Notification 6786-01-0130

Summary of the risk assessment of genetically modified potato plants (*Solanum tuberosum* L.) (3 independent lines) carried out by the German Competent Authority within the framework of a proposed deliberate release, Berlin, 13 June 2001

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.

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

(a) The *alcR* transactivator gene and the ß-glucuronidase gene

A cDNA of the *alc*R gene from *Aspergillus nidulans* fused to the CaMV 35S promoter, linked with the ß-glucuronidase (*uid*A, GUS) gene from *E. coli*, which is under the transcriptional control of a chimeric *Aspergillus nidulans alcA*/CaMV 35S promoter, was inserted into the genome of the potato plants.

The *alcR* gene codes for a protein with a zinc-finger motif which binds to specific DNA sequences in the presence of ethanol. In addition to the *ald*A gene (aldehyde dehydrogenase) and 5 other structural protein genes, the *alcA* gene (alcohol dehydrogenase) is contained in the ethanol regulon of *Aspergillus nidulans*. It is activated by the transcriptional *alcR* activator protein by the induced activator binding specifically to the transcription start region and stimulating the transcription of the gene. To this end, the *alcA* promoter exhibits three *alcR* binding sites near the transcription start, which are required for the expression of the alcohol dehydrogenase in *Aspergillus nidulans*. In the genetically modified potatoes, the *alcR* gene is expressed constitutively under the control of the 35S CaMV promoter and the terminator of the nopaline synthase (*nos*) gene from *A. tumefaciens*.

The GUS gene was introduced into the potato genome as a reporter gene. The enzyme β-glucuronidase cleaves glucuronides and is found in the tissue of vertebrates and invertebrates as well as in bacteria. Plants also exhibit minor endogenous β-glucuronidase activity, which can, however, be suppressed using appropriate methods. The expression of the GUS gene is controlled by a chimeric *alcA*::35S promoter. The chimeric promoter consists of a deleted 35S promoter of the CaMV and a fragment of the *alcA* promoter that contains the *alcR* binding sites and is responsible for the ethanol regulation of the promoter. The 35S terminator of the CaMV was inserted to control the transcription termination.

An ethanol treatment of the genetically modified plants leads to the expression of the GUS gene. Distinct enzyme activity was detected in protein extracts of genetically modified potato plants treated with gaseous ethanol. The expression of the GUS gene is not expected to confer a selective advantage to the plants.

Under normal growth conditions, ethanol is not formed in potatoes. Only under stress (e.g. lack of oxygen) can small amounts of ethanol form under natural conditions. The system consisting of the *alcR* gene and the *alcA* promoter is not activated by endogenous plant transcription factors. No information is available on the development of the genetically modified potatoes under inducing conditions in the greenhouse. However, there is evidence to suggest that ß-glucuronidase expressed by genetically modified potato plants affects the development of these plants. Under non-inducing conditions, the genetically modified potatoes do not differ from the parent variety regarding growth, size, leaf morphology and colour, root and tuber formation, development of structures for reproduction and spread as well as survivability.

The AlcR activator protein binds to specific sequences. To influence the expression of endogenous plant genes, corresponding specific gene fragments must be present in the potato genes. There is no evidence to suggest the presence of such gene fragments.

These genetic modifications in the potato plants are not expected to pose any risk to human or animal health or to the environment.

After the end of the trial, the harvested tubers of the genetically modified plants will partly be stored for replanting or used for analytical tests. Surplus potatoes will be inactivated.

(b) The *npt*II gene

The *npt*II gene transferred into the genetically modified plants codes for the enzyme neomycin phosphotransferase. It was inserted as a marker gene for the selection of transformed plant cells.

Neomycin phosphotransferase is a type-II aminoglycoside-3'-phosphotransferase (APH(3')II) that catalyses 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. The clinically relevant gentamicin and other aminoglycosides and aminocyclitols used in human medicine do not belong to the substrate spectrum of the APH(3')II enzymes. Kanamycin and neomycin are also widely used in veterinary medicine. The enzyme is non-toxic to plants, animals, microorganisms and humans.

