Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

Notification 6786-01-0160

Summary of the risk assessment of the genetically modified potatoes

(*Solanum tuberosum*; Albatros)

lines pCB301-Kan-MaSpI-100xELP-3 und -6; pCB301-Kan-MaSpII-100xELP-26, -27 und -28; pCB301-Kan-So1-100xELP-31, -33, -37, -38, -39, -44 und -50

within the framework of a proposed deliberate release

carried out by the German Competent Authority

Berlin, 10 May 2005

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

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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) Constructs for the production of the spider silk proteins MaSpI, MaSpII and SO1 from *Nephila clavipes*

The genes *MaSp*I and *MaSp*II, which were transferred to the genetically modified potato plants, encode the spider silk proteins in the dragline core fibre of the golden silk orbweaver spider *Nephila clavipes*. The *SO*1 gene comprises the repetitive part of the *MaSp*I gene; the 3´-localised non-repetitive sequence of ca. 180 bp was removed.

All three target genes (*MaSp*I, *MaSp*II and *SO*1) were combined with the same functional elements in the respective transformation plasmids. These comprise: the gene for a synthetic elastin, the nucleic acid sequence for the c-myc tag, the LeB4 signal sequence and the ER retention signal KDEL. These constructs are expressed under the control of the CaMV35S promoter and termination signal in all plant parts of potato.

As a result of the transformation the genetically modified potato plants produce spider silk proteins fused with a synthetic elastin protein. The elastin protein 100xELP is a synthetic protein similar to human elastin. It is made up of oligomeric repeats of the pentapeptide valine-proline-glycine-Xaa-glycine and displays solubility dependent on salt content and temperature. This trait is transferred to the fusion partner, thereby enabling efficient isolation and purification of the spider silk protein. “Xaa” denotes any amino acid except proline. The N-terminally linked legumin A LeB4 signal sequence is thought to induce the transport of the fusion proteins MaSaI-100xELP, MaSaII-100xELP and SO1-100xELP into the endoplasmic reticulum, where the signal sequence is cleaved by peptidases. The ER retention signal KDEL was fused to the C-terminal. As a result, the recombinant protein is retained in the lumen of the endoplasmic reticulum and is not secreted by the cell. The c-myc tag serves as immunochemical evidence of the fusion protein in Western blot analyses using antibodies. Expression of the fusion proteins MaSpI-100xELP, MaSpII-100xELP and SO1-100xELP in the leaves and tubers of the genetically modified potatoes proposed for release was demonstrated by Western blotting.

The golden silk orbweaver spider *Nephila clavipes* is commonly found in the southern US states (e.g. Florida) and in Central America. The female has a number of different spinning glands for producing a range of fibres with different properties. The most stable fibre is the dragline, which displays high tensile strength and is therefore of interest for use as a biomaterial. The spider silk protein produced by *Nephila clavipes* is not known to have any toxic effect on humans.

Human elastin, a fibrillous, elastic protein rich in glycine and proline, is the main component of elastic connective tissue. It is made up of long, twisted polypeptide chains. Elastin is secreted into the extracellular matrix in the form of proelastin by fibroblasts and smooth muscle cells, where it is enzymatically crosslinked. Elastic fibres have a long survival time; even in boiling water they remain insoluble and are resistant to acids and bases.

The c-myc tag from *Homo sapiens* is a sequence of 11 amino acids and is used for the immunochemical detection of the SO1-100xELP fusion protein. The short amino acid sequence originates from the c-MYC protein, a transcription factor from mammalian cells with cell growth and proliferation functions. It does not possess any enzymatic activity.

The ER retention signal KDEL from *Homo sapiens* is a short amino acid sequence (Lys-Asp-Glu-Leu) that induces retention of BiP (heavy chain binding protein) in the endoplasmic reticulum. The KDEL retention signal is not specific to human BiP but, in addition to HDEL (His-

Asp-Glu-Leu), ensures the retention of proteins in the ER of eukaryotic cells in general. In the case of the genetically modified potatoes the recombinant protein is thus retained in the ER.

