OBJECTIONS TO THE APPLICATION BY PIONEER HI-BRED FOR COMMODITY CLEARANCE OF GRAN AND DERIVED PRODCUTS OF GENETICALLY MODIFIED (GM) SOYBEAN 356043

PREPARED BY

25 FEBRUARY 2008

Independent scientific biosafety assessment of Genetically Modified *Glycine max*: Soybean 356043. (Optimum GAT ™, Pioneer)

The application is for the commodity clearance of grain and derived soy products of **genetically modified (GM) Soybean 356043**. The GM soybean 356043 has been genetically modified to express the **GAT4601** and **GM-HRA** proteins for resistance to two herbicides. GAT4601 protein is a glyphosate acetyltransferasederived from the *gat* gene from *Bacillus licheniformis* that confers tolerance to the herbicide glyphosate. The GM-HRA protein is an acetolactate synthase (ALS) derived from the *als* gene of *Glycine max* that confers tolerance to ALS-inhibiting herbicides such as chlorimuron and thifensulfuron.

GM cassette integrity and stability

Soybean line 356043 was genetically modified by means of particle acceleration. There are two expression cassettes:

- *gat4601* gene is driven by the SCP1 promoter (synthetic constitutive promoter consisting of sequences of the cauliflower mosaic virus, CaMV- 35S promoter and the Rsyn7-Syn II core consensus promoter) as well as the , tobacco mosaic virus (TMV) omega 5'-UTR, that enhances translation. The pinII terminator is from *Solanum tuberosum* (tomato).
  The gat4601 gene gene is based on glyphosate acetyltransferase protein sequences from the bacteria, *Bacillus licheniformis*, and optimised in the laboratory by gene shuffling. Different gat4601 gene variants were combined to produce a novel, synthetic sequence with optimised glyphosate acetyltransferase activity. The gat4601 gene product modifies the glyphosate herbicide by acetylation thereby inactivating it.
- *gm-hr*a gene is driven by S-adenosyl-L-methionine synthetase (SAMS) constitutive promoter from *Glycine max*, and the 5'UTR and the terminator region from soybean endogenous acetolactate synthase gene.
  The *gm-hra* gene is a modified form of the endogenous acetolacte synthase (*als*) gene from *Glycine max*. The enzyme catalyzes the first step in the biosynthesis of the essential branched amino acids (valine, leucine and isoleucine). The mode of action of several of herbicides (e.g. chlorimuron and thifensulfuron) is based on the inhibition of this enzyme resulting in the inability of the plant to synthesise branched-chain amino acids required for plant growth. The modified or mutated *gm-hra* is tolerant to these herbicides due to reduced binding.

The cassette integrity and gene stability was assessed by Southern blotting using probes to the cassette and flanking regions. The data indicates that there is probably one intact insertion of the cassette, but the results are unclear and not conclusive. They rely on Southern blots with insufficient sensitivity and a limited number of frequently-cutting restriction enzymes. In addition, the positive control (plasmid cassette) did not produce the expected pattern with *Xba1* enzyme probably because it was methylated. This means that fragment sizes of the cassette from the Soybean 356043 plant could not be accurately compared to that of the original construct and the integrity of the cassette cannot be accurately determined. Furthermore, the copy number was determined by visual reference to known copies of plasmid DNA; however, only 1 and 3 copies were used on the Southern blots (figure 3-12) and no quantification was made (scanning

densitometry).

The applicant states that the cassette is genetically and phenotypically stable, but do not have sufficient data to support this. What is required is a detailed molecular analysis for populations (>20 individual plants) of Soybean 356043 and non-GM but only a few plants were studied using basic Southern blots with a few restriction enzymes (generally three Xga1 Bgl11 and Hind 111- all of which cut too infrequently to reveal sequence variation such as deletions, mutations or re-arrangements (see fig 4-11 Annex 2). Furthermore, the analysis was limited to only two generations. Several (3 -5) generations should be analysed to be confident of stable inheritance; as is commonly employed in agronomic trait selection by backcrossing. A more appropriate method would be the used of PCR with internal and flanking primer and the DNA sequencing of the PCR products. This would determine the integrity (order of genes and sequence) of the cassette and can be used to accurately quantify the gene copy number (using real time PCR) over several generations. The sequence of the flanking site and the homologous region in the non-parent line also needs to be compared so that one can determine if they are any unintended rearrangements at the site of integration; yet there is no data to address this.

