Notification 6786-01-0138

Summary of the risk assessment of genetically modified potato plants

(*Solanum tuberosum* L.*)* (a total of three transformation events)

carried out by the German Competent Authority within

the framework of a proposed deliberate release,

Berlin, 24 June 2002

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]
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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.

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

(a) The sucrose isomerase gene from *Erwinia rhapontici,* fused to the transit peptide sequence of the proteinase inhibitor II from *Solanum tuberosum*

The chimeric gene, which was derived from the sucrose isomerase and the proteinase inhibitor II transit peptide genes and transferred into the genetically modified potato plants, is specifically expressed in the potato tubers under the control of the B33 promoter. In the genetically modified potatoes, the transit peptide of the proteinase inhibitor II from potato should enable the transport of the sucrose isomerase into the apoplast. For this purpose, the fusion protein is transported into the endoplasmic reticulum where the signal sequence is cleaved off by peptidases. Secretion of sucrose isomerase subsequently takes place in the cell wall. Evidence that the sucrose isomerase is expressed only in the tubers and not in other plant parts of the potato plants proposed for release here was not provided by the applicant. However, it is stated that when the protein is expressed using a constitutively expressed promoter in autotrophic tissues of tobacco plants, phenotypic changes occur due to the change in photoassimilate availability. In the genetically modified potato plants grown in the greenhouse, on the other hand, no phenotypic differences to wild-type plants were observed by the applicant.

Expression of sucrose isomerase in the genetically modified plants was demonstrated in the potato tubers by western blot and by carbohydrate analysis.

The donor organism for the sucrose isomerase gene is the bacterium *Erwinia rhapontici*. The genus *Erwinia* belongs to the *Enterobacteriaceae* family and includes gram-negative, motile, facultatively anaerobic bacteria, which occur as pathogens, saprophytes or constituents of plant-associated epiphytic flora. The genus is characterised by strong heterogeneity. *Erwinia rhapontici* lives in association with rhubarb (*Rheum rhaponticum*) and causes crown rot. But the bacterium also appears on other plants (e.g. wheat). It is not known to have a pathogenic effect on humans.

As a consequence of the genetic modification the carbohydrate metabolism in the genetically modified potato plants was changed in such a way that a foreign carbohydrate, palatinose, is synthesised in the potato tubers. To a lesser extent an isomeric compound, trehalulose, is formed in addition to palatinose. According to tests carried out by the applicant, the carbohydrate analysis demonstrated a decrease in sucrose concentration and an accumulation of palatinose in the genetically modified potato tubers. The trehalulose content was not determined.

The palatinose (6-*O*-α-D-glucopyranosyl-D-fructose) is an isomer of sucrose. It is used in the food industry as a sweetener in diet foods and is non-cariogenic. To date palatinose is microbially produced in bioreactors. According to the applicant’s statements, with the synthesis of palatinose in genetically modified potato plants a more efficient system for the production of this alternative sweetener is to be tested under field conditions.

The execution of the planned experimental trial is unlikely to pose any threat to human or animal health or to the environment as a result of the formation of the sucrose isomerase, the changes in the carbohydrate composition and the synthesis of foreign carbohydrates in the genetically modified potato plants in the context of the proposed deliberate release.

(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 paromomycin 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, in the absence of substrate under field conditions, no new metabolic products are expected to 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 selective advantage on the genetically modified plants. There is no evidence to suggest that this enzyme is toxic to plants, animals, micro-organisms or humans.

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

The plasmid used to generate the genetically modified potato lines is a derivative of the pBIN19 vector.

In this plasmid, the multiple cloning site is located within the coding sequence of the α-fragment of β-galactosidase from *E. coli*. The native enzyme β-galactosidase cleaves β-D-galactoside into galactose and the corresponding alcohol compound. The most important physiological substrate is lactose, which is hydrolised into galactose and glucose. The α-fragment refers to the first 146 amino terminal amino acids of β-galactosidase. The α-fragment is not enzymatically active by itself, but it may be complemented in suitable hosts.

The coding sequence for the α-fragment of the β-galactosidase was interrupted by inserting different expression cassettes in the multiple cloning site so that in this form, *inter alia* in *E. coli* bacteria, it is no longer able to code for an α-fragment which is capable of complementation. The interrupted sequence of the α-fragment of β-galactosidase is under the control of a bacterial promoter. A functional gene product is not encoded by this sequence. Changes in the genetically modified potatoes are not expected to result from the presence of this sequence.

In addition, the genetically modified plants also contain 5'- and 3'-sequences of the repressor gene *lac*I. However, these 5'- and 3'-sequences are separated from one another by the *lacZ* and M13 *ori* sequences. The *lac*I sequences are unlikely to have any functional capacity in the genetically modified plants.