(c) The coding sequence of the α-fragment of the ß-galactosidase, *lac*I sequences

The genetically modified plants were created by using vectors derived from the plasmid pBIN19, the multiple cloning site of which is located within the sequence coding for the α fragment of the ß-galactosidase from *E. coli*.

The native enzyme β-galactosidase splits β-D-galactosides into galactose and the related alcohol compound. The physiologically most important substrate is lactose, which is hydrolysed into galactose and glucose. The first 146 amino-terminal amino acids of the β-galactosidase are referred to as the α fragment. The α fragment by itself is not enzymatically active; however, complementation in suitable hosts is possible.

The sequence coding for the α fragment of the β-galactosidase was interrupted by the insertion of the chimeric *alcR*::GUS DNA sequence into the multiple cloning site, preventing it from coding for an α fragment capable of complementation in *E. coli* bacteria. The interrupted sequence of the α fragment of the β-galactosidase is expressed under the control of a bacterial promoter. This sequence does not code for a functional gene product. The presence of this sequence is not expected to cause any changes in the genetically modified potatoes.

The genetically modified plants additionally contain 5’ and 3’ sequences of the repressor gene *lacI*. However, these 5’ and 3’ sequences are separated from each other by the *lacZ* and M13 *ori* sequences. The *lacI* sequences are not expected to be functional in the genetically modified plants.

(d) M13 sequences

The genetically modified plants created by transformation using a derivative of the vector pBIN19 contain two fragments from M13mp19, namely a 440-bp fragment, which encompasses one part of an open reading frame of a structural protein of M13, and a 433-bp fragment, which contains the origin of replication of phage M13.

If transcription of the fragment of the open reading frame of the structural protein were to occur in the genetically modified potato plants, no functional protein would result, since the fragment only codes for 167 of the total 423 amino acids of the complete phage protein. The presence of this fragment is thus not expected to affect plant metabolism.

The origin of replication of M13 causes the phage to replicate in *E. coli*, if *E. coli* is infected with M13, f1 or fd phages. The origin of replication is not expected to be functional in plants.

(e) The fragment of the *ocd* gene

The plants created by transformation using derivatives of the vector pBIN19 contain a fragment of the *ocd* gene (ornithine cyclodeaminase), which is located between the 3' terminal end of the translated sequence of the *npt*II gene and the *nos* terminator sequence. Since this sequence is transcribed as part of the mRNA of the *npt*II gene, but is located downstream of the termination codon of the *npt*II gene, this sequence is not expected to be translated.

(f) Border sequences from Ti plasmids and regulatory sequences

The genetically modified plants contain sequences of the left and right border region of the TL-DNA of the plasmid pTiB6S3 from *A. tumefaciens*. Depending on the gene products of the *vir* region of the helper plasmid pGV2260 that is contained in the *Agrobacterium* strain GV2260 used for transformation and is not transferred into the plants, these sequences cause the genes located between the border regions to integrate into the chromosomes of the potato 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:

- 35S promoter of the cauliflower mosaic virus (CaMV);
- Promoter and terminator of the nopaline synthase gene from *A. tumefaciens*;
- *alcR* binding site of the *alcA* gene from *Aspergillus nidulans.*

In the genetically modified plants, the promoter and terminator sequences regulate the expression of the chimeric *alcR*::GUS DNA sequence as well as the *npt*II gene. Further information on the effects associated with the expression of these sequences in the plants can be found in III.1.2.1 (a) and (b).

(g) Sequences located outside the T-DNA

As a general 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 border regions has been reported and cannot be ruled out based on the information provided in the application.

