Notification 6786-01-0059

Summary of the risk assessment of genetically modified potato plants (*Solanum tuberosum* L.) (lines Ka1 to Ka4; DHL 59/1 to DHL 59/20; fusions- and crossing products of these with dihaploid breeding material) carried out by the German Competent Authority within the framework of a proposed deliberate release, Berlin, 29 April 1997

Explanatory note to this document:

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

I. Consent [to the application]
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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

III.1.2.1.1. The cDNA for the coat protein of Potato virus Y (PVY)

The genetically modified potato plants contain the cDNA for the coat protein (cp) of Potato virus Y (PVY) expressed under the control of the 35S promoter of Cauliflower mosaic virus (CaMV). The inserted cDNA for the PCV-cp contains, in front of the open reading frame of the PVY-cp gene, three ATG start codons in combination with corresponding stop codons so that in the case of expression only short peptides would result from this arrangement; only the double start codon, which was removed from the transcription start at nucleotide 124, could be responsible for initiating translation of the PVY-cp. As a result of this large gap, the translation efficiency of the construct is assumed to be extremely low. Extremely low amounts of the recombinant PVY-cp can be detected in the genetically modified potato plants only after stress induction.

The transcript of the cDNA for the PVY-cp is supposed to confer resistance to infection by PVY to the genetically modified plants. Comparable studies have shown that the degree of this resistance is proportional to the transcription rate of the transgene and inversely proportional to the accumulation rate of the transgenic protein.

An infection of the genetically modified potato plants with RNA viruses with the resulting possibility of recombination with the transcript of the PVY-cp gene, would not result in a situation that is fundamentally different from that already present in non-genetically modified potato plants which are simultaneously infected with PVY and other viruses.

Gene products of Potato viruses enter the food chain via naturally infected potatoes without any harmful effects on animals or humans being reported. As also basically explained and assessed in the OECD document OECD/GD(96)162 [OECD Series on Harmonization of Regulatory Oversight in Biotechnology, No. 5], in the event of consumption of parts of the genetically modified potatoes, these gene products would be degraded in the digestive tract of humans and animals.

III.1.2.1.2. The *gus* gene

The *gus* gene in the genetically modified potato plants codes for the enzyme β-glucuronidase (GUS). The enzyme cleaves glucuronides hydrolytically. Certain substrates such as 4-nitrophenyl β-D-glucuronide or 4-methylumbelliferyl β-D-glucuronide produce coloured reaction products following hydrolysis. The *gus* gene from *E. coli* was inserted as part of the T-DNA into the genome of the potato plants as a reporter gene to provide histochemical proof of successful transformation. Expression of the gene is controlled by the 35S promoter of CaMV. Based on current knowledge of the catalytic activity of the GUS enzyme, plants are not expected to gain a selective advantage through expression of the *gus* gene under field conditions. The enzyme β-glucuronidase is widespread in nature. It is found in tissues of vertebrates, invertebrates and in bacteria, but not in plant tissues. The genetically modified plants are not intended for consumption. Nevertheless, should parts of the plants be consumed, adverse effects on human or animal health would not be expected to result because the GUS enzyme would be degraded in the digestive tract.

III.1.2.1.3. 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 genetically modified plants under field conditions. There is no evidence to suggest that this enzyme is toxic to plants, animals, microorganisms or humans.

III.1.2.1.4. Other DNA fragments located within the T-DNA

(a) The vector pBIN19, in which the multiple cloning site is located within the coding sequence of the α-fragment of the β-galactosidase from *E. coli*, was used to generate the genetically modified plants.

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. The α-fragment by itself is not enzymatically active, but complementation may occur in suitable hosts.

Through the insertion into the multiple cloning site of the GBSS gene in antisense orientation, 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 expressed under the control of a bacterial promoter. A functional gene product is not encoded by this sequence. Changes in the genetically modified potato plants 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.

(b) 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 a 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.

(c) 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.

(d) 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.

Integrated into the genome, the genetically modified plants contain the 35S promoter of Cauliflower mosaic virus (CaMV) as well as the promoter of the nopaline-synthase gene from *A. tumefaciens* as regulatory sequences. Both promoters regulate the expression of the cDNA or genes located downstream of them. The consequences of the expression of these sequences in the plants are described in detail in sections III.1.2.1.1. to III.1.2.1.3.

III.1.2.1.5 Sequences located outside the T-DNA

As a general rule, with *Agrobacterium*-mediated transformation, only DNA located within the border regions is integrated into the plant genome. 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.

According to the information provided in the notification, 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 (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 insertion element 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. The gene is therefore 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, respectively, provided the mobilisation functions are supplied by a helper plasmid. There is no evidence to suggest that *oriV* or *oriT* 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).

