Notification 6786-01-0079

Summary of the risk assessment of genetically modified potato plants (*Solanum tuberosum* L.) (five independent lines of two different transformation events) carried out by the German Competent Authority within the framework of a proposed deliberate release, Berlin, 21 April 1998

Explanatory note to this document:

The following text reflects the summary of the risk assessment of (a) genetically modified organism(s) to be used for experimental field trials (deliberate releases) in Germany. The text forms part of the official authorisation regarding applications for the permit of deliberate releases (field trials) of genetically modified organisms in Germany under the legal framework of Directive 2001/18/EC and the German Gene Technology Act (Gentechnikgesetz, GenTG). The authorisation is issued by the Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, BVL [*Federal Office of Consumer Protection and Food Safety*], as the German Competent Authority. It comprises the chapters

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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 gene for the cytosolic fructose 1,6-bisphosphatase from *Solanum tuberosum* in antisense orientation as well as the gene for the anorganic pyrophosphatase from *Escherichia coli* in sense orientation.

The coding region for the cytosolic fructose 1,6-bisphosphatase (FBPase) which is contained in the genetically modified potato plants under the control of the CaMV 35S promotor is expressed constitutively, i.e. not tissue-specifically. The coding region of the FBPase was arranged in reverse (antisense) orientation relative to the promoter. In the genetically modified plants, this causes the formation of an antisense RNA which inactivates the transcript of the endogenous FBPase gene, thus preventing formation of the FBPase. Inhibition of the cytosolic FBPase in the transgenic potato plants leads to a reduction of their sucrose content. An increase in the level of toxic compounds is not anticipated.

The coding region for the anorganic pyrophosphatase (PPase) which is contained in the genetically modified potato plants under the control of the promoter of the FBPase gene is expressed tissue-specifically, primarily in the photosynthetically active mesophyll cells. Expression of the PPase gene from *E. coli* leads to the production of this enzyme in the genetically modified potato plants. Removal of the cytoplasmic pyrophosphate favours the production of sucrose in the metabolism of the transgenic potato plants. The possibility of conversion of the soluble sugar into secondary plant compounds cannot be ruled out. However, this is not expected to affect the composition of the potato tubers.

As a result of the genetic modifications, the capacity for sucrose biosynthesis in the transgenic potato plants is either reduced by switching off expression of the FBPase gene in a targeted manner or increased through the expression of the heterologous PPases. The tubers of the transgenic potato plants were not examined, inter alia, for alterations in their starch or sucrose content in comparison to non-transgenic potato tubers. However, on the basis of the characteristics of the enzymes encoded by the inserted DNA, it can be assumed that no safety-relevant changes have occurred.

After the end of the trial, the harvested tubers of the genetically modified plants will be transferred to an appropriate facility and are not intended for use as food or feed. However, even in the event of unintentional consumption by animals or humans, no harmful health effects would be expected to result.

(b) The *nptII* gene

The *nptII* gene transferred to the genetically modified plants encodes the enzyme neomycin phosphotransferase. It was inserted as a marker gene for the selection of transformed plant cells.

The neomycin phosphotransferase is a type II aminoglycoside 3'-phosphotransferase (APH(3')II), which 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 as well as paromomycin belong to the APH(3')II enzyme substrates. The clinically relevant antibiotic gentamicin 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. Due to the substrate specificity of neomycin phosphotransferase, in the absence of substrate under field conditions no new metabolic products are expected to arise 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 to the genet-

ically 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

To generate the genetically modified plants, vectors which derive from the plasmid pBIN19 were used. In these vectors, the multiple cloning site is located within the coding sequence of the α-fragment of the β-galactosidase from *E. coli*.

The native enzyme β-galactosidase cleaves β-D-galactoside into galactose and the corresponding alcohol compound. The most physiologically important substrate is lactose, which is hydrolysed into galactose and glucose. The α-fragment refers to the first 146 amino terminal amino acids of β-galactosidase. By itself, the α-fragment is not enzymatically active, but complementation may occur in suitable hosts.

