Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

Notification 6786-01-0176

Summary of the risk assessment of the genetically modified

potato (*Solanum tuberosum L.*; Désirée, Albatros)
within the framework of a proposed deliberate release

carried out by the German Competent Authority

Berlin, 14 June 2006

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]
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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 sequences

(a) The *vp60* construct

The viral capsid protein VP60 is a structural protein of the RHD virus (rabbit haemorrhagic disease virus). This virus only attacks adult rabbits and infection results in death within 48 hours. The virus belongs to the Caliciviridae group of viruses. Post-infection immune responses in humans and some animals have been observed, but no symptoms of illness have been identified to date. The VP60 protein alone does not trigger the disease in rabbits. In domestic rabbit populations, the virus is combated by slaughter and vaccination. The vaccines used in this case contain inactivated virus and thus the VP60 protein. According to the current literature, the vaccines are well-tolerated and can even be administered to pregnant animals.

To enable expression of the VP60 gene in potatoes, a technically synthesised gene was introduced into potato plants of the Désirée variety via *Agrobacterium tumefaciens*-mediated gene transfer. This gene contains the sequence information of the *vp60* gene from the RHDV virus isolate R-592, utilizing the tobacco codon preference. A DNA fragment that encodes the SEKDEL amino acids was added to the *vp60* sequence, thereby effecting the return transport of the protein to the endoplasmic reticulum in the secretory pathway of protein synthesis. This is aimed at stabilising the protein. In the plant, the expression is regulated by the 35S promoter and terminator from the cauliflower mosaic virus, leading to expression in all plant parts throughout the entire lifecycle of the potato. The expression of VP60 protein was verified by ELISA and Western blot tests using a VP60-specific antibody.

There have been reports of vaccinations with the VP60 protein in heterologous expression systems. Subcutaneous and oral immunisation experiments with VP60 extracted from genetically modified potatoes have also been published. In the latter, attempts to immunise rabbits by feeding them fresh plant mass have proved unsuccessful. So far, an immune response could only be achieved by administering plant extracts or protein extracts from other expression systems. The genetically modified potatoes proposed for release have been tested on rats and rabbits in feeding experiments at the FBN Dummerstorf and the Friederich-Löffler Institute. The feeding of fresh tuber mass to rabbits did not lead to immunisation in this case either. Feeding studies on rats produced no change whatsoever in histological, biochemical and haematological parameters. Consequently, there is no reason to expect health damage or immunisation in wild animals grazing on the GM plants used in this trial.

The genetically modified potatoes grown during the proposed deliberate release are not intended for use in the production of food or feed beyond the indicated laboratory tests and the trials are to be conducted on a marked and fenced plot, so that no health risks to animals or humans are to be expected in the context of the experimental release.

(b) The *ctxB* construct

The cholera toxin is a member of the AB family of toxins. The function of the non-toxic B subunit is to assemble into a ring-shaped, homologous pentamer and to adhere to membrane receptors on the intestinal epithelial cell surface (GM1 gangliosides). The pathogenic effect stems from the A subunit. This is split into two domains, an anchor domain A2 and a toxic domain A1. The anchor domain binds the toxic A1 domain to the B subunit. Through a series of further intermediate activation steps, the A1 domain ultimately causes a massive secretion of chloridions in the gut lumen, causing diarrhoea. It follows that the A1 subunit is toxic, but the B subunit is non-toxic.

To enable expression of the non-toxic B subunit, a construct was introduced into the Albatros variety of potato by *Agrobacterium tumefaciens*-mediated gene transfer. This construct contains a synthetic gene that corresponds to 71% with the gene sequence of the *ctxB* gene from *Vibrio cholerae* that codes for the non-toxic unit of the cholera toxin. The gene was adapted to the codon preference of higher plants.

A DNA fragment coding for the SEKDEL group of amino acids was added to the *ctxB* sequence, causing the protein to return to the endoplasmic reticulum in the secretory pathway of protein synthesis. This is intended to stabilise the protein. In the plant, expression is regulated by the 35S promoter and terminator from the cauliflower mosaic virus, which leads to an expression in all plant parts throughout the entire lifecycle of the potato. The ELISA technique was used to verify CtxB protein expression.

