# Plant Pest Risk Assessment for DAS-68416-4 Soybean

## Contents

## A. Introduction

Dow AgroSciences LLC (DAS) has petitioned APHIS (APHIS number 09-349-01p) for a determination that genetically engineered (GE) soybean (*Glycine max*) event DAS-68416-4 is unlikely to pose a plant pest risk (DAS 2010) and, therefore, should no longer be a regulated article under APHIS' 7 Code of Federal Regulations (CFR) part 340. APHIS administers 7 CFR part 340 under the authority of the plant pest provisions of the Plant Protection Act of 2000[1]. This plant pest risk assessment was conducted to determine whether DAS-68416-4 soybean is unlikely to pose a plant pest risk.

DAS-68416-4 was produced by transformation of cotyledonary node explants of soybean (*Glycine max* cv Maverick) with *Agrobacterium tumefaciens*. Because *A. tumefaciens* is a plant pest and some of the regulatory sequences (promoter from cassava vein mosaic virus and terminator from *A. tumefaciens*) used to facilitate expression of the genes in soybean were derived from plant pests, this soybean has been considered a regulated article under APHIS regulations at 7 CFR part 340.

Potential impacts considered in this Plant Pest Risk Assessment are those that pertain to the use of DAS-68416-4 and its progeny in the absence of confinement. APHIS regulation 7 CFR 340.6(c) specifies the information needed for consideration in a petition for nonregulated status. APHIS will evaluate information submitted by the applicant, in addition to current literature, related to plant pest risk characteristics, disease and pest susceptibilities, expression of the gene product, new enzymes, or changes to plant metabolism, weediness of the regulated article, any impacts on the weediness of any other plant with which it can interbreed, potential changes to agricultural or cultivation practices, potential effects to non-target organisms, and transfer of genetic information to organisms with which it cannot interbreed, to determine if DAS-68416-4 is unlikely to pose a plant pest risk. If APHIS determines that a GE organism is not a plant pest risk, then APHIS has no regulatory authority over that organism.

[1] Section 403 (14) of the Plant Protection Act (7USC Sec 7702(14) defines plant pest as: "Plant Pest - The term "plant pest" means any living stage of any of the following that can directly or indirectly injure, cause damage to, or cause disease in any plant or plant product: (A) A protozoan. (B) A nonhuman animal. (C) A parasitic plant. (D) A bacterium. (E) A fungus. (F) A virus or viroid. (G) An infectious agent or other pathogen. (H) Any article similar to or allied with any of the articles specified in the preceding subparagraphs."

## B. Development of DAS-68416-4 herbicide tolerant[2] soybean

In the U.S. soybean was grown on 75.0 million acres in 2011 (Figure 1; USDA NASS 2011a) with a value of $29.6 billion in 2008/2009 (USDA ERS 2011a). Growers select soybean lines adapted to the different environmental and climatic features, operator's education, weed and disease pressures, cost of seed and other inputs, technology fees, human safety, ease and flexibility of the productions system and marketing reasons (USDA ERS 2002; Brookes 2011).

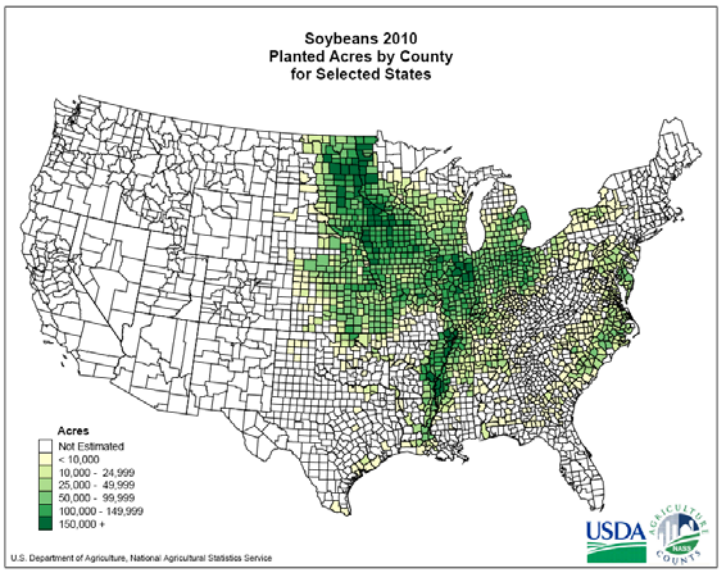


Figure 1. Soybean production areas in the U.S. (USDA NASS 2011b).

