Import of genetically modified soybean DAS-44406-6 with three herbicide tolerance traits

COGEM advisory report CGM/130627-01

Summary

The present application (EFSA/GMO/NL/2012/106) concerns import and processing for use in feed and food of the genetically modified soybean line DAS-44406-6. Cultivation is not part of this application.

Soybean DAS-44406-6 was generated by Rhizobium *mediated transformation of the conventional soybean cultivar 'Maverick' and expresses the* 2mepsps, aad-12 *and* pat *genes. As a result, soybean DAS-44406-6 is tolerant to glyphosate, 2,4-D and glufosinate-ammonium containing herbicides.*

In Europe, there are no wild relatives of soybean and therefore, hybridisation with other species is not possible. Soybean does not possess any of the attributes commonly associated with problematic weeds such as seed shattering, dormancy and cold resistance. Establishment of feral soybean populations has never been observed in Europe.

The molecular characterization of DAS-44406-6 soybean meets the criteria of COGEM. COGEM considers the General Surveillance plan adequate for import of DAS-44406-6, although there are some aspects of the General Surveillance plan that could be improved.

In conclusion, COGEM is of the opinion that import and processing of soybean line DAS-44406-6 poses a negligible risk to the environment. COGEM abstains from giving advice on the potential risks of incidental consumption since a food/feed assessment is already carried out by other organisations.

Introduction

The present application by Dow AgroSciences LLC (EFSA/GMO/NL/2012/106) concerns import, food, feed and processing of genetically modified (GM) soybean DAS-44406-6. This soybean was produced by *Rhizobium radiobacter* (previously known as *Agrobacterium tumefaciens*[1]) mediated transformation and expresses the *2m epsps* gene derived from *Zea mays*, the *aad-12* gene derived from *Delftia acidovorans*, and the *pat* gene isolated from *Streptomyces viridochromogenes*. As a result, soybean DAS-44406-6 is tolerant to glyphosate, 2,4-D and glufosinate-ammonium containing herbicides.

Previous COGEM advice

In 2011, COGEM advised positively on the import and processing of soybean DAS-68416-4. This line expresses the same *aad-12* and *pat* genes used in the current application.[2] In 2012, COGEM advised positively on the import and processing of genetically modified soybean FG72 expressing the *2m epsps* gene.[3]

Aspects of the crop

Soybean (*Glycine max*) is a member of the genus *Glycine* and belongs to the Fabaceae (Leguminosae) family. Soybean is grown from equatorial to temperate zones. The optimum temperature for soybean growth is between 25°C and 30°C. Soybean seeds will germinate when the soil temperature reaches 10°C and under favourable conditions a seedling will emerge in a 5-7 day period. Soybean is sensitive to frost and therefore does not survive freezing conditions.[4,5]

In the Netherlands, frost is common. On average 58 days in a year have a minimum temperature below 0°C.[6,7] In the summer days are long, whereas soybean is a quantitative short-day plant that needs short days for induction of flowering. The Dutch climate is therefore not optimal for cultivation of soybean. However, field trials with a number of soybean varieties have shown that cultivation of soybean under temperate climatic conditions is possible.[8,9] Further improvement of these varieties may result in soybean varieties suited for commercial cultivation in the Netherlands. The soybean plant is not weedy in character.[4] As for all domesticated crops, plant breeders aimed to reduce seed shattering in order to increase yield during harvesting. Soybean seeds rarely display dormancy and poorly survive in soil.[10] Soybean volunteers are rare throughout the world and do not effectively compete with other cultivated plants or primary colonisers.[4] In addition, volunteers are easily controlled mechanically or chemically.[4] COGEM is not aware of any reports of feral soybean populations in Europe.

Soybean is predominantly a self-pollinating species. The cross-pollination rate of soybean is less than 1%.[4] The dispersal of pollen is limited because the anthers mature in the bud and directly pollinate the stigma of the same flower. In Europe, hybridisation with other species is not possible because there are no wild relatives of soybean.[4]

Molecular characterization

Soybean line DAS-44406-6 was produced from the conventional soybean cultivar ‘Maverick’. The disarmed *Rhizobium* strain EHA101 was used to generate transformants. This *Rhizobium* strain was produced from strain A281 by inactivation of the T-DNA *onc* genes and carried the helper plasmid pTiBo542 and the binary vector pDAB8264. The *Rhizobium* mediated transformation of the conventional soybean cultivar ‘Maverick’ was followed by conventional breeding.

