Notification 6786-01-0127

Summary of the risk assessment of genetically modified potato plants (*Solanum tuberosum* L.) (transformation events VR/T18; VR/T21; VR/T23 and amf/T85; amf/T103; amf/T121) carried out by the German Competent Authority within the framework of a proposed deliberate release, Berlin, 13 June 2001

Explanatory note to this document:

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

I. Consent [to the application]
II. Provisions [to be respected in execution of the field trials]
III. Justification
III.1. Requirements for approval according to section 16 GenTG [German Gene Technology Act]
III.1.1. Requirements for approval according to section 16 (1) Nr. 1 GenTG
III.1.2. Requirements for approval according to section 16 (1) Nr. 3 GenTG
III.1.3. Requirements for approval according to section 16 (1) Nr. 2 GenTG
III.1.4. Formal requirements according to section 16 (4, 5) GenTG
III.2 Appraisal of and reply to objections
IV. Costs
V. Legal instruction

Only the original German document is legally binding. The following passage is a courtesy translation of the chapter III.1.2. and was prepared for the Biosafety Clearing-House.

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

(a) The modified *pr17* gene of the potato leafroll virus (PLRV)

During the development cycle of the virus, the gene codes for a 17-kDa protein (pr17). This is assumed to be a transport protein which spreads the viral genome as a ribonucleoprotein complex by the passage through plasmodesmata in the phloem of infected plants. It is probably non-essential for virus replication. The *pr17* gene is a part of the coat protein gene (*cp*) located on ORF3 of the PLRV genome, but it is encoded by a second reading frame.

A modified *pr17* gene under the control of the 35S promoter and terminator of the cauliflower mosaic virus was inserted into the potato plants by *Agrobacterium*-mediated transformation. The modification of the *pr17* gene consists in a 5' terminal extension by fusion to the multiple cloning site (polylinker) of the vector pBluescript. By targeted mutagenesis, the first two AUG translation start codons of the *pr17* gene were changed into ACG codons and an AUG codon was inserted into the polylinker sequence.

In the genetically modified potato plants, this construct causes the formation of a pr17 protein which is extended at the N terminus. As a result of the extension, the amphipathic character of the protein oligomerisation domain is changed. In the event of a virus infection, the protein so modified could inhibit the formation of the viral transport structures in the genetically modified plants by forming hetero-oligomers with the wild-type transport protein encoded by the viral genome. This could prevent the long-distance transport of viral RNA in the phloem and consequently the spread and further multiplication of the virus in the plant. Greenhouse experiments revealed that the genetically modified plants were resistant to the potato viruses PLRV, PVX and PVY. This result suggests a similar phloem transport mechanism of non-related plant viruses and could mark the starting point for the generation of "broad-spectrum virus resistance" in plants using genetic engineering methods.

Basically, there is a possibility of recombination with the wild-type *pr17* gene in the phloem of non-genetically modified potato plants that are simultaneously infected with both the PLRV and other viruses. Sequence homologies between the PLRV and other widespread potato viruses which could promote the recombination of these viruses with PLRV sequences were not identified. Since these viruses differ fundamentally from the PLRV in regard to their genome structures and translation strategies and, in addition, are already capable of systemic spread in the plant, the development of virulent viruses is not expected even if recombination events were to occur.

Recent experiments with genetically modified tobacco plants which express a modified "movement protein" MP17 of the PLRV have shown that the protein changes the plasmodesmata in the phloem. This has an influence on the distribution of assimilates. The accumulation of assimilates can lead to the induction of specific defence genes. This would then also explain the resistance to various phytopathogenic organisms, such as *P. infestans*.

Under greenhouse conditions, no phenotypic changes in the genetically modified plants were observed that would suggest any effects of the expressed extended pr17 protein on plant metabolism.

The tubers of the genetically modified plants are not intended for consumption. Even in the event of unintentional consumption by animals or humans, the specified changes are not expected to have any adverse health effects.

In potato cultivation, PLRV infections of plants occur regularly. Tubers harvested from such plants contain not only the virus but also the pr17 protein. Such tubers are consumed by humans without this causing any adverse health effects. In many cases, the concentration of the wild-type pr17 protein in plants infected with the PLRV is higher than that of the modified pr17 protein in the genetically modified plants.

(b) The cDNA for the granule-bound starch synthase (GBSS) from potato in antisense orientation

The genetically modified plants contain cDNA of the granule-bound starch synthase (GBSS) under the control of the GBSS promoter *Wx*, including an "untranslated leader (ul)" sequence from potato as well as the 35S terminator of the cauliflower mosaic virus (CaMV).

The cDNA is arranged in antisense orientation relative to the promoter. In the genetically modified plants, this causes the formation of an antisense RNA which inactivates the endogenous transcript of the respective gene, reducing or inhibiting the production of the corresponding endogenous potato enzyme.