The presence of weeds in soybean fields can cause greater production losses than either insects or diseases (Gibson 2005; Oerke 2006). Before the development of effective herbicides for the selective control of weeds in soybeans in the early 1960's, cultural practices including tillage, use of weed free seed, row spacing and crop rotation were the only ways to control weeds (Wax 1973). By 1987, over 30 herbicides were being used on soybeans (Jordan 1987). With the 1996 commercial introduction and rapid adoption of glyphosate tolerant soybeans, a major change in herbicide usage occurred with an increasing use of glyphosate concurrent with the increased planting of glyphosate tolerant soybeans and a decrease in use of other soybean herbicides (Figure 2; NRC 2010; Young 2006). Consequently, the diversity of herbicides used for weed management has declined in soybean (Table 1; Young 2006) resulting in weed species shifts (Johnson 2009). Determination of nonregulated status for

[2] The applicant has described DAS-68416-4 soybean as "herbicide tolerant" and historically APHIS has also referred to GE plants with diminished herbicide sensitivity as "herbicide tolerant." However, the phenotype would fall under the Weed Science Society of America's (WSSA) definition of "herbicide resistance" since DAS-68416-4 has an inherited ability to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type variety (WSSA 1998). By the WSSA definition, "resistance [to an herbicide] may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis." Herbicide tolerance, by the WSSA definition, only applies to plant species with an "inherent ability" to survive and reproduce after herbicide treatment.

DAS-68416-4 soybean would provide soybean growers with additional options for the post-emergent control of both broadleaf and grass weeds. The integration of other herbicides with different modes of action with glyphosate has been encouraged to improve the duration of weed control, to enhance control of glyphosate tolerant weeds, to reduce the risk of developing glyphosate resistant weeds and to control glyphosate-resistant weeds (WSSA 2010). This soybean product would also provide another tool to use against increased incidence of weed species that are more tolerant to glyphosate ( NRC 2010; WSSA 2010).

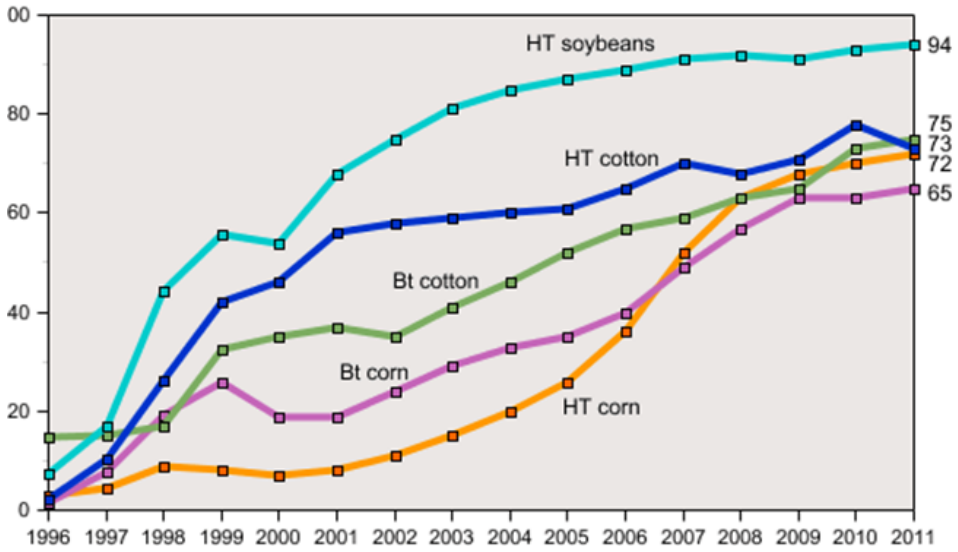


Figure 2. Percent acreage of genetically engineered crops in the U.S. (USDA ERS 2011b).