An overview of the T-DNA introduced in DAS-44406-6 (10,280 bp) is given below:

- Intervening sequence;
- RB7 MAR; matrix attachment region (MAR) from the 7-5A gene of *Nicotiana tabacum*. MARs are thought to attach to the matrix or scaffold of the nucleus. Some MARs have been shown to stabilise expression of transgenes and to reduce the incidence of gene silencing;
- Intervening sequence;
- Histone H4A748 3’UTR; 3’ untranslated region (UTR) sequence comprising the transcriptional terminator and polyadenylation site of the histone H4A748 gene from *A. thaliana;*
- Intervening sequence;

- *2m epsps* gene; 5-enolpyruvylshikimate-3-phosphate synthase (*epsp*) gene from *Z. mays* containing two mutations providing glyphosate tolerance;
- TPotp C; an optimized chloroplast transit peptide from *Z. mays* and *Helianthus annuus* ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO);
- Intervening sequence;
- Histone H4A748 promoter, constitutive promoter and 5 'untranslated region from the Histone H4A748 gene *A. thaliana* including an intron from the histone 3 gene from *A. thaliana*;
- Intervening sequence; sequence used for DNA cloning;
- AtUbi10 constitutive promoter and the 5'untranslated region and intron from the *A. thaliana* polyubiquitin 10 (UBQ10) gene;
- Intervening sequence; sequence used for DNA cloning;
- *aad-12* gene; codon optimized version of the aryloxyalkanoate dioxygenase (*aad*) gene from *D. acidovorans*, this gene encodes an enzyme with an alpha ketoglutarate-dependent dioxygenase activity which results in metabolic inactivation of certain herbicides*;*
- Intervening sequence; Sequence used for DNA cloning;
- AtuORF23 3'UTR, 3' UTR sequence comprising the transcriptional terminator and polyadenylation site of open reading frame 23 (ORF23) of plasmid pTi15955 from *R. radiobacter;*
- Intervening sequence;
- CsVMV constitutive promoter and 5' UTR from the *Cassava vein mosaic virus*;
- Intervening sequence;
- *pat* gene; codon optimized version of the phosphininothricin N-acetyl transferase (*pat*) gene derived from *S. viridochromogenes* providing tolerance to glufosinate ammonium;
- Intervening sequence;
- AtuORF1 3'UTR; 3' UTR sequence comprising the transcriptional terminator and polyadenylation site of open reading frame 1 (ORF1) of plasmid pTi15955 from *R. radiobacter;*
- Intervening sequence;
- T-DNA border A; necessary to transfer the T-DNA from *R. radiobacter* into plant cells.

The applicant used Southern blot analyses to demonstrate that soybean DAS-44406-6 contains a single intact T-DNA insert and that no backbone sequences from the plasmid or T-DNA fragments are present in the DAS-44406-6 soybean genome. Southern blot analyses also demonstrated that the insert is stably inserted in the soybean genome and inherited in a Mendelian manner.

The applicant analysed the sequences of the T-DNA insert (10,280 bp), its 5' flanking region (1,494 bp) and its 3' flanking region (1,885 bp). In addition, the applicant determined the sequence of the locus in which the T-DNA insert was inserted from the parental conventional soybean cultivar 'Maverick'. A comparison of the obtained sequences showed that the sequence of the inserted T-DNA region was identical to the sequence of the T-DNA region of plasmid pDAB8264.

In addition, the comparison showed that three basepairs were inserted at the 5'end of the insert and that 4,383 basepairs were deleted from the genome at the integration site.

Analysis of the 5' and 3' flanking regions using the GenBank nucleotide and protein databases as well as the soybean scaffold sequence collection (which is a sequence assembly of the soybean genome from soybean variety William 82) indicates that the T-DNA in soybean DAS-44406-6 is flanked by genomic sequences and that it is present on chromosome 6.

The applicant performed several bioinformatic analyses (BLASTx and BLASTp) on the region that was deleted during integration of the insert in the soybean genome. Part of the deleted sequence showed similarity to a putative reverse transcriptase family member, most likely derived from a retro-transposable element.

The applicant also examined whether endogenous genes or regulatory elements had been deleted or disrupted by the T-DNA integration. According to the applicant, the combined analyses provide no indication that a known endogenous soybean gene or regulatory element was disrupted or deleted by the integration of the T-DNA.