The ul sequence serves to optimise the transcription of the transgene. Just like the promoter, it is commonly found in the GBSS gene of non-genetically modified potatoes and thus involves no hazard potential.

As a result of the genetic modifications, the starch metabolism in the genetically modified potato plants was altered by targeted inhibition of the expression of the enzyme involved in starch metabolism to the effect that the synthesised starch was modified in terms of structure and/or composition. Based on previous experiments with plants cultivated in the greenhouse, the modified starch differed from normal potato starch in its amylose content.

After the end of the trial, the harvested tubers of the genetically modified plants will be transferred to a genetic engineering facility and are not intended for consumption or use as animal feed. Even in the event of unintentional consumption by animals or humans, no adverse health effects are expected. The starch contained in the genetically modified potato plants consists of the same basic components (amylopectin and amylose) as conventional potato starch. The altered starch composition and structure may have a (positive or negative) influence on the digestibility of the potato starch. Even in the event of consumption, however, the change in digestibility would not give reason to fear any adverse health effects.

(c) The *npt*II gene

The *npt*II gene was inserted into all genetically modified potato clones under the control of the nopaline synthase promoter and terminator. The *npt*II gene serves as a marker gene for the selection of transformed plant cells and codes for the enzyme neomycin phosphotransferase.

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.

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

The genetically modified plants of the recipient variety "Tomensa" were created by using derivatives of the vector pBIN19, the multiple cloning site of which is located within the sequence coding for the α fragment of the ß-galactosidase from *E. coli*.

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

The sequence coding for the α fragment of the β-galactosidase was interrupted by the insertion of the modified *pr17* gene 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 potato plants.

The genetically modified plants are likely to 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.

(e) M13 sequences

The genetically modified plants created by transformation using a derivative of the vector pBIN19 are likely to 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.

(f) The fragment of the *ocd* gene

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

(g) Border sequences from Ti plasmids and regulatory sequences

The genetically modified plants contain sequences of the left and right border region of the TL-DNA of the pBIN19 derivatives p17N (I.) and pWx/ul-asGBSS (II.) from *A. tumefaciens*. Depending on the gene products of the *vir* region of the helper plasmid pAL4404 that is contained in the *Agrobacterium* strain 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:

(I.)

- The 35S promoter of the cauliflower mosaic virus (CaMV),
- The 35S terminator of the CaMV,

- Promoter and terminator of the nopaline synthase gene from *A. tumefaciens*.

In the genetically modified plants, the promoter and terminator sequences regulate the expression of the modified *pr17* gene as well as the *npt*II gene. Further information on the effects associated with the expression of these sequences in the plants can be found in III.1.2.1 (a) and (c).

(II.)

- Promoter region of the GBSS gene (*Wx*), including the ul sequence from potato,
- 35S terminator of the cauliflower mosaic virus (CaMV),
- Promoter and terminator of the nopaline synthase gene from *Agrobacterium tumefaciens*,

In the genetically modified plants, the promoter and terminator sequences regulate the expression of the cDNA for the GBSS enzyme of starch metabolism from potato in antisense orientation as well as for the *npt*II gene located between them. Further information on the effects associated with the expression of these sequences in the plants can be found in III.1.2.1 (b) and (c).

(h) 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. The transformation vector used was developed from pBIN19. The vector backbone contains the *npt*III gene, amongst others. A PCR analysis demonstrated the absence of the antibiotic resistance gene *npt*III in the 3 genetically modified, virus-resistant potato lines intended for release (I.), whereas the results of Southern blot analyses of the 3 amylose-free lines (II.) presented by the applicant did not allow for the presence of the *npt*III gene to be ruled out beyond doubt.

The risk assessment of the latter lines is therefore carried out under the assumption that the entire vector has been integrated:

- The *aphAIII* (=*npt*III) gene from *Streptococcus faecalis* (= *Enterococcus faecalis*), interrupted by the transposon *IS*1, under the control of its own promoter, is only functional in bacteria;
- The *tetA* gene ("repressor of tetracycline resistance") 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 *ori*T of the plasmid RP4 from *E. coli*;
- The origin of replication *ori*V 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 *npt*III gene, the expression of which is controlled by a bacterial promoter.

The origins of replication *ori*V and *ori*T of the plasmid RK2 allow for the 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.

(j) 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 regarding their resistance to fungal or viral pathogens are not altered to the same degree in the field as they are under climate-controlled or greenhouse conditions, i.e. their resistance in the field may be increased or reduced. 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 in the greenhouse and in the field as part of previous deliberate release trials with comparable constructs conducted by different applicants, 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 other plants that express the same genes, no evidence was found to suggest an increased allergenic potential of these 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.9. 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 "Tomensa" can flower and produce seeds. Under the present Central European climate conditions, it cannot be ruled out that potato seeds or tubers 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 phenotype of the genetically modified potatoes did not differ from that of the control plants. The expression of the granule-bound starch synthase (GBSS) in the genetically modified potatoes influences the starch composition in the tubers. A possible change in the frost sensitivity of the tubers as a result of the genetic modifications 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.

