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Decision Document DD2004-49

Determination of the Safety of Bayer CropScience's Herbicide Tolerant LibertyLink® Cotton Event LLcotton25 (*Gossypium hirsutum* L.)

This Decision Document has been prepared to explain the regulatory decision reached under the regulatory directive Dir95-03 *Guidelines for the Assessment of Livestock Feed from Plants with Novel Traits* and based on the environmental criteria in regulatory directive Dir94-08 *Assessment Criteria for Determining Environmental Safety of Plants with Novel Traits*.

The Canadian Food Inspection Agency (CFIA), specifically the Feed Section of the CFIA, with advice from the Plant Biosafety Office of the CFIA has evaluated information submitted by Bayer CropScience regarding LibertyLink® cotton event LLcotton25, a herbicide tolerant cotton. CFIA has determined that feed derived from this plant with a novel trait does not present a significant risk to the environment, nor does it present livestock feed safety concerns when compared to currently commercialized cotton varieties in Canada

Livestock feed use of LibertyLink® cotton event LLcotton25 is therefore authorized as of August 31, 2004. LibertyLink® cotton event LLcotton25 and any cotton lines derived from it may be imported and/or released, provided (i) no inter-specific crosses are performed, (ii) the intended use(s) are similar, (iii) it is known following based on characterization that these plants do not display any additional novel traits and are substantially equivalent to currently commercialized cotton, in terms of their specific use and safety for the environment and for human and animal health.

LibertyLink® cotton event LLcotton25 is subject to the same phytosanitary import requirements as its unmodified counterparts.

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I. Brief Identification of Plant with Novel Traits (PNT)

Designation(s) of the PNT:	LibertyLink® cotton event LLcotton25 OECD identifier ACS-GH001-3
Applicant:	Bayer CropScience
Plant Species:	Cotton (*Gossypium hirsutum* L.)
Novel Traits:	Resistance to the herbicide glufosinate ammonium
Trait Introduction Method:	*Agrobacterium* mediated gene transfer
Proposed Use of PNT's:	Production of cotton for fibre, cottonseed and cottonseed meal for livestock feed, and cottonseed oil for human consumption. These materials will be grown outside Canada, in the usual production areas for cotton. Cottonseed and cottonseed meal will be imported into Canada for livestock feed use only.

II. Background Information

Bayer CropScience has developed a cotton line resistant to glufosinate ammonium. The cotton line, designated as LibertyLink® cotton event LLcotton25, was developed to impart novel tolerance to the herbicide glufosinate ammonium. This herbicide tolerance trait allows for the control or suppression of weeds in cotton production.

LibertyLink® cotton event LLcotton25 was developed using *Agrobacterium* mediated gene transfer technology, resulting in the introduction of a *bar* gene isolated from the soil bacterium *Streptomyces hygroscopicus.* The *bar* gene codes for the production of the enzyme Phosphinothricin Acetyl-Transferase (PAT) which acetylates glufosinate ammonium, thereby detoxifying the herbicide, conferring herbicide resistance to the cotton plant.

Bayer CropScience has provided data on the identity of LibertyLink® cotton event LLcotton25, a detailed description of the transformation method, data and information on the gene insertion site, gene copy number and levels of gene expression in the plant and the role of the inserted genes and regulatory sequences. The novel protein was identified and the mode of action was described and characterized. Data was provided for the evaluation of the potential toxicity of the novel protein to livestock and non-target organisms and potential allergenicity of the novel protein to humans.

LibertyLink® cotton event LLcotton25 was field tested in multiple locations (14) in the United States under confined research field trial conditions in 2000 and 2001.

Agronomic characteristics of LibertyLink® cotton event LLcotton25 such as plant morphology, disease susceptibility, agronomic performance and reproductive fitness were compared to those of unmodified cotton counterparts.

Nutritional components of LibertyLink® cotton event LLcotton25 such as proximates, amino acids and fatty acids were compared with unmodified cotton counterparts.