The nature of the cassette also raises questions of stability since the cassettes have viral promoters and synthetic elements (SCP1 promoter is derived from CaMV- 35S promoter and the synthetic Rsyn7-Syn II core consensus promoter) that are new to nature and have not been tested for biosafety in terms of stability and horizontal gene transfer (HGT, see below). There is existing concern over the use of CaMV-35S promoter due to increased rearrangements/ deletions affecting cassette integrity and genome stability. These biosafety risks include:

- Increased recombination (rearrangements, deletions, insertions). There is evidence from the laboratory (Koholi *et al.* 1999) and field studies (Quist and Chapela 2001, Collonier *et al., Ho et al.* 2000) that the *35S-CaMV* is a recombination 'hotspot'.
- The *35S-CaMV* results in very high expression levels that may result in unintended (pleiotropic) effects from the expressed transgenes.
- Increased recombination with other viral elements and the creation of new viruses (Wintermantel *et al.* 1996, Vaden and Melcher 1990, Greene *et al*. 1994).

The effects on genome stability, global gene expression and HGT have not been addressed by the applicant A comparative assessment has not been made (by comparative genome hybridisation or repPCR/RAPD, RNA microarrys and/or proteomics) to establish if there are any other unintended genome changes. Established techniques such as repPCR, RAPD and comparative genome hybridization (CGH) could have been used to establish the genome similarity between the Soybean 356043 and non-GM parent line (Bao et al. 1993, Pinkel and Albertson 2005).

Nutritional equivalence and safety

A detailed study was carried out to compare Soybean 356043 and non-GM plants that were grown at several locations. The levels of palmitic, heptadecanoic and heptadecenoic and eicosadecenoic acids of the Soybean 356043 were significantly different to the non-GM (Annexe 4 PHI-2005-056/000). There were also significant differences of the calcium and magnesium (only at one or two locations) and the isolflavones malonyldiadzin. and glycitin (the latter only at only one location). All these differences are significant (P

<0.05) even after the application of false discovery rate (FDR). Therefore, the applicant cannot claim equivalence of Soybean 356043 compared to non-GM soybean.

The activity of the GAT4601 protein is also a biosafety issue since the enzyme modifies (acetylates) amino acids. The applicant therefore measured the levels of N-acetyl-glutamate (NAG) and N-acetyl aspartate (NAA) in Soybean 356043 and non-GM soy as well as the processed products (Annexe 14). The NAA and NAG in whole cooked soybean are also considerable indicating litle loss of NAA or NAG in processing (380ug/g and18.5ug/g compared to < 4ug/g for the non-GM while for soy flour and defatted toasted flakes had 498 \ug/g NAA and 18.4ug/g and NAG (Annexe 17) . The s NA A and NAG are thought to be non-toxic since some other foods contain considerable amounts (i.e. yeast extract 10-15ug/gNAA and 155-165ug/g NAG; chicken approx. 3ug/g NAA and 1ug/g NAG and mushroom approx. 2-3ug/g NAG, 1-2 NAA). However, it should be noted that soy may consist a large proportion of a meal (defatted toasted flakes a.k.a. soy mince) while the other foods known to contain high NAG and NAA will usually constitute a small portion of the meal (e.g. yeast extract) so the exposure is likely to increase hundred or more fold with the ingestion of Soybean 356043. The health effects of consuming large quantities of NAA and NAG are currently uncertain. However, acetylated amino acid neurotransmitters are important for brain function and the administration of N-acetyl-l-aspartyl-l-glutamate (NAAG) to 12-day-old rats was shown to cause extensive neuro-degeneration and the development of abnormal behaviour (Bubeníková-Valešová *et al.*2006).