The proposed isolation distance of at least 10 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.

Microorganisms that would express the extended pr17 protein would not have a significant selective advantage, since pr17 is a protein of a plant-specific virus, the primary function of which is assumed to be to change the exclusion limit of plasmodesmata. The horizontal gene transfer of the modified *pr17* gene inserted into the genetically modified plants to microorganisms is not expected to have any adverse effects on the environment.

The GBSS gene and the GBSS promoter (*Wx*), including the ul sequence, are derived from potatoes, i.e. are already widespread in the environment. Horizontal gene transfer from non-genetically modified organisms to microorganisms in the environment is thus far more likely to occur.

As already elaborated in III.1.2.1 (c), the antibiotics inactivated by the neomycin phosphotransferase are of little relevance in human medicine but are widely used in veterinary medicine. It was thus necessary to examine whether the clinical use of the relevant antibiotics would be affected by a potential horizontal gene transfer of the *npt*II gene.

In soil microorganisms, the inactivation of aminoglycoside antibiotics by phosphorylation is a naturally occurring resistance mechanism. APH(3')II enzymes have also been found in 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.

Even if the other regulatory sequences used in the constructs were to be transferred, there is no reason to fear that the overall frequency of the corresponding DNA sequences will increase. The GBSS promoter occurs naturally in potatoes. The other regulatory sequences are derived from *A. tumefaciens* and CaMV. *A. tumefaciens* are ubiquitously present in the soil and in wild-type agrobacteria the specified sequences are located on Ti plasmids, which can be exchanged between different strains of *Rhizobiaceae* by conjugation. The theoretical possibility of transfer of the CaMV sequences from the genetically modified plants would not constitute a new situation compared to that found in nature, because CaMV as a double-stranded plant-infecting DNA virus is commonly found in plants.

As a rule, only the sequences located within the border regions are integrated into the plant genome in *Agrobacterium*-mediated transformation events. However, the transfer of sequences outside the border regions cannot be ruled out based on the information provided in the application. The genetically modified potato plants of the recipient variety "Tomensa" (at least the constructs with the antisense GBSS; II.) thus may also contain nucleic acid sequences from the regions of the binary plasmids outside the T-DNA (see III.1.2.1 (h)). Regarding the origins of replication of the plasmids pBR322 and pVS1, it should be noted that they are found in gram-negative bacteria. In the case of these nucleic acid sequences, the probability of genetic spread by transfer between bacteria is thus far higher than the probability of horizontal gene transfer from the genetically modified plants to microorganisms.

The genetically modified potato plants contain the α fragment of the β-galactosidase gene, which is interrupted by insertion of the modified *pr17* gene, 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 (f), the fragment of the *ocd* gene is not likely to be translated.

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

The sequences inserted into the potatoes to regulate the transferred genes are derived from *A. tumefaciens*, *S. tuberosum* and CaMV. The theoretical possibility of transfer of the CaMV sequences from the genetically modified plants would not constitute a new situation compared to that found in nature, because CaMV as a double-stranded plant-infecting DNA virus is commonly found in plants.

The following additional DNA fragments may have been integrated into the genetically modified plants by the transfer of sequences located outside the border regions:

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

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

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

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

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

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

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

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

A PCR analysis performed by the applicant did not confirm the presence of the *npt*III gene in the lines VR/T18, VR/T21 and VR/T23, whereas its presence in the lines amf/T85, amf/T103 and amf/T121 could not be ruled out. According to literature references, the *npt*III gene (i), which may be contained in the genetically modified plants under the control of its own promoter, confers resistance not only to kanamycin and neomycin, but also to the antibiotic amikacin. In Germany, amikacin is not authorised for use as a veterinary medicinal product but it may be employed in human medicine as a so-called reserve antibiotic. Because of its status as a reserve antibiotic and its attendant infrequent use, amikacin resistance is so far not widespread. Given the low probability of a horizontal gene transfer from plant DNA to microorganisms and the absence of selection pressure on the release sites, it can also be assumed that the presence of this gene in the genetically modified potato plants would not lead to a significant increase in the overall frequency of this resistance mechanism in microorganisms.

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

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

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

III.1.2.5 Agrobacteria used to generate the genetically modified plants

In order to generate the genetically modified plants, sterile potato leaves were incubated with agrobacteria which harbour the genes to be transferred between the border regions of the binary vector plasmid. Following transformation, antibiotic treatment was carried out to eliminate the agrobacteria. The regenerated plants were examined for the presence of agrobacteria by incubating plant homogenates in suitable growth media. The agrobacteria used for transformation were not detected. 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.