As a result of the genetic modification, protein metabolism in the genetically modified potato plants is altered in such a way that non-native plant proteins, namely the spider silk fusion proteins SO1-100xELP, MaSpI-100xELP or MaSPII-100xELP are synthesised in the potato plants. In the applicant's estimation, the recombinant protein accounts for about 4% of the total soluble protein content.

In greenhouse studies the applicant did not observe any phenotypic differences (size, leaf morphology, colour, tuber yield, tuber size) in the genetically modified potato plants in comparison to wild-type plants. Field trials conducted in 2003 and 2004 by the Leibniz Institute of Plant Genetics and Crop Plant Research (*IPK*) with comparable genetically modified potatoes which express SO1-100xELP did not reveal any significant difference in tuber yield. The genetically modified potatoes referred to in this application are not intended for human or animal consumption.

Within the context of the proposed experiments, no risks to human or animal health or to the environment are expected to result from the production of spider silk elastin fusion proteins, or from the resulting changes in protein composition or from the synthesis of non-native plant proteins in the genetically modified potatoes.

(b) The *npt*II gene

The *npt*II gene transferred to the genetically modified plants encodes the enzyme neomycin phosphotransferase. It was introduced as a marker gene for selecting transformed plant cells.

The neomycin phosphotransferase gene is a type II aminoglycoside 3'-phosphotransferase (APH(3')II), which catalyses the ATP-dependent phosphorylation of the 3'-hydroxyl group of the aminohexose ring of specific aminoglycoside antibiotics, causing these to become inactivated. The enzyme is characterised by its high substrate specificity. The antibiotics kanamycin, neomycin, geneticin, butirosin, gentamicin A and B, and paramycin belong to the APH(3')II enzyme substrates. Clinically relevant gentamicins and other aminoglycosides and aminocyclitoles used in human medicine do not belong to the substrate spectrum of the APH(3')II enzymes. However, both kanamycin and neomycin are widely used in veterinary medicine.

Given the substrate specificity of neomycin phosphotransferase, it is expected that in the absence of substrate under field conditions no new metabolic products will be synthesised in the genetically modified potato plants. Since the relevant antibiotics are not present in the soil in high concentrations, the neomycin phosphotransferase does not confer any selection advantage on the genetically modified plants. There is no evidence to suggest that this enzyme is toxic to plants, animals, microorganisms or humans

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

The pCB301-Kan-MaSpI-100xELP, pCB301-Kan-MaSpII-100xELP and pCB301-Kan-SO1-100xELP plasmids used in the transformation of the potato plants are derivatives of the binary vector pCB301, which was developed from pBIN19 and which has been fully sequenced.

Besides the specific spider silk constructs, the expression cassette of the *npt*II gene, and the multiple cloning site of the pBluescriptII plasmid, the transformation plasmids also contain about 160 nucleotides with the right border sequence and about 140 nucleotides with the left border sequence from pBIN19 within the T-DNA. These are not functional in plants.

(d) Sequences located outside the T-DNA

As a rule only DNA located within the T-DNA border regions is integrated into the plant genome during Agrobacterium-mediated transformation. However, the transfer of DNA fragments outside the border regions has been reported. The pCB301 plasmid contains the following outside the border regions:

- the replication origin *ori*V of the plasmid RK2 from *E. coli*;
- the *aphA*III (= *npt*III) gene from *Streptococcus faecalis* (= *Enterococcus faecalis*);
- a DNA fragment with sequence homologies to the trfA gene of the plasmid RK2 for replication in *E. coli* and *A. tumefaciens*.

