Bundesamt für
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Opinion of the ZKBS on application EFSA-GMO-NL-2005-24 from the company Monsanto on approval of placing the genetically modified soybean 40-3-2 on the market for the purpose of cultivation as genetically modified food and feed according to Regulation (EG) No. 1829/2003

1. Subject matter of the application and purpose for introduction onto the market

The company Monsanto submitted application EFSA-GMO-NL-2005-24 (BVL-Ref. No. 6787-01-0024) to the Competent Authority of the Netherlands, who forwarded it on to the European Food Safety Authority (EFSA) on November 04, 2005.

The scope of the application is introducing the genetically modified soybean (*Glycine max* L.) of the strain GTS 40-3-2, which has tolerance to glyphosate-containing herbicides, on the market.

The aim referred to in the application is agricultural cultivation in the EU. Cultivation of glyphosate-tolerant soybean GTS 40-3-2 in connection with using the corresponding herbicide allows a new form of weed management.

In 1994, Monsanto submitted an application to the Competent Authority of the UK (C/UK/94/M3/1) to introduce the soybean 40-3-2 on the market, to which the ZKBS gave a positive opinion in 1995. The application was approved in 1996 according to Regulation 90/220/EG valid at that time. This previous approval was carried over as valid approval according to the new law (notated as “existing product”), which pertains to its use as food and feed as well as food and feed additives. The time period for the approval expired on April 18, 2007. Since the company Monsanto has submitted an application for further approval of an “existing product” according to Regulation (EG) No. 1829/2003 for genetically modified food and feed (EFSA-GMO-RX-40-3-2), the products are currently still marketable. Therefore, the current application exclusively applies to approval for agricultural cultivation in the EU.

2. Description of the genetically modified organism

The plasmid PV-GMGT04 was introduced into plant tissues from the soybean line A5403 using the micro-projectile bombardment method. The vector is derived from plasmid pUC119 (Vieira and Messing, 1987) and thus contains a replication origin (*ori*-pUC) for replication in *Escherichia coli*. In addition, the plasmid carries the bacterial kanamycin resistance gene *npt* II under the control of its own promoter, which codes for aminoglycoside-3’-phosphate II and comes from the *E. coli* transposon Tn5 (Beck *et al*., 1982). Moreover, the plasmid vector PV-GMGT04 carries both an *uidA* expression cassette and two *cp4 epsps* expression cassettes. Expression of b-D-glucuronidase from the *uidA* gene from *E. coli* (Jefferson *et al.*, 1986) serves as the initial marker for a successful transformation event (R_0 shoots). Due to natural genetic segregation the R_2 progeny (40-3-2) derived from an original R_0 transformant no longer contained the *uidA* gene, as was confirmed by molecular characterization of the line GTS 40-3-2.

The soybean line GTS 40-3-2 was more closely characterized by Southern blot and PCR analyses. One can conclude from the results of these analyses that one of the two *cp4 epsps*

expression cassettes, but not functional sequences of the vector backbone, nor another expression cassette present in the plasmid RV-GMGT04, is stably integrated into the genome of line GTS 40-3-2. In addition to the *epsps* gene encoding 5-enoylpyrovyl shikimate-3-phosphate synthase (EPSPS) from the *Agrobacterium* strain CP4, the integrated expression cassette contains the 35S promoter with a duplicated enhancer region from Cauliflower Mosaic Virus (CaMV), the coding region for the N-terminal chloroplast transit peptide (CTP4) sequence, the *epsps* gene from *Petunia hybrida* and the termination signal of the nopaline synthase gene (*nos* gene) from *Agrobacterium tumefaciens*.

A detailed molecular characterization of the insert, which included sequence analysis in addition to Southern blot and PCR analyses, also gave the following results:

A 354 base pair (bp) long region of the duplicated enhancer from the 35S promoter of CaMV was not transferred to the soybean genome. The promoter is however sufficiently active to provide the genetically modified soybean plant with a useful agronomic tolerance to the herbicide agent glyphosate conferred by the expression of the *cp4 epsps* gene.

