Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

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Summary of the risk assessment of the genetically modified pea plants

(*Pisum sativum* L.) pGPTV-BAR::USPPAMYLI

within the framework of a proposed deliberate release

carried out by the German Competent Authority

Berlin, 27 January 2000

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.

III.1.2.1. Evaluation of changes in the genetically modified peas effected by the transferred nucleic acid sequences

(a) The α–amylase gene

The genetically modified pea plants contain 1800 bp of the 1948-bp AmyLi sequence (α–amylase gene) from *B. licheniformis* that is controlled by the seed-specific USP promoter of broad beans (*Vicia faba*) and the terminator sequence of the *ocs* gene from *A. tumefaciens*.

α–amylase helps to degrade starch by hydrolysing the α-1,4-glycosidic bonds of oligo- and polysaccharides. Besides in bacteria, it is also found in fungi, higher plants and animals. Amylase is commonly used in the fermentation and baking industry. According to the information provided by the applicant, the transferred α–amylase from *B. licheniformis* is characterised by high heat resistance and a broad pH optimum (stable at 25 °C at pH 6 – 11 as well as at the optimum temperature of 76 °C at pH 9).

The USP promoter is a constituent of the *usp* gene, which was isolated by the applicant from mature cotyledons of *V. faba*. Proteins that are comparable to the USP protein are also found in other plant species. Although the function of the protein has so far been unknown, information is available on the function of the promoter. It is a strong promoter that, according to the information provided by the applicant, becomes active at an early stage of seed development.

According to the information provided by the applicant, genetically modified pea plants in which the bacterial α–amylase gene is present in homozygous condition do not show any significant changes in configuration and general appearance, even in the fifth generation in the greenhouse. The gene is stably passed on and expressed.

Nevertheless, it cannot be excluded that the effects of the recombinant α–amylase on the plant will become noticeable under field conditions. The recombinant α–amylase is located in protein bodies. However, the endogenous seed starch, the main constituent of pea seeds (approx. 70%), is assumed to be degraded also at suboptimal temperatures, resulting in the formation of mainly maltose, maltotriose and α–dextrin. The partial change in the carbohydrate composition may, amongst other things, change the osmotic value of the seeds and increase their frost resistance. However, the undesirable dissemination of the pea seeds will be prevented by the proposed safety measures.

This is not expected to have any no adverse effects on the objects of legal protection pursuant to Section 1 No. 1 GenTG.

(b) The *bar* gene

In the genetically modified plants, the *bar* gene is controlled by the promoter of the *nos* gene and the terminator sequence of the *g*7 gene from *A. tumefaciens*. It was used for the selection of transformed plant cells.

The *bar* gene codes for an acetyltransferase (PAT) that selectively catalyses the acetylation of L-phosphinothricin. L-phosphinothricin, a glutamic acid analogue, is the active ingredient of the herbicidal agent glufosinate ammonium (= ammonium-D,L-phosphinothricin) and it blocks glutamine synthetase by competitive inhibition. This is why phosphinothricin is used as the active ingredient in the non-selective herbicide Basta®. Basta® contains the enantiomers D- and L-phosphinothricin in a 1:1 ratio. D-phosphinothricin does not act as a glutamine synthetase inhibitor.

In non-genetically modified tissues, the application of glufosinate ammonium leads to cell death resulting from accumulated ammonium.

In the genetically modified tissues, L-Phosphinothricin is converted by acetylation into its derivative N-acetyl-phosphinothricin, which has no herbicidal effect. This makes the genetically modified plants tolerant to the herbicide Basta®. The substrate specificity of phosphinothricin acetyltransferase is high. Even the phosphinothricin analogue glutamate is hardly converted. In the absence of the substrate, the expression of the *bar* gene in the plants is therefore unlikely to give rise to the formation of any additional metabolic products. D-phosphinothricin is not metabolised by phosphinothricin acetyltransferase.