The applicant performed PCR analysis on the genetically modified potatoes proposed for release using primers which specifically amplify the replication origin, the *npt*III gene or the *trf*A gene. In these studies neither the *npt*III gene nor the replication origin were demonstrated in the genetically modified potato lines proposed for release. Therefore it is highly unlikely that the *npt*III gene is present any of the potato lines intended for release. In one genetically modified potato line a region in the *trf*A gene was demonstrated. Therefore the risk assessment is performed under the assumption that these sequences may be present in the plants.

However, the formation of functional gene products based on these sequences is not to be expected in the genetically modified plants since they are not controlled by plant-specific promoters.

(e) 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 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 potato plants are not modified to the same degree in the field as under climate-controlled 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 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 have an effect on plant metabolic pathways. In this respect the genetically modified plants proposed for release here do not differ fundamentally in their characteristics 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. From numerous releases of plants that express the *npt*II gene under the control of non-tissue-specific promoters no evidence has been found to indicate an increased allergenicity of the plants. In any case 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

The cultivation of potatoes in Central Europe goes back several hundred years. In areas where potatoes have been cultivated, tubers or seeds may remain in the soil after harvesting. Depending on temperatures in the winter following cultivation, these may give rise to volunteer potato plants in the following year. In Europe the establishment of potatoes in natural ecosystems has not been observed, since potatoes compete poorly against wild plants and they are not frost resistant. From time to time potato plants are found beyond cultivated areas, but only on non-natural sites such as verges and other ruderal areas. Owing to the lack of frost hardiness the cultivated potato does not establish in these areas either.

The trial site lies within an area officially declared as the natural floodplain of a minor river which runs some distance from the site. Therefore the risk assessment must take into account the possible consequences of flooding. In fact, potato tubers are heavy and do not float. Moreover, the stolons, scion and roots ensure that the potato plant remains firmly anchored in the soil. Flood water would have to flow a very high rate to wash away tubers, plants or daughter tubers, or fruit growing on the plants from the plant stock. The release area is to a large extent level and the river flows at a distance of about 600m from the site. Under these areal conditions it seems unlikely, if not impossible, that viable plant parts would be washed away by flooding. During the trial period, the formation of viable seeds is to be prevented by gathering the flowers and/or young fruit from the genetically modified potatoes, thereby ensuring that plant parts with a capacity to reproduce are not conveyed by flood water to another site where they might take root and establish or spread. Should the trial area be threatened by flooding, appropriate measures (e.g. enclosure of the site with sandbags or removal of the plants) should be taken to prevent viable plant parts from being carried away from the trial area by the water current.

After the tubers of the genetically modified potatoes have been manually harvested they are graded, weighed and transported to a genetic engineering facility for further analysis. Surplus potato tubers are inactivated by appropriate methods. The potato haulms are left to decompose on the release site.

Subsequent to harvesting the surface of the release site will be levelled by harrowing. Crop rotation on the trial site is designed in such a way that no potatoes are cultivated on that site for at least two vegetation periods after the deliberate release of genetically modified potatoes. During this time the site is monitored for volunteer potatoes. In the event that genetically modified potato volunteers do emerge on the release site during a given period of observation, post-trial monitoring will be extended by a further year.

Potato plants can flower and bear fruit. Under Central European climate conditions it is unlikely that potato seeds will overwinter and produce plants. In the event that tubers or seeds remain in the soil, the resulting plant growth would be detected during post-trial monitoring.

In greenhouse studies no phenotypic differences (size, leaf morphology, colour, tuber yield, tuber size) were observed in the genetically modified potato plants in comparison to the wild-type plants. Field trials conducted in 2003 and 2004 by the Leibniz Institute of Plant Genetics and Crop Plant Research (*IPK*) with comparable genetically modified potatoes which express SO1-100xELP did not reveal any significant difference in tuber yield. These findings did not reveal any evidence of altered competitiveness or increased invasiveness in comparison to the wild-type plants.