In addition to the complete *epsps* gene from *Agrobacterium* strain CP4, contained in the *cp4 epsps* expression cassette integrated into the plant genome as described above (primary insert), the soybean line GTS 40-3-2 and its progeny contain two additional fragments of the *epsps* gene, namely:

a) a 72 bp long secondary fragment that is inserted separately from the primary fragment in the soybean genome, but still segregates together with it in subsequent progeny,

b) a 250 bp long fragment attached to the 3' end of the *nos* gene terminator signal (from *Agrobacterium tumefaciens*) contained in the primary insert. The 3' end of the 250 bp *epsps* fragment described in b) is linked to a 534 bp long DNA fragment that represents genomic soybean DNA that has been rearranged during the transformation process.

Bioinformatics-based analyses were carried out to investigate possible open reading frames (ORF) that may have arisen as a consequence of the integration of the inserts contained in soybean line 40-3-2. This includes identifying and evaluating all putative peptides that could be formed, starting 5' and 3' from the overlapping regions between genomic DNA and the described inserts, in terms of their allergenic, toxic or other bio-active potential. Sequence analysis of the primary insert gave six possible polypeptides for the 5' overlap region and five for the 3' overlap, and these were included in the bioinformatics analyses. Analysis of the 72 bp secondary fragment gave eight possible polypeptides, whereby due to the small size of the insert, several peptides stretch over the region of the complete insert and both flanking sequences. The results of the bioinformatics analyses of the predicted putative protein sequences were that none of these polypeptides show significant similarity to known allergens, toxins or other bioactive peptides.

Northern blot analyses not only showed the presence of an mRNA of the expected length (ca. 1.5 kb) but also the presence of a secondary transcript that results from the *nos* termination signal located within the *cp4 epsps* expression cassette not being recognized. This gives an over-length transcript that in addition to the complete *cp4 epsps* sequence also contains part of the 3' flanking sequence region, including the downstream 250 bp *epsps* fragment. Posttranscriptional splicing of this fusion product results in four different mRNA variants that all show the same reading frame (Rang *et al*., 2005). The results of Western blot analyses, however, indicate that the secondary transcript is not translated, since only the expected full-length CP4 EPSPS protein with a size of about 46 kDa, but no other CP4 EPSPS fusion protein, could be detected. Even in the case where the observed secondary transcript might be translated, based on the results of the bioinformatics analysis and lack of similarity of the sequences to known allergens and toxins, no adverse effects would be expected. To investi-

gate the expression of the insert, the average CP4 EPSPS concentrations in leaves and seeds from soybean 40-3-2 were determined using ELISA. The measured concentration determined in plant material collected from field studies carried out in 1998 in the EU lay between 0.321 µg/mg and 0.618 µg/mg FG (fresh weight) in the leaves and between 0.086 µg/mg and 0.270 µg/mg FG in seeds. Similar expression amounts were obtained with material from US American field trials. Expression of the introduced *cp4 epsps* gene confers the genetically modified plants with tolerance towards the herbicide agent glyphosate, which is present for example in the commercial product Roundup®. Inheritance of the herbicide tolerance follows a monogenic pattern.

3. Experience from previous field trials

Release of genetically modified soybean 40-3-2 has occurred since 1991, particularly in the USA, but also in Puerto Rico (1991-1994), Argentina (1993-1994) and Canada (1993-1994). In the context of cultivation in North and South America, comprehensive tests were carried out to determine the spread of herbicide tolerance, as well as harvest parameters and the characteristics of the genetically modified plants in open fields (ability to germinate, persistence, reproduction, capacity to spread, susceptibility to pathogens and pests, etc.). Based on the results of these investigations it can be concluded that with the exception of herbicide tolerance, the genetically modified soybean plants do not significantly differ from non-genetically modified soybean plants in their properties and behaviour. In addition, field trials were carried out in Italy and France in 1994, as well as in Italy in 1996 and 1997, to investigate the feasibility of cultivating the genetically modified soybean in the these areas. This included carrying out an investigation of the phenotypic and agronomic properties of soybean 40-3-2 compared to conventional soybean sorts, which confirmed the results of the American studies. Thus altogether, there is no evidence for any changes in terms of survival, replication, and ability to spread. Moreover, in the 1998 growing period in Europe, European cultivation experiments were carried out at seven locations in France and Italy, as well as in 2005 at five locations in Romania (Harrigan *et al*., 2007) where comparative content analyses were carried out. The results of the content analyses based on soybean cultivation in Europe confirmed the previous studies in the USA (Padgette *et al.*, 1996), which showed that soybean 40-3-2 is substantially equivalent to traditional soybeans. Also any unintentional effect on the plant's metabolism through a genetic change in the sense of a so-called positional effect or through pleiotropic effects could not be detected in the genetically modified plants based on the parameters analyzed. All in all, the total comparative content analyses presented in the application – also in terms of the issues addressed in the application – are enough to confirm the substantial equivalence of soybean 40-3-2 with sufficient confidence. Altogether, the range and number of cultivation experiments reiterate the experiences under field conditions in a representative selection of geographical locations over more than one vegetative period.

