Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

Notification 6786-01-0182

Summary of the risk assessment of the genetically modified

peas (*Pisum sativum*; Eiffel)

within the framework of a proposed deliberate release

carried out by the German Competent Authority

Berlin, 25. April 2007

Explanatory note to this document:

The following text reflects the summary of the risk assessment of (a) genetically modified organism(s) to be used for experimental field trials (deliberate releases) in Germany. The text forms part of the official authorisation regarding applications for the permit of deliberate releases (field trials) of genetically modified organisms in Germany under the legal framework of Directive 2001/18/EC and the German Gene Technology Act (Gentechnikgesetz, GenTG). The authorisation is issued by the Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, BVL [*Federal Office of Consumer Protection and Food Safety*], as the German Competent Authority. It comprises the chapters

I. Consent [to the application]
II. Provisions [to be respected in execution of the field trials]
III. Justification
III.1. Requirements for approval according to section 16 GenTG [German Gene Technology Act]
III.1.1. Requirements for approval according to section 16 (1) Nr. 1 GenTG
III.1.2. Requirements for approval according to section 16 (1) Nr. 3 GenTG
III.1.3. Requirements for approval according to section 16 (1) Nr. 2 GenTG
III.1.4. Formal requirements according to section 16 (4, 5) GenTG
III.2 Appraisal of and reply to objections
IV. Costs
V. Legal instruction

Only the original German document is legally binding. The following passage is a courtesy translation of the chapter III.1.2. and was prepared for the Biosafety Clearing House.

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III.1.2.1. Evaluation of changes in the genetically modified plants effected by the transferred nucleic acid sequence

a) The coding sequences for the single-chain antibody BA11, the signal peptide LeB4, the hexahistidine anchor and the retention signal KDEL

The coding sequences for the LeB4 signal peptide, the single-chain antibody BA11, a hexahistidine anchor and the retention signal KDEL were transferred into the GM peas. The genetic modification causes the production of the scFv antibody BA11 in the GM pea seeds.

ScFv antibody is a synthetic product derived from the heavy and light chain variable regions of a complete antibody, combined by a synthetic peptide linker. The ability to bind to antigens is retained. ScFv antibodies are used in diagnostic and therapeutic applications.

The nucleotide sequence that encodes the scFv antibody BA11 is derived from mouse (*Mus musculus*). Phage display technology was used to isolate an scFv antibody-encoding nucleotide sequence that targets the F4 fimbriae of *E. coli* cells (single-chain antibody BA11). F4 is the most significant and most frequently occurring fimbrial antigen of pathogenic *E. coli* strains of porcine origin. These fimbriae facilitate the adhesion of enterotoxic *E. coli* (ETEC) to the pig intestinal wall – a precondition for the development of infections that cause diarrhoea. In particular, ETECs play a role in triggering postweaning diarrhoea (PWD) in piglets. These bacterial diarrhoeal infections can result in the loss or delayed growth of infected animals. *In vitro* studies have shown that the antibody fragment BA11 blocks the adhesion of F4 fimbriae-carrying *E. coli* to the intestinal villi.

The nucleotide sequence that encodes the LeB4 signal peptide was isolated from the field bean *V. faba*, which is used in animal feed and as a vegetable. The N-terminal signal peptide regulates the transport of the fusion protein into the endoplasmic reticulum (ER). Signal sequences are cleaved by peptidases in the ER lumen. In combination with the C-terminal retention signal KDEL, the signal peptide causes the accumulation of the recombinant antibody fragment in the endoplasmic reticulum. The KDEL retention signal is a short amino acid sequence (Lys-Asp-Glu-Leu) which, in addition to HDEL (His-Asp-Glu-Leu), causes the retention of proteins in the endoplasmic reticulum of eukaryotic cells. The single-chain antibody is held back in the ER by the KDEL retention signal, thereby preventing secretion and leading to a higher level of accumulation.

A hexahistidine anchor made up of six sequential histidine residues was attached for tagging and purification purposes.

Southern blot analysis and an analysis of the segregation behaviour demonstrated the presence of a copy of the insert in the genome of line BA11-2 plants and showed that the plants are homozygous.

In the GM peas, the introduced nucleotide sequences are expressed under the control of the USP(+) promoter from *Vicia faba* and the terminator of the CaMV 35S transcript. The USP(+) promoter regulates the seed-specific expression of the genes under its control, although expression in pollen has also been observed.

Expression of the target protein in seeds of the line BA11-2 was demonstrated using polyacrylamide gel electrophoresis and subsequent silver staining or western blot analysis. Up to 2 g of the BA11 antibody fragment accumulate in each kg of line BA11-2 pea seed. ELISA (enzyme-linked immunosorbent assay) and western blot analyses were used to study expression in the leaves, tendrils, stalks and roots of the GM peas. According to the application dossier, in the case of both methods cross-reactions interfered with the detection of expression in flower extracts. Formation of the antibody fragment in flowers and pollen cannot be ruled out.