The Feed Section, CFIA, with input from the Plant Biosafety Office, CFIA, has reviewed the above information. The following assessment criteria as described in regulatory directives Dir95-03 and Dir94-08 were used to determine the safety and efficacy as livestock feed and the environmental safety of feed from this plant with a novel trait:

- potential impact of LibertyLink® cotton event LLcotton25 on livestock nutrition,
- potential impact of LibertyLink® cotton event LLcotton25 cotton on livestock and workers/by-standers,
- potential of LibertyLink® cotton event LLcotton25 cotton to become a weed of agriculture or be invasive of natural habitats,
- potential for gene flow from LibertyLink® cotton event LLcotton25 cotton to wild relatives whose hybrid offspring may become more weedy or more invasive,
- potential of LibertyLink® cotton event LLcotton25 cotton to become a plant pest,
- potential impact of LibertyLink® cotton event LLcotton25 cotton or their gene products on non-target species, including humans, and
- potential impact of LibertyLink® cotton event LLcotton25 cotton on biodiversity.

Additionally, CFIA has reviewed a method submitted by Bayer CropScience for the detection and identification of cotton containing the PAT protein.

III. Description of the Novel Traits

1. Development Method

The cotton variety Coker312 was transformed with a plasmid vector containing the *bar* gene to confer the glufosinate ammonium tolerance trait. The DNA sequence was introduced by *Agrobacterium* mediated gene transfer. Whole plant selection with glufosinate ammonium was employed to select an appropriate event which was designated LLcotton25.

2. Glufosinate Ammonium Tolerance

Phosphinothricin, the active ingredient of glufosinate ammonium, inhibits glutamine synthetase, which results in the accumulation of lethal levels of ammonia in susceptible plants within hours of application. Plants produce ammonia as a result of normal metabolic processes.

The *bar* gene engineered into LibertyLink® cotton event LLcotton25 codes for the production of the enzyme Phosphinothricin Acetyl-Transferase (PAT) which acetylates glufosinate ammonium, thereby detoxifying the herbicide, conferring herbicide resistance to the cotton plant. The PAT enzyme has extremely high substrate specificity.

The introduced *bar* gene was originally isolated from *Streptomyces hygroscopicus*. *S. hygroscopicus* is a common gram-positive soil-borne bacterium. The PAT enzyme is therefore naturally occurring in the soil. More generally, acetyltransferases are ubiquitous in nature.

The *bar* gene expressed in LibertyLink® cotton event LLcotton25 is linked to a constitutive promoter, (i.e., results in expression in all cotton tissues). PAT protein expression was determined from plants grown in various locations across the USA. Mean PAT expression in LibertyLink® cotton event LLcotton25 was 127 µg/g fresh weight and 69.9 µg/g fresh weight in cleaned seed and fuzzy seed respectively, 7.97 µg/g fresh weight, 52.9 µg/g fresh weight and 36.8 µg/g fresh weight in root, leaf and stem tissue respectively. PAT expression was 19.2 µg/g fresh weight in pollen.

Unlike typical allergens, PAT protein is present at low levels in the plant (0.019% to 0.036% in seeds). Protein allergens are normally resistant to digestion unlike the PAT protein which was shown to degrade readily in simulated gastric fluid (digested within 30s) and in simulated intestinal fluid (digested within 5 minutes). The PAT protein from the *bar* gene is not degraded when incubated at 60, 75 and 90 ºC for up to 60 minutes. However the enzymatic activity of PAT is lost when incubated at 60 ºC for 10 minutes.

A search for amino acid sequence similarity between the PAT protein and known allergens, using a database assembled from the public domain databases SwissProt, trEMBL, GeneSeq-Prot, PIR, PDB, DAD and GenPept revealed no significant amino acid sequence homologies (based on sequence identity of 8 or more contiguous amino acids) and no overall homology with known allergens (based on 35% identity on a window of 80 amino acids). PAT only had high similarity with other Acetyl transferase proteins. A search of a similarly constructed database of known toxins indicated no amino acid sequence homologies between known toxins and the PAT protein.