(d) M13 sequences

The genetically modified plants contain two fragments derived from M13mp19, namely, a 440-bp fragment comprising a component of an open reading frame of a structural protein from M13 as well as a 433-bp fragment containing the origin of replication of the M13 phage.

Should transcription of the fragment of the open reading frame of the structural protein occur in the genetically modified potato plants, a functional protein would not result because the fragment encodes only 167 amino acids of a total of 423 amino acids of the complete phage protein. Effects on the metabolism of the plants due to the presence of this fragment are therefore not expected.

The origin of replication of M13 causes replication of the phage in *E. coli* when *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 contain a fragment of the *ocd* gene (ornithine cyclodeaminase), which is located between the 3' end of the translated sequence of the *nptII* gene and the *nos* terminator sequence. Because this sequence is transcribed as part of the mRNA of the *npt*II gene, but is located behind the termination codon of the *npt*II gene, the sequence is not expected to be translated.

(f) Border sequences from Ti plasmids and regulation sequences

The genetically modified plants contain sequences from the left and right border regions of the T-DNA of the plasmid pTiB6S3 from *A. tumefaciens*. These sequences, in conjunction with the gene products of the *vir* region of the helper plasmid pGV2260 present in the *Agrobacterium* strain GV2260, which was used in the transformation but was not transferred to the plants, are responsible for integrating the genes located between the border regions 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.

The following regulation sequences have been integrated into the genome of the genetically modified plants:

- the patatin B33 promoter from *S. tuberosum*,
- the promoter and terminator of the nopaline synthase gene from *Agrobacterium tumefaciens*,
- the terminator of the octopine synthase gene from *Agrobacterium tumefaciens*.

These promoter and termination sequences regulate the expression of the DNA sequences located between them in the genetically modified plants. The effects of the expression of these sequences in the plants are described in detail in section III.1.2.4.

(g) Sequences located outside the T-DNA

As a general rule, only DNA located within the border regions is integrated into the plant genome during Agrobacteria-mediated transformation. However, the transfer of DNA fragments outside the border regions has been reported. PCR analysis showed that all three genetically

modified potato lines intended for release contain the antibiotic resistance gene *npt*III and that therefore the T-DNA border regions were not respected in the transformation.

The transformation vector pBIN19 contains the following outside the border regions:

- the *aph*AIII (= *npt*III) gene from *Streptococcus faecalis* (= *Enterococcus faecalis*), interrupted by the transposon *IS*1, but functional in prokaryotic systems;

- the *tetA* gene of the pRK2 plasmid, interrupted by the T-DNA;

- the *trfA* gene of the pRK2 plasmid for replication in *E.coli* and in *A. tumefaciens*;

- a fragment of the *klaC* gene from *Klebsiella aerogenes*;

- a *traF* fragment containing the *ori*T of the RP4 plasmid from *E. coli*;

- the origin of replication *ori*V of the RK2 plasmid from *E. coli*;

- the origin of replication of the pUC plasmid (ColE1 *ori*) from *E. coli*.

The formation of functional gene products based on these sequences is not expected in the genetically modified plants because they are not controlled by plant-specific promoters. This also applies to the *npt*III gene, whose expression is controlled by a bacterial promoter.

The replication origins *oriV* and *oriT* of the plasmid RK2 facilitate the replication of the plasmid in a broad host range of gram-negative bacteria or its transfer by conjugation, provided the mobilisation functions are supplied by a helper plasmid. There is no evidence to suggest that the replication origins of RK2, the replication origin of pMB1 or the other DNA fragments of bacterial origin have a function in higher plants. Moreover, some of the DNA fragments are incomplete or interrupted.

(h) Position effects and context changes; allergenicity

Genes which have been integrated into the plant genome by genetic engineering are expressed at different levels, depending on the integration site on the chromosome or rather on the sequences neighbouring the integration site (“position effect”). Under field conditions the level of expression may also 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 does not pose a risk to the environment or to human or animal health.

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

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. They were first discovered 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 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, pollen of potato plants is only dispersed to a limited 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 become established in the environment

The cultivation of potatoes in Central Europe goes back several hundred years. The establishment of potatoes in natural ecosystems has not been observed in Europe. Potato plants are occasionally found outside cultivated areas, but only on non-natural sites such as verges and other ruderal areas. Because potatoes are not frost hardy they do not form persistent populations in these areas either. 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.