Table 1. Percent of U.S. Soybean Acres Treated with Herbicides in 1990, 1995, 2001 and 2006 (USDA NASS 2010).

| Herbicide | Percent Soybean Acres Treated | | | |
|---|---|---|---|---|
| | 1990 | 1995 | 2001 | 2006 |
| 2,4 D | 3 | 10 | 4 | |
| 2,4-D Dimethlyl salt | -- | -- | -- | 3 |
| Acetic acid (2,4 D) | -- | -- | -- | 7 |
| 2,4-DB | -- | 1 | -- | -- |
| Acifluorfen | -- | -- | 3 | -- |
| Alachlor | 13 | 4 | -- | -- |
| Bentazon | 16 | 12 | 1 | -- |
| Chloramben | 1 | -- | -- | -- |
| Chlorimuron, ethyl | 20 | 16 | 5 | 4 |
| Clethodim | -- | 5 | 4 | 3 |
| Clomazone | 7 | 4 | | |
| Cloransulam, methyl | -- | -- | 5 | 1 |
| Dimethenamid | -- | 1 | -- | -- |
| Ethalfluralin | 5 | 1 | -- | -- |
| Fenoxaprop | -- | 6 | 3 | -- |

| Herbicide | Percent Soybean Acres Treated | | | |
|---|---|---|---|---|
| | 1990 | 1995 | 2001 | 2006 |
| Fluazifop, P, butyl | 6 | 10 | 3 | 1 |
| Flumetsulam | -- | 2 | -- | -- |
| Flumiclorac, pentyl | -- | -- | -- | 1 |
| Fomesafen | 2 | 4 | 7 | 2 |
| Glyphosate | 5 | 21 | 73 | 92 |
| Imazamox | -- | -- | 5 | -- |
| Imazaquin | 16 | 15 | 2 | 1 |
| Imazethapyr | 11 | 44 | 9 | 3 |
| Lactofen | 1 | 5 | 1 | -- |
| Linuron | 6 | 2 | -- | -- |
| Metolachlor | 10 | 7 | -- | -- |
| Metribuzin | 19 | 11 | 2 | 2 |
| Paraquat | 2 | 2 | -- | 1 |
| Pendimethalin | 14 | 26 | 10 | 3 |
| Quizalofop | 3 | 6 | -- | -- |
| S-Metolachlor | -- | -- | -- | 1 |
| Sethoxydim | 4 | 7 | 1 | -- |
| Sulfentrazone | -- | -- | 5 | 1 |
| Sulfosate | -- | -- | 3 | 1 |
| Thifensulfuron | 4 | 12 | 2 | 1 |
| Tribenuron, methyl | -- | -- | -- | 1 |
| Trifluralin | 37 | 20 | 7 | 2 |

DAS-68416-4 soybean is a GE soybean line that has been developed to increase tolerance to the herbicides 2,4-D and glufosinate. The introduced genetic material results in the production of aryloxyalkanoate dioxygenase-12 (AAD-12) that degrades the herbicide 2,4-D into herbicidally-inactive 2,4-dichlorophenol (Müller 1999; Westendorf 2002 and 2003; Wright 2010a) and phosphinothricin acetyltransferase (PAT) that metabolizes the L-isomer of glufosinate into non-phytotoxic N-acetyl-L-glufosinate (2-acetamido-4-methylphosphinico-butanoic acid) (OECD 2002). If given non-regulated status, DAS-68416-4 soybean would be the first soybean variety with increased tolerance to both 2,4-D and glufosinate.