Analysis of the 5' flanking region, however, revealed a sequence close to the 5' junction which is similar to the beginning of a gene that potentially encodes for a vacuolar ATPase or kinase-like protein from *Arabidopsis*. The same similarity was found with other plant species, indicating that it is a functional region. This putative kinase-like gene consists of several introns, which are less conserved, explaining why only a small part of the 5' flanking region showed a significant similarity to the kinase-like gene from *Arabidopsis* and other plants. If the sequence close to the 5' junction is indeed the 5' end of gene, it implies that transcription occurs away from the T-DNA insertion and that the insertion may have removed regulatory promoter elements. Thus, contrary to the applicants' interpretation, COGEM is of the opinion that it cannot be excluded that the T-DNA insertion affects the expression of an endogenous gene. However, since DAS-44406-6 is in all tested aspects equivalent to the parental soybean, it is unlikely that the insertion has an environmental impact.

The applicant screened the junctions between elements within the T-DNA insert and the junctions between the insert and its borders for the presence of putative open reading frames. A total of 12 putative open reading frames were identified between the insert and its borders (the so-called putative 'across-junction' open reading frames) and 651 putative open reading frames were identified between elements within the T-DNA. Putative open reading frames were defined as any open reading frame spanning the junctions regardless of the presence of a start codon and the number of amino acids. All of the putative 'across-junction' open reading frames were evaluated for potential similarity to known proteins. The analysis of similarity to known proteins of putative open reading frames between elements within the T-DNA was limited to a length equal or greater than 8 amino acids. No significant amino acid sequence similarities with known allergens or toxic proteins harmful to humans or animals were identified.

In summary, DAS-44406-6 soybean contains a single intact T-DNA insert in its nuclear genome. Three additional basepairs were included at the 5' end of the insert and 4,383 basepairs were deleted from the integration locus. No backbone sequences or T-DNA fragments are present. Although it cannot be excluded that a functional endogenous gene is disrupted by the integration of the T-DNA, such a disruption is not expected to have an environmental effect.

When novel reading frames across the junctions between the T-DNA insert and the genome, and across the junctions between the various elements within the T-DNA insert were analysed, no significant amino acid sequence similarities with known allergens or toxic proteins harmful to humans or animals were identified.

The molecular characterisation of soybean DAS-44406-6 meets the criteria laid down by COGEM.[11]

Properties of the introduced genes conferring herbicide tolerance

DAS-44406-6 soybean expresses the *aad-12* gene and as a result produces the aryloxyalkanoate dioxygenase-12 (AAD-12) enzyme originating from the soil bacterium *D. acidovorans*. AAD-12 is capable of deactivating several aryloxyalkanoate based herbicides, including phenoxy auxin (e.g., 2,4-D, MCPA) and pyridyloxy auxins (e.g., fluroxypyr, triclopyr). The *aad-12* gene was modified to allow optimal expression in plants. According to the applicant the wild type and plant optimised DNA sequences of the *aad-12* gene are 79.7% identical.

DAS-44406-6 also expresses the phosphinothricin acetyltransferase (PAT) protein from *S. viridochromogenes*. This protein acetylates L-phosphinothricin, the active isomer of glufosinate ammonium. The resulting compound N-acetyl-L-phosphinothricin does not inhibit the activity of glutamine synthetase.[12] As a result DAS-44406-6 is tolerant to glufosinate-ammonium based herbicides. The *pat* gene was also used as a selectable marker in the process of transgenic soybean regeneration.

In addition, DAS-44406-6 contains the *2m epsps* gene. This gene was generated by introducing mutations into the wild-type *epsps* gene from maize, leading to a modified 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) with two amino acid substitutions (2mEPSPS). A chloroplast transit peptide sequence (TPotp C) is fused to the *2m epsps* gene, resulting in the transport of the 2mEPSPS protein to the chloroplast.[13] In addition, a methionine amino acid is added at the N-terminal site of the protein to restore the cleavage site of the chloroplast transit peptide.

EPSPS is a natural occurring enzyme involved in the biosynthesis of aromatic amino acids and is active in the chloroplasts of a plant cell. Glyphosate inhibits EPSPS, resulting in a lack of amino acids essential for growth and development of plants.[14,15] The 2mEPSPS enzyme has a decreased binding affinity of the protein for glyphosate.