The intention is to destroy the above-ground parts of the potato plants by mechanical or chemical methods before they reach maturity in order to prevent the formation of viable seeds. After harvesting, the tubers will be analysed or stored for replanting in the following year. Surplus tubers are to be inactivated. Transgenic plant residues that remain on the release site are to be left there to decompose. Potatoes will not be cultivated during the two-year post-trial monitoring period. During this period, volunteer potato plants are to be identified and destroyed. The likelihood of the genetically modified plants persisting as a result of tubers possibly remaining in the soil after harvesting will be minimised by the measures stipulated under provision II.8. (to the present application). In order to eliminate tubers remaining in the soil, the experimental area is to be loosened to a depth of 15 cm after harvesting and again in spring of the following year. Any tubers found are to be inactivated.

Potato plants of the “Solara” variety can flower and produce seeds. Potato seeds are unlikely to overwinter and give rise to plants under Central European climate conditions. Should tubers or seeds remain in the soil, the resulting plant growth would be detected during the planned post-trial monitoring. According to information provided by the applicant, no phenotypic differences between the genetically modified plants and control plants were observed in greenhouse trials. However, the possibility of a change in the frost sensitivity of the tubers as a result of the genetic modification cannot be completely ruled out.

Expression of the sucrose isomerase in the genetically modified potatoes affects the carbohydrate composition in the tubers. An increase in the content of soluble sugars and the associated lowering of the freezing point could enhance the frost resistance and persistence capacity of the potato tubers.

This possibility is adequately taken into account by the planned cultivation gap of two years and by the post-trial monitoring.

During the post-trial monitoring period after completion of the release, no plants or only plants that do not interfere with monitoring shall be planted on the site. This ensures that volunteer potatoes can be detected easily.

For the reasons stated above, the genetically modified plants are not expected to persist or become established 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. However, these turned out to be sterile. The potato and *Solanum dulcamara* (bittersweet or woody nightshade) proved to be strictly bilaterally incompatible species; in crossbreeding experiments pollination of the ovule was not achieved. Similarly, potato does not crossbreed with tomato (*Lycopersicon esculentum*). In agricultural practice, potatoes are propagated vegetatively via tubers.

Hence the following passage deals solely 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 takes place only over short distances. Potatoes are primarily self-fertilizing. Cross-fertilization, even within a field of flowering potato plants, is rare. It is most likely to occur between neighbouring plants.

On the test site located at Gatersleben, the Leibniz Institute for Plant Genetics and Crop Plant Research are cultivating genetically modified peas and potatoes within the scope of various deliberate release trials. If the genetically modified potato plants from the various deliberate release trials are cultivated at the same time in close proximity, then the common area can be regarded as the release site and the separation distance to the next field with non-genetically modified potatoes may apply to the entire area.

The applicant plans to maintain a distance of at least 20 metres to other agricultural areas where non-genetically modified potatoes are grown. This is considered adequate for the purposes of the proposed trial. Should pollen nevertheless be transferred to potato plants being cultivated for the production of table potatoes, no adverse effects would be expected. Planting material for the agricultural cultivation of potatoes is 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 the course crop rotation, such plants would be eliminated by conventional soil preparation practices. Even if the tubers of such plants were to be consumed, this would not be expected to pose a health hazard, as indicated by the evaluation undertaken in section III.1.2.1.

III.1.2.4. Assessment of the possibility of a transfer of the inserted foreign genes from the genetically modified plants to micro-organisms by horizontal gene transfer

The inserted sequences are stably integrated into the chromosomes of the recipient organisms. There is no evidence that a transfer of genetic information from plants or its expression in micro-organisms 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.

If it is assumed that an exchange of genetic material between organisms which are so distantly related in terms of taxonomy is actually possible, it must 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 gene for the sucrose isomerase from *Erwinia rhapontici,* fused to the transit peptide sequence of the proteinase inhibitors II from *Solanum tuberosum*

The gene for the sucrose isomerase originates from *Erwinia rhapontici* and the gene for the transit peptide originates from potato itself. These genes are already commonly found in the environment and therefore there is a much higher probability of horizontal gene transfer occurring from non-genetically modified organisms to micro-organisms.

(b) The *npt*II gene

As already outlined under point III.1.2.1, the antibiotics inactivated by the neomycin-phosphotransferase are of little relevance in human medicine, but they are widely used in veterinary medicine. It was therefore 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 is a naturally occurring resistance mechanism in soil micro-organisms. 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 use of these antibiotics, and by the fact that these genes are often located on plasmids, enabling effective transfer between micro-organisms by conjugation. Even if a horizontal gene transfer from the genetically modified potatoes to micro-organisms were to occur, the overall frequency of this resistance mechanism would not be noticeably increased.

(c) Additional sequences within the border regions of the plasmid used for the transformation

The gene for the α-fragment of the β-galactosidase is interrupted so that no functional gene product can be formed. This would also be the case in bacteria that receive the gene by a horizontal gene transfer. The same applies to the 3'- and 5'-sequences of the *lac*I gene.

