



Notification 6786-01-0137

Summary of the risk assessment of genetically potato plants

(Solanum tuberosum L.), 6 different independent lines,

carried out by the German Competent Authority within

the framework of a proposed deliberate release,

Berlin, 24 June 1996

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 genes for enzymes of the gibberellin metabolism from *Arabidopsis thaliana*

The coding regions of heterologous genes for enzymes of the gibberellin metabolism (GA20-oxAT, GA2-oxAT) contained in genetically modified potato plants are expressed constitutively – i.e. non-tissue-specifically – under the control of the CaMV-35S promoter. The expression of the respective DNA fragment leads to the production of the corresponding enzyme in the genetically modified potato plants.

As a result of the genetic modifications, the expression of the GA20-oxidase causes an increased formation of biologically active gibberellins in the genetically modified potato plants. The GA20-oxidase catalyses one of the final steps in gibberellin biosynthesis, converting a C₂₀-gibberellin into a physiologically active C₁₉-gibberellin. Active gibberellins have a stimulating effect on the elongation growth of the plant stem – especially the internodes – in vegetative tissues, flowering induction as well as flower and fruit development. According to the information provided by the applicant, the genetically modified potato plants that express an increased level of the GA20-oxidase, phenotypically grew to a greater height in the greenhouse than the control plants.

Due to the increased expression of the GA2-oxidase in the genetically modified potatoes, intermediate compounds of gibberellin biosynthesis are metabolised into physiologically inactive gibberellins.

According to the information provided by the applicant, based on observations made in greenhouse experiments the genetically modified potato plants intended for release that express the GA2-oxidase are characterised by a considerable growth retardation.

A change in the flower morphology of the genetically modified potato plants intended for release was not observed. The tuber yields of the GA2-oxidase-expressing plants under greenhouse conditions do not differ significantly from those of the wild-type plants or are slightly reduced in the GA20-oxidase-expressing plants.

The expression of the two genes and the related changes in the genetically modified potato plants with genes from *Arabidopsis thaliana* are not expected to pose any risk to human and animal health or to the environment within the scope of the deliberate release trial.

(b) The *nptII* gene

The *nptII* 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. However, kanamycin and neomycin are widely used in veterinary medicine.

Given the substrate specificity of neomycin phosphotransferase, it is not expected that new metabolic products will form in the genetically modified potatoes in the absence of substrate under field

conditions. Since the relevant antibiotics are not present in the soil in elevated concentrations, the neomycin phosphotransferase does not confer any selective advantage to the genetically modified plants under field conditions. There is no evidence to suggest that this enzyme is toxic to plants, animals, microorganisms or humans.

(c) The coding sequence of the α -fragment of the β -galactosidase, *lacI* sequences

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

The multiple cloning site of this vector is located within the sequence coding for the α fragment of the β -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 various expression cassettes 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 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 contain a fragment of the *ocd* gene (ornithine cyclodeaminase), which is located between the 3' terminus of the translated sequence of the *nptII* gene and the *nos* terminator sequence. Since this sequence is transcribed as part of the mRNA of the *nptII* gene, but is located downstream of the termination codon of the *nptII* 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 T-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 *Agrobacterium tumefaciens*,
- Terminator of the octopine synthase gene from *Agrobacterium tumefaciens*.

In the genetically modified plants, the promoter and terminator sequences regulate the expression of the DNA sequences located between them. Further information on the effects associated with the expression of these sequences in the plants can be found in 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 in *Agrobacterium*-mediated transformation events. However, the transfer of DNA fragments outside the border regions has been reported. A PCR analysis demonstrated the presence of the antibiotic resistance gene *nptIII* in all 6 genetically modified potato lines intended for release, which means that the T-DNA border regions were not taken into regard during transformation.

Outside the border regions, the transformation vector pBIN19 contains:

- The *aphAIII* (= *nptIII*) gene from *Streptococcus faecalis* (= *Enterococcus faecalis*), interrupted by the transposon *IS1*, but functional in prokaryotic systems;
- The *tetA* gene of the plasmid RK2, interrupted by the T-DNA;
- The *trfA* gene of the plasmid pRK2 for replication in *E. coli* and in *A. tumefaciens*;
- A fragment of the *klaC* gene from *Klebsiella aerogenes*;
- A *traF* fragment, containing the *oriT* of the plasmid RP4 from *E. coli*;
- The origin of replication *oriV* of the plasmid RK2 from *E. coli*;
- The origin of replication of the plasmid pUC (ColE1 *ori*) from *E. coli*.

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

The origins of replication *oriV* and *oriT* 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 or the remaining 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 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 *npftII* 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 the formation of viable seeds. The tubers will be analysed after harvest or stored for replanting in the following year. Surplus tubers 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. 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 proposed post-trial monitoring.

In previous greenhouse experiments carried out by the applicant, the genetically modified plants differed from non-genetically modified potato plants by their enhanced or retarded stem elongation growth. A change in the flower morphology of the genetically modified potato plants was not observed. The flowers of potato plants are often sterile, which is why potatoes are usually propagated vegetatively via tubers. The risk for the environment as a result of possible changes in flowering time or flower morphology is thus negligibly low. The tuber yields under greenhouse conditions either do not differ significantly from those of wild-type plants or are slightly reduced. The possible effects of a temporal shift in tuber development caused by the genetic modification on the persistence of the plants are planned to be examined within the scope of this deliberate release trial.

A possible change in the frost sensitivity of the tubers as a result of the genetic modifications in the various lines cannot be ruled out. This possibility is adequately addressed by the proposed two-year cultivation gap and post-trial monitoring.

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 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.

(a) The genes of the gibberellin metabolism

The genes for the enzymes of gibberellin synthesis are derived from *Arabidopsis thaliana*, i.e. are already widespread in the environment. Horizontal gene transfer from non-genetically modified organisms to microorganisms is thus far more likely to occur

(b) The *nptII* gene

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 *nptII* 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 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 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.

(c) Other sequences within the border region of the plasmid used for transformation

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.

(d) Sequences located outside the T-DNA

In the case of the lines created 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 *nptIII* was confirmed in all 6 genetically modified potato lines intended for release.

Outside the border regions, the transformation vector pBIN19 contains:

- The *aphAIII* (= *nptIII*) gene from *Streptococcus faecalis* (= *Enterococcus faecalis*), interrupted by the transposon *IS1*, but functional in prokaryotic systems;
- The *tetA* gene of the plasmid pRK2, interrupted by the T-DNA;
- The *trfA* gene of the plasmid pRK2 for replication in *E. coli* and in *A. tumefaciens*;
- A fragment of the *klaC* gene from *Klebsiella aerogenes*;
- A *traF* fragment, containing the *oriT* of the plasmid RP4, from *E. coli*;
- The origin of replication *oriV* of the plasmid RK2 from *E. coli*;
- The origin of replication of the plasmid pUC (ColE1 *ori*) from *E. coli*.

According to literature references, the *nptIII* gene, which is 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, 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 or interrupted.

The insertion element *IS1* 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 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.