T A T P L R P L V K V H P E T G R P S L L	210
211	I G R H A H A I P G M d a a e s e r f l e g l v D W A C Q A	240
241	P R V H A H Q W A A G d v v v w D N R C L L H R A E P W d f	270
271	k l p r v m w h s r l a g r p e t e g a a l v	293

Figure 40. Sequence coverage in the tryptic and Asp-N peptide mapping analysis of plant-derived AAD-12 protein with MALDI-TOF and ESI/LC MS

Note: The numbers on the left and right sides of the protein sequence indicate the amino acid residue numbers. Letters highlighted in gray represent tryptic peptide sequence detected by MALDI-TOF MS and ESI-LC/MS. Underlined letters represent Asp-N peptide sequence detected. The overall sequence coverage was 73.4%. The down arrow indicates the N-terminal methionine was removed by an aminopeptidase and the N-terminal alanine was N-acetylated.

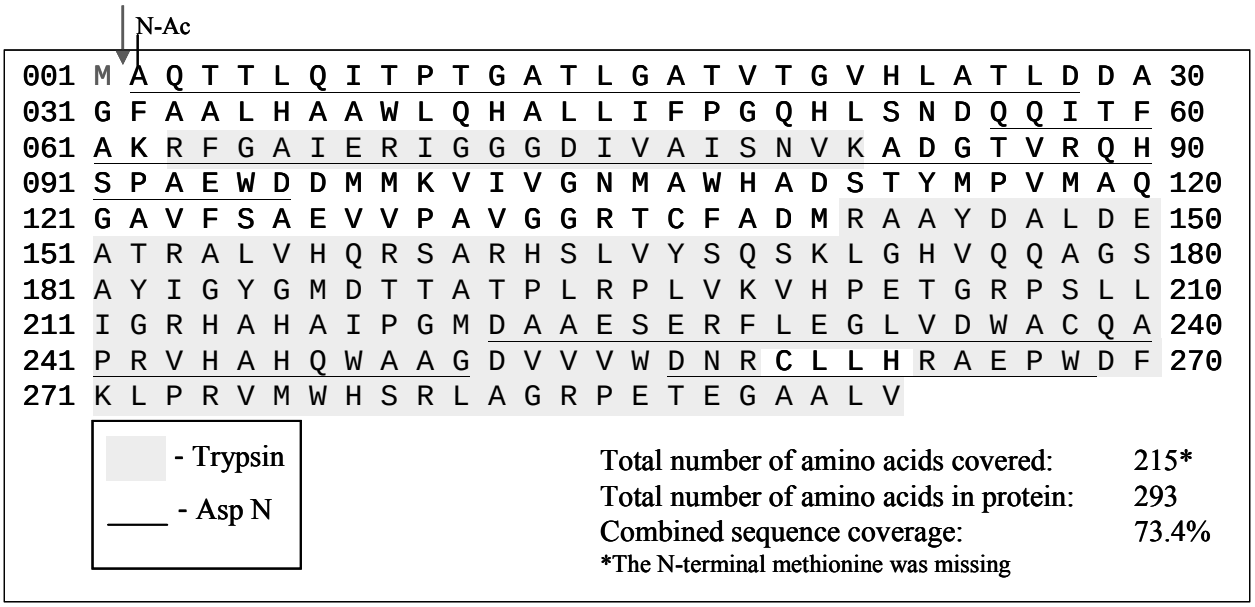


Table 5. Summary of N-terminal sequence data of AAD-12 soybean- and microbe-derived proteins

Source	Expected N-terminal Sequence[1]
P. fluorescens	M^{1} A Q T T L Q I T P T G A T L G A T V T G V H L A T L D^{27}
Soybean Event DAS-68416-4	M^{1} A Q T T L Q I T P T G A T L G A T V T G V H L A T L D^{27}
Source	**Detected N-terminal Sequence[2]**
P. fluorescens	A^{2} Q T T L Q I T P T G A T L G A T V T G V H L A T L D^{27}
Soybean Event DAS-68416-4[3]	$^{N\text{-}Ac}A^{2}$ Q T T L Q I T P T G A T L G A T V T G V H L A T L D^{27}

[1]Expected N-terminal sequence of the first 27 amino acid residues of *P. fluorescens*- and soybean-derived AAD-12.

[2]Detected N-terminal sequences of *P. fluorescens*- and soybean-derived AAD-12.

[3]The MALDI-TOF MS data for the N-terminal peptide revealed that the soybean-derived AAD-12 protein was acetylated (*N-Acetyl*-A Q T T L Q I T P T G A T L G A T V T G V H L A T L D).

Notes:

Numbers in superscript (R^{x}) indicate amino acid residue numbers in the sequence. Amino acid residue abbreviations:

A:	alanine	D: Aspartate	G: glycine
H:	histidine	I: isoleucine	L: leucine
M:	methionine	P: proline	Q: glutamine
T:	threonine	V: valine	

Table 6. Summary of C-terminal sequence data of AAD-12 soybean- and microbe-derived proteins

Source	Expected C-terminal Sequence[1]
P. fluorescens	^{281}L A G R P E T E G A A L V^{293}
Soybean Event DAS-68416-4	^{281}L A G R P E T E G A A L V^{293}

Source	Detected C-terminal Sequence[2]
P. fluorescens	^{281}L A G R P E T E G A A L V^{293}
Soybean Event DAS-68416-4	^{281}L A G R P E T E G A A L V^{293}

[1]Expected C-terminal sequence of the last 13 amino acid residues of *P. fluorescens*- and soybean-derived AAD-12.
[2]Detected C-terminal sequences of *P. fluorescens*- and soybean-derived AAD-12.
Notes:
Numbers in superscript (R^x) indicate amino acid residue numbers in the sequence.
Amino acid residue abbreviations:

A:	alanine	E: glutamate	G: glycine
L:	leucine	P: proline	R: arginine
T:	threonine	V: valine	

The biochemical identity of microbe-derived AAD-12 protein was equivalent to the protein purified from leaf tissue of event DAS-68416-4. The plant- and microbe-derived AAD-12 proteins showed the expected molecular weight of ~32 kDa by SDS-PAGE and were immunoreactive to AAD-12 protein specific antibodies by western blot analysis. The amino acid sequence of both proteins was confirmed by enzymatic peptide mass fingerprinting by MALDI-TOF MS and ESI-LC/MS. In addition, the lack of glycosylation of the plant-derived AAD-12 protein provided additional evidence that the AAD-12 protein produced by *P. fluorescens* and DAS-68416-4 soybean are biochemically equivalent.

Toxicity assessment

Safety of donor organism

The donor organism, *Delftia acidovorans*, which has previously been identified as *Pseudomonas acidovorans* and *Comamonas acidovorans*, is a non glucose-fermenting, gram-negative, non spore-forming rod present in soil, fresh water, activated sludge, and clinical specimens (von Gravenitz 1985; Tamaoka *et al*., 1987; Wen *et al*., 1999). *D. acidovorans* can be used to transform ferulic acid into vanillin and related flavor metabolites (Toms and Wood, 1970; Ramachandra and Ravishankar, 2000; Shetty *et al*., 2006). This utility has led to a history of safe use for *D. acidovorans* in the food processing industry.

Bioinformatic searches

The following studies were performed in order to assess the safety of the insert and its flanking regions (Table 7).

Table 7. Summary of bioinformatic searches (Attachments CBI-DELETED: Section 68(a),(b) and (c)ii of the Promotion of Access to Information Act)

Flanking sequences (both against DNA and protein databases)								
General Database		Date	Algorithms	Study	EST Database	Date	Algorithms	Study
Nucleotide: GenBank non-redundant nucleotide collection		February 12, 2010	BLASTn	Song, 2010a-Attachment F	GanBank No-mouse No-human ESTs	February 12, 2010	BLASTn	Song, 2010a
Protein: GenBank non-redundant protein sequences		February 12, 2010	BLASTx	Song, 2010a				
ORF analyses ☒ insert-plant (a)/ ☐ insert-insert (b)/ ☐ whole insert (c)								
Allergen database		Date	Algorithms	Study	General (and toxin) Database	Date	Algorithms	Study
(a) FARRP v10		January, 2010	FASTA and 8-mer match	Song, 2010b-Attachment G	GenBank non-redundant protein sequences	April 23, 2010	BLASTp	Song, 2010b
Newly expressed proteins								
Allergen database		Date	Algorithms	Study	General (and toxin) Database	Date	Algorithms	Study
AAD-12	FARRP v10	March, 2010	FASTA and 8-mer match	Song, 2010c-Attachment H	GenBank non-redundant protein sequences	March 18, 2010	BLASTp	Song, 2010d-Attachment I
PAT	FARRP v10	January, 2010	FASTA and 8-mer match	Song, 2010e-Attachment J	GenBank non-redundant protein sequences	January, 2010	BLASTp	Song, 2010f-Attachment K

Amino acid sequence comparison to known toxins

To assess potential toxicity of the newly expressed protein in DAS-68416-4 soybean, a search for similarity of protein sequences was conducted using the BLASTp program. Amino acid sequences of AAD-12 were queried using the BLASTp (Version 2.2.22+) against a non-redundant protein dataset (update to January 8, 2010), which incorporates non-redundant entries from all GenBank and RefSeq nucleotide translations (Genpept "nr") along with protein sequences from SWISS-PROT (http://www.expasy.org/sprot/), PIR (http://pir.georgetown.edu/), PRF (http://www.prf.or.jp/aboutdb-e.html), and PDB (http://www.wwpdb.org/). The search was done through the BLAST website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in the NCBI (National Center of Biotechnology Information) with default settings (Matrix = BLOSUM62, Gap Costs = Existence: 11, Extension: 1) except that a cutoff expectation E-value of 1.0 was used to generate biologically meaningful similarity between the query sequences and proteins in the database, the low complexity filtering was turned off, and the sequence output was set to 1000 alignments. Although a statistically significant sequence similarity generally requires a match with an expectation value less than 0.01, a cutoff of E()< 1.0 insures that proteins with even limited similarity will not be overlooked in the search (Song, 2010d).