A similar situation applies to the fragment of the gene for a structural protein of the M13 phage and the fragment of the *ocd* gene. These fragments are not expected to be functional in bacteria. In addition, the fragment of the *ocd* gene is unlikely to have been translated, as explained in III.1.2.1.

The genetically modified potatoes contain the origin of replication of M13. M13 belongs to the F-specific *E. coli* phages. Therefore, the likelihood of this origin of replication being spread by transfer between bacteria is far greater than the likelihood of being spread by horizontal gene transfer from the genetically modified plants to micro-organisms.

(d) Sequences located outside the T-DNA

In the case of the lines generated by transformation with pBIN19 derivatives, sequences located outside the border regions may have been integrated into the genetically modified

plants. The presence of the antibiotic resistance gene *npt*III was demonstrated in all three genetically modified potato lines intended for release.

The transformation vector pBIN19 contains the following outside the border regions:

- the *aph*AIII (= *npt*III) gene from *Streptococcus faecalis* (= *Enterococcus faecalis*), interrupted by the transposon *IS*1, but functional in prokaryotic systems;

- the *tetA* gene of the pRK2 plasmid, interrupted by the T-DNA;

- the *trfA* gene of the pRK2 plasmid for replication in *E.coli* and in *A. tumefaciens*;

- a fragment of the *klaC* gene from *Klebsiella aerogenes*;

- a *traF* fragment containing the *ori*T of the RP4 plasmid from *E. coli*;

- the origin of replication *ori*V of the RK2 plasmid from *E. coli*;

- the origin of replication of the pUC plasmid (ColE1 *ori*) from *E. coli*.

According to the literature, the *npt*III gene, which is contained in the genetically modified plants along with its native promoter, confers resistance not only to kanamycin and neomycin, but also to the antibiotic amikacin. Amikacin is not approved for use as a veterinary drug in Germany, but it may be used in human therapy as a so-called reserve antibiotic. Because of its status as a reserve antibiotic and its attendant infrequent use, resistance to amikacin is so far not widespread. Due to the low probability of a horizontal gene transfer from plant DNA to micro-organisms and the absence of selection pressure on the trial sites, however, the presence of this gene in the genetically modified potato plants is unlikely to cause a significant increase in the overall frequency of this resistance mechanism in micro-organisms.

RK2 belongs to a group of broad-host-range plasmids (including RP1, RP4, R18 and R68) which are capable of replication in numerous gram-negative bacteria. Therefore, for DNA fragments derived from RK2, the likelihood of spreading by transfer between bacteria is far greater than the likelihood of spreading by horizontal gene transfer from the genetically modified plants to micro-organisms. Moreover, some of the DNA fragments are incomplete or interrupted.

The insertion element *IS*1 occurs naturally in different species of Enterobacteriaceae. It has, for example, been found in species of the genera *Escherichia, Shigella, Klebsiella, Serratia* and *Salmonella*. In *IS*1 the number of copies per bacterial genome can amount to more than 40. Copies of *IS*1 may be localised on the chromosome or the plasmid and were also found in prophages. These insertion elements are likely to be easily spread by horizontal gene transfer between bacteria. Therefore, in comparison, the theoretically conceivable risk of a spread by horizontal gene transfer from the genetically modified plants to micro-organisms is negligible.

The pMB1 replicon belongs to the ColE1-type plasmids, which have a host range that is limited to a small number of gram-negative bacteria. Essentially, it has the ability to replicate in *E. coli* and other closely related species of bacteria. In most gram-negative soil bacteria, replication does not occur. ColE1 plasmids occur quite commonly in enterobacteria. The likelihood of gene transfer from enterobacteria to other bacteria is considered to be far greater than the likelihood of horizontal gene transfer from the genetically modified plants to bacteria.

The potential presence of the replication origin of pMB1 in the plant chromosome is therefore 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

To generate the genetically modified plants sterile potato leaves were inoculated with Agrobacteria containing the genes to be transferred between the border regions of the binary vector plasmid. After transformation had occurred, the plant parts were treated with antibiotics to eliminate the Agrobacteria. Only potato plants that were free of Agrobacteria were used.

In contrast to the ubiquitous wild-type *A. tumefaciens*, the *Agrobacterium* strains used for the transformation 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 are transferred to a cell of another plant by these Agrobacteria, that 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 offspring of the plant. 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, a potential transfer by conjugation of the binary plasmids contained in the Agrobacteria to wild-type Agrobacteria (*A. tumefaciens* or *A. rhizogenes*) present in the environment must also be considered, since these could, in turn, pass on the foreign genes to individual cells of other plants.

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

A possible transfer of the inserted genes from Agrobacteria to other soil bacteria must also be considered. The potential impact of such a transfer has already been addressed in III.1.2.4.