There are also uncertain effects of consuming these novels proteins in terms of allergenic potential. The applicant has determined that there is no sequence similarity to database allergens and the proteins are rapidly degraded in simulated gastric or intestinal fluids (Annex 19 and Annex 23). There is, however no data to observe the immune response using live animal feeding studies to support this *in vitr*o system. Also, the animal feeding study used bacterially expressed protein to determine toxicity to mice. Although there seems to be no difference in glycosylation pattern of the bacterially-expressed compared to the plant-expressed HRA protein, the peptide fingerprints reveal that there may be differences (the microbial had 18 peptides while the plant HRA had 14 peptides). This may indicate differences in sequence, protein modifications or folding but has not been addressed or explored further. The MALDI-TOF analysis only allowed 26-38% match to the theoretical sequence- meaning that the data is insufficiently detailed enough to make a complete comparison. Interestingly, the Western blot (figure24, pg 69) shows a more diffuse band for plant HRA suggesting some type of protein modification. More MALDI-TOF data or support from 2-D peptide mapping would help resolve this issue. The data from the animal feeding study (mice toxicity) that did not find any toxic effects (annexe 9 and 12) lacks relevance because of the uncertainty that the bacterially expressed protein is that same as the plant derived one.

Escape and monitoring

Relatively little of the soy imported into South Africa is the whole soybean (seed):-1 600 tones of soybean seed was imported in to SA while import of processed soybean products were 1.2 million tonnes (2007). The importation of seed carries risks in escape and hybridisation with other soy varieties in the field that may occur through human error (spillage or planting of some soybean seed that was intended for processing and food or feed). There is no monitoring system proposed which is a requirement of legislation,

namely the Convention on Biological Diversity (CBD), NEMA (1998) and the Genetically Modified Organisms Act. Either a monitoring system needs to be put in place or the commodity import restricted to processed soy (cracked soybeans or any further processed soybean products) to reduce this risk.

Additionally, the issue of gene escape and horizontal gene transfer (HGT) has not been addressed despite the fact the HGT can occur to soil bacteria (wastes disposed of to the environment at soy processing factories) as well as to human intestinal bacteria (when Soybean 356043 products are consumed). Current evidence indicates that HGT to bacteria does occur and occurs at a high frequency when sequence homology is present (de Vries J, Wackernagel W 1998). The *gat4601* gene has many homologs in bacteria found in the soil and other environments (*Bacillus, Shigella, Enterococcus* with 30-85% protein identity). The same is true for that has *gm-hra* gene (homolgs in *Klebsiella, Shigella Escherichia coli* with 30-50 % protein identity) and therefore there is an increased risk for HGT to occur to soil bacteria. Similarly, HGT can take place between ingested Soybean 356043 in food and intestinal bacteria. A study carried out by the British Food Standards Agency to determine if transgenic DNA transferred to bacteria of the human gut by HGT found that this did indeed occur (Netherwood 1990).

Genes acquired through HGT will be retained only if there is a suitable positive selective pressure. The selective pressures that would confer advantage to bacteria are poorly understood, but have clearly contributed to the ability of bacteria to cause disease (Salyers and Shoemaker, 1994; and Witte, 1997). The obvious selective pressure is the application of herbicide (glyphosphate and/or chlorimuron, thifensulfuron) that will positively select for bacteria containing the *gat4601* and *gm-hrs* genes.

There may be several consequences of gene escape and hybridisation including the spread of herbicide resistance, and non-target effects on other plants, animals (Cui and Xia 1999, Hillbeck 1999) and soil microorganisms important for soil function such as nutrient availability and cycling (Benbrook 1999 and Kowalchuk 2003). After almost three decades of world-wide use, confirmed resistance to glyphosate exists in *Lolium rigidum* (annual ryegrass) in Australia and South Africa; *Lolium multiflorum* (Italian ryegrass) in Chile, *Eleusine indi*ca (goosegrass) in Malaysia; and *Conyza canadensis* (marestail) in eastern USA. http://www.cropscience.org.au/icsc2004/symposia/2/5/2166_killmer.htm

In summary, there are uncertainties in the stability of the genetic cassette and lack of nutritional equivalence of the Soybean 356043 compared to non-GM soybean. There are additional uncertain health risks with the consumption of aceylated amino acids that are considerably elevated in Soybean 356043 compared to non-GM soy (and other food products). Finally, there are risks of Soybean 356043 gene escape (HGT) that have not been addressed. It is therefore recommended that the precautionary principle be applied and Soybean 356043 not be approved for commodity clearance.