Due to the low levels of PAT protein expressed in the cotton plant it was necessary to produce PAT protein by bacterial fermentation to obtain sufficient quantities to conduct some of the safety studies (acute intravenous mouse toxicity, glycosylation, heat stability and simulated gastric and intestinal fluid digestion studies). The bacterial produced protein was compared to the plant produced protein and shown to be of similar molecular weight and immunological reactivity. Neither the plant or bacterially expressed PAT is glycosylated.

3. Stable Integration into the Plant's Genome

Southern blot analysis of LibertyLink® cotton event LLcotton25 indicated that there is one site of integration of the introduced DNA which includes a single copy of the *bar* gene. The data demonstrates that the *bar* coding region and associated promoter and terminator sequences are intact.

Southern blot analysis over 3 generations (T4, T5, BC3/F3) and over different environments (11 different locations) probed with the complete T-DNA coding region demonstrated the stability of the DNA insert. Segregation analysis was performed on three stages in the breeding process (T1, F1, BC1 and F2) and follows Mendelian genetics.

Southern blot analysis and Mendelian segregation data provides evidence of the stable inheritance of the genetic elements introduced into LibertyLink® cotton event LLcotton25.

IV. Criteria for the Environmental Assessment

Lines derived from LibertyLink® cotton event LLcotton25 will not be grown in Canada. However, Canada imports cottonseed, as well as a wide range of other cotton products, that are used as human food, livestock feed or other industrial products.

1. Potential of LibertyLink® Cotton Event LLcotton25 to Become a Weed of Agriculture or Invasive of Natural Habitats

Cotton (*Gossypium hirsutum*) is a member of the family Malvaceae. It is a perennial species cultivated as an annual and grown in the United States, mostly in areas from Virginia southward and westward to California. Cotton is not grown in Canada as it is not adapted to environmental conditions found at these latitudes.

Cotton is not considered a weed pest in the regions where it is grown, nor is it invasive of unmanaged habitats in Canada. LibertyLink® cotton event LLcotton25 has not been modified to have altered cold-tolerance and information supplied by Bayer CropScience indicates that the reproductive and survival biology of LibertyLink® cotton event LLcotton25 are unchanged compared to unmodified counterparts.

CFIA has concluded that LibertyLink® cotton event LLcotton25 is unlikely to become a weed of agriculture or invasive of natural habitats.

2. Potential for Gene Flow to Wild Relatives Whose Offspring May Become More Weedy or More Invasive

Cotton is predominately self-pollinated. Although cross-pollination may occur at low levels, particularly in the presence of pollinators such as honeybees, cotton has no wild relatives native to Canada. Wild relatives of commercial cotton (*G. barbadense and G. tomentosum*) are found only in tropical and sub-tropical regions.

The CFIA has therefore determined that gene flow from LibertyLink® cotton event LLcotton25 to wild relatives in Canada is not possible.

3. Altered Plant Pest Potential

Cotton is not a plant pest in Canada and the intended effect of the novel trait is unrelated to plant pest potential. In addition, agronomic characteristics of LibertyLink® cotton event LLcotton25 are similar to those described for currently commercialized cotton varieties. Susceptibilities to diseases such as *Rhizoctonia sp.* and *Phymototricum sp.* were unchanged, leading to the conclusion that plant pest potential was not inadvertently altered. Similarly, no differences were noted in the susceptibility of the transformed cotton line to insect pests when compared to non-transformed cotton lines. Comparable responses between the LibertyLink® cotton event LLcotton25 and the controls were noted for budworm, boll weevil, thrips, cotton aphids, plant bugs, whiteflies, cutworms, stinkbug, and cotton bollworm.

The CFIA has therefore determined that LibertyLink® cotton event LLcotton25 does not present a plant pest concern.