No adverse effects are expected to result from the consumption of parts of the genetically modified plants containing phosphinothricin acetyltransferase by animals or humans. In the event of oral intake, it can be assumed that this enzyme would be fully degraded in the digestive tract, as is generally the case with proteins. Phosphinothricin acetyltransferase does not possess any properties typical of allergenic proteins in food (heat stability, stability in the digestive tract) and no sequence homology with known allergens.

Treatment of the release site with phosphinothricin is not planned.

(c) The *uidA* gene (*gus* gene)

The *uidA* gene that codes for the enzyme β-glucuronidase is promoterless and contains the nopaline synthase termination region of *A. tumefaciens*. It is a constituent of the Ti region of the transformation vector pGPTV-BAR and is used as a reporter gene for promoter examinations. The *uidA* gene is not expected to be expressed in the genetically modified pea plants.

(d) Border sequences from Ti plasmids and regulatory sequences

The genetically modified plants contain sequences of the left and right T-DNA border region of the pTiT37 from *A. tumefaciens*. Depending on the gene products of the *vir* region of the deletion variant of the helper plasmid pTiBo542 that is contained in the *Agrobacterium* strain EHA105 used for transformation and was not transferred into the plants, these sequences caused the genes located between the border regions to integrate into the genome of the pea plants. These border regions of the Ti plasmid are non-functional in the genetically modified plants and are not expected to cause any changes in the plants.

Integrated into the genome, the genetically modified plants contain the following regulatory sequences:

- The promoter region of the *usp* gene from *Vicia faba*,
- The promoter of the nopaline synthase gene from aus *A. tumefaciens*,
- The terminators of the octopine synthase gene and the gene 7 of the T-DNA from *A. tumefaciens*.

In the genetically modified plants, the promoter and terminator sequences regulate the expression of the coding DNA sequences located between them. Further information on the ef-

fects associated with the expression of these sequences in the pea plants can be found in III.1.2.1 (a) and (b).

(e) Sequences located outside the T-DNA

As a general rule, only sequences of the transformation vector located within the border regions are integrated into the plant genome in *Agrobacterium*-mediated transformation events. However, the transfer of sequences outside the border regions has been reported. In the case of the pea transformant “pGPTV-BAR::USPPAMYLI”, the results of southern blot analyses and available data on the expression of the transferred genes suggest that no sequences of the vector were transferred outside the border regions.

(f) Position effects and context changes; allergenicity

Genes 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 neighbouring sequence at 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 proportion of various carbohydrates is shifted in the seeds of the genetically modified plants. This does not represent a risk to the environment or to human and animal health.

The insertion of foreign genes may influence the expression or regulation of native plant genes at or near the site of insertion. Such processes can affect plant metabolic pathways. However, during the propagation of the genetically modified plants in the greenhouse, 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. In this regard, the genetically modified plants to be deliberately released do not differ fundamentally from non-genetically modified plants.

Allergenic proteins are found naturally in peas. However, the available information does not suggest an increased allergenic potential of the genetically modified pea plants. The promoterless *uid*A gene is not assumed to be expressed in the plants. Since the newly introduced α-amylase gene is controlled by a seed-specific promoter in the genetically modified plants, α-amylase is not expected to be formed in the pea pollen. In a great number of previous deliberate release trials with plants that express the *bar* gene under the control of non-tissue-specific promoters, no evidence was found to suggest an increased allergenic potential of the plants.

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

Peas have been cultivated in Central Europe for several thousands of years. The establishment of peas in natural ecosystems of Europe has not been observed. Peas

are a highly developed crop plant type that cannot survive outside cultivated areas since germinating peas have a competitive disadvantage compared to the vegetation on the germinal spot (weed infestation). They are therefore not capable of permanently establishing at such locations.

The employed pea type “Erbi” is a summer annual crop plant type that propagates by seed. Vegetative propagation, e.g. by bulbs or storage roots, does not take place. Seeds in the stock can be dispersed if the pods open before harvesting. The summer pea types used in Germany are susceptible to frost. However, pea seeds can persist under certain climatic conditions (mild winter). If seeds were to remain in the soil, any plants that would emerge in favourable weather conditions would be indentified and disposed of within the scope of the post-trial monitoring period proposed by the applicant.