The non-toxic B subunit of the cholera toxin has been used for more than 20 years as a component of human vaccines against cholera and other diarrheal diseases. Both subcutaneous and oral applications are administered. Expression of this protein in potatoes has also been accomplished already in order to determine whether oral administration in food stimulates an immune response. This proved to be the case after the potatoes were fed to mice. Furthermore, findings exist from one feeding study on test subjects who ingested genetically modified potatoes containing a protein very similar to the CtxB protein. These studies did not reveal any effects that resulted in adverse reactions.

Against this background, the emergence of harmful metabolic products is not to be expected. The genetically modified potatoes grown during the proposed deliberate release are not intended for use in the production of food or feed outside the notified laboratory trials and the proposed release is to be conducted on an isolated, designated test site, so that no risks to human health or to the environment are to be expected.

(c) The *psbY-cyel* construct

The introduced *cyel* gene codes for a cyanophycin synthetase from the cyanobacterium (blue-green algae) *Thermosynechococcus elongatus*. The enzyme catalyzes the synthesis of the polymer cyanophycin, which consists of an aspartate backbone and an arginine sidechain. This nonribosomal protein presumably serves as a storage protein for the bacterium. A wide range of cyanobacteria are capable of synthesising cyanophycin. Since cyanobacteria are ubiquitous on exposed surfaces in the environment, cyanophycin is constantly released into the environment from dying blue algae. Cyanophycin is decomposed by cyanobacterial enzymes and enzymes from other soilborne bacteria, e.g. streptomycetes.

A construct with a cyanophycin synthetase gene was introduced into the genetically modified potatoes. At the 5' end, the construct contains an additional, *Arabidopsis thaliana*-derived sequence encoding a transit peptide for import into chloroplasts. By binding the structural gene to the regulatory 35S promoter and terminator sequences, expression is driven in all tissues and at all developmental stages of the potato, whereby the synthetase in the leaf and in the tuber is transferred to different plastids. This expression was verified using electron microscopy aided by gold-labelled antibodies against cyanophycin.

A report is on hand of a feeding study with rats conducted by the FBN Dummerstorf to study the effects of orally ingesting cyanophycin on rats. The report contains indications that the consumption of feed containing cyanophycin additives is associated with a reduced uptake of nutrients. However, no acute toxic effects or health damage were detected during the observation period. The concentrations of cyanophycin in the feed used for this study were higher than those present in the tubers of the potatoes to be used in the proposed field trial. Furthermore, wild animals (in particular small mammals, due to the planned fencing-in of the plot) are not expected to feed exclusively on these potatoes during the vegetation period. Given this limited consumption, a further reduction in the level of cyanophycin in the total food intake by wild animals can be assumed. Under these conditions, the potential consumption of the genetically modified potatoes is not expected to have any health-damaging effects.

(d) The *npt*II gene

The *npt*II gene codes for a neomycin phosphotransferase. It was introduced into the genetically modified plants as a marker gene for selecting transformed plant cells.

The neomycin phosphotransferase is a type II aminoglycoside 3'-phosphotransferase (APH(3')II), which catalyses the ATP-dependent phosphorylation of the 3'-OH 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 paromomycin belong to the APH(3')II enzyme substrates. Clinically relevant gentamicins (predominantly C_1, C_α and C_2) and other aminoglycosides and aminocyclitoles used in human medicine do not belong to the substrate spectrum of the APH(3')II enzyme. Kanamycin and neomycin are, however, widely used in veterinary medicine.
Given the substrate specificity of the neomycin phosphotransferase, it can be assumed that, in the absence of substrate under field conditions, no new metabolic products can be synthesised in the genetically modified potato plants. Since high concentrations of the relevant antibiotics are not present in soil, the neomycin phosphotransferase does not confer any selection advantage to the genetically modified plants under field conditions. No evidence has been recorded to suggest that this enzyme is toxic to plants, animals, microorganisms or humans.