4. Potential Impact on Non-Target Organisms

The detailed characterization of the novel gene and resulting enzyme, as briefly summarized in Part III of the present document, led to the conclusion that LibertyLink® cotton event LLcotton25 does not result in altered toxic or allergenic properties. The PAT enzyme responsible for glufosinate ammonium tolerance has very specific enzymatic activity and does not affect the metabolism of the plant. Furthermore, PAT is rapidly inactivated in mammalian stomach and intestinal fluids by enzymatic degradation and pH-mediated proteolysis. Raw seeds of the LibertyLink® cotton event LLcotton25 were shown to be substantially equivalent to conventional cotton varieties for their content of antinutritional factors. Seed protein content, amino acid, fibre, oil and fatty acid compositions fall within the range of those of the unmodified counterparts.

LibertyLink® cotton event LLcotton25 will not be grown in Canada and exposure to the novel gene and resulting enzyme is expected to be minimal to non-existent. In the event that LibertyLink® cotton event LLcotton25 seed was accidentally released into the environment, any resulting plants would not be expected to set seed.

Based on the above, CFIA has determined that the use of LibertyLink® cotton event LLcotton25 will not result in altered impacts on interacting organisms, including humans, when compared to currently commercialized cotton varieties

5. Potential Impact on Biodiversity

No varieties of cotton, or wild relatives that can readily interbreed with cotton, grow in the Canadian environment. Cotton is not grown in Canada and is not adapted to the

environmental conditions encountered in Canadian agricultural environments. LibertyLink® cotton event LLcotton25 has not been modified to have altered cold-tolerance, and therefore is not expected to enter or survive in unmanaged ecosystems.

The CFIA has therefore concluded that LibertyLink® cotton event LLcotton25 does not present any adverse impacts on biodiversity in Canada.

V. Criteria for the Livestock Feed Assessment

1. Potential Impact on Livestock Nutrition

Nutritional Composition and Anti-Nutritional Factors

Cottonseed for the compositional analysis (nutrients and antinutrients) was obtained from 15 trial sites over 2 years in the United States. In the first year (2000), 6 sites were used. In the second year, 9 sites were used.

At each site, samples were obtained from 3 control (Coker 312) and 6 LL25 plots (3 treated with traditional herbicides (or untreated in a few cases), 3 treated with Liberty®). In the first year, cottonseed and lint samples were analysed. In the second year, cottonseed samples only were analysed. Seed sample analyses included protein, fat, ash, crude fibre, ADF, NDF, amino acids, fatty acids, Ca, P, K, Mg, Fe, Zn, vitamin E, phytic acid, gossypol, cyclopropenoid fatty acids. Lint sample analysis included protein, fat, ash, CF, ADF, NDF.

In addition, cottonseed from two of the trial sites (one from 2000 and one from 2001) was used to generate samples of cottonseed byproducts (linters, delinted seed, meal, toasted meal, hulls, crude oil, deodorized oil) and to produce toasted cottonseed meal for animal feeding studies. These samples were analysed for the various nutrients and antinutrients mentioned above, as appropriate for the particular byproduct.

Nutritional composition of LLcotton25 was shown to be equivalent to the parental control (Coker312). Results were also compared with literature values, and all but a very few were shown to be within the literature ranges. These exceptions included free gossypol was higher in both control and LLcotton25 than the reported literature values; phytic acid was lower in control and transgenic than reported in the literature; the amino acids Cys and Glu, Tyr, Val were all slightly below literature values, but there were no differences between Coker312 and LLcotton25.

LLcotton25 was further assessed in a 33-day broiler chicken feeding study. Treatment diets included a commercial non transgenic variety, Coker312 parental variety, LLcotton25 not sprayed with Liberty®, and LLcotton25 sprayed with Liberty®. Diets all contained 10% cottonseed meal. There were no differences in animal performance (weight gain, feed efficiency) feed consumption, or chilled carcass weight among the treatments.

The applicant has demonstrated through compositional analysis and a broiler feeding study that LLcotton25 is equivalent to cotton varieties currently used as livestock feed.