A possible change in the frost resistance of the bulbs as a result of changes in the carbohydrate composition induced by the activity of the α–amylase cannot be excluded. This is sufficiently taken into account by the planned one-year cultivation gap and the proposed post-trial monitoring period. This makes it possible to easily identify any volunteer peas.

The packaging of the seed pods before maturity and the manual harvesting proposed in the trial planning of the present application prevent unintentional spread of the genetically modified pea plants. There are no objections to the planned rotting of harvested, mechanically destroyed pea plants on the release site, since these plants can no longer regenerate.

The genetically modified plants are thus 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 pea plants to other plants

P. sativum is almost exclusively self-pollinating. The proportion of cross-pollination can amount to 1-3% and takes place mainly by insects (predominantly wild bees). Thus, the possibility of pollen transfer to sexually compatible garden peas *(P. sativum ssp. hortense*) or field peas (*P. sativum ssp. arvense*) cannot be excluded, but is largely limited due to the predominant self-pollination. The proposed separation distance of 20 m in all directions to agricultural areas and 800 m to cultivation sites for pea lines of the applicant’s gene bank is regarded as sufficient. In Central Europe, wild types of the species *Pisum* that could hybridise with *P. sativum* do not occur. There are no indications of hybridisation with other plant species.

The pollination of individual flowers of non-genetically modified peas and the one-time saving of these pea seeds would result in the temporary emergence of individual α-amylase-producing, phosphinothricin-tolerant pea plants in the surroundings of the release site. Since without the application of phosphinothricin the inserted genes do not confer any selective advantage to the plants, this is not associated with any risks for the environment or agriculture.

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

The inserted coding DNA sequences were integrated into the chromosomes of the recipient organisms in the course of the transformation. 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 and extremely rare event.

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

Besides in bacteria, α–amylase also occurs in fungi, higher plants and animals. The transfer of the recombinant α–amylase gene to these organisms would not confer any novel traits to them. A selective advantage is not expected. Moreover, the exchange of α–amylase between microorganisms (e.g. rhizobium) is far more likely to occur than horizontal gene transfer from the genetically modified pea plants.

In soil microorganisms, the inactivation of phosphinothricin by acetylation with the help of phosphinothricin acetyltransferase encoded by the *bar* gene is a naturally occurring process. Bacteria with a corresponding resistance are commonly found in the environment. The *bar* gene can therefore also be spread by horizontal gene transfer from non-genetically modified microorganisms. Even if the *bar* gene were to be transferred from the genetically modified plants to microorganisms, the overall frequency of this resistance in the environment would not be significantly increased.

The *uid*A gene is found naturally in bacteria. The transfer of the promoterless *uid*A gene from the plants to bacteria in which the *uid*A gene occurs naturally and its integration by recombination would not confer a fundamentally new phenotype to them. This also applies to microorganisms in which the *uid*A gene is missing, since the transferred promoterless gene is not expressed.

DNA fragments of the vector located outside the T-DNA could not be detected by southern blot analysis.

III.1.2.5. Agrobacteria used to generate the genetically modified pea plants

In order to generate the genetically modified pea plants, agrobacteria containing the genes to be transferred between the border regions of a binary vector plasmid were used. In contrast to the common wild-types of *A. tumefaciens*, the agrobacteria used are disarmed, i.e. they no longer have the capacity to induce tumours. Following transformation, antibiotic treatment is usually carried out to eliminate the agrobacteria. No agrobacteria were detected.

Even if a few agrobacteria were to remain in the genetically modified plant material, this would represent no risk. In this case, the possibility of *Agrobacterium*-mediated transfer of transgenes to other plants would have to be considered. Even is such a transfer were to oc-

cur, it would have no consequences, since the plant cell would have to spontaneously regenerate into a fertile plant after its transformation by the modified agrobacteria to pass on the transgenes to the plant progeny. Such an event is not expected to occur under natural conditions.

Furthermore, the possible horizontal transfer of the transgenes from agrobacteria to other bacteria present in the environment would have to be considered (cf. III.1.2.4).