Consumption of the seeds or any other parts of the GM pea plants is not planned within the framework of the proposed deliberate release. Use of the harvested pea seeds as fodder within the scope of animal experiments is planned. Animal trials have already been conducted with single-chain antibody-producing GM pea seeds derived from the BA11-2 line.

Any unintended human or animal consumption of the GM pea seeds is not expected to have adverse effects. The antibody fragment BA11 specifically binds to the F4 fimbriae of porcine pathogenic *E. coli* cells; it is not expected to bind to any other epitopes. Even if unspecific protein-binding of the antibody fragment were to occur, no hazard would be expected to result. Immunoglobulins are ubiquitous in animal-derived foodstuffs; the consumption of immunoglobulins has not been reported to be hazardous to animals or humans, nor is such a hazard expected. It is assumed that only a small amount of consumed scFv antibody is not denatured during passage through the stomach. Since the function of the antibody fragment BA11 is limited to the receptor domain with translocation potential, any random docking to cells of the intestinal epithelium in humans or animals is not expected to result in cytotoxic or altered effects.

The performance of the proposed experiments is not expected to pose a threat to human or animal health or to the environment.

(b) Additional DNA fragments located within the T-DNA

The plasmid pPZP-USP(+)-BA11 used for the cotransformation of the peas only contains the target construct within the T-DNA borders.

The other plasmid employed in the cotransformation, the pPZP-bar plasmid, contains a cassette for expression of the phosphinothricin acetyltransferase gene (*bar* gene) under the control of regulatory sequences of the *nos* gene from *Agrobacterium tumefaciens.* The *bar* gene was integrated into the plant genome at a different locus to the target construct.

Following the generation of fertile plants, a breeding technique was used to select the BA11-2 line, in which the *bar* gene is not present.

(c) Sequences located outside the T-DNA

As a rule, only DNA located within the border regions is integrated into the plant genome in *Agrobacterium*-mediated transformations. However, the transfer of DNA fragments outside the border regions has been reported. The plasmids pPZP-USP(+)-BA11 and pPZP-bar are derived from the vector pPZP200.

The results of Southern blot studies submitted with the application show that regions outside the T-DNA of the transformation plasmids were not transferred to the GM peas.

(d) Position effects and context changes; allergenicity

Genes which have been integrated into the plant genome by genetic engineering methods are expressed at different levels, depending on the site of integration on the chromosome and on the nucleotide sequences neighbouring the integration site (“position effect”). Under field conditions the level of expression may be influenced by environmental factors, for instance, by temperature. In this particular case this could mean that the characteristics of the genetically modified peas are not modified to the same degree in the field as under climate chamber or greenhouse conditions. This is not expected to pose a risk to the environment or to human or animal health.

The insertion of foreign genes may influence the expression or regulation of the plant’s own genes at or near the site of insertion. Such processes may alter plant metabolic pathways. However, during the course of the work carried out to date on these genetically modified plants, no observations were made that would suggest such an event.

Mobile genetic elements (transposable elements), which when transposed within the genome can exert effects on existing plant genes at the target site, occur naturally in plants. The inactivation of genes or alterations in gene regulation also take place in a range of other naturally occurring processes such as point mutations, deletions or translocations and are traditionally used in plant breeding. Therefore, even in non-genetically modified plants such events can always influence plant metabolic pathways. With regard to these properties the genetically modified plants do not differ fundamentally from non-genetically modified plants.

Given the current state of knowledge, it is not possible to make reliable predictions about the possible allergenic action of a protein on the basis of the amino acid sequence.

The GM peas harvested within the scope of the proposed deliberate release are not intended for use in the production of food or feed. Animal feeding studies with the GM peas are planned. The pollen of potato plants is only dispersed to a small extent by wind and does not generally play a noteworthy role in triggering pollen allergies.

III.1.2.2. Evaluation of the ability of the genetically modified plants to persist or establish in the environment

P. sativum is a summer annual plant with a one-year life cycle. Sexual reproduction takes place by seed. Seeds in the stock can be dispersed if the pods open before harvesting. To prevent dispersal through seeds falling from the pod or being spread by animals, the applicant intends to either wrap the plants individually in nets (in the less densely planted area of the release site) or to cover the plants with a larger net (in the densely planted area). In addition, a bird protection net is to be put in place. The applicant also plans to erect a close-meshed wire fence around the entire plot. The establishment of crops such as the pea outside the propagation area is unlikely, because germinating peas compete poorly against the vegetation (weed growth) present at the site of germination. Plants of the summer pea varieties found in Germany are sensitive to frost and die off at temperatures of between -5 and -9°C. In frost-free regions seeds that have germinated in autumn have the potential to overwinter. Under certain climate conditions (mild winter) pea seeds may persist in the soil. The emergence of seedlings from such seeds would, however, be rare and these would be only weakly competitive. Furthermore, they would be identified during the planned post-trial monitoring of the release site. Vegetative reproduction does not take place. There is no evidence for the spread of cultivated peas in the environment. Thus establishment of these plants outside cultivated areas is not anticipated.