Potential Impact on Livestock and Workers/By-standers

PAT is a highly substrate specific enzyme that has been well defined. Exposure to PAT protein is not new. The *bar* gene is isolated from *S. hygroscopicus* a common soil bacterium. The *bar* gene is present in the environment with no known adverse effects on humans and animals. In addition, PAT from the *pat* gene has been expressed in various crops authorized in Canada. The amino acid sequence of the PAT protein encoded by the *pat* gene shares 85% homology with the PAT sequence encoded by the *bar* gene. PAT from both the *pat* and *bar* genes show high functional similarity.

Cotton expressed PAT protein has been demonstrated to be rapidly digested under simulated gastric and intestinal fluid conditions. This protein has been shown to have no sequence similarities with known allergens or toxins. Given that the PAT protein is heat stable, further examination of the safety of the protein was warranted. The acute intravenous mouse toxicity study with PAT protein supports safety of the PAT protein, with no treatment related adverse effects demonstrated at 10 mg/kg body weight, the highest doses tested. Safety of PAT was further supported by the subchronic rodent study, which also showed no treatment related adverse effects. The subchronic study utilized PAT from the *pat* gene as opposed to the *bar* gene. In addition, no adverse affects were demonstrated in the broiler chicken feeding study utilizing cottonseed meal (10% dietary incorporation).

Based on the expected exposure levels and the results of the above tests, the CFIA concludes that, the introduced gene and its corresponding novel trait is unlikely to be a novel toxin or allergen.

Based on the detailed characterization provided (nutritional composition, and agronomic data of the modified plant compared to the unmodified comparator) it is unlikely that modifications causing unintended effects have occurred in the cotton genome.

Cotton is not known for the production of endogenous allergens and the transformation event which produced LibertyLink® cotton event LLcotton25 would not be expected to induce their synthesis.

VI. New Information Requirements

If at any time, Bayer CropScience becomes aware of any information regarding risk to the environment, or risk to human or animal health that could result from release of these materials in Canada, or elsewhere, Bayer CropScience will immediately provide such information to the CFIA. On the basis of such new information, the CFIA will re-

evaluate the potential impact of the proposed use and will re-evaluate its decision with respect to the livestock feed authorization of LibertyLink® cotton event LLcotton25.

VII. Regulatory Decision

Based on the review of data and information submitted by Bayer CropScience, including comparisons of LibertyLink® cotton event LLcotton25 with the unmodified parental counterparts, the Feed Section, CFIA, has concluded that the novel gene and its corresponding trait does not confer to the plants any characteristic that would raise any concerns regarding the safety or nutritional composition of LibertyLink® cotton event LLcotton25. Cottonseed and cottonseed meal and hulls are currently listed in Schedule IV of the *Feeds Regulations* and are, therefore approved for use in livestock feeds in Canada. LibertyLink® cotton event LLcotton25 has been assessed and found to be as safe and as nutritious as traditional cotton varieties. LibertyLink® cotton event LLcotton25 and its products are considered to meet the present ingredient definitions and are approved for use as livestock feed ingredients in Canada. LibertyLink® cotton event LLcotton25 will not be grown in Canada nor can the seed overwinter, therefore the release of the feed into the environment would result in neither intended nor unintended environmental effects.

Livestock feed use of LibertyLink® cotton event LLcotton25 is therefore authorized as of August 31, 2004. LibertyLink® cotton event LLcotton25 and any other cotton lines derived from it may be imported and/or released, provided no inter-specific crosses are performed, provided the intended uses are similar, and provided it is known, based on characterization that these plants do not display any additional novel traits and are substantially equivalent to currently grown cotton, in terms of their specific use and safety for the environment and for human and animal health.

LibertyLink® cotton event LLcotton25 is subject to the same phytosanitary import requirements as its unmodified counterparts.

Please refer to Health Canada's Decisions on Novel Foods for a description of the food safety assessment of LibertyLink® cotton event LLcotton25. The food safety decisions are available at the following Health Canada web site:

http://www.hc-sc.gc.ca/food-aliment/mh-dm/ofb-bba/nfi-ani/e_novel_foods_and_ingredient.html