There is no reason to assume that the genetically modified potato plants possess different plant ecological traits in comparison to conventionally cultivated potatoes, nor are they ex-

pected to have the ability to colonise natural ecosystems. Therefore, even if the fruit, seeds or tubers of the genetically modified plants were to be dispersed by animals or as a result of flooding, the GM potato plants would not be expected to spread in the environment.

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

Attempts to crossbreed potatoes with solanaceous plants found in Central Europe were not successful. Under field conditions no incrossing took place from genetically modified potatoes to *Solanum nigrum* (black nightshade). The artificial transfer of pollen to *S. nigrum* also failed to produce viable seeds. Only under conditions that do not occur naturally and with the help of artificial methods (embryo rescue) was it possible to regenerate a small number of hybrids. These, however, turned out to be sterile. The potato and *Solanum dulcamara* (bittersweet or woody nightshade) proved to be strictly bilaterally incompatible; in crossbreeding experiments pollination of the ovule was not achieved. Similarly, the potato does not crossbreed with the tomato (*Lycopersicon esculentum*). In agricultural practice, potatoes are propagated vegetatively via tubers.

The following passage, therefore, deals only with a possible pollen transfer from the genetically modified potato plants to other potato plants. The pollen of the potato plant can be transferred by insects or by wind. However, wind dispersal only takes place over short distances. Potatoes are largely self-pollinating; even within a flowering potato field cross-pollination is rare. The likelihood of this occurring is greatest between neighbouring plants.

The proposed minimum isolation distance of 20 m to other agricultural areas - which might also be used for potato cultivation - is considered sufficient. If the transfer of pollen to potato plants intended for the production of table potatoes were to occur despite these measures, no adverse effects are anticipated, since in an agricultural setting potato plants are propagated vegetatively, i.e. not via seeds.

In any case, the planned timely removal of flowers and/or young fruit during the trial period as a measure to prevent the formation of viable seeds on the genetically modified potatoes should guard against the possible emergence of plants from seeds.

III.1.2.4. <u>Assessment of the possibility of transfer of the inserted foreign genes from the genetically modified plants to microorganisms by horizontal gene transfer</u>

The inserted sequences are stably integrated into the chromosomes 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 possible in theory, 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 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 such an exchange could always result in the uptake of all forms of heterologous genetic material, including all forms of plant DNA.

(a) Constructs for expression of the spider silk proteins MaSpI, MaSpII and the synthetic spider silk protein (SO1) from *Nephila clavipes,* each fused with the gene for a syn-

thetic elastin (100xELP) as well as the c-myc tag from *Homo sapiens*, the LeB4 signal sequence from *Vicia faba* and the ER retention signal KDEL from *Homo sapiens*

The genes that encode the spider silk proteins are derived from *Nephila clavipes*. The coding region used in this case for the SO1 protein is a synthetic spider silk protein with a high degree of similarity to the repetitive parts of the sequence of the dragline protein MaSpl. The synthetic protein SO1 displays a homology of 94% to the natural protein. The sequence for the signal peptide originates from *Vicia faba*. The gene that encodes the synthetic elastin mimics the human elastin gene. The sequences for the c-myc tag and the ER retention signal are derived from *Homo sapiens.* Therefore these genes are already widespread in the environment and a horizontal gene transfer in microorganisms is far more likely to occur from non-genetically modified organisms.

(b) The *npt*II gene

As already described under III.1.2.1., antibiotics that are inactivated by neomycin phosphotransferase do not play a significant role in human medicine, but they do have manifold uses in veterinary medicine. Therefore, it was necessary to examine whether the therapeutic use of the respective antibiotics would be compromised by the possible horizontal gene transfer of the *npt*II gene.

The resistance mechanism for inactivation of aminoglycoside antibiotics through phosphorylation occurs naturally in soil microorganisms. APH(3')II enzymes have also been found in human clinical isolates. The widespread distribution of genes that confer resistance to aminoglycoside antibiotics can be explained by the frequent use of these antibiotics, and also by the fact that these genes are often localised on plasmids, enabling effective transfer by conjugation. Even in the case of horizontal gene transfer from the genetically modified potatoes to microorganisms, the overall frequency of this resistance mechanism would not increase noticeably.