The BLASTp search returned a total of 1577 alignments (E() < 1). By their annotations, all of the proteins associated with those alignments can be grouped into the following 10 categories (Table 8): 2,4-D/alpha-ketoglutarate dioxygenase, alkylsulfatase AtsK, alpha-ketoglutarate (dependent) dioxygenase, alpha-ketoglutarate-dependent sulfonate dioxygenase, taurine catabolism dioxygenase, taurine dioxygenase, dioxygenase, oxidoreductase, pyoverdine biosynthesis protein, and hypothetical (putative) or unnamed proteins. AAD-12 (aryloxyalkanoate dioxygenase-12) itself is an alpha-ketoglutarate dependent dioxygenase. Hypothetical and unnamed proteins are derived from conceptual translation of DNA sequences generated from massive genome sequencing projects of various fungi and bacteria. Those proteins have functional annotations such as "probable taurine catabolism dioxygenase", "clavaminic acid synthetase (CAS) –like", and "putative alpha-ketoglutarate dependent dioxygenase". None of these proteins returned by the BLATSp search is associated with toxicity.

In conclusion, AAD-12 protein expressed in soybean event DAS-68416-4 contains no significant sequence similarity with any known toxic protein that is harmful to humans or animals (Song, 2010d).

Table 8. BLASTp search summary of proteins in the alignments with AAD-12

Description	Number of alignments	E-value range
2,4-D/alpha-ketoglutarate dioxygenase	91	$1.00 \times 10^{-169} - 0.002$
Alkylsulfatase AtsK	6	$8.00 \times 10^{-22} - 4.00 \times 10^{-16}$
Alpha-ketoglutarate dioxygenase	254	$1.00 \times 10^{-33} - 5.70 \times 10^{-01}$
Alpha-ketoglutarate-dependent sulfonate dioxygena	29	$8.00 \times 10^{-18} - 0.044$
Taurine catabolism dioxygenase	155	$1.00 \times 10^{-82} - 0.33$
Taurine dioxygenase	468	$1.00 \times 10^{-32} - 0.32$
Dioxygenase	295	$2.00 \times 10^{-69} - 0.9$
Oxidoreductase	5	$1.00 \times 10^{-07} - 0.011$
Pyoverdine biosynthesis protein	29	$1.00 \times 10^{-06} - 0.18$
Hypothetical or unnamed proteins	245	$3.00 \times 10^{-31} - 0.75$

Assessment of gene disruption on the parental genomic locus and structural similarity of potential novel reading frames of the insert and its flanking regions to known toxins or other biologically active proteins that could cause adverse effects in humans or animals was performed.

All the junctions across the insert and its flanking borders were identified and screened for "novel" reading frames spanning the junction sites. A total of 12 "novel" reading frames were identified and 8 of them were evaluated for potential allergenicity and toxicity using bioinformatics tools since 4 of them are only 4 amino acids long. Searches of those putative "novel" reading frames against a peer reviewed allergen database (FARRP Allergen Database Version 10, Released in January, 2010) did not generate any significant amino acid sequence similarities with known allergens. Similarly, the search against the GenBank non-redundant protein sequences "nr" did not detect any protein sequence similarity with toxic proteins harmful to humans or animals (Song, 2010d).

Conversely, to update the characterization of the soybean genomic DNA sequences surrounding the insert location of event DAS-68416-4 soybean, the sequences of the flanking borders and parental locus were analyzed using BLAST search algorithms along with up-to-date GenBank nucleotide collection [Nucleotide collection (nr/nt)], Non-Human and Non-Mouse ESTs (est_others), and protein [Non-redundant protein sequences (nr)] databases. BLASTn and BLASTx analysis of the sequences comprising the insert of DAS-68416-4 and its 5' and 3' flanking border regions revealed identities only to soybean derived or pDAB4468 T-DNA derived sequences. The insert of DAS-68416-4 most likely integrated into a locus close to the 3' end downstream of a putative peroxidase gene in the soybean genome (Song, 2010b).

Assessment of toxicity to mammals

An acute oral toxicity study with AAD-12 protein was conducted in mice at a level of 2000 mg AAD-12/kg after adjustment for purity (Wiescinski and Golden, 2008-Attachment L [**CBI-DELETED: Section 68(a),(b) and (c)ii of the Promotion of Access to Information Act**]). All animals survived and no clinical signs were observed during the

study. All animals gained weight by study termination on day 15. There were no treatment-related gross pathological observations. Therefore the acute oral LD_{50} and no-observed-effect level (NOEL) of AAD-12 in male and female mice was greater than 2000 mg/kg based on the fact that no mortality was observed and there were no observable effects (adverse or non-adverse effects) with the AAD-12-treated animals. AAD-12 protein displays very low acute toxicity potential.

Safety assessment of the PAT protein

The PAT protein has already been found safe to human health during the assessment of glufosinate-ammonium tolerant maize (OECD, 1999). The *pat* gene was originally obtained from *Streptomyces viridochromogenes* strain Tü494 which has no known toxic or pathogenic potential. The PAT protein is enzymatically active but it has high substrate specificity to the active ingredient of glufosinate-ammonium (L-PPT). A toxicity study consisting of feeding rats with the PAT protein has been carried out (Health Canada, 1997; EFSA, 2007).

Biochemical characterisation

Mode of action

The active ingredient in glufosinate-ammonium herbicide is L-phosphinothricin (L-PPT). L-PPT binds to glutamine synthetase in plants preventing the detoxification of excess ammonia resulting in plant death (OECD, 2002). The activity of the PAT protein (phosphinothricin-N-acetyltransferase) is specific to catalysing the conversion of L-PPT to N-acetyl-L-PPT. This is an inactive form which does not bind to glutamine synthetase (De Block *et al*., 1987). As a consequence, the expression of the PAT protein in 59122 maize results in the absence of phytotoxic physiological effects when glufosinate-ammonium herbicides are applied.

The activity of the PAT protein has been described in detail in OECD (1999).

Equivalence of microbially-derived PAT protein DAS-68416-4 soybean expressed protein

In order to have sufficient amounts of purified PAT protein for the multiple studies required to assess their safety, PAT protein with equivalent biochemical structure and biological activity to the structure and activity of DAS-68416-4 soybean expressed AAD-12 protein was produced in *Pseudomonas fluorescens* (Schafer and Embrey, 2009b).

Greenhouse-grown DAS-68416-4 T4 plants were used as the plant source of the PAT protein. Prior to use, individual plants were leaf tested to confirm expression of the PAT protein using a rapid lateral flow test strip according to the manufacturer's instructions. Leaves (and some stems) from PAT expressing plants were harvested, lyophilized, ground to a fine powder, and stored frozen until needed.

Control soybean material

Control soybean line Maverick had a genetic background representative of the DAS-68416-4 soybean plants, but did not contain the *pat* gene. Absence of PAT expression in the control plants was confirmed by immunoassay using a PAT specific rapid lateral flow

test strip. Leaves (and some stems) of control plants were harvested, lyophilized, ground and stored under the same conditions as the DAS-68416-4 soybean.

Reference material
Recombinant PAT microbial protein was produced in *Pseudomonas fluorescens* (*Pf*) and purified to homogeneity. The microbe-derived PAT protein preparation was aliquoted and stored at -80 ºC to maintain activity.

SDS-PAGE and western blot analysis of crude extracts
Lyophilized leaf tissue from event DAS-68416-4 and Maverick was mixed with PBST buffer containing ~2.0% protease inhibitor cocktail (Sigma) and the protein was extracted by grinding with ball bearings in a Geno-Grinder. The samples were centrifuged and the supernatants were mixed with Laemmli sample buffer, heated and briefly centrifuged. The samples were loaded directly on to a Bio-Rad Criterion SDS-PAGE gel. The positive reference standard, microbe-derived PAT, was also mixed with sample buffer and loaded on to the gel. Electrophoresis was conducted with Tris/glycine/SDS buffer (Bio-Rad). Following electrophoresis, the gel was cut in half, with one half stained with Pierce GelCode Blue protein stain and the other gel half was electro-blotted onto a nitrocellulose membrane. The nitrocellulose membrane was then cut in half with one probed with a PAT specific polyclonal rabbit antibody and the remaining half probed with a PAT specific monoclonal antibody. A chemiluminescent substrate was used to visualize the immunoreactive bands.

Lateral flow test strip assay
The presence of the PAT protein in the pooled leaf tissue (T4) of DAS-68416-4 was confirmed using commercially prepared lateral flow test strips from EnviroLogix. The strips easily discriminated between transgenic and non-transgenic plants as the non-transgenic extracts of Maverick did not contain detectable amounts of immunoreactive protein. This result was also confirmed by western blot analysis.

SDS-PAGE and western blot analysis
In the toxicology-lot preparation of *P. fluorescens*-produced PAT protein (TSN105742), the major protein band, as visualized on Coomassie stained SDS-PAGE gels, was approximately 20.5 kDa (Figure 41 Panel A). As expected, the corresponding soybean-derived PAT protein was visualized by immunospecific polyclonal and monoclonal antibodies at an identical size to the microbe-expressed proteins (Figure 41, Panel B and C). The microbe-derived PAT protein also showed a positive signal of the expected size by polyclonal and monoclonal antibody western blot analysis (Figure 41, Panel B and C). In the PAT western blot analysis, no immunoreactive proteins were observed in the control Maverick extract and no alternate size proteins (aggregates or degradation products) were seen in the transgenic samples (Figure 41, Panel B and C). The monoclonal antibody did detect a small amount of the PAT dimer in the microbe-derived protein. These results add to the evidence that the protein expressed in soybean is not post-translationally modified which would have added to the overall protein molecular weight (Schafer and Embrey, 2009b).