2,4-D (2,4-dichlorophenoxyacetic acid) is an herbicide in the phenoxy or phenoxyacetic acid family used for selective control of broadleaf weeds since the mid-1940s in over 600 products in agricultural and residential applications (USEPA 2005). The mode of action of 2,4-D for broadleaf plants is unclear, but it is believed to function as a plant growth regulator[3] with synthetic auxin hormone-like properties. When applied as an herbicide 2,4-D causes abnormal cell division and growth leading to plant injury and death.

Glufosinate (phosphinothricin; DL-homoalanin-4-yl(methyl) phosphinic acid) is a non-selective foliar herbicide used for pre-plant and post-emergent control of broadleaf plants and

[3] Plant Growth Regulators are synthetic plant hormones that regulate cellular processes, plant growth and development. Auxin compounds represent a class of hormones that along with other plant hormones determine patterns of plant development.

annual and perennial grasses (OECD 2002; USEPA 2008). Glufosinate acts by inhibiting the enzyme glutamine synthetase, which leads to poisoning in plants because of the overproduction of ammonia. Glufosinate was first registered by EPA for use in 2000 as a non-selective foliar herbicide that is used for pre-plant and post-emergent control of broadleaf weeds (USEPA 2008). EPA registration authorizes use on many crops including; apples, berries, canola, corn, cotton, currants, grapes, grass grown for seed, potatoes, rice, soybeans, sugar beets, and tree nuts and in non-crop areas including lawns and residential areas (USEPA 2008). APHIS reached a determination of nonregulated status for the first soybean line genetically engineered with the glufosinate-tolerance trait in 1996 (USDA APHIS 2011).

## C. Description of the modification

Soybean DAS-68416-4 was produced by transformation using disarmed *Agrobacterium tumefaciens* (DAS 2010). Soybean (cultivar Maverick) cotyledonary nodes were infected with *Agrobacterium* strain EHA101 (Hood 1986) containing plasmid pDAB4468. Plants containing the introduced DNA were selected based on growth in the present of glufosinate (DAS 2010, p. 20).

The plasmid pDAB4468 contained two gene expression cassettes flanked by T-DNA border sequences from the *Agrobacterium* Ti-plasmid (DAS 2010, p. 21).

Aryloxyalkanoate dioxygenase

- RB7 MAR (Matrix attachment region[4]) from the *Nicotiana tabacum* rb-7-5A gene (Hall 1991).
- Polyubiquitin promoter (AtUbi10) from *Arabidopsis thaliana* (Norris 1993).
- *aad-12* gene from *Delftia acidovorans* encoding aryloxyalkanoate dioxygenase-12 (AAD-12) that degrades 2,4-D into herbicidally-inactive 2,4-dichlorophenol (DCP) (Wright 2010a and 2010b). The native gene was modified for better expression in plants (DAS 2010). *Delftia acidovorans* is a bacterium commonly found in the environment (Wen 1999) has a history of safe use.
- 3' untranslated transcriptional terminator and polyadenylation site of open reading frame 23 (AtuORF23) from *Agrobacterium tumefaciens* pTi15955 (Barker 1983).

Phosphinothricin N-acetyltransferase (PAT)

- Promoter and the 5'UTR (CsVMV) from the cassava vein mosaic virus (Verdagurer 1996).

[4] Chromosomal DNA is organized into looped domains through interactions with and attachment to a proteinaceous structure, called the nuclear scaffold, nuclear scaffold, or matrix. Specific chromosomal DNA sequences are thought to contain attachment regions that anchor the DNA to the matrix. Attachment regions may increase levels of expression, reduce transformant-to-transformant variation of expression, stabilize transgene expression and minimize transgene silencing (Abranches 2005; Allen 2000; Spiker 1996).

- *pat* (phosphinothricin N-acetyltransferase) gene from *Streptomyces viridochromogenes* inactivates the herbicide glufosinate (Wohlleben 1988). The native gene was modified for better expression in plants (DAS 2010).
- Transcriptional terminator and polyadenylation site of open reading frame 1 (AtuORF1) of *Agrobacterium tumefaciens* pTi15955 (Barker 1983).