Based on the information provided in the application, the following functional units may have been transferred into the genetically modified potato plants in this particular case as a result of the integration of DNA fragments located outside the border regions:

(i) The *npt*III gene (codes for a type-III aminoglycoside-3'-phosphotransferase) for resistance to aminoglycoside antibiotics;

(ii) The origin of replication *oriV* of the plasmid RK2;

(iii) The *traF* region, containing the *oriT* of the plasmid RK2;

(iv) The *trfA* locus of the plasmid RK2 (codes for two proteins required for the replication of the plasmid);

(v) A non-functional fragment of the *klaC* gene from the plasmid RK2;

(vi) The *tetA* gene of the plasmid RK2 (interrupted by insertion of the T-DNA region);

(vii) The transposon IS1 within the *npt*III gene;

(viii) The origin of replication of the plasmid pMB1.

Since the *npt*III gene (i) is under the control of a bacterial promoter, it is not assumed to be expressed in plants. Even if the gene were to be expressed, it is not expected to affect plant metabolism.

The origins of replication *ori*V (ii) and *ori*T (iii) of the plasmid RK2 allow replication of the plasmid in a broad host range of gram-negative bacteria and/or its conjugative transfer, as long as the mobilisation functions are provided by a helper plasmid. There is no evidence to suggest that the origins of replication of RK2, the origin of replication of pMB1 (viii) or the remaining DNA fragments of bacterial origin (iv, v, vi, vii) have a function in higher plants. Moreover, some of the DNA fragments are incomplete (v) or interrupted (vi).

(h) 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 expression level 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 altered to the same degree in the field as under climate-controlled or greenhouse conditions. 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 endogenous plant genes at or near the site of insertion. Such processes can affect plant metabolic pathways. In previous work with the genetically modified plants, however, 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 and were first identified in maize. 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.

Given the current state of knowledge, it is not possible to make reliable predictions about the potential allergenicity of a protein on the basis of its amino acid sequence. In previous experiments with the genetically modified plants as well as in earlier deliberate release trials with genetically modified plants that express the *npt*II gene under the control of non-tissue-specific promoters, no evidence was found to suggest an increased allergenic potential of the plants. Pollen of potato plants is dispersed by wind only to a little extent and generally does not 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 Europe, the establishment of potatoes in natural ecosystems during this period has not been observed. From time to time, potato plants are found beyond cultivated areas, but only on non-natural sites such as roadsides and other ruderal areas. Owing to the lack of frost hardiness, potatoes do not establish in these areas either. As a result of potato cultivation, "volunteer potatoes" can, depending on winter temperatures, emerge in the subsequent cultivation period from tubers or seeds that have overwintered in the soil.

The above-ground parts of the potato plants are planned to be mechanically or chemically destroyed prior to reaching maturity to prevent seed maturation. The tubers will be analysed after harvest or stored for replanting in the following year. Surplus potatoes will be inactivated. The remaining transgenic plant parts will be left on the field to decompose. Potatoes will not be cultivated during the two-year post-trial monitoring period. Volunteer potatoes that emerge during this period will be identified and destroyed. The probability of persistence of genetically modified plants due to any tubers remaining in the ground after harvest is minimised by the measures pursuant to the supplementary provision II.8. To remove any tubers remaining in the ground, the soil on the trial site will be tilled to a depth of about 15 cm after harvesting the tubers as well as in the spring of the following year. Any tubers found will be inactivated.

Potato plants of the variety "Solara" can flower and produce seeds. It is conceivable that the genetically modified potatoes may be fertilised by the introduction of foreign potato pollen, which is why the formation of seeds cannot be ruled out completely, but is unlikely. Under Central European climate conditions, it is unlikely that potato seeds will overwinter and produce plants.

If tubers or seeds were to remain in the soil, any plants that would emerge from them would be identified within the scope of the post-trial monitoring proposed by the applicant. A possible change in the frost sensitivity of the tubers as a result of the genetic modifications is not expected. During the post-trial monitoring period following the deliberate release, no plants or at least only plants which will not obstruct the monitoring will be cultivated on the control sites. This makes it possible to easily identify any volunteer potatoes.

For the reasons stated above, 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

Attempts to crossbreed 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 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 mainly self-pollinating; cross-pollination is uncommon even within one flowering potato field and is most likely to occur between neighbouring plants.