Through the insertion of the PL3 gene into the multiple cloning site, the coding sequence for the α-fragment of the β-galactosidase was interrupted so that in this form, *inter alia* in *E. coli* bacteria, it no longer has the ability to code for an α-fragment that 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 probably also contain 5' and 3' sequences of the repressor gene *lacI*. However, these 5' and 3' sequences are separated from one another by the *lacZ* and M13 *ori* sequences. The *lacI* sequences are unlikely to have any functional capacity in the genetically modified plants.

(d) M13 sequences

The genetically modified plants, which were created by transformation with derivatives of the vector pBIN19, probably contain two fragments from M13mp19, namely, a 440-bp fragment comprising part of an open reading frame of a structural protein of M13 as well as a 433-bp fragment containing the origin of replication of the M13 phage.

If transcription of the fragment of the open reading frame of the structural protein were to occur in the genetically modified potato plants, a functional protein not would not result because the fragment encodes only 167 amino acids of a total of 423 amino acids of the complete phage protein. Therefore, the presence of this fragment is not expected to affect the metabolism of the plants.

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. This origin of replication in not expected to be functional in plants.

(e) The fragment of the *ocd* gene

The plants, which were created by transformation with derivatives of the vector pBIN19, probably 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 termination sequence. Because this sequence is transcribed as part of the mRNA of the *nptII* gene, but is located behind the termination codon of the *nptII* gene, the sequence is not expected to be translated.

(f) Border sequences from Ti plasmids and regulatory sequences

The genetically modified plants contain sequences from the left and the right border region of the TL-DNA of the plasmid pTiT37 from *A. tumefaciens*. These sequences, dependent on the gene products of the *vir* regions of the helper plasmid pGV2260 present in the *Agrobacterium* strain used in the transformation, which was not transferred to the plants, cause the genes located between the border regions to integrate into 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 genetically modified plants contain the following regulatory sequences integrated into the genome:

- the promoter region of the FBPase gene from potato,
- the 35S promoter and terminator of Cauliflower mosaic virus (CaMV),
- the promoter and terminator of the nopaline synthase gene from *A. tumefaciens,*
- the terminator of the octopine synthase gene from *A. tumefaciens.*

The promoter and termination sequences regulate the expression of the DNA sequences located between them for the cytosolic fructose 1,6-bisphosphatase from potato in antisense orientation and for the anorganic pyrophosphatase from *E. coli* in sense orientation as well as the expression of the *nptII* gene in the genetically modified plants. Further information on the effects of the expression of these sequences in the plants are found under point 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 during *Agrobacterium*-mediated transformation. However, individual cases of the transfer of sequences outside the borders have been reported and, on the basis of the information contained in the notification, this possibility cannot be ruled out. Therefore, the risk assessment also considers those areas of the vectors used for the transformation that are located outside the border regions. These include, in particular, the following sequences:

(i) the *nptIII* 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 which are required for 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 *nptIII* gene;

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

Because the *nptIII* gene (i) is under the control of a bacterial promoter, it is not expected to be expressed in plants. Even in the event of its expression, the gene is not expected to affect the metabolism of the plant.

The origins of replication *oriV* (ii) and *oriT* (iii) of the RK2 plasmid 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 origins of replication of RK2, the origin of replication of pMB1 (viii) or the other DNA fragments of bacterial origin (iv, v, vi, vii) have any function in higher plants. Moreover, some of the DNA fragments are incomplete (v) or interrupted (vi).