Data from Southern blot analysis demonstrate that DAS-68416-4 soybean contains (DAS 2010, tables 2 & 3): (1) a single copy of RB7 (DAS 2010, figures 24 & 26), AtUbi10 (DAS 2010, figures 13, 14, 25 & 26) *add-12* (DAS 2010, figures 8, 9, 10, 11), AtuORF23 (DAS 2010, figures 13 & 15); and (2) a single copy of CsVMV (DAS 2010, figures 21 & 22), *pat* (DAS 2010, figures 16, 17, 18, 19, 20 & 21), AtuORF1 (DAS 2010, figures 21 & 23).

## D. Potential for DAS-68416-4 to have altered disease and pest susceptibilities

APHIS assessed whether DAS-68416-4 soybean is likely to have significantly altered disease and pest susceptibility. This assessment encompassed consideration of the introduced trait and disease and pest susceptibility data from DAS-68416-4 soybean field trials.

DAS-68416-4 soybean has been field tested in the U.S. since 2008. Agronomic data was collected in 2008 and 2009 in 27 locations that represented a diverse range of environmental conditions where DAS-68416-4 soybean is expected to be grown. No statistically significant differences were observed for stand count, emergence, seedling vigor, days to flower, lodging, disease incidence, insect damage and yield (DAS 2010, Section VII-B, VII-C, VII-D). No qualitative or quantitative observations indicated any biologically meaningful differences from control lines or differences outside the range of conventional soybean norms.

The descriptions of the introduced genetic elements, expression of the gene products and their functions of DAS-68416-4 soybean have been summarized above. The *Agrobacterium* transformed plants used in the generation of DAS-68416-4 soybean were treated with an antibiotic to kill the *Agrobacterium* cells. Furthermore, DNA sequences derived from plant pests that were incorporated in DAS-68416-4 do not result in the production of infectious agents or disease symptoms in plants, and so it is unlikely that DAS-68416-4 soybean could pose a plant pest risk.

Given the interactions between the environment, the genetic backgrounds of the cultivars used and some inherent genetic variability within soybean varieties, APHIS concludes that these results do not indicate an increased pest risk. Expression of AAD-12 and PAT in DAS-68416-4 soybean is not expected to cause plant disease or influence susceptibility of DAS-68416-4 soybean or its progeny to diseases or other pests.

## E. Potential for effect on non-target organisms, including those beneficial to agriculture

There is no reason to believe that deleterious effects or significant impacts on non-target organisms, including beneficial organisms, would result from the cultivation of DAS-68416-4. Field observations of DAS-68416-4 soybean (DAS 2010, Section VII-B) revealed no negative

effects on non-target organisms, suggesting that the production of the ADD-12 and PAT proteins in the plant tissues are not toxic to organisms. The introduced genetic material does not result in the production of novel proteins, enzymes, or metabolites in the plant that are known to have toxic properties. The lack of known toxicity of ADD-12 and PAT suggests no potential for deleterious effects on beneficial organisms such as bees and earthworms. The use of 2,4-D and glufosinate herbicides in the cultivation of DAS-68416-4 soybean or its offspring is regulated by EPA under its existing regulations for the registration of pesticide use. EPA considers the impacts on the environment, including effects on non-target organisms in establishing application rates and residue tolerances for herbicides, including 2,4-D and glufosinate tolerant lines (USEPA 1997; USEPA 2005). APHIS has not identified any other potential mechanisms for deleterious effects on non-target organisms.

## F. Potential for enhanced weediness or invasiveness

APHIS assessed whether DAS-68416-4 soybean is any more likely to become a weed than the non-transgenic recipient soybean line, or other soybean currently cultivated. The assessment encompasses consideration of the basic biology of soybean and an evaluation of unique characteristics of DAS-68416-4 soybean.

Weediness for the purposes of this part of the plant pest risk assessment is an attribute, which causes a crop to act as a weed due to the addition of genes, in comparison to the non-GE comparator (parental line Maverick). If the fitness of DAS-68416-4 soybean improves in natural or agricultural ecosystems due to the inserted DNA, the potential for weediness could increase. The following analysis of the inserted DNA is intended to document that DAS-68416-4 soybean has a negligible likelihood of increased weediness.