Owing to the planned wrapping or covering of the plants before maturation and the manual harvesting of the plants described in the experimental design of the present application, an unintentional spread of the genetically modified pea plants is not to be expected. In the case that plant material remains on the release site, no plants would be able to regenerate from the residue. Consequently, the genetically modified plants are not expected to persist or establish in the environment.

III.1.2.3. Assessment of the possibility of pollen-mediated transfer of the inserted genes from the genetically modified plants to other plants

P. sativum is almost exclusively self-fertilising. The predominance of self-pollination is mainly due to the fact that peas are cleistogamic.

The percentage of cross-pollination, which is primarily carried out by insects (mainly wild bees), can be up to 1-3%. In the case of commercial cultivars, the cross-pollination rate typically quoted is less than 1%. In pea, the flowering period of a single blossom lasts about 3 days; the duration of flowering in individual plants is 2-3 weeks. The possibility of pollen transfer from GM peas to non-GM peas is largely limited by the prevalence of self-pollination. The applicant intends to observe a minimum separation distance of 1 km to the next nearest area cultivated with peas. The application dossier also describes plans to observe a distance of 500m to pea cultivations from the gene bank stock belonging to the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK). Moreover, the applicant plans to sow the GM peas at least 2-3 weeks later than the gene bank peas. In the event of an overlap in the flowering periods of the GM peas and the pea cultivations from the IPK gene bank, the provisions [of the decision on this application] stipulate that during this period isolation measures are to be enforced to prevent potential pollinators from visiting the GM pea plants.

The IPK itself does not plan to sow or cultivate peas in its fields in the 2007 vegetation period.

The measures described in the application dossier coupled with the provisions of the decision on this application are considered sufficient to prevent pollen transfer from the GM pea plants to non-GM pea plants.

In Central Europe, no wild species of the genus *Pisum* occur that could hybridise with *P. sativum*. There is no evidence of hybridisation with other plant species.

III.1.2.4. Assessment of the possibility of horizontal gene transfer of the inserted foreign genes from the genetically modified plants to microorganisms

The inserted sequences are stably integrated into the genome of the recipient organisms. There is no evidence that the transfer of genetic information from plants or its expression in microorganisms takes place under natural conditions. However, studies on the transformation ability of soil bacteria under natural conditions suggest that the transfer of plant genetic material to soil bacteria is theoretically possible, although it is assumed that a gene transfer of this type would constitute an extremely rare event.

Insofar as we assume that an exchange of genetic material between organisms which are so distantly related in terms of taxonomy as plants and bacteria is actually possible, it could be concluded that the occurrence of an exchange of heterologous genetic material does not in itself represent a safety criterion, since there is always the possibility that such an exchange would result in the uptake of all forms of heterologous genetic material, including all forms of plant DNA.

(a) The coding sequences for the single-chain antibody BA11, the signal peptide LeB4, the hexahistidine anchor and the KDEL retention signal

The sequence that encodes the single-chain antibody BA11 is derived from mouse. The sequence that codes for the LeB4 signal peptide originates from the field bean (*Vicia faba*). The regulatory sequences were isolated from the field bean and from the cauliflower mosaic virus. These nucleotide sequences are already widespread in the environment and, therefore, the likelihood of a horizontal gene transfer to microorganisms is much more likely to occur from the donor organisms.

The nucleotide sequence encoding the KDEL retention signal is synthetic. C-terminal KDEL protein sequences generally signal for protein retention in the endoplasmic reticulum of higher eukaryotes and are therefore common in the environment.

The sequence that encodes the hexahistidine anchor is also synthetic. A hexahistidine anchor comprises six sequential histidine residues and serves to purify or detect recombinant proteins.

A transfer of these nucleotide sequences is not expected to confer a selective advantage to microorganisms; therefore no potential adverse affects are to be expected.

(b) Sequences located outside the T-DNA

Based on the study results presented in the application dossier, it is assumed that nucleic acid sequences of the plasmids pPZP-USP(+)-BA11 and pPZP-bar, which are located outside the T-DNA border regions, were not transferred into the genome of the GM peas.