The results of this study demonstrated that both the transgenic soybean-plant extract and

the microbe-derived PAT toxicological lot contained the intact, full-length PAT protein. This was confirmed by SDS-PAGE molecular-weight approximation, western blot analysis and commercially available lateral flow strip tests. Together, these biochemical tests indicate that the plant- and microbe-derived proteins are substantially equivalent, and therefore the microbe-derived protein is acceptable for use in regulatory studies.

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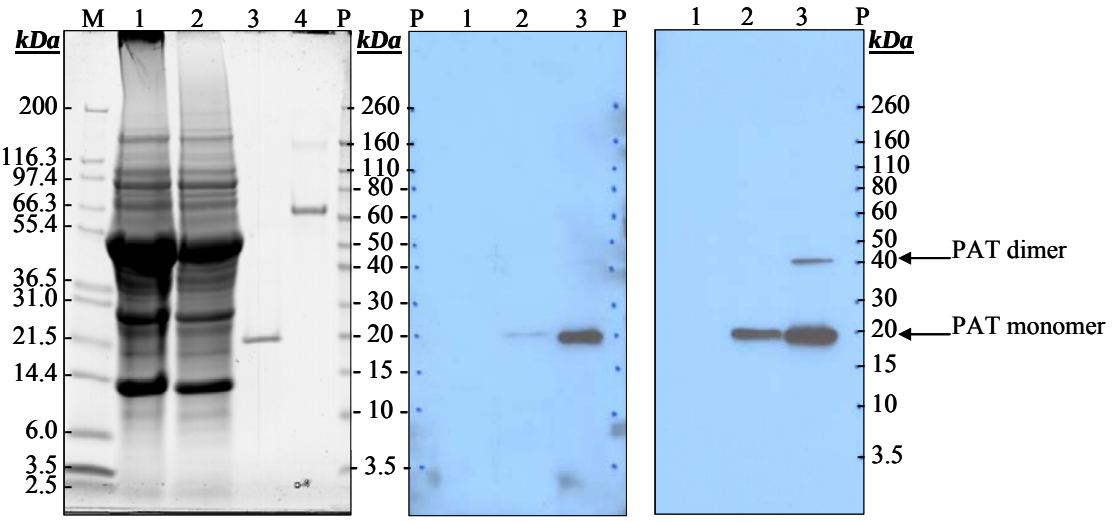
Figure 41. SDS-PAGE and western blots of transgenic soybean event DAS-68416-4 and non-transgenic Maverick



Panel A: SDS-PAGE

Panel B: Polyclonal antibody western blot

Panel C: Monoclonal antibody western blot

Lane	Sample	Amount
M	Invitrogen Mark12 molecular weight markers	10 µL
1	Non-transgenic (Maverick) soybean extract	40 µL
2	Transgenic (Event DAS-68416-4) soybean extract	40 µL
3	Microbe-derived PAT protein (TSN105742)	750 ng gel, 35 ng blot
4	Bovine serum albumin (BSA)	780 ng gel
P	Novex Sharp prestained molecular weight markers	10 µL

SDS-PAGE analysis of the transgenic and non-transgenic soybean extracts was performed with Bio-Rad Criterion gels (Cat #: 345-0123) fitted in a Criterion Cell gel module (Cat #: 165-6001) with MES running buffer (Bio-Rad Cat #: 161-0789). Extracts were prepared by Geno-Grinding (Spex, Model #: 2000) ~70 mg/mL of tissue with steel ball bearings in a PBST based buffer for 3 minutes in a chilled Teflon microfuge tube holder. The supernatants were clarified by centrifuging the samples for 5 minutes at 20,000×g and 120 µL of each extract was mixed with 30 µL of 5x Laemmli sample buffer containing 10% freshly added 2-mercaptoethanol and heated for 5 minutes at ~95 °C. After a brief centrifugation, 40 µL of the supernatant was loaded directly on the gel. The reference standard, microbe-derived PAT (TSN105742), and control standard, BSA, were diluted with 2x Laemmli sample buffer containing 5% 2-mercaptoethanol and processed as described earlier. The electrophoresis was conducted at a constant voltage of 150 V for ~60 minutes. After separation, the gel was cut in half and one half was stained with Pierce GelCode Blue protein stain (Cat #: 24592) and scanned with a densitometer (Molecular Dynamics, Personal Densitometer Si) to obtain a permanent record of the image. The remaining half off the gel was electro-blotted to a nitrocellulose membrane (Bio-Rad, Cat #:162-0233) with a Criterion trans-blot electrophoretic transfer cell for 60 minutes under a constant voltage of 100 volts. The transfer buffer contained 20% methanol and Tris/glycine buffer from Bio-Rad. After transfer, the membrane was cut in half and one half was probed with a PAT specific polyclonal rabbit antibody (EnviroLogix Lot #: 69:74A, 1.0 mg/mL) and the remaining half was probed with a PAT specific monoclonal antibody (DAS Lot #155AD4, 1.2 mg/mL). A conjugate of goat anti-rabbit IgG (H+L) and horseradish peroxidase (Pierce Chemical, Cat #: 31460) and goat anti-mouse IgG (H+L) and horseradish peroxidase (Bio-Rad, Cat#: 170-6516) were used as the secondary antibodies respectively. GE Healthcare chemiluminescent substrate (Cat #: RPN2132) was used for development and visualization of the immunoreactive protein bands. The membranes were exposed to Thermo Scientific CL-XPosure detection film (Cat #: 34091) for various time points and subsequently developed with an All-Pro 100 Plus film developer.

Toxicity assessment

Amino acid sequence comparison to known toxins

In addition, to further assess potential toxicity of the newly expressed proteins in DAS-68416-4 soybean, a search for similarity of protein sequences was conducted using the BLASTp program. Amino acid sequences of PAT protein were queried using the BLASTp (Version 2.2.22+) against a non-redundant protein dataset (update to January 8, 2010), which incorporates non-redundant entries from all GenBank and RefSeq nucleotide translations (Genpept "nr") along with protein sequences from SWISS-PROT (http://www.expasy.org/sprot/), PIR (http://pir.georgetown.edu/), PRF (http://www.prf.or.jp/aboutdb-e.html), and PDB (http://www.wwpdb.org/). The search was done through the BLAST website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in the NCBI (National Center of Biotechnology Information) with default settings (Matrix = BLOSUM62, Gap Costs = Existence: 11, Extension: 1) except that a cutoff expectation E-value of 1.0 was used to generate biologically meaningful similarity between the query sequences and proteins in the database, the low complexity filtering was turned off, and the sequence output was set to 1000 alignments. Although a statistically significant sequence similarity generally requires a match with an expectation value less than 0.01, a cutoff of E()< 1.0 insures that proteins with even limited similarity will not be overlooked in the search (Song, 2010f).

Bioinformatics analysis of the PAT protein expressed in DAS-68416-4 soybean using BLASTp search against an up-to-date GenBank non redundant protein database did not generate any significant sequence similarity with any known proteins that are harmful to humans or animals (Song, 2010f).

Toxicity studies in mammals

The PAT protein has also been tested in an additional acute toxicity study in mice (Brooks, 2000). As before, the relatively high dose tested did not give rise to any toxicity and therefore the acute LD_{50} of PAT protein could not be determined other than estimated to be higher than 5000 mg PAT per kg body weight.

In conclusion, a thorough evaluation of the safety of the AAD-12 and PAT proteins establishes that it is highly unlikely that these proteins would cause any toxic effects on human or animal health. Moreover, a confirmatory animal feeding experiment was conducted using whole-grain DAS-68416-4 fed to broiler chickens, which are known to be a sensitive animal model. As expected, this study did not indicate any nutritional effects or safety concerns for DAS-68416-4.

Allergenicity

Assessment of allergenicity of the newly expressed proteins

The assessment of allergenic potential of proteins compares the biochemical characteristics of these proteins to characteristics of known allergens (Metcalfe *et al.*, 1996; CODEX,

2009). With that in mind, the allergenic potential of the AAD-12 and PAT proteins expressed in DAS-68416-4 soybean was assessed as follows: i) safety of the donor organism; ii) homology with known allergens; and iii) *in vitro* simulated gastric fluid digestibility.

Safety assessment of the AAD-12 protein

Amino acid sequence comparison to known allergens

For the allergenicity assessment, the amino acid sequence of the AAD-12 and PAT proteins were compared with a peer-reviewed database containing 1471 known and putative allergens as well as celiac-induction proteins residing in the FARRP dataset (Version 10, Released in January 2010, University of Nebraska, http://www.allergenonline.org). Potential identities between the AAD-12, PAT and proteins in the allergen database were evaluated with the FASTA program (v34) using the default algorithm parameters (Matrix = BLOSUM50; Expect = 10; Gap Penalties = -12/-2). The FASTA search was run by an in-house Perl script in a UNIX computer with Linux operation system. If a query sequence is longer than 80 amino acids, the script parses the query sequence into a complete (overlapping) set of 80 amino acid long fragments and each fragment is subjected to a FASTA search. A greater than 35% identity threshold over any 80 or more amino acid sequences between a query sequence and an allergen was used to indicate the potential for cross-reactivity. The AAD-12 and PAT sequence was also screened for any matches of 8 contiguous amino acids to the allergens contained in the database noted above. This was done using the Fuzzpro program (Emboss Package v2.10.0) that generates all sequentially possible 8-residue peptides from a query protein and compares each query "word" with all allergen sequences in the database for perfect matches (Song, 2010c).

When the amino acid sequence of the AAD-12 protein was compared with the FARRP allergen dataset (Version 10), no over threshold identities (greater than 35% identity over greater than or equal to 80 amino acid residues) were detected in the FASTA search outputs (Song, 2010c). No matches of eight or greater contiguous identical amino acids with known allergens in the database were observed in the entire AAD-12 sequence.