References

Bao, P.H.; Castiglione, S.; Giordani, C.; Li, W.; Wang, G.; Datta, S.K.; Datta, K.; Potrykus, I.; Sala, F. (1993). State of the foreign gene and of the genome in transgenic rice *(*Orvza sativa). Cytotechnology 11: S123125,

Benbrook, C.M. 1999. Impacts on soil microbial communities needs further study. AgBioTech InfoNet. June 24. http://www.biotechinfo.net/microbial_communities2.html Venkateswerlu G. and

Collonier C, Berthier G, Boyer F, Duplan M-N, Fernandez S, Kebdani N, Kobilinsky A, Romanuk M, Bertheau Y. Characterization of commercial GMO inserts: a source of useful material to study genome fluidity. Poster courtesy of Pr. Gilles-Eric Seralini, Président du Conseil Scientifique du CRII-GEN, www.crii-gen.org

de Vries J, Wackernagel W (1998) Detection of nptII (kanamycin resistance) genes in genomes of transgenic plants by marker-rescue transformation. Molecular and General Genetics 257:606-613

Gendel S. (1998). The use of amino acid sequence alignments to assess potential allergenicity of proteins used. Advances in Food and Nutrition Research 42, pp. 45-6

Greene, A.E. and Allison, R.F. (1994). Recombination between viral RNA and transgenic plant transcripts. Science 263, 1423-5.

Hilbeck, A.,W.J. Moar, M. Pusztai-Carey, A. Filippini, and F. Bigler. 1999. Prey-mediated effects of Cry1Ab toxin and protoxin and Cry2A protoxin on the predator *Chrysoperla carnea. Entomologia Experimentalis et Applicata* 91: 305-316.

Ho MW, Ryan A and Cummins J (2000). CaMV35S promoter fragmentation hotspot confirmed and it is active in animals. Microbial Ecology in Health and Disease, 12, 189.

Ho MW, Ryan A and Cummins J (2000). Hazards of transgenic plants with the cauliflower mosaic viral promoter. Microbial Ecology in Health and Disease, 12, 6-11.

Ho, M-W. 1998. Genetic Engineering: Dream or Nightmare? The Brave New World of Bad Science and Big Business. Penang, Malaysia: Third World Network.

Kohli A., Griffiths S, Palacios N, Twyman R, Vain P, Laurie D and Christou P. (1999) Molecular characterization of transforming plasmid rearrangements in transgenic rice reveals a recombination hot spot in the CaMV 35S promoter and confirms the predominance of microhomology mediated recombination" Plant.J. 17,591-601

Kowalchuk G.A., Bruinsma, M., and van Veen, J.A. (2003) Assessing responses of soil microorganisms to GM plants. Trends in Ecology and Evolution Vol.18 No.8 .

Marvier, M. 2001. Ecology of transgenic crops. *American Scientist* 89: 160-167. United States Environmental Protection Agency. 2001a.

Netherwood T, Martín -Orúe SM, O'Donnell AG, Gockling S, Graham J, Mathers JC, Gilbert HJ (2004). Assessing the survival of transgenic plant DNA in the human gastrointestinal tract. Nature Biotechnol. 22:204-209.

Pinkel D, and Albertson D.G. (2005). Comparative genome hybridization. Annual Review of Genomics and Human Genetics, Vol. 6, Pages 331-354

Vaden V.S. and Melcher, U. (1990). Recombination sites in cauliflower mosaic virus DNAs: implications for mechanisms of recombination. Virology 177, 717-26

Věra Bubeníková-Valešováa, Vladimir J. Balcarb, , , Hana Tejkalováa, Miloš Langmeierc and František Šťastnýa, d Neurochemistry International. (2006). Neonatal administration of N-acetyl-l-aspartyl-l-glutamate induces early neurodegeneration in hippocampus and alters behaviour in young adult rats Volume 48, Issues 6-7, May-June 2006, Pages 515-522

Wintermantel, W.M. and Schoelz, J.E. (1996). Isolation of recombinant viruses between cauliflower mosaic virus and a viral gene in transgenic plants under conditions of moderate selection pressure. Virology 223, 156-64.