On the trial site of the Gatersleben location, genetically modified peas and potatoes are being released until 2002 as part of various deliberate release trials of the Institute for Plant Genetics and Crop Plant Research, Gatersleben. Should genetically modified potato plants from different deliberate release trials be cultivated on the same plot in close proximity, the shared site will be treated as the release site and the isolation distance to the nearest field with non-genetically modified potatoes will be applied to the entire site.

The proposed isolation distance of at least 20 m to other agricultural potato cultivation areas is considered adequate. However, should pollen be transferred to potato plants cultivated to produce table potatoes, no adverse effects are to be expected, since in an agricultural environment potato plants are propagated vegetatively, i.e. not 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. Even if the tubers of these plants were to be consumed, no health hazards would be expected to result – as stated in the evaluation summarised in III.1.2.1.

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 in the chromosomes of the recipient organisms. No evidence exists to suggest 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 that are as distantly related in terms of taxonomy as 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 any heterologous genetic material, including all forms of plant DNA.

The genes for the *alcR* transactivator and the ß-glucuronidase are derived from the ubiquitous *Aspergillus nidulans* and *E. coli*, i.e. are already widespread in the environment. Horizontal gene transfer from non-genetically modified organisms, such as *Aspergillus nidulans* and *E. coli*, to microorganisms is thus far more likely to occur under natural conditions.

As already elaborated in III.1.2.1 (b), the antibiotics inactivated by the neomycin phosphotransferase 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.

In soil microorganisms, the inactivation of aminoglycoside antibiotics by phosphorylation is a naturally occurring resistance mechanism. APH(3')II enzymes have also been found in clinical isolates of human microorganisms. 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 effective transfer by conjugation. Even in the event of horizontal gene transfer from the genetically modified potatoes to microorganisms, the overall frequency of this resistance mechanism in the environment would not be noticeably increased.

The gene for the α fragment of the β-galactosidase is interrupted, preventing the formation of a functional gene product. This would also be the case in bacteria receiving the gene by horizontal gene transfer. The same applies to the 3’ and 5’ sequences of the *lacI* gene.

The situation is similar with the fragment of the gene for a structural protein of the phage M13 and the fragment of the *ocd* gene. These fragments are not expected to be functional in bacteria. In addition, as elaborated in III.1.2.1 (e), the fragment of the *ocd* gene is not likely to be translated.

The genetically modified potatoes contain the origin of replication of M13. M13 belongs to the F-specific *E. coli* phages. In the case of this origin of replication, the probability of genetic spread by transfer between bacteria is thus far higher than the probability of horizontal gene transfer from the genetically modified plants to microorganisms.

The sequences inserted into the potatoes to regulate the transferred genes are derived from *A. tumefaciens, Aspergillus nidulans* and CaMV. Regarding the horizontal gene transfer of these sequences to microorganisms, it should be noted that *A. tumefaciens* and *Aspergillus nidulans* are widespread in the environment and the transfer of the corresponding sequences from *Agrobacterium* and *Aspergillus* is far more likely than their transfer from the genetically modified plants. The theoretical possibility of transfer of the CaMV sequences from the genetically modified plants would not constitute a new situation compared to that found in nature, because CaMV as a double-stranded plant-infecting DNA virus is commonly found in plants.

As a rule, only the sequences 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 cannot be ruled out based on the information provided in the application. In this particular case, the following DNA fragments may have been integrated into the genetically modified plants by the transfer of sequences located outside the border regions:

(i) The *npt*III gene from *Streptococcus faecalis* (codes for a type-III aminoglycoside-3'-phosphotransferase) for resistance to aminoglycoside antibiotics;

(ii) The origin of replication *ori*V of the plasmid RK2;

(iii) The *tra*F region, containing the *ori*T of the plasmid RK2;

(iv) The *trf*A locus of the plasmid RK2 (codes for two proteins required for the replication of the plasmid);

(v) A non-functional fragment of the *kla*C gene from the plasmid RK2;

(vi) The *tet*A gene of the plasmid RK2 (interrupted by insertion of the T-DNA region);

(vii) The transposon IS1 within the *npt*III gene;

(viii) The origin of replication of the plasmid pMB1.