(h) 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 insertion on the chromosome, and on the environment at the site of insertion (position effect). Under field conditions the level of expression may be additionally influenced by environmental factors, for instance by temperature. In the present case this means that the starch properties of the genetically modified potato plants might not be altered to the same extent under field conditions as under climate-chamber or greenhouse conditions. This does not imply any risks 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 with these genetically modified plants in the greenhouse and – in the case of one of the lines – in the field, no observations were made that would indicate 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 detected in maize. The inactivation of genes or alterations in gene regulation also take place in a range of other naturally occurring processes, e.g. 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 those 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 its amino acid sequence. However, in previous experiments with the genetically modified plants, as well as in deliberate releases carried out in other countries with plants which express the *nptII* gene under the control of non-tissue-specific promoters, no evidence of increased allergenicity of the plants was found.

As a result of the expression of the DNA sequence for the cytosolic fructose 1,6-bisphosphatase in antisense orientation, the native plant protein is present in low concentrations or not at all. There is no evidence for allergenicity of this protein or of the anorganic pyrophasphatase.

In any case, the pollen of potato plants is dispersed only to a limited extent by wind and in general 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 become established in the environment

The cultivation of potatoes in Central Europe goes back several hundred years. During this time, establishment of potato in natural ecosystems in Europe has not been observed. Potato plants are occa-

sionally found outside cultivated areas, but only on non-natural sites such as waysides and other ruderal areas. Because potato is not frost resistant, no permanent establishment occurs in in those areas either.

As a result of potato cultivation, on land used for agricultural purposes, "volunteer potatoes" can emerge from tubers left in the soil after harvesting. Potato tubers are sensitive to frost. Their survival is therefore affected primarily by winter temperatures.

The likelihood of the genetically modified plants surviving as a result of tubers potentially remaining in the ground after harvesting will be minimised by the measures stipulated in supplementary provision II.6. To remove any remaining tubers from the soil, after the tuber harvest as well as in the spring of the following year, the soil on the trial site is to be loosened to a depth of approx. 15 to 20 cm. Any tubers found are to be destroyed.

Some of the potato varieties intend to be released will flower and produce seeds which are rarely capable of germination under field conditions. Under Central European climate conditions, it is unlikely that potato seeds will overwinter and produce plants.

In the event that tubers or seeds remain in the soil, the resulting plant growth would be detected during the post-trial monitoring period planned by the applicant and stipulated in supplementary provision II.7. The frost sensitivity of the tubers is unlikely to change as a result of the genetic modification. During the monitoring period following completion of the deliberate release, no plants or only plants that do not interfere with monitoring may be cultivated on the trial sites. This means that volunteer potatoes can be detected easily.

For the reasons stated above, an establishment or uncontrolled spread of these genetically modified plants is not expected.

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, genetically modified potato did not hybridise with *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 which, however, turned out to be sterile. Potato and *S. dulcamara* (bittersweet or woody nightshade) proved to be strictly bilaterally incompatible; in crossbreeding experiments, fertilisation of the ovules was not achieved. Potato is also not cross-compatible with tomato (*Lycopersicon esculentum*). In agricultural practice, potatoes are propagated vegetatively using 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 potato plants can be transferred by insects or by wind. However, wind dispersal takes place over short distances only. Potatoes are primarily self-pollinating; foreign pollination is rare, even within a field of flowering potato plants. If at all, it is most likely to occur between neighbouring plants.

The applicant plans to maintain a distance of 20 m between the release trial and nearby potato crops and this is considered adequate. However, should pollen be transferred to potato plants cultivated to

produce table potatoes despite this measure, no adverse effects are to be expected. Planting material for the cultivation of potatoes is propagated vegetatively, i.e. not via seeds. As already explained above, the probability that potentially generated seeds could give rise to plants under the given climatic conditions is very slight. Such plants would be eliminated in the course of conventional soil preparation practices during crop rotation. Even if the tubers of such plants were to be consumed, no health hazards would be expected to result, as follows from the evaluation undertaken in section III.1.2.1.

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 integrated into chromosomes of the recipient organisms. Studies on the transformation ability of soil bacteria under natural conditions suggest that the transfer of plant genetic material to soil bacteria is, in principle, possible, although it is assumed that a gene transfer of this nature would constitute an extremely rare event.