In the U.S., soybean is not listed as a weed in the major weed references (Crockett 1977; Holm 1979; Muenscher 1980) nor is it designated as noxious weed by the federal government (USDA NRCS 2012a). Soybean does not possess any of the attributes commonly associated with weeds (Baker 1965), such as long persistence of seed in the soil, the ability to disperse, invade, and become a dominant species in new or diverse landscapes, or the ability to compete well with native vegetation. Furthermore, mature soybean seeds have no innate dormancy, are sensitive to cold, are not expected to survive in freezing winter conditions and do not reproduce vegetatively (Hermann 1962; OECD 2000; Padgette 1996; Raper Jr.1987).

In 2008 and 2009, Dow conducted field trials to evaluate phenotypic characteristics comparing DAS-68416-4 soybean with the non-transgenic soybean variety “Maverick.” Agronomic performance characteristics, including stand count, emergence, seedling vigor, days to flower, days to maturity, plant height, lodging, and shattering were evaluated. There were no significant differences except that DAS-68416-4 took one less day (128.4 compared to 129.3) to reach seed maturation and had a slightly lower seed size (3.5%) (DAS 2010, page 85). However this lower seed size did not affect yield. Based on analysis of data on all these parameters, soybean DAS-68416-4 soybean is unlikely to pose any more of a plant pest risk from weediness than the conventional soybean from which it was derived.

APHIS also assessed whether DAS-68416-4 soybean is any more likely to become a weed than its parental comparator line "Maverick" or other soybean varieties currently under cultivation. The assessment encompasses consideration of the basic biology of soybean and an evaluation of the unique characteristics of DAS-68416-4 soybean under field conditions. To increase weediness of the soybean plant there would have to be selection pressure on the line (Tiedge 1989). Because 2,4-D or glufosinate will not affect the survival of DAS-68416-4 soybean and because soybean is not itself weedy, this type of selection pressure does not now and is unlikely ever to exist.

Results on growth characteristics, seed production and germination indicate that DAS-68416-4 soybean is not significantly different from its comparators (DAS 2010, pages 79 - 85). There is no indication that DAS-68416-4 soybean possesses a selective advantage that would result in increased weediness. DAS-68416-4 soybean lacks the ability to persist as a troublesome weed, and there would be no significant impact on current weed management practices for soybean cultivation.

## G. Potential of DAS-68416-4 to impact the weediness of other plants with which it can interbreed

The genus *Glycine*, a member of the Fabaceae (= Leguminosae or pea family), consists of two subgenera, *soja* and *glycine* (OECD 2000; USDA NRCS 2012b). Perennial species in the subgenus *glycine* do not occur in the U.S. (USDA NRCS 2012b), except in the U.S. territories in the South Pacific (Hymowitz 1987). The subgenus *soja* consists of three annual species: *G. soja* Sieb. and Zucc., the wild form of soybean; *G. gracilis* Skvortz., the weedy form of soybean; and *G. max* (L.) Merr., the cultivated soybean. *G. soja* and *G. max* do not occur naturally in the U.S. (Hermann 1962; Hymowitz 1987; USDA NRCS 2012b). Hybrids from crosses between the subspecies have generally been sterile, and further progeny have only been obtained with extreme difficulty (OECD 2000).

Cultivated soybean is highly self-pollinating (Ahrent 1994). When soybean plants are grown directly adjacent to other soybean plans, the amount of natural cross pollination has generally been found to be 0.5 - 1 percent (Fehr 1980; OECD 2000), although higher values (2.5 percent) occur in some varieties (Abud 2007). Outcrossing can be reduced to 0 – 0.01 percent with a separation distance of 10 meters (Abud 2007).