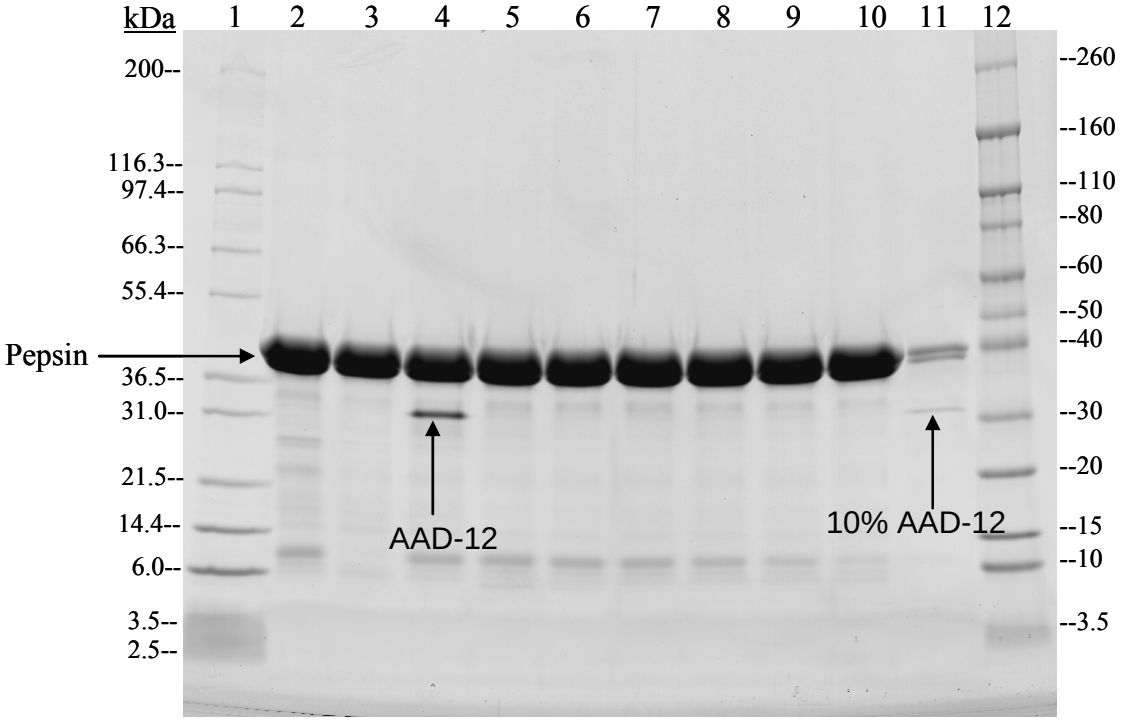
In conclusion, the results of this study show that AAD-12 does not share any significant amino acid sequence similarity with known protein allergens and is considered to have a low risk of allergenic potential.

Lability in simulated gastric fluid

The digestibility of the AAD-12 protein was tested *in vitro* using simulated gastric fluid (SGF) (Embrey and Shafer, 2008-Attachment M [**CBI-DELETED: Section 68(a),(b) and (c)ii of the Promotion of Access to Information Act**]). For the SGF method, the microbially-produced AAD-12 protein was incubated in SGF (0.32% w/v pepsin at pH 1.2; U.S. Pharmacopeia) at a ratio of enzyme to protein of 1.5 mg pepsin to 1.0 nM test substance solution (AAD12: 1.0 nM equals 33 µg). At each time point (0.5, 1, 2, 4, 8, and 16 minutes), 0.1 mL of the reaction mixture was removed and placed into a microcentrifuge tube containing 0.04 mL stop solution (200 mM Na2CO3, pH ~11.0). For

the zero time point samples, 2.85 mL SGF solution was neutralized with 1.2 mL stop solution and then the AAD-12 protein sample was added. All samples were kept on ice after the stop solution was added. After all digestion time points were completed, the samples were mixed with Laemmli sample buffer and heated at 95 ºC for 5 minutes. The samples were then analyzed via SDS-PAGE and western blot analysis using an antibody specific to AAD-12. The results demonstrated that the AAD-12 protein was readily digested (not detectable at 30 seconds) in SGF (Figure 42 and Figure 43).

Figure 42. SDS-PAGE analysis of AAD-12 (M.W. ~32 kDa) protein subjected to digestion in simulated gastric fluid



The neutralized and digested AAD-12 samples and SGF controls were held frozen for two days following the digestion. Samples were mixed with equal volumes of Laemmli sample buffer (containing 5% freshly added 2-mercaptoethanol) and heated for 5 minutes at ~95 °C. The samples were loaded into a Bio-Rad 4-20% Tris-HCl Criterion gel and electrophoresed at a constant voltage of 180 V for ~45 minutes using Tris/Glycine/SDS buffer from Bio-Rad. After separation, the gel was stained with GelCode Blue stain from Pierce Chemical. Invitrogen Mark 12 molecular weight markers 3.5 and 2.5 kDa represent Insulin A and B chains which are unresolved when separated on Tris-Glycine buffer systems.

Lane	Sample	Amount Loaded
1	Invitrogen Mark 12 MW markers	10 µL
2	SGF Reagent Blank, 0 minute incubation	40 µL
3	SGF Reagent Blank, >16 minute incubation	40 µL
4	Neutralized AAD-12 digestion	~1.67 µg
5	30-second AAD-12 digestion	~1.67 µg
6	1-minute AAD-12 digestion	~1.67 µg
7	2-minute AAD-12 digestion	~1.67 µg
8	4-minute AAD-12 digestion	~1.67 µg
9	8-minute AAD-12 digestion	~1.67 µg
10	16-minute AAD-12 digestion	~1.67 µg
11	10% Neutralized AAD-12 digestion	~0.17 µg
12	Invitrogen Novex Sharp Prestained MW markers	10 µL

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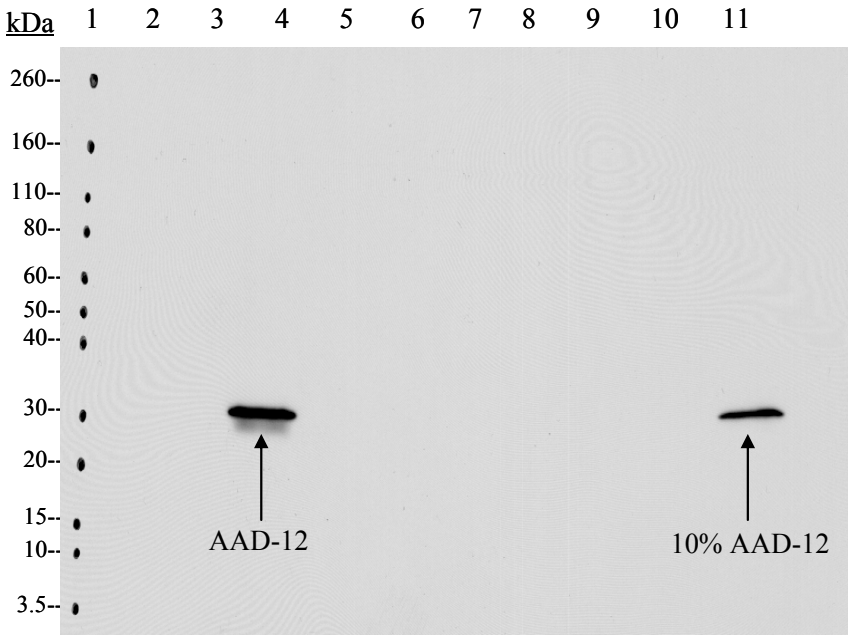
Figure 43. Western blot analysis of AAD-12 protein subjected to digestion in simulated gastric fluid



The neutralized and digested AAD-12 samples and SGF controls were held frozen for two days following the digestion. Samples were mixed with equal volumes of Laemmli sample buffer (containing 5% freshly added 2-mercaptoethanol) and heated for 5 minutes at ~95 °C. The samples were loaded into a Bio-Rad 4-20% Tris-HCl Criterion gel and electrophoresed at a constant voltage of 180 V for ~45 minutes using Tris/Glycine/SDS buffer from Bio-Rad. After separation, the gel was electro-blotted to a nitrocellulose membrane for 60 minutes under a constant charge of 50 volts. For immunodetection, the membrane was probed with an AAD-12 specific polyclonal rabbit antibody (Protein A purified: Lot #: DAS F1197-167-2, 4.3 mg/mL). A conjugate of goat anti-rabbit IgG (H+L) and horseradish peroxidase was used as the secondary antibody. GE Healthcare chemiluminescent substrate was used for development and visualization of the immunoreactive protein bands. The membrane was exposed to film and subsequently developed with a film developer. The molecular weight markers were manually transferred to the film after development.

Lane	Sample	Amount Loaded
1	Invitrogen Novex Sharp Prestained MW markers	10 µL
2	SGF Reagent Blank, 0 minute incubation	40 µL
3	SGF Reagent Blank, >16 minute incubation	40 µL
4	Neutralized AAD-12 digestion	~0.17µg
5	30-second AAD-12 digestion	~0.17µg
6	1-minute AAD-12 digestion	~0.17µg
7	2-minute AAD-12 digestion	~0.17µg
8	4-minute AAD-12 digestion	~0.17µg
9	8-minute AAD-12 digestion	~0.17µg
10	16-minute AAD-12 digestion	~0.17µg
11	10% Neutralized AAD-12 digestion	~0.017µg

Safety assessment of the PAT protein

Amino acid sequence comparison to known allergens

For the allergenicity assessment, the amino acid sequence of the AAD-12 and PAT proteins were compared with a peer-reviewed database containing 1471 known and putative allergens as well as celiac-induction proteins residing in the FARRP dataset (Version 10, Released in January 2010, University of Nebraska, http://www.allergenonline.org). Potential identities between the AAD-12, PAT and proteins in the allergen database were evaluated with the FASTA program (v34) using the default algorithm parameters (Matrix = BLOSUM50; Expect = 10; Gap Penalties = -12/-2). The FASTA search was run by an in-house Perl script in a UNIX computer with Linux operation system. If a query sequence is longer than 80 amino acids, the script parses the query sequence into a complete (overlapping) set of 80 amino acid long fragments and each fragment is subjected to a FASTA search. A greater than 35% identity threshold over any 80 or more amino acid sequences between a query sequence and an allergen was used to indicate the potential for cross-reactivity. The AAD-12 and PAT sequence was also screened for any matches of 8 contiguous amino acids to the allergens contained in the database noted above. This was done using the Fuzzpro program (Emboss Package v2.10.0) that generates all sequentially possible 8-residue peptides from a query protein and compares each query "word" with all allergen sequences in the database for perfect matches (Song, 2010e).