Insofar as we assume that an exchange of genetic material between organisms which are so distantly related in terms of taxonomy as plants and bacteria actually occurs, it follows 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.

The inserted genes, including the regulatory sequences, derive from *S. tuberosum*, *E. coli* and *A. tumefaciens*, respectively, and are thus already widespread in the environment. Therefore, they could also – with a far higher probability – enter microorganisms in the environment by horizontal gene transfer from non-genetically modified organisms.

As already elaborated under point 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. Therefore, it was necessary to examine whether a potential horizontal gene transfer of the *nptII* gene would affect the therapeutic use of the relevant antibiotics.

The inactivation of aminoglycoside antibiotics by phosphorylation has been demonstrated as a natural resistance mechanism in microorganisms in a range of different environments. 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 their 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 noticeably increase.

The gene for the α-fragment of the β-galactosidase is interrupted so that no functional gene product can be produced. This would also be the case in bacteria that receive the gene through horizontal gene transfer. The same applies to the 3’ and 5’ sequences of the *lacI* gene.

A similar situation applies to 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, the fragment of the *ocd* gene is unlikely to be translated, as explained under III.1.2.1.(e).

The genetically modified potatoes probably 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 microorganisms.

The sequences inserted into the potatoes to regulate the transferred genes derive from *A. tumefaciens* and CaMV. Regarding a horizontal gene transfer of these sequences to microorganisms, it should be noted that *A. tumefaciens* is widespread in soils and that a transfer of the relevant sequences from *Agrobacterium* is far more likely than their transfer from the genetically modified plants. The theoretical possibility of a transfer of the CaMV sequences from the genetically modified plants would not constitute a new situation compared to the naturally occurring situation because CaMV, as a double-stranded plant-infecting DNA virus, is already present in plants.

As a general rule, only sequences located within the borders are integrated into the plant genome in *Agrobacterium*-mediated transformation events. However, on the basis of the information contained in the notification, a transfer of sequences outside the borders cannot be ruled out. In the present case, the following DNA fragments could have been integrated into the genetically modified plants through the integration of sequences located outside the borders:

(i) the *nptIII* gene from *Streptococcus faecalis* (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 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 insertion element IS1 within the *nptIII* gene;

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

According to the literature references, the *nptIII* gene (i), which may be contained in the genetically modified plants with 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 where it serves 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 selective 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 to vi), the probability of a spread by transfer between bacteria is far higher than the probability of a spread via 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) is naturally present in various species of *Enterobacteriaceae*. For example, it has been found in species of the genera *Escherichia*, *Shigella*, *Klebsiella*, *Serratia* and *Salmonella*. In the case of IS1, the number of copies per bacterial genome can amount to > 40 copies. Copies of IS1 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 between bacteria via horizontal gene transfer. By comparison, the risk of spreading from the genetically modified plants to microorganisms via horizontal gene transfer, although theoretically conceivable, is negligible.

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 replicate 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

To generate the genetically modified plants, the explants of the two potato varieties were inoculated with agrobacteria which contained the genes to be transferred between the border regions of binary vector plasmids. Following transformation, the regenerated potato plants were tested to determine whether they were free of agrobacteria. Only potato plants that were free of agrobacteria were used further.

In contrast to the ubiquitous wild forms of *A. tumefaciens*, the *Agrobacterium* strain used for the transformation is disarmed, i.e. it no longer has the capacity to induce tumours. In the unlikely but theoretically conceivable event of an *Agrobacterium*-mediated transfer of the inserted foreign genes to a cell of another plant, that cell would have to spontaneously regenerate into a whole, fertile plant for the foreign genes to enter the germ cells and thereby be passed on the progeny 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 vector plasmids contained in the agrobacteria to wild-type agrobacteria present in the environment (*A. tumefaciens* or *A. rhizogenes*) 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 by 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 under III.1.2.4..