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

In addition to the above-mentioned genes and the expression cassettes of the *npt*II gene, the plasmids used in the transformation of the GM potatoes contain the regulatory promoter and terminator sequences 35S from the cauliflower mosaic virus necessary for expression. These are non-coding and regulate expression of the DNA sequences located between them in the genetically modified plants. Further functions have not been identified and further effects on the GM plants are not anticipated.

(f) 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 transformation events. However, the transfer of DNA fragments outside the borders has been reported in the literature.

The transformation plasmid p35S that underlies the constructs is a derivative of the binary vector pLH9000 and contains the following genetic elements outside of the border regions:

- the origin of replication pVS1 from *Pseudomonas aeruginosa;*
- the origin of replication of the plasmid pBR322 (ColE1 *ori*) from *E. coli;*
- the *aadA* gene of the Tn7 transposon from *E. coli,* which confers resistance to the antibiotics streptomycin and spectinomycin.

The presence or absence of these sequences in the genetically modified potatoes was not demonstrated. However, the formation of functional gene products based on these sequences is not expected, as they are not controlled by plant-specific promoters.

(g) 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 potato plants 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 the GM 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 changes 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. Nevertheless, none of the trials conducted so far with GM plants or the existing results of feeding studies indicate an increased allergenicity in the plants. The pollen of potato plants is only dispersed over short distances by wind and generally plays a negligible 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. As a result of potato cultivation, volunteer potatoes can, depending on winter temperatures, emerge in the subsequent cultivation period from seeds or tubers that have overwintered in the soil. In Europe the establishment of potatoes in natural ecosystems during this period has not been observed, as potatoes compete poorly with wild plants and are not frost-resistant either. 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 a lack of frost hardiness the cultivated potato does not establish in these areas either.

The leaves and stalks of the potatoes grown on the trial site are to be destroyed by chemical dessicants before harvesting. The tubers of the trial plants are to be harvested and transferred to a genetic engineering facility for further investigations or for storage as reference samples. Surplus tubers are inactivated by autoclaving or steam sterilisation. The potato haulms are left to decompose on the trial site.

After harvesting, the trial site will be tilled to a depth of 15-20 cm, in order to force any residual tubers to the surface. Crop rotation on the site is designed in such a way that no potatoes will be cultivated on the trial site for at least two years after the release of genetically modified potatoes (grass fallow or winter crops). In the year after the release, the site will be monitored for potato volunteers. The monitoring period will be extended until the site on which the genetically modified potatoes were cultivated has been declared free of volunteers for one whole vegetation period. If volunteers are detected during post-trial monitoring, the monitoring period will be extended by another year.

Potato plants can flower and bear fruit. However, under Central European climate conditions it is unlikely that potato seeds will overwinter and produce plants. Seed-bearing berries should be removed before applying a chemical dessicant. In the event that tubers or seeds remain in the soil, the resulting plant growth would be detected during post-trial monitoring.

Even if the berries, seeds or tubers of the genetically modified plants were dispersed by animals, which is highly unlikely, the GM potato plants would not be expected to establish in the environment.

Within the framework of the release trial, the possible post-emergence of tubers will be adequately monitored by post-trial measures.

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

Attempts to cross-breed potatoes with solanaceous plants found in Central Europe were unsuccessful. 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 cross-breeding experiments pollination of the ovule was not achieved. Similarly, the potato does not cross-breed 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. The potato is capable of cross- and self-fertilisation, with cross-fertilisation most likely to occur between neighbouring plants.

The proposed distance of at least 150 metres to neighbouring potato fields is considered adequate for the purposes of the proposed trial. Outcrossing from *Solanum tuberosum* to other potatoes over a distance of more than 20 m is unlikely. However, should pollen be transferred to potato plants cultivated to produce table potatoes, no adverse effects are to be expected, since potato plants for cultivation are propagated vegetatively, i.e. no via seeds.