The cultivated soybean, *G. max*, lacks sexually compatible wild relatives in the U.S. and its territories. Consequently, there is no potential for gene flow from cultivated soybean plants to wild relatives in the U.S. Therefore, it is not likely that gene flow and introgression will occur between DAS-68416-4 soybean and other species of soybean. APHIS has determined that any adverse consequences of gene flow from DAS-68416-4 soybean to wild or weedy species in the U.S. are highly unlikely.

## H. Potential changes to agricultural or cultivation practices

None of the management practices currently employed for soybean production is expected to change if DAS-68416-4 soybean is determined to be no longer subject to the regulatory requirements of 7 CFR part 340 or the plant pest provisions of the Plant Protection Act. DAS's (2010) studies demonstrate that the agronomic characteristics and cultivation practices employed when growing DAS-68416-4 soybean are essentially indistinguishable from practices used to grow other soybean varieties, including other herbicide-tolerant varieties (DAS 2010, Section IX-E). The geographic range or seasonality of soybean cultivation is not expected to change to accommodate the cultivation of DAS-68416-4 soybean (DAS 2010). DAS-68416-4 soybean is comparable to currently available soybean varieties in terms of resistance to insects and disease (DAS 2010, Section VII B.). Therefore, no changes are expected for insect and disease control practices with DAS-68416-4 soybean. Because agricultural and cultivation practices would not be significantly different than that of conventional soybean, APHIS does not foresee changes in on insects or diseases damage or control measures employed due to agricultural or cultivation practices with DAS-68416-4 soybean.

## I. Potential impacts from transfer of genetic information to organisms with which DAS-68416-4 cannot interbreed

APHIS examined the potential for the new genetic material inserted into DAS-68416-4 soybean to be horizontally transferred to other organisms without sexual reproduction and whether such an event could lead directly or indirectly to disease, damage, injury or harm to plants. Horizontal gene transfer and expression of DNA from a plant species to other species is highly unlikely to occur based on the following reasons.

The horizontal gene transfer (HGT) between unrelated organisms is one of the most intensively studied fields of science. HGT and expression of DNA from a plant species to bacteria or animal species is unlikely to occur (Keese 2008).

1. Many genomes (or parts thereof) from bacteria that are closely associated with plants have been sequenced, including *Agrobacterium* and *Rhizobium* (Kaneko 2000; Kaneko 2002; Wood 2001). There is no evidence that these organisms contain genes derived from plants. Therefore, the likelihood of any impact or new HGT that is not already capable of taking place in the soil is extremely unlikely.
2. No evidence has been identified for any mechanism by which soybean genes could be transferred to humans or animals, or any evidence that such gene transfer has occurred for any plant species during evolutionary history, despite animals and humans eating large quantities of plant DNA. In cases where review of sequence data implied that HGT occurred, these events are inferred to occur on an evolutionary time scale on the order of millions of years (Brown 2003; Koonin 2001).
3. Transgene DNA promoters and coding sequences are optimized for plant expression, not prokaryotic bacterial expression. Thus, even if HGT occurred, proteins corresponding to the transgenes are not likely to be produced.

4. FDA has evaluated HGT from the use of antibiotic resistance marker genes, and concluded that the likelihood of transfer of antibiotic resistance genes from plant genomes to microorganisms in the gastrointestinal tract of humans or animals, or in the environment, is extremely unlikely (FDA 1998).

Therefore, APHIS concludes that HGT is highly unlikely to occur and thus poses no significant plant pest risk.

## J. Conclusion

APHIS has prepared this plant pest risk assessment in order to determine if DAS-68416-4 soybean is likely to pose a plant pest risk. Based on the information provided by the applicant and the lack of atypical responses to disease or plant pests in the field, the lack of weedy characteristics of the DAS-68416-4 soybean or other plants with which it can interbreed, that there are no anticipated changes to agricultural or cultivation practices that would result in increased diseases or pests, the lack of effects on non-targets or beneficial organisms in the agro-ecosystem, the lack of any indirect effects on other agricultural products and the unlikelihood of horizontal gene transfer, APHIS has concluded that soybean DAS-68416-4 soybean is highly unlikely to pose a plant pest risk.

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