III.1.2.1.6. 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 resistance trait of the genetically modified potato plants might not be pronounced to the same extent under field conditions as under climate-chamber or greenhouse conditions, i.e. resistance could be enhanced or reduced in the field. This is not expected to pose a risk to the environment or to human or animal health.

The insertion of foreign genes may influence the expression or regulation of the plant's own genes at or near the site of insertion. Such processes may alter plant metabolic pathways. However, during the course of the work carried out to date with these genetically modified plants in the greenhouse, 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 *npt*II gene under the control of non-tissue-specific promoters, no evidence of increased allergenicity of the plants was found.

Expression of the cDNA for the PVY-cp in the genetically modified potato plants is not expected to result in increased allergenic potential because after PVY infection this viral coat protein is also present in non-transgenic potato plants and allergenicity due to its presence has not been observed.

In any case, the pollen of potato plants is dispersed only to a small extent by wind and generally it 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. However, potato plants are sometimes found outside cultivated areas, for example on non-natural sites such as waysides and other ruderal areas. But because potato is not frost hardy, it does not become established in these areas.

As a result of potato cultivation, on surfaces used for agriculture, "volunteer potatoes" can emerge in the subsequent growing season from tubers which have remained in the ground 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. After tubers have been harvested, the soil on the release site is to be loosened to a depth of approx. 15 to 20 cm in order to remove any remaining tubers from the soil. Any tubers found are to be destroyed.

Even if the potato lines proposed for release flower and produce seeds, under Central European climate conditions it is unlikely that they would survive the winter and give rise to 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 post-trial monitoring period, no plants or only plants that do not impede monitoring may be cultivated on the field sites. This means that volunteer potatoes can be identified easily.

For the reasons stated above, the genetically modified plants are not expected to establish or persist 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 hybridisation between the genetically modified potatoes and *Solanum nigrum* (black nightshade) took place. 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 pollination of the ovule was not achieved.

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 only over short distances. 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 site and neighbouring potato cultivation plots and this is considered adequate for the purposes of the proposed trial. However,

should pollen be transferred to potato plants cultivated to produce table potatoes despite this measure, no adverse effects are to be expected. Potato plants for the cultivation of potatoes are 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 stated in 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 the 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 as 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.

PVY is a naturally occurring virus. Therefore, its cp gene could also – with a far higher probability – enter microorganisms in the environment by horizontal gene transfer from PVY-infected, non-genetically modified organisms.

As already elaborated under III.1.2.1.3., the antibiotics inactivated by the neomycin-phosphotransferase are of little relevance in human medicine but are widely used in veterinary medicine. Whether a potential horizontal gene transfer of the *nptII* gene would compromise the therapeutic use of the relevant antibiotics was examined as a precautionary measure.

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 the effective transfer between microorganisms by conjugation. Even in the event of a horizontal gene transfer from the genetically modified potatoes to microorganisms, the overall frequency of this resistance mechanism would not be noticeably increased.

The β-glucuronidase gene from *E. coli* that was transferred to the potato plants codes for β-D-glucuroniside-gluconohydrolases, which cleave glucuronides hydrolytically. They are widespread in nature and found e.g. in vertebrates, invertebrates and bacteria. Therefore, a gene transfer of the *gus* gene from the genetically modified potato plants to microorganisms is unlikely to noticeably increase the prevalence of this gene.

Also, in the case of a transfer of the other regulatory sequences used in the constructs, there are no grounds for concern that the overall frequency of the relevant DNA sequences would be increased. The regulatory sequences derive from *A. tumefaciens* and CaMV. *A. tumefaciens* is widespread in the

soil and the sequences in question are found on Ti-plasmids in wild-type agrobacteria which can be exchanged between different *Rhizobiaceae* by conjugation. 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.

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.1.2.1.4.(c).

The genetically modified potatoes probably contain the origin of replication from 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 (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.

For the *nptIII* gene (i), as for the *nptII* gene (see above), the corresponding resistance mechanism is widespread in bacteria.

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. In comparison, (the probability of) a theoretically conceivable spread from the genetically modified plants to microorganisms via horizontal gene transfer 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

In order to generate the genetically modified plants, sterile potato leaves were inoculated with agrobacteria which contained the genes to be transferred between the border regions of the binary vector plasmid. After transformation had occurred, antibiotic treatment was carried out to eliminate the agrobacteria. In order to demonstrate that the propagation material of the plants proposed for deliberate release was free from agrobacteria, tissue homogenates were spread onto appropriate culture media. No agrobacteria were detected in the cultures.

In contrast to the ubiquitous wild-type *Agrobacterium*, 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 that the inserted foreign genes are transferred 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. 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 vector plasmid 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..