Environmental risk assessment

The current application concerns import and processing of soybean line DAS-44406-6. In case of spillage, soybean seed may be released into the environment. Soybean seeds rarely display dormancy, poorly survive in soil and do not survive freezing winter conditions. The Dutch climatic

conditions are not optimal for growth of soybean. In the summer, days are long, whereas soybean is a quantitative short-day plant that needs short days for induction of flowering. Due to the characteristics of soybean, COGEM is of the opinion that the development and improvement of soybean varieties more suited for commercial cultivation in the Netherlands does not affect the environmental risk assessment of DAS-44406-6 at this time.
Soybean volunteers are rare throughout the world and do not effectively compete with other cultivated plants, weeds or primary colonisers.[4] In addition, volunteers are easily controlled mechanically or chemically.[4]

There is no reason to assume that soybean line DAS-44406-6 has an increased potential to survive or establish feral populations in case of incidental spillage.

COGEM abstains from giving advice on the potential risks of incidental consumption since a food/feed assessment is already carried out by other organisations.[16] This application is submitted under Regulation (EC) 1829/2003, therefore a food/feed assessment is carried out by EFSA and national organisations involved in the assessment of food safety. In the Netherlands, a food and/or feed assessment for Regulation (EC) 1829/2003 applications is carried out by RIKILT. Regarding the risks for food and feed, the outcome of the assessment by other organisations (EFSA, RIKILT) was not known at the moment of the completion of this advice.

General surveillance
General surveillance (GS) has been introduced to be able to observe unexpected adverse effects of genetically modified (GM) crops on the environment. The setting or population in which these effects might occur is either not, or hardly predictable. A GS plan is required for every application for market authorisation.

The current GS plan states that unanticipated adverse effects will be monitored by existing monitoring systems which include the authorization holder and operators involved in the handling and use of viable soybean. The third parties (operators) involved in GS will report adverse effects to the authorization holder. COGEM points out that it depends on the person what is considered an *adverse* effect. In COGEM's view a list of specific questions that would help operators to identify adverse effects, would further improve the GS plan.

In 2010, COGEM published a report on the principles that should be followed for general surveillance.[17] COGEM concluded that the GS plans could be improved by a guarantee that operators will monitor for unanticipated effects. In the present GS plan, the authorization holder states that the operators have agreed to provide information relevant to the monitoring of DAS-44406-6 to the authorization holder. More important, it is stated that the authorization holder will be able to give evidence that the operators collect this information. This is in line with the criteria laid down by COGEM.[17] COGEM points out that a commitment of the applicant to make raw monitoring data and the analyses thereof available to the Competent Authorities and the European Commission, could further improve the GS plan.[18] This request is also made by EFSA in the guidance document on post-market environmental monitoring.[19]

The GS plan states that if the authorisation holder identifies an unexpected adverse effect caused by the GM plant, he will inform the European Commission immediately. COGEM is of the opinion that Member States should also be directly informed of these effects by the authorisation holder, to ensure that appropriate measures for protection of humans and the environment can be implemented immediately.

Advice

COGEM has been asked to advice on import and processing for use in food and feed of herbicide tolerant soybean line DAS-44406-6. This genetically modified soybean line expresses the *2m epsps*, *aad-12* and *pat* genes providing tolerance to glyphosate, aryloxyalkanoate or glufosinate-ammonium containing herbicides. The molecular characterization of DAS-44406-6 soybean meets the criteria of COGEM.

Although field trials have indicated that some soybean varieties can be cultivated in the Netherlands, the Dutch climate is not optimal for soybean growth.

Soybean volunteers are rare throughout the world and do not effectively compete with other cultivated plants or primary colonisers. Modern soybean cultivars do not possess any of the characteristics commonly associated with problematic weeds. There is no reason to assume that expression of the introduced *2m epsps, aad-12* and *pat* genes will increase the potential of soybean to establish feral populations. In addition, establishment of feral soybean populations in Europe has never been observed.

COGEM is of the opinion that the risk of spread of soybean DAS-44406-6 within the Netherlands due to incidental spillage of this soybean line is negligible. Wild relatives of soybean are not present in Europe and therefore introgression of the inserted gene into closely related species cannot occur. COGEM considers the current GS plan sufficient for import and processing of soybean line DAS-44406-6.

In conclusion, COGEM is of the opinion that import and processing of soybean DAS-44406-6 poses a negligible risk to the environment. A food/feed safety assessment is carried out by other organisations. Therefore, COGEM abstains from advice on the potential risks of incidental consumption.

Additional remark

A GS plan was provided that describes how the applicant will monitor the occurrence of unanticipated adverse effects of DAS-44406-6 soybean. COGEM noted that the hyperlinks in this document are no longer functional. COGEM points out that it is of utmost importance that the website which contains information for operators working with genetically modified crop products is easily retrievable and accessible at all times. Therefore, COGEM urges the applicant to update the hyperlinks in the GS plan.

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