According to literature references, the *npt*III gene (i), which may be contained in the genetically modified plants under the control of its own promoter, confers resistance not only to kanamycin and neomycin, but also to the antibiotic amikacin. In Germany, amikacin is not authorised for use as a veterinary medicinal product but it may be employed in human medicine as a so-called reserve antibiotic. Because of its status as a reserve antibiotic and its attendant infrequent use, amikacin resistance is so far not widespread. Given the low probability of a horizontal gene transfer from plant DNA to microorganisms and the absence of selection pressure on the release sites, it can also be assumed that the presence of this gene in the genetically modified potato plants would not lead to a significant increase in the overall frequency of this resistance mechanism in microorganisms.

RK2 belongs to a group of broad host-range plasmids (incl. RP1, RP4, R18, R68), which are replicable in numerous gram-negative bacteria. Hence, in the case of the RK2-derived DNA fragments (ii – vi), the probability of genetic spread by transfer between bacteria is far higher than the probability of horizontal gene transfer from the genetically modified plants to microorganisms. Moreover, some of the DNA fragments are incomplete (v) or interrupted (vi).

The insertion element IS1 (vii) occurs naturally in various species of *Enterobacteriaceae*. It has been found, for example, in species of the genera *Escherichia*, *Shigella*, *Klebsiella*, *Serratia* and *Salmonella*. In the case of IS1, the number of copies per bacterial genome can be up to > 40. IS1 copies can have either a chromosomal or a plasmid location and have also been detected in prophages. It can be assumed that this insertion element would be easily spread by horizontal gene transfer between bacteria. In comparison, the probability of spread by horizontal gene transfer from the genetically modified plants to microorganisms, although theoretically conceivable, would be negligibly low.

The pMB1 replicon (viii) belongs to the ColE1-type plasmids, whose host range is limited to a number of gram-negative bacteria. Basically, this replicon can be replicated in *E. coli* and closely related species of bacteria such as *Serratia* or *Salmonella*. In most gram-negative soil bacteria, replication does not take place. ColE1 plasmids occur frequently in enterobacteria. Gene transfer from enterobacteria to other bacteria is considered far more likely than a horizontal gene transfer from the genetically modified plants to bacteria. Therefore, the potential presence of the origin of replication of

pMB1 in the plant chromosome is not expected to contribute to an increase in the overall frequency of horizontal gene transfer.

III.1.2.5 Agrobacteria used to generate the genetically modified plants

In order to generate the genetically modified plants, sterile potato leaves were incubated with agrobacteria which harbour the genes to be transferred between the border regions of the binary vector plasmid. Following transformation, antibiotic treatment was carried out to eliminate the agrobacteria. Only potato plants that were free of agrobacteria were used.

In contrast to the common wild types of *A. tumefaciens*, the *Agrobacterium* strains used are disarmed, i.e. they no longer have the capacity to induce tumours. In the unlikely but theoretically conceivable event that the inserted foreign genes were transferred to a cell of another plant by these agrobacteria, this cell would have to spontaneously regenerate into a whole, fertile plant for the foreign genes to enter the germ cells. This is the only way that these genes could be passed on to the plant progeny. Such an event is not expected to occur under natural conditions.

Assuming that the presence of small amounts of recombinant agrobacteria in the genetically modified plants cannot be ruled out, the potential transfer by conjugation of the binary vector plasmids contained in the agrobacteria to wild-type agrobacteria (*A. tumefaciens* or *A. rhizogenes*) present in the environment would also have to be considered, since these could, in turn, pass on the foreign genes to individual cells of other plants.

In the event of infection and subsequent transformation by wild-type *A. tumefaciens* or *A. rhizogenes*, a crown gall or hairy root would develop from the transformed plant cell. Under natural conditions, such a tumour would not be expected to give rise to a plant.

Furthermore, the transfer of the inserted genes from agrobacteria to other soil bacteria would have to be considered. The potential effects of such a transfer were already addressed in III.1.2.4.