As elaborated above, the probability that potentially generated seeds could give rise to plants under the given climatic conditions is very slight. In agricultural areas such plants would be eliminated in the course of conventional soil preparation practices.

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

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 also 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 is actually possible between organisms as distantly related in terms of taxonomy as plants and bacteria or plants and viruses, 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.

(a) The *vp60* construct

Plant-pathogenic caliciviruses do not exist. In principle, however, a heterologous transcapsidation cannot be ruled out if the genetically modified potatoes are infected by a phytopathogenic virus. This would give the virus no obvious advantage and a loss of the absorbed foreign protein can be expected in the following propagation cycle. Moreover, the likelihood that the three-dimensional structure of the VP60 protein would fit into the spatial capsid structure of a plant virus so that the chimeric capsid remains functional is extremely slight. The possibility of a gene exchange between an over-infecting virus and a genetically modified plant in the case of the *vp60* gene is also unlikely, since nucleotide sequence homologies between the *vp60* gene and genes from plant viruses have not been described.

Furthermore, an expansion of the host area through the uptake of the *vp60* gene would not give a phytopathogenic virus an advantage, since the synthetic *vp60* gene in the GM potatoes is adapted to the codon usage of higher plants. The regulatory sequences in the construct (35S promoter and terminator) come from the cauliflower mosaic virus and are commonly found in the environment. This also applies for the *vp60* gene itself, which enters the environment from the carcasses of infected wild rabbits. Thus, for each part of the construct, a horizontal gene transfer to microorganisms is more likely to result from non-genetically modified plants than from the GM potatoes used in the proposed field trial. Even in the unlikely event of a horizontal gene transfer, it would have no function in microorganisms.

(b) The *ctxB* construct

The ctxB gene codes for a non-toxic protein. It is also a synthetic gene whose codon structure is adapted to higher plants. This would result in an inefficient conversion to protein in the unlikely scenario of a horizontal gene transfer to microorganisms. No evidence exists to indicate a selection advantage in such a case. *Vibrio cholerae* itself mainly thrives in freshwater and salty brackwater habitats. Transfer of the gene to other microorganisms from non-GM organisms due to, say, floods, would be far more likely than a transfer from genetically modified potatoes within the framework of a deliberate release subject to temporal and spatial constraints. The conditions described in (a) also apply to the regulatory sequences.

(c) The *psb-cyel* construct

The *psb-cyel* construct effects the synthesis of the enzyme cyanophycin synthetase. In the unlikely event of a horizontal gene transfer to microorganisms, it is hypothetically possible that this would activate the enzyme in the microorganisms. This does not imply a selective advantage. Moreover, cyanobacteria are ubiquitously found on exposed sites in the environment. Thus, the deliberate release of the GM plants does not appear to increase the entry of this gene into the environment. Furthermore the product of the cyanophycin synthetase, the protein-like polymer cyanophycin, is non-toxic and biodegradable via the activity of other bacteria.

(d) The *npt*II gene

In the genetically modified plants the *npt*II gene is under the control of the *nos* promoter. This gene codes for the enzyme aminoglycoside 3'-phosphotransferase II (APH(3')II), which catalyses the ATP-dependent phosphorylation of certain aminoglycoside antibiotics (kanamycin, neomycin, geneticin), causing their inactivation.

As already elaborated in III.1.2.1. (b), the antibiotics inactivated by the aminoglycoside 3'-phosphotransferase II are of little relevance in human medicine but are widely used in veterinary medicine. It was thus necessary to examine whether the clinical use of the relevant antibiotics would be affected by a potential horizontal gene transfer of the *npt*II gene.

The inactivation of aminoglycoside antibiotics by phosphorylation has been demonstrated as a natural resistance mechanism in soil microorganisms. APH(3')II enzymes have also been found in human clinical isolates. The prevalence of genes which confer resistance to aminoglycoside antibiotics can be explained by the frequent application of these antibiotics, and by the fact that these genes are often located on plasmids, enabling the effective transfer between microorganisms by conjugation. Even in the event of a horizontal gene transfer from the genetically modified potatoes to microorganisms, the overall frequency of this resistance mechanism would not be noticeably increased.