4. Approval granted for placing on the market outside the EU

The RoundupReady® soybean (Event GST 40-3-2) was first approved for commercial cultivation in the USA and Argentina in 1996, and since then has received the corresponding approval for cultivation in a range of other countries (e.g. Canada, Brazil, Uruguay, Paraguay, South Africa). In 2007, genetically modified soybeans, the largest proportion being the RoundupReady® soybean (Event GST 40-3-2), were cultivated on about 58.6 million hectares worldwide, corresponding to 64% of the total soybean cultivation area (James, 2007). Furthermore, the import of soybeans with Event GST 40-3-2 and the food and feed produced from them, is not only approved in the EU but also in 13 other countries (China, Mexico, Ja-

pan, Taiwan, Korea, Thailand, Australia, New Zeeland, the Philippines, Columbia, Bolivia, Russia and Switzerland).

5. Risk assessment

The subject matter of the application is approval to cultivate soybeans from the line GST 40-3-2 in the EU. In their opinion on RoundupReady® soybean (Event GST 40-3-2) the ZKBS took into account relevant points to be checked from Annex II of Directive 2001/18/EG.

5.1. Assessment of the capability of the genetically modified soybean for persistence or invasiveness and the potential for gene transfer via pollen to other plants.

The soybean is an annual cultivated herbaceous plant of sub-tropical origin. Its cultivation is limited to warm climates due to its temperature requirements and lack of tolerance to the cold. In the European Union soybean is only cultivated in appreciable amounts in southern countries, particularly Italy, France, and Romania. Since soybean is a domesticated plant, it depends on human cultivation, and cannot establish itself in the natural flora. Soybean plants are not winter hardy and the seeds show no secondary dormancy. Seeds that are lost during growth or harvesting germinate directly, if there is sufficient moisture. It is however expected that the emerging plants will die during winter time or will be destroyed by common soil preparation methods. Soybeans are self-pollinators, and cross-pollination occurs only in rare cases (<1%) in cultivated fields. According to Council Directive 69/208/EWG on the marketing of seeds from oil and fibre plants, no minimal distance is stipulated between reproductive soybean plants and neighbouring soybean fields. The few wild relatives of soybean (*Glycine gracilis, Glycine soja*) are not native to Europe, so hybridizations are not expected. It is not expected that the soybean characteristics described above are affected by the genetic modifications and/or the resulting trait (herbicide resistance) described in the application. The applicant has carried out analyses on numerous chemical components, as well as various agronomic and phenotypic properties (e.g. ability to germinate, persistence, reproduction, dispersal capacity, susceptibility to pathogens and pests) of the genetically modified plants from field trials. These tests confirmed that the genetically modified plants do not significantly differ from non-genetically modified soybean cultivars in terms of these characteristics. The possibility of persistence, dispersal, invasiveness and pollen transfer by the genetically modified plants is not assessed as being any different from traditionally bred soybeans.