When the amino acid sequence of the PAT protein was compared with the FARRP allergen dataset (Version 10), no over threshold identities (greater than 35% identity over greater than or equal to 80 amino acid residues) were detected in the FASTA search outputs. No matches of eight or greater contiguous identical amino acids with known allergens in the database were observed in the entire PAT sequence (Song, 2010e).

PAT protein is readily degradable in simulated digestive juice, (United States Environmental Protection Authority [US EPA], 1995; US EPA, 1997; CFIA, 1998; OECD, 1999 and EFSA, 2007) and inmunoblot detection demonstrated the lack of glycosylation when expressed in soybeans (EFSA, 2007).

In conclusion, based on the lack of significant amino-acid-sequence homology to known allergens, and the lack of enzymatic and heat stability, the PAT protein is considered to have a low risk of allergenic potential.

Assessment of allergenicity of the whole GM plant or crop

As soybean is one of the top eight important allergenic foods (Sampson, 1999; Chapman *et al*., 2006; Sicherer and Sampson, 2006), a study was conducted to determine if the genetic modification used to generate DAS-68416-4 soybean altered the endogenous allergen content. In order to assess the potential impact of the genetic transformation on the overall allergenicity of DAS-68416-4 soybean, two different studies were performed: (i) one dimensional IgE immunoblot and ELISA inhibition and (ii) Western blot analysis of the 2D gels to assess the IgE reactivity with human plasma derived from soybean allergic patients.

One dimensional IgE immunoblot and ELISA inhibition

The purpose of this study was to assess the relative allergenicity of DAS-68416-4 soybeans compared with an isogenic line and commercially available soybeans using sera and plasma from soybean-allergic patients. DAS-68416-4 soybean was compared with its non-transgenic counterpart (Maverick) and commercially available soybeans by one dimensional (1D) IgE immunoblot (qualitative analysis) and ELISA inhibition (a quantitative assessment).

The protein profiles between DAS-68416-4 and the non-transgenic soybean line, Maverick were compared using SDS-PAGE analysis with Coomassie blue staining, which did not reveal any differences in protein banding patterns between the two soybean extracts or any of the commercial non-GM varieties (Stagg, 2010-Attachment N [**CBI-DELETED: Section 68(a),(b) and (c)ii of the Promotion of Access to Information Act**]). The IgE binding profiles of DAS-68416-4 and Maverick were compared in the one-dimensional immunoblot analysis with 20 different samples of human soy-allergic serum/plasma and no differences were detected (Stagg, 2010). All samples were within the normal range of variability seen with the commercial varieties.

The ELISA inhibition data with the individual and pooled human soybean-allergic serum/plasma samples showed the same IgE binding (inhibition) response between the non-transgenic Maverick soybean and DAS-68416-4 soybean extracts against 2 µg/well of immobilized Maverick extracts on the plate (Stagg, 2010). Furthermore, the associated EC_{50} values for Maverick and DAS-68416-4 were similar and fall within the calculated 95% confidence intervals.

Collectively, the SDS-PAGE, 1D immunoblot and ELISA inhibition data demonstrate that the genetic modification used to generate DAS-68416-4 soybean did not alter the endogenous allergenicity compared with its non-transgenic control, Maverick.

Western blot analysis of the 2D gels to assess the IgE reactivity with human plasma derived from soybean allergic patients

Initial experiments were conducted to assess the spot pattern of Maverick and event 416 after separation on IPG strips from pH 3-11 in the 1st dimension and on 4-20% gradient SDS-PAGE gels in the second dimension. Samples were loaded at two different proteins concentrations to assess that the loading indicated from the BCA assay was compatible with the 2D-SDS-PAGE separation. Results from this experiment are shown in Harpham and Stagg (2010) [Attachment O [**CBI-DELETED: Section 68(a),(b) and (c)ii of the Promotion of Access to Information Act**].

The results of the 2D-SDS-PAGE show that the sample separation is similar between event 416 and Maverick, and the majority of proteins in the samples are found between 10 and 110 kDa. The results also show that the loading at 63 µg per IPG strip gave good separation and visualization. Therefore, it was decided to run future experiments at a load of 63 µg per IPG strip and to use 12% SDS-PAGE gels in the second dimension. This type of gel is also widely used in published results (2,4), and the 2D separations detected are comparable to those found previously (Harpham and Stagg, 2010).

The 2D western blot analysis of the soybean seed extracts was carried out in two experimental batches. Within each batch, six gels were run, three of Maverick and three of event 416. Pairs of Maverick and event 416 gels were run and blotted together to improve comparability. The results are presented in Harpham and Stagg (2010).

The results of the 2D western blots with the 6 soy-allergic human IgE samples show similar IgE binding between event 416 and Maverick. These results are consistent with the 1D immunoblot and ELISA inhibition testing of event 416 and Maverick.

The 2D-SDS-PAGE western blots with well-characterized soy allergic plasma show similar IgE binding between Maverick and event-416. This data demonstrates that the insertion of the AAD-12 gene did not alter the levels of endogenous allergens and no new allergenic proteins were expressed as a consequence of the genetic modification.

Nutritional assessment

The herbicide tolerance trait in DAS-68416-4 is of agronomic interest and not intended to change any nutritional aspects of this soybean, nor is the presence of this trait expected to alter patterns or volumes of soybean consumption. Several studies have been conducted to demonstrate equivalence of DAS-68416-4 to conventional soybean. These studies are summarised below.

Nutritional assessment of GM food

Composition analyses of DAS-68416-4 soybean have shown that the contents of protein, fiber, carbohydrates, fat, ash, minerals, fatty acids, amino acids, vitamins, secondary metabolites and anti-nutrients are equivalent to that found in non-GM control soybean with comparable genetic background, representative commercial lines, and to the published range of values in the literature (Phillips and Lepping, 2010b-Attachment P [**CBI-DELETED: Section 68(a),(b) and (c)ii of the Promotion of Access to Information Act**]). The comparable composition of DAS-68416-4 soybean together with the results of the assessment of dietary intake and nutritional impact (see subsequent sub-section) confirm that food products derived from DAS-68416-4 soybean are nutritionally equivalent to food products derived from commercial soybean. Hence, anticipated dietary intake, of soybean derived foods and feeds from DAS-68416-4 soybean, is not expected to change.

Nutritional assessment of GM feed

A study was conducted to evaluate the nutritional and metabolic value of feed containing a genetically-modified (GM) soybean, a non-modified near isogenic soybean, or standard commercially available soybeans (Fletcher, 2010-Attachment Q [**CBI-DELETED: Section 68(a),(b) and (c)ii of the Promotion of Access to Information Act**]). All birds received their respective diets for 42 days. Effects on mortality and weight gain, feed conversion efficiencies, and market dressed carcass, muscle (breast, thigh, leg and wing), liver and abdominal fat pad weights were determined.

There were no adverse effects of the consumption of DAS-68416-4 soybean on mortality or moribundity, general clinical observations, body weight, body weight gain, or feed conversion. Daily feed intake was 3.7% less for male birds fed diets containing the transgenic soybean

meal compared with those fed the non-transgenic near-isogenic soybean meal, but this did not occur for females and was not manifested in any significant change in performance for the male birds. This difference may have been an artifact related to the loss of male birds in all treatments (which were typical of commercial production), which can affect estimation of feed intake. These results indicate that DAS-68416-4 soybean is nutritionally equivalent to the non-transgenic near-isogenic control.

Consumption

There are three major soybean commodity products: seeds, oil, and meal. There is animal feed use, and no food use for unprocessed soybeans, since they contain anti-nutrient factors, such as trypsin inhibitors and lectins. Considering that the high temperatures used in processing degrade the proteins, we anticipate negligible amounts of proteins being present in products derived from DAS-68416-4 soybean. Whilst DAS-68416-4 soybean also expresses the PAT protein, our dietary risk assessment does not focus further on it, because the PAT protein is a safe protein for humans, animals and the environment that is well-understood and risk assessed, with a long list and history of regulatory approvals. Hence, this report presents a dietary exposure assessment for the AAD-12 protein from DAS-68416-4 soybean for humans and livestock.

Anticipated intake/extent of use by humans

The highest possible intake estimates for both single serving (acute, or short term intake (STI)) and repeat dose (chronic or average daily intake) soybean exposure were used for purposes of the Human Dietary Assessment. For a dietary risk/safety assessment the STI consumption is typically compared to an acute toxicity endpoint while the repeat dose consumption is compared to a repeat dose toxicity endpoint (Cleveland, 2010-Attachment R [**CBI-DELETED: Section 68(a),(b) and (c)ii of the Promotion of Access to Information Act**]). Each of these scenarios is discussed separately.