The Scientific Panel on Genetically Modified Organisms (GMO Panel) of the European Food Safety Authority (EFSA) has allocated the *npt*II gene to the group of genes which, in terms of safety, provide no grounds to restrict or ban their usage, either for field trials or for the purpose of placing on the market. In its statement of 6.7.1999 on the biological safety of antibiotic resistance genes in the genome of genetically modified plants, the Central Committee on Biosafety (ZKBS) allocated the *npt*II gene to the group of antibiotic resistant genes which “(a) are already widespread in soil and enterobacteria and (b) whose relevant antibiotics have no, or only little significance in human and veterinary medicine, so that one can assume that the

presence – if any - of these antibiotic resistance genes in the genome of transgenic plants will have no effect on the spread of these antibiotic resistance genes in the environment".

(e) Further sequences located outside the T-DNA

The genetically modified potatoes can contain the following genetic elements which are located on the pLH900 derivates outside the border regions:

- the replication origin pVS1 from *Pseudomonas aeruginosa*;
- the replication origin of the plasmid pBR322 (ColE1 *ori*) from *E. coli*;
- the *aadA* gene from *E. coli*.

The replication origin of the plasmid pVS1 stems from *Pseudomonas aeruginosa* and contains the genetic information for the stability and replication of the plasmid. In the case of this DNA fragment, the likelihood of spreading via propagation between pseumonades and other microorganisms is far higher than between the GM plants and microorganisms.

The pBR322 replicon belongs to the ColE1-type plasmids, whose host range is restricted to certain gram-negative bacteria. Basically, the replicon can replicate in *E. coli* and closely-related bacterial species. No replication occurs in the majority of gram-negative soil bacteria. ColE1 plasmids are quite frequently present in enterobacteria. A gene transfer from enterobacteria to other bacteria is considered far more likely than a horizontal gene transfer from the genetically modified plants to bacteria. There is therefore no reason to expect that the potential presence of the replication origin of pBR322 in the plant chromosome contributes to an increase in the overall frequency of horizontal gene transfer.

The *aadA* (Strep/SpecR) gene dervies from the transposon Tn7 from *E. coli* and codes for an aminoglycoside adenyltransferase. The *aadA* gene is located on the transformation plasmid outside the T-DNA borders, and a transfer to the GM potatoes has not yet been investigated. It is therefore necessary to consider the risk of a horizontal gene transfer from the plant to microorganisms. The *aadA* gene confers resistance to streptomycin and spectinomycin. These antibiotics have only restricted use in human medicine, but are still clinically relevant for the treatment of tuberculosis (streptomycin) or gonorrhoea (spectinomycin). Bacteria with a resistance to streptomycin are commonly found in the environment. A resistance to this antibiotic can also spread via horizontal gene transfer from non-genetically modified microorganisms. The Central Committee on Biosafety (ZKBS) thus classified the *aadA* gene in group II of antibiotic-resistant genes which "(a) are widespread in microorganisms and (b) whose relevant antibiotics are still clinically used only in limited areas of human and veterinary medicine, so that one can assume that the presence – if any - of these antibiotic resistance genes in the genome of transgenic plants has very little effect on the spread of these antibiotic resistance genes in the environment". In its report of 2.4.2004 on the use of antibiotic-resistant genes as marker genes in GM plants, the Scientific Panel on Genetically Modified Organisms (GMO Panel) of the European Food Safety Authority (EFSA) allocated the *aadA* gene to the group of genes which should be restricted to experimental field trials and not occur in GM plants destined for placing on the market. The GM potato plants should only be released on a limited area for a limited period of time. The use of the plants in animal feed or for human consumption is banned. Given the negligible likelihood of a horizontal gene transfer from plant DNA to microorganisms and the absence of selection pressure on the trial sites, it is not expected that the presence of the *aadA* gene in the genetically modified potato plants would significantly increase the overall frequency of this resistance mechanism in organisms.