5.2. Assessment of the effects on the environment due to the changes conferred by the transferred gene (*cp4 epsps*)

The *epsps* gene codes for a 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS). In plants and microorganisms this enzyme catalyses the reaction of shikamate-3-phosphate with phosphoenol pyruvate resulting in the formation of 5-enolpyruvyl shikimate-3-phosphate, an intermediate step in the biosynthesis of aromatic amino acids and other aromatic substances in plant secondary metabolism. The natural EPSPS present in soybeans is inhibited by the herbicide agent glyphosate, which is why the plants die after application of the corresponding herbicide. In contrast, the equivalent enzyme from *Agrobacterium* strain CP4 is not inhibited, so the biosynthesis of aromatic metabolites is maintained at sufficient levels even after treatment of the plants with glyphosate-containing herbicides (Malik *et al.,* 1989; Steinrücken & Amrhein, 1980). In the construction of the introduced expression cassette, the *epsps* gene from *Agrobacterium* strain CP4 was fused with the DNA sequence for the chloroplast transit peptide (CTP4) of EPSPS from *Petunia hybrida.* This ensures the trans-

port of EPSPS into the chloroplasts. The transit peptide is usually cleaved off during import into the chloroplasts. Regulatory sequences are the 35S promoter of CaMV and the termination signal of the *nos* gene from *Agrobacterium tumefaciens*. The newly formed CP4 EPSPS in the genetically modified soybean catalyzes the same reaction as equivalent enzymes naturally present in soybeans and other cultivated plants. Since no adverse health potential is known for the EPSPS chloroplast transit peptide from *Petunia hybrida,* as well for other currently known signal peptides, whether processed or unprocessed, it can be assumed that this also applies to the fusion protein of transit peptide and enzyme, here CTP4 and CP4 EPSPS. There are no grounds for expecting an adverse effect from the newly formed EPSPS protein.

5.3. Assessment of potential effects of the genetically modified soybean on non-target vertebrates

As an indicator for the presence of possible risks for non-target vertebrates, one can assess the results of rat feeding studies, presented both in the form of a study carried out and documented by the applicant, and in the form of peer reviewed articles (Teshima *et al.*, 2000; Zhu *et al.*, 2004). These studies were carried out with processed as well as raw soybean material and focused on toxicological end points. They confirmed overall that the GM soybean corresponds to conventional soybeans in terms of its safety as feed. In addition, the applicant carried out feeding studies with soybean 40-3-2 and corresponding control diets in chickens, catfish, dairy cows, pigs and quail. The results of these studies confirmed the nutritional equivalence of the genetically modified soybean 40-3-2 compared to conventional soybeans, and thus also confirmed the substantial equivalence of soybean 40-3-2 detected in the compositional analyses. Also no unexpectedly,, accompanying pleiotropic effects could be detected. The application addresses the reason for cultivating the GM soybean. The data obtained with feeding studies are altogether sufficient to exclude adverse effects of the genetically modified soybean on non-targeted vertebrates with sufficient certainty.

5.4. Assessment of potential effects of the genetically modified soybean on further non-target organisms

Due to the genetic modification, the molecular characterization, the detected substantial equivalence and lack of interactions posing environmental risks, no adverse effects on further non-target organisms, particularly arthropods, are expected due to cultivation of soybean 40-3-2. This is confirmed by the many years of large area cultivation of this soybean outside the EU, where there has been no report of direct adverse effects on further non-targeted organisms as yet. The studies on pathogens and symbiotic organisms presented by the applicant confirm with sufficient certainty the assumption that adverse effects of soybean 40-3-2 are not to be expected. The ZKBS notes critically that many of the accompanying studies in the application are only of limited suitability to confirm a lack of effects on non-target arthropods. A large proportion of the studies do not correspond to up-to-date scientific standards. Several of the studies show poor experimental design, including an insufficient exposure of non-target arthropods, an inadequate replications or an insufficient statistical evaluation. Many of the tested organisms do not represent organisms that occur in the biocoenosis of growing soybeans or do not occur in Europe. Furthermore, not all relevant trophic levels have been tested with conclusive studies. Considering the total available information, the ZKBS considers the potential adverse effects on further non-target organisms as unlikely. No adverse cause and effect interactions could be identified that would necessitate a case specific monitoring.

5.5. Assessment of horizontal gene transfer from the genetically modified soybean to microorganisms

Horizontal gene transfer from plants to microorganisms is not observed under natural conditions, but it cannot be excluded (Gebhard & Smalla, 1998; Nielsen *et al*., 2000; De Vries *et al*., 2004). When assessing the safety of possible horizontal gene transfer of the *cp4 epsps* gene to microorganisms it should be taken into account whether this gene is present in the gene pool of the receiving population. The *cp4 epsps* gene occurs naturally in soil bacteria. Ecologically adverse consequences are therefore not expected from the probably very rare event of horizontal transfer of the *cp4 epsps* gene from genetically modified soybean to soil bacteria.