Short term intake/Acute assessment

A conservative acute consumption (i.e. exposure) estimate is made based on global data published by the World Health Organization (WHO). WHO has established a maximum consumption of each food commodity for acute exposures for the entire world, based on maximum inputs from multiple countries[1] (WHO, 1999). Table 9 includes 97.5th percentile values for various commodities associated with soybean. For DAS-68416-4 soybean, the appropriate maximum consumption value is associated with dry soybean with an upper limit for soybean (dry) reported by Japan. Consumption information for soybean oil is not used for the analysis as it is known that oils and other highly refined fractions do not contain significant amounts of protein. Consumption information for immature seeds is also excluded from the analysis as this is an application for commodity clearance of dry soybean grain and the presence of immature seeds is not envisaged. Moreover, total acute consumption for all commodities associated with soybean cannot be calculated, because it is not appropriate to add 97.5th percentile values for individual commodities for survey results from different countries.

[1] FAO WHO, http://www.who.int/foodsafety/chem/en/acute_hazard_db1.pdf

Table 9. Estimates of acute soybean consumption from the GEMS/Food highest 97.5th percentile "eater-only" worldwide

		Consumption[a] (g/kg/day)	
Commodity	Country with Reported Maximum	General Population	Children ≤6 years
Soybean (dry)	**Japan**	**3.03**	**5.55**
Soybean (immature seeds)	Thailand	2.41	3.86
Soybean oil[b] (refined)	USA	1.51	2.36

[a] Total acute consumption across these entities cannot be calculated because, it is not appropriate to add 97.5th percentile values for individual commodities survey results from different countries.
[b] Even though information on soybean oils is provided, for completeness, it is understood that oil will not contain appreciable amounts of protein.

When the soybean (dry) acute consumption information is coupled to the the AAD-12 field expression level of 21.49 ng/mg tissue, the potential acute exposure to AAD-12 protein *via* soybean is estimated as:

- 0.0651 mg protein/kg bw/day, for general population (i.e. adults)
- 0.1192 mg protein/kg bw/day, for children of 6 years or younger

Acute margin of exposure calculation
Acute risk assessments are typically not required for substances with acute NOEL values above 500 mg/kg bw/day or for compounds which have no associated mortalities below 1000 mg/kg bw in single dose studies (Solecki *et al*., 2005). Nonetheless, to place the AAD-12 protein exposure estimate in context, a comparison of the exposure information to the lower limit NOEL has been made to provide margins of exposure (MOE) in Table 10 for AAD-12 protein where:

$$MOE = \frac{NOEL}{Exposure}$$

The larger the MOE value, the less likelihood there is for adverse effects, because the exposure is well below the established NOEL threshold. The calculated MOE values for AAD-12 protein in soybean are extremely large, indicating no concern for adverse effects from acute dietary exposure through soybean.

Table 10. Margins of exposure for AAD-12 protein in soybean based on WHO 97.5th percentile consumption

	Exposure[a] (mg AAD-1 /kg bw/day)	NOEL (mg/kg bw)	MOE
General Population	0.0651	>2000	>30 720
Children <6 year	0.1192	>2000	>16 775

[a] Based on WHO 97.5th percentile consumption of dry soybean.

Repeat dose assessment

The GEMS/Food consumption cluster diets[1] give estimates of regional dietary patterns of raw and semi-processed food commodities. The diets are built with the FAO Stats data but are expressed on the 1993 Codex classification basis. South Africa is classified under WHO Cluster I[2] which predicts consumption of dry soybean among the general population (adults) as 11.2 g soybean/day or 0.187 g/kg bw/day (assumes 60kg as body weight for an adult). When the WHO soybean (dry) chronic consumption information is coupled to the AAD-12 field expression level of 21.49 ng/mg tissue, a repeat exposure to AAD-12 protein via soybean is estimated as:

- 0.00402 mg protein/kg bw/day, for general population

The corresponding repeat dose MOE is made from a comparison of the consumption exposure to the lower limit repeat dose NOEL of 47 mg/kg (the NOEL for AAD-12 protein in male and female Crl:CD1(ICR) mice, following 28 days of dietary administration, was the highest targeted dose of 47 mg/kg-bw/day, Cleveland, 2010). The resulting ratios indicate that a MOE value greater than 11 600 fold has been demonstrated, which in turn indicates the likelihood for adverse effects from exposure to AAD-12 protein from soybean is very low, as shown in Table 11. Additionally, the size of these margins of safety provides a further cushion for the use of short-term toxicity data to cover longer or chronic exposure.

Table 11. Margins of exposure for AAD-12 protein in soybean based on WHO/GEMS Cluster I consumption

Soybean consumption levels	Exposure[a] (mg AAD-12 /kg bw/day)	NOEL (mg/kg bw)	MOE
General Population	0.00402	>47	>11 600

[a] Based on chronic consumption of dry soybean.

Estimated consumption by animals

A dietary exposure estimate for novel feed in livestock diets based on traditional use of the unmodified feeds is provided here by coupling field expression information for AAD-12 protein from DAS-68416-4 soybean plants with livestock dietary consumption assumptions for soybean seed, soybean meal and soybean hulls. In addition, the relevance of the exposure estimate is placed into context, based on the mammalian toxicity information.

Animal feed exposure

This livestock assessment has been constructed based on the traditional use of the unmodified counterpart as per the diets listed in the Annex 4 Harmonized Feedstuffs Table of the 2009 OECD Guidance for residue studies (OECD, 2009). Even though the South African estimates are not given in the OECD estimates, a worst case scenario is taken by using figures from

[1] http://www.who.int/foodsafety/chem/gems/en/index1.html
[2] http://www.who.int/foodsafety/chem/countries.pdf

those regions or countries with the highest amounts of exposure. In addition, consumption of soybean hull is included in the assessment even though consumption of soybean hulls of the product of this application is not envisaged. Furthermore, the estimates of dietary exposure to AAD-12 in this application are conservative (and protective) because in the calculations a 100% replacement of the unmodified counterpart is assumed, while in actual practice DAS-68416-4 would be mixed with other soybean before this commodity is processed and consumed. Furthermore, not all imported soybean will contain the AAD-12 protein. In addition maximum potential exposure to soybean for the reference animals was assumed. The resulting intake dietary burden for animal feeds is totalled in (Table 12) for the four typical representative animals of Beef and Dairy Cattle, Poultry (broiler) and Swine (finishing). Use of the reference animal weight and feed consumption allows for a translation to daily dose by animal in (Table 13).

$$\text{Animal Dietary Burden} = \frac{\text{Dietary Contribution} \times \text{AAD-12 concentration}}{\text{Dry Matter}}$$

Table 12. Intake animal dietary burdens for livestock

	Dry Matter (%)	Dietary Contribution (%)					Animal Dietary Burden (ppm)			
Feedstuff		**Beef**	**Dairy**	**Swine**	**Poultry**	**AAD-12 (ppm)[a]**	**Beef**	**Dairy**	**Swine**	**Poultry**
Soybean seed	89	20	20	20	20	**21.49**	4.83	4.83	4.83	4.83
Soybean meal	92	65	60	30	40	**16.6**[b]	11.73	10.83	5.41	7.22
Soybean hulls	90	15	10	10	10	**21.49**	3.58	2.39	2.39	2.39
						Total	**20.14**	**18.05**	**12.63**	**14.44**

[a] Estimated content of AAD-12 protein in grain used for all assessments except for soybean meal
[b] Herman *et al*. (2011)

The Daily Dose Estimates (Table 13) are then computed as:

$$\text{Daily Dose Estimate} = \frac{\text{Maximum AAD-12 Intake} \times \text{Daily Maximum Feed}}{\text{Body weight}}$$

Table 13. Livestock daily dose estimates of AAD-12 protein from soybean

	Cattle		Swine	Poultry
	Beef	**Dairy**	**Finishing**	**Broiler**
Body weight (kg)	500	600	266	1.7
Daily maximum feed [kg Dry Matter (DM)]	9.1	17	3	0.12
Maximum AAD-12 intake (mg/kg feed)	20.14	18.05	12.63	14.44
Maximum intake (mg/kg bw)	**0.37**	**0.51**	**0.14**	**1.02**

The highest potentially exposed reference animal is poultry with 1.02 mg AAD-12/kg bw. When this value is compared to the repeat dose mammalian NOEL of >47 mg/kg bw, there is an adequate margin of safety for livestock. Variations in calculated livestock feed diets or reference animals could results in slight changes in the calculated values, but would not alter the conclusion regarding the large margin of safety afforded livestock animals for AAD-12 protein in DAS-68416-4 soybean.

In conclusion, results of the overall safety assessment of the AAD-12 protein indicate that it is unlikely to cause adverse effects in humans or animals. AAD-12 protein has been demonstrated to pose a low risk of toxicity to mammals with an acute NOEL value of >2000 mg/kg bw and a repeat dose NOEL of >47 mg/kg bw/day. The assessments presented here are known to be conservative and an overprediction of actual dietary exposure to the protein in South Africa. Actual exposure and impact will be lower because: 1) there may be protein degradation during transport and storage, 2) soybean containing AAD-12 will be mixed with non-AAD-12 soybean, 3) for humans, consumption of soybean products is often in food forms which are cooked and heat is known to denature this protein.

6.3 If the foreign gene products are toxic or allergenic in any way, detail how the commodity clearance will be managed to prevent contact with animals or humans that will lead to discomfort or toxicity.

Based on the characteristics of the introduced genetic material and of the newly expressed protein, AAD-12, together with the evidence provided throughout this application, it can be concluded that DAS-68416-4 soybean does not introduce any new allergens and that the inherent allergenicity of soybean has not been altered. Consequently, DAS-68416-4 soybean is substantially equivalent to conventional soybean and no special management of this product is required.