The GMO Panel of the European Food Safety Authority (EFSA) has allocated the *npt*II gene to the group of genes for which, with respect to safety, there are no grounds for banning or limiting use - either for field trials or for the purpose of placing on the market. In its position statement of 6.7.1999 on the biological safety of antibiotic resistance genes in the genome of genetically modified plants, the German Central Commission on Biological Safety (ZKBS) assigned the *npt*II gene to the group of antibiotic resistance genes “that (a) are already widespread in soil microorganisms and enterobacteria and (b) their relevant antibiotics have no, or very little, therapeutic significance in human or veterinary medicine, so that it can be assumed that, if at all, the presence of these antibiotic-resistance genes in the genome of transgenic plants would have no effect on the distribution of these antibiotic resistance genes in the environment”.

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

In addition to the expression cassette mentioned under a) and b), the transformation plasmids contain about 160 nucleotides with the right border sequence and about 140 nucleotides with the left border sequence from pBIN19 within the T-DNA. These are non-functional in plants. They are derived from *Agrobacterium tumefaciens,* a soil bacterium commonly found in the environment.

(d) Sequences located outside the T-DNA

The following genetic elements are located outside the T-DNA border regions of the transformation plasmids used:

- the origin of replication *oriV* of the plasmid RK2 derived from *E. coli*,
- the *aphA*III (= *npt*III) gene from *Streptococcus faecalis* (= *Enterococcus faecalis*),
- a DNA fragment with sequence homologies to the *trf*A gene of the plasmid RK2 for replication in *E. coli* and *A. tumefaciens*.

The results of the PCR studies performed show that a region of the *trf*A gene was transferred in one of the genetically modified potato lines proposed for release. The *trf*A gene is derived from the plasmid RK2. RK2 belongs to a group of broad host range plasmids (including, among others, RP1, RP4, R18, R68) that can be replicated in numerous gram negative bacteria. Therefore, in the case of RK2-derived DNA fragments, the probability of spreading by transfer between bacteria is far greater than the probability of spreading by horizontal gene transfer from the genetically modified plants to microorganisms.

Based on the results of all other PCR analyses presented, it is extremely unlikely that additional genetic elements outside the T-DNA border regions were transferred to the genetically modified potatoes proposed for release. However, since it is not possible to rule out the presence of the *npt*III gene, all of the potato lines released should be checked for presence of the *npt*III gene after the leaves appear. This should be done using primers that exclude the presence of the coding sequence. In the event that the *npt*III gene is found to be present in a potato line, the Federal Office for Consumer Protection and Food Safety is to be notified and the plants of the respective line are to be removed immediately, leaving the soil residue-free. The plants are to be inactivated and disposed of.

In its position statement of 02.04.2004 the European Food Safety Authority's Scientific Panel on Genetically Modified Organisms classified the *npt*III gene present in the genetically modified potatoes proposed for release among the group III antibiotic resistance marker genes. The Scientific Panel recommends that antibiotic resistance genes belonging to this group not be contained in plants intended for release or placing on the market.

Indeed the EFSA statement was developed in connection with the implementation of Article 4, Section 2 of Directive 2001/18/EC. According to this directive environmental safety assessments must give special consideration to antibiotic resistance genes, in particular with regard to the identification and gradual phasing out of the use of antibiotic resistance markers in GMOs. This phasing out shall take place by the 31 December 2004 in the case of GMOs placed on the market according to part C and by 31 December 2008 in the case of GMOs authorised under part B.

However, the EFSA's statement of 02.04.2004 is not limited to a specific time frame but represents a general recommendation to abandon the use of these antibiotic resistance marker genes.