Inactivation of glyphosate and the expression of glyphosate tolerant EPSPS synthase are naturally occurring process in soil bacteria. Bacteria with a resistance to glyphosate are widespread in the environment. Also DNA sequences that code for chloroplast transit peptides – particularly for the *epsps* gene – have been described in a range of different plant species. Even if the *cp4 epsps* gene or the *ctp4* sequence was transferred from genetically modified plants to microorganisms, this would not noticeably increase the total frequency of either this gene or the associated phenotype conferred by a glyphosate tolerant variant of EPSPS in the environment.

Transfer of other regulatory sequences used in the construct and an increase in the total frequency of the corresponding DNA fragment should also give no reason for concern. These regulatory sequences derive from CaMV and *Agrobacterium tumefaciens*. CaMV is a plant-infecting double stranded DNA virus that is found in plants naturally. *Agrobacterium tumefaciens* is a bacterium occurring in soils.

5.6. Effects due to the use of complementary glyphosate-containing herbicides on cultivation and management of the soybean

Effects of the use of glyphosate-containing herbicides were not evaluated in this application, but rather are the subject of approval for plant protection products. The ZKBS notes critically that potential differences between the cultivation of soybean 40-3-2 and conventional soybean under European conditions are presented in only a very limited context in the application, since the applicant considers the use of glyphosate-containing herbicides comparable to conventional cultivation methods. In the opinion of the ZKBS it cannot be excluded that cultivation and planting management will change through the use of GM soybeans. Glyphosate-containing herbicides can be applied after germination of the soybean plants and thus could have effects on the accompanying weed flora. Based on experience from using conventional plant protection products it is to be expected that sooner or later tolerance to the active ingredient of glyphosate-containing herbicides will develop in the weed flora. It has already been shown in the cultivation of herbicide tolerant (HT) soybeans in the USA that continuous and repeated application of glyphosate to HT cultivars causes weed changes and resistance or tolerance development in herbs and grasses (Fernandez-Cornejo J. & Caswell M. 2006). The ZKBS is of the opinion that there is possibly also an indirect interaction between the use of glyphosate-containing herbicides and nitrogen-fixing symbiotic partners of the soybean (e.g. *Bradyrhizobium japonicum,* Moorman *et al.*, 1992, King *et al.*, 2001), which could lead to a reduction in harvest yield (King *et al.*, 2001). To compensate, potential increased application of nitrogen fertilizer might be necessary with the cultivation of HT soybeans. The ZKBS recommends that herbicide and cultivation management of soybean 40-3-2 should be adapted to minimize possible negative effects.

The ZKBS assumes that indirect effects of the complementary herbicide use will be recorded, documented and evaluated within the monitoring program (general surveillance). The *Stewardship Program* presented by the applicant should be harmonized with the pesticide permission authorities and should consider the efficacy of the measures included in the monitoring plan. This proposed procedure would allow herbicide application conditions (such as used amount, intensity of weed control or management to prevent resistance developing against glyphosate-containing herbicides) to be better adapted to the regional requirements of the European Union.

5.7. Monitoring plan

The applicant has presented a complete plan for monitoring the environmental effects of cultivating soybean 40-3-2, and with the implementation of the national monitoring plan is committed to taking into account data collected and published from existing monitoring programs in the evaluation of the general surveillance. In addition, for the safety assessment of soybean 40-3-2 there is no direct reason for case specific monitoring by the applicant.

6. Recommendation of the ZKBS

The Central Commission for Biological Safety came to the conclusion that no adverse effects on the protected items mentioned in §1 No. 1 of the Genetic Engineering Act are to be expected by the cultivation of soybean 40-3-2.

The ZKBS assumes that possible indirect effects of complementary herbicide application will be taken into account by the applicant in the context of a *Stewardship Program* harmonized with the authorities assessing plant protection products. This should ensure that unexpected effects (in general surveillance) can be detected.¶

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