6.4 What are the common/major allergens present in the recipient organism before modification?

Soybeans have a relatively high intrinsic allergenicity. They possess as many as 15 proteins recognized by IgEs from sensitive people (Burks *et al*., 1988). The immunodominant soybean allergens are the β-subunit of conglycinin and a member of the papain family of cysteine proteases termed P34 or Gly m Bd 30k. The P34/Gly m Bd 30k protein is a unique member of the papain superfamily lacking the catalytic cysteine residue that is replaced by a glycine. These two allergens account for the large majority of the IgE cross-reactivity for soybean-sensitive people with the P34/Gly m Bd 30k protein alone accounting for about two-thirds of the IgE cross-reactivity in the two populations that have been studied, Japanese nationals and babies in the US. A database of the allergenic proteins found in soybean can be found online at http://www.allergome.org/.

6.5 What evidence is there that the genetic modification described in this application did not result in over-expression of the possible allergens indicated in 6.4 i.e. is

the expression of the possible allergens in the non-GM counterpart substantially equivalent to that in the GM organism?

Experiments have directly tested the allergenicity of herbicide-tolerant soybeans using immunological tests with samples from soybean-sensitive people. These assays have shown that herbicide-tolerant GM soybeans do not present any measurable differences in allergenicity compared with non-GM soybeans (Burks and Fuchs, 1995) and are, therefore, substantially equivalent by allergenic criteria. Sensitive people remain allergic to GM soybeans, but there is no additional allergenic risk to others.

6.6 What are the implications of the proposed activity with regard to the health and safety of the workers, cleaning personnel and any other person that will be directly or indirectly involved in the activity? Please take into consideration the provisions of the Occupational Health and Safety Act, 1993 (Act No. 181 of 1993) and accompanied regulations.

The grain produced from DAS-68416-4 is substantially equivalent to conventional soybean grain. All activities regarding the health and safety of individuals associated either directly or indirectly with grain/food products derived from DAS-68416-4 will be the same as for conventional soybean.

6.7 Indicate the proposed health and safety measures that would be applied to safeguard employees during the proposed activity.

Considering that DAS-68416-4 is substantially equivalent to conventional soybean, no measures are required.

7. ENVIRONMENTAL IMPACT AND PROTECTION

7.1 Detail any long-term effect the commodity clearance of the genetically modified organism is likely to have on the biotic and abiotic components of the environment.

This is an application for commodity clearance approval of DAS-68416-4 and is not intended for release into the environment of South Africa.

7.2 Provide data and information on ecosystems that could be affected by use of the plant or its products.

As indicated above, this is not an application for release of DAS-68416-4 into the environment of South Africa.

7.3 Specify what effect the general release of the genetically modified plant will have on biodiversity.

As indicated above, this is not an application for release of DAS-68416-4 into the environment of South Africa.

Based on centuries of experience with conventional, domesticated soybean in Africa, there is no potential for soybean to be invasive of natural habitats or persist in the agricultural environment without the aid of human intervention. Soybean is a poor competitor, which outside of cultivation has no meaningful impact on biodiversity or the environment (Abel, 1970; OECD, 2000).

7.4 Specify the measures to be taken in the event of the plant or product being misused or escaping into an environment for which it is not intended.

This is an application for commodity clearance approval of DAS-68416-4, i.e. use as food, feed or in processing. As indicated previously, grain imports are made by international grain traders, with the time of importation dependent on the local or regional need for grain. The grain traders would, as per the requirements in terms of the Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997) obtain the necessary permits from the Registrar prior to importation. Importation activities would thus be subjected to the conditions (such as milling) prescribed in the relevant import permits.

Information and data provided in this application support the conclusion that, except for the specifically introduced herbicide tolerance trait, DAS-68416-4 is substantially equivalent to conventional soybean. In the unlikely event that some grain may end up in the environment of South Africa before being crushed, the impact would be negligible as soybean is known to be a poor competitor outside of the agricultural environment. Cultivated soybean has been domesticated to the extent that it cannot survive outside managed agricultural environments. In addition, lack of dormancy prevents soybean seed from readily surviving from one growing season to the next. Furthermore, South Africa is not the centre of origin for *Glycine max* and there are no wild relatives in South Africa with which soybean can outcross.

7.5 If the foreign genes give rise to crops resistant to agrochemicals, provide information on the registration of the agrochemicals to be used on the crop.

As indicated above, this is not an application for release of DAS-68416-4 into the environment of South Africa.

8. SOCIO-ECONOMIC IMPACTS

8.1 Specify what, if any, positive or negative socio-economic impacts the genetically modified plant will have on communities in the proposed region of release.

As indicated above, this is not an application for release of DAS-68416-4 into the environment of South Africa.

9. WASTE DISPOSAL

9.1 Where only a portion of the genetically modified plant will be used for the product, how will the unused plant parts be disposed of?

This is an application for commodity clearance approval of DAS-68416-4. DAS-68416-4, like any other soybean grain contained in an imported consignment, would be used as food, feed or in processing.

10. MONITORING AND ACCIDENTS

10.1 Indicate the methods and plans for monitoring of the GMO (also refer to Environmental Risk Assessment Framework for genetically modified organisms)

This is an application for commodity clearance approval of DAS-68416-4, *i.e.* use as food, feed or in processing. Intentional release of DAS-68416-4 into the environment of South Africa is not intended.

As indicated previously, grain imports are made by international grain traders, with the time of importation dependent on the local or regional need for grain. The grain traders would, as per the requirements in terms of the Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997) obtain the necessary permits from the Registrar prior to importation. Importation as well as use of the grain would thus be subjected to the conditions prescribed in the relevant import and use-as-commodity permits. Adherence to these permit conditions would be monitored by the Registrar's office.

10.2 Indicate any emergency procedures that will be applied in the event of an accident.

Information and data provided in this application supports the conclusion that except for the specifically introduced herbicide tolerance trait, DAS-68416-4 is substantially equivalent to conventional soybean. In the unlikely event that some grain may end up in the environment of South Africa before being crushed, the impact would be negligible as soybean is known to be a poor competitor outside of the agricultural environment. Cultivated soybean has been domesticated to the extent that it cannot survive outside managed agricultural environments. In addition, lack of dormancy prevents soybean seed from readily surviving from one growing season to the next. Furthermore, South Africa is not the centre of origin for *Glycine max* and there are no wild relatives in South Africa with which soybean can outcross.

11. PATHOGENIC AND ECOLOGICAL IMPACTS

11.1 Submit an evaluation of the foreseeable impacts, in particular any pathogenic and ecologically disruptive impacts.

As indicated before, except for the specifically introduced herbicide tolerance trait, DAS-68416-4 is substantially equivalent to conventional soybean.

With this application, DAS-68416-4 is destined for use as food, feed and in processing. No environmental release is proposed. In the unlikely event that some grain should inadvertently end up in the environment, the potential of any adverse environmental impact, including any pathogenic or ecologically disruptive impact, is negligible.

12. RISK MANAGEMENT

12.1 Please indicate any risk management measures that would be required for commodity clearance.

With this application, a comprehensive data package is provided to demonstrate that DAS-68416-4 is, except for the specifically introduced traits, substantially equivalent to conventional soybean and has no negative impacts on human and animal health.

This commodity clearance approval is to support the potential shortage of soybean in South Africa and the subsequent need to import grain from exporting countries that may be commercially growing DAS-68416-4 and other genetically modified soybean.

Grain imports are done by grain traders. Grain imported into South Africa for food and feed use would contain different GM events, depending on the events commercially grown in the exporting country. The measures pertaining to monitoring of the imported grain and emergency measures to be taken in an event of accidental spillage would be prescribed by the Executive Council and contained in the permits issued by the Registrar to the importers and the users (*i.e.* millers), as these are the parties that will handle and use the grain.

Accidental release of DAS-68416-4 would be negligible. Soybean plants are unable to survive without the intervention of man; they are totally dependent on cultivation and care to be able to grow, mature and produce seed. Thus, even if any grain from an imported consignment, which could contain DAS-68416-4, accidentally found their way into the environment, the grain would still require the typical agronomic practices required to ensure a sustainable crop. Furthermore, South Africa is not the centre of origin for soybean and there are no close relatives with which soybean can outcross. Therefore, should there be an accidental release into the environment, it is extremely unlikely that the traits contained in the consignment of grain would move into existing crop populations and survive.

13. COMPLETE THE AFFIDAVIT

The completed affidavit is provided at the end of this application.

CBI-DELETED

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COMMON FORMAT FOR Risk Assessment
(In accordance with Annex III of the Cartagena Protocol on Biosafety)

	Risk assessment details
1. Country Taking Decision:	Republic of South Africa.
2. Title:	Application for Commodity Clearance of DAS-68416-4 in the Republic of South Africa.
3. Contact details:	Dow AgroSciences South Africa, P.O. Box 76129 Lynnwoodridge, Pretoria 0040, South Africa. Telephone: (+27) 12 361 8120; Fax (+27) 12 361 8126.
	LMO information
4. Name and identity of the living modified organism:	The LMO is DAS-68416-4. DAS-68416-4 soybean was developed using *Agrobacterium*-mediated transformation to stably incorporate the *aad*-12 gene from *Delftia acidovorans* and *pat* gene from *Streptomyces viridochromogenes* into soybean. The *aad*-12 gene encodes the aryloxyalkanoate dioxygenase-12 (AAD-12) enzyme which, when expressed in plants, degrades 2,4-D into herbicidally-inactive 2,4-dichlorophenol (DCP). The *pat* gene encodes the enzyme phosphinothricin acetyl transferase that inactivates glufosinate.
5. Unique identification of the living modified organism:	DAS-68416-4
6. Transformation event:	DAS-68416-4
7. Introduced or Modified Traits:	**Chemical tolerance** - Herbicide tolerance.
8. Techniques used for modification:	*Agrobacterium*-mediated transformation.
9. Description of gene modification:	Transgenic soybean (*Glycine max*) DAS-68416-4 was generated through *Agrobacterium* mediated transformation, using the disarmed *Agrobacterium tumefaciens* strain EHA101 carrying the binary vector pDAB4468 that leads to the transfer and insertion of its TDNA into the genome of cells from soybean cotyledonary node explants.
	Characteristics of modification

10. Vector characteristics (Annex III.9(c)):	The vector pDAB4468 is a binary one derived from *Agrobacterium tumefaciens*. DAS-68416-4 was generated by *Agrobacterium*-mediated transformation using the plasmid pDAB4468. The T-DNA insert in the plasmid contains a synthetic, plant-optimized sequence of the *aad*-12 gene from *Delftia acidovorans* and the *pat* gene from *Streptomyces viridochromogenes*.
11. Insert or inserts (Annex III.9(d)):	The transgene insert in soybean event DAS-68416-4 occurred as a simple integration of the T-DNA insert from plasmid pDAB4468, including a single, intact copy of the *aad*-12 and *pat* expression cassettes. The event is stably integrated as shown by its stable inheritance pattern across several breeding generations, and no plasmid backbone sequences are present.
Recipient organism or parental organisms (Annex III.9(a)):	
12. Taxonomic name/status of recipient organism or parental organisms:	Family; Leguminosae: Genus; *Glycine:* Species; *Glycine max*
13. Common name of recipient organism or parental organisms:	Soybean
14. Point of collection or acquisition of recipient or parental organisms:	Privately owned germplasm.
15. Characteristics of recipient organism or parental organisms related to biosafety:	Soybean is a one of the oldest cultivated crops and a well-known crop plant worldwide. Soybean is a common source of food and feed with a centuries-long history of safe use and consumption around the world. Soybean is widely grown in South Africa and has a history of safe use. Soybean seed is however known to contain a number of natural anti-nutritional components, which are completely or partially inactivated during processing. Trypsin (proteinase) inhibitors are known to have anti-nutritive properties in animals fed unprocessed soybeans. Other anti-nutrients include lectins, stachyose and raffinose, phytoestrogens and phytate. Some of these anti-nutrients relate to their impact on human nutrition, while others relate to animal nutrition in general including livestock.
16. Centre(s) of origin of recipient organism or parental organisms:	North and Central China. Specific geographic coordinates are unknown.

17. Centres of genetic diversity, if known, of recipient organism or parental organisms:	Centers of genetic diversity of soybean are the same as its centre of origin.
18. Habitats where the recipient organism or parental organisms may persist or proliferate:	Soybean does not persist or proliferate outside of agriculture in South Africa. There are no known populations in any natural habitat in the country.
Donor organism or organisms (Annex III.9(b)):	
19. Taxonomic name/status of donor organism(s)	1. *Nicotiana tobacum*: donor of the Matrix Attachment Region v3: Magnoliopsida; Solanales; Solanaceae; *Nicotiana* 2. *Arabidopsis thaliana* donor of the Ubi10 promoter: Rosids; Brassicales; Brassicaceae; *Arabidopsis* 3.*Delftia acidovorans*: donor of the *aad*-12 gene: Betaproteobacteria; Comamonadaceae; *Delfia* 4. *Agrobacterium tumefaciens* donor of the 3' UTR of the ORF23 and ORF1: Alfa Proteobacteria; Rhizobiales; Rhizobiaceae; *Agrobacterium* 5. *Cassava vein mosaic virus* donor of the CsVMV promoter: *Caulimoviridae; Cavemovirus;* Species: Cassava vein mosaic virus 6. *Streptomyces viridochromogenes*: donor of the *pat* gene: Betaproteobacteria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; *Streptomyces*
20. Common name of donor organism(s):	Tobacco; Arabidopsis; Bacteria; Virus
21. Point of collection or acquisition of donor organism(s):	None of the donor organisms was collected from a specific geographical location.

22. Characteristics of donor organism(s) related to biosafety:	1. *Nicotiana tobacum*: *N. tabacum* is a native of tropical and subtropical America but it is now commercially cultivated worldwide (its leaves are commercially grown in many countries to be processed into tobacco). 2. *Arabidopsis thaliana:* is native to Europe, Asia, and northwestern Africa. It is an annual (rarely biennial) plant usually growing to 20–25 cm tall. The small size of its genome makes *Arabidopsis thaliana* useful as a model plant for plant biology study and has been widely used safely for studies including genetic mapping and sequencing, *etc.* 3. *Delftia acidovorans*: is a non glucose-fermenting, gram-negative, non spore-forming rod prevalent in soil and fresh water. Some species have also been isolated from activated sludge and clinical specimens. *Delftia acidovorans* can be used to transform ferulic acid into vanillin and related flavor metabolites. This utility has led to a history of safe use for *D. acidovorans* in the food processing industry. This strain also produces polyhydroxyalkanoates that are being developed as biomaterials for medical applications. There are limited reports of *D. acidovorans* causing infections in compromised patients. There are no reports of this strain producing any allergens. 4. *Agrobacterium tumefaciens*: is a bacterium that is found in soils worldwide. It infects plants but does not infect humans or animals. 5. *Cassava vein mosaic virus:* is a plant pathogenic virus that infects many crops and is therefore a commonly ingested virus which however does not infect humans or animals, or even plants outside its host range. 6. *Streptomyces viridochromogenes*: is a common soil bacterium that produces the tripeptide L-phosphinothricyl-L-alanyl-alanine (L-PPT), which was developed as a non-selective herbicide by Hoechst Ag. It is ubiquitous in nature with no known adverse effects on human and animal health.
Intended use and receiving environment	
23. Intended use of the LMO (Annex III 9(g)):	For all uses as for any other soybean, excluding cultivation.
24. Receiving environment (Annex III.9(h)):	This is not an application for release of DAS-68416-4into the environment of South Africa.
Risk assessment summary	

25. Detection/Identification method of the LMO (Annex III.9(f)):	PCR detection methods to confirm the molecular identity of DAS-68416-4 soybean as well as certified reference materials have been developed (see http://www.sumobrain.com/patents/wipo/Detection-aad-12-soybean-event/WO2011066360A1.pdf).
26. Evaluation of the likelihood of adverse effects (Annex III.8(b)):	The scope of this application does not include cultivation of of DAS-68416-4 soybean in South Africa. Any exposure to the environment will be limited to any unintended release of DAS-DAS-68416-4 soybean, which could occur via accidental spillage during loading/unloading of the vessels, trains and trucks carrying the load of commodity grain, including DAS-68416-4 soybean, destined for processing into animal feed or human food products. However, this limited exposure is highly unlikely to give rise to any adverse effect and, if necessary, any spillage could be easily controlled by the application of current agricultural practices used for the control of volunteer soybean plants. Furthermore, soybean is known to be a weak competitor in the wild, which cannot survive outside cultivation without human intervention. Environmental conditions at the sites of handling are unlikely to be conducive to germination, growth and reproduction of soybean grain that is incidentally released.
27. Evaluation of the consequences (Annex III.8(c)):	In the unlikely event that some grain containing DAS-68416-4 soybean (from grain imports) may end up in the environment of South Africa before being crushed, the impact would be negligible as data supported a conclusion that DAS-68416-4 soybean is substantially equivalent to conventional soybean; soybean plants cannot survive without human intervention. Furthermore, South Africa is not the centre of origin for *G. max* and there are no wild relatives in South Africa with which soybean can outcross.
28. Overall risk (Annex III.8(d)):	The overall risk posed by this GMO is negligible.
29. Recommendation (Annex III.8(e)):	The overall risk is negligible. No recommendations other than procedures that may apply to conventional soybean are applicable.
30. Actions to address uncertainty regarding the level of risk (Annex III.8(f)):	There is no uncertainty regarding the risk profile.
Additional information	
31. Availability of detailed risk assessment information:	All data relating to this risk assessment have been presented in this dossier.

32. Any other relevant information:	To the best of our knowledge, all relevant information has been supplied in this dossier.
33. Attach document:	*Not applicable to applicant* <Specific types of entry: option to choose a file from the local source and 'upload' a copy to the BCH server>
34. Notes:	<Text entry>
35. Notes:	<Text entry>

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AFFIDAVIT/VERKLARING/STATEMENT
(moet ingevul word in teenwoordigheid van 'n Kommissaris van Ede / to be completed in the presence of a Commissioner of Oaths)

Ek/I……

ID-Nommer/Number…… Ouderdom/Age ……..

Woonadres/ Residing address ……

Werkadres/working address ……..

Tel ……..(w) ……(h) ……(cell)

Verklaar onder eed in afrikaans / bevestig in afrikaans -
Declare under oath in English / confirm in English –

……
……
……
……

Ek is vertroud met die inhoud van bostaande verklaring en begryp dit. Ek het geen beswaar/het beswaar teen die aflê van die voorgeskrewe eed. Ek beskou die voorgeskrewe eed/bevestiging as bindend vir my gewete.
I am familiar with, and understand the contents of this declaration. I have no objection/have objection to taking the prescribed oath. I consider the prescribed oath as binding to my conscience.

Plek/Place: …….. Datum/Date: ……..

Tyd/Time: ……

Handtekening/Signature: ……

Ek sertifiseer dat bostaande verklaring deur my afgeneem is en dat die verklaarder erken dat hy/sy vertroud is met die inhoud van hierdie verklaring and dit begryp. Hierdie verklaring is voor my beëdig en verklaarder se handtekening/merk/duimafrduk is in my teenwoordigheid daarop aangebring.
I certify that the above statement was taken from me and that the deponent has acknowledge that he/she knows and understands the contents of the statement. The statement was sworn to/affirmed before me and deponents signature/mark/thumb print was placed thereon in my presence.

Te/At: ……op/on ……om/at ……

……..
Kommisaris van Ede/Commissioner of Oaths
(inligting i.v.m. fisiese en posadres moet verskaf word, bv. Stempel van die polisiestasie
details to be provided on physical and postal address e.g. stamp of police station)

……
Magsnommer /Rang/Naam – drukskrif
Force number/Rank/Name - print