# Notification 6786-01-0106

## Summary of the risk assessment of genetically modified potato plants (*Solanum tuberosum* L.) (lines DARA5 and DARA12) carried out by the German Competent Authority within the framework of a proposed deliberate release, Berlin, 23 July 1999

**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]
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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

The genetically modified potato lines DARA5 and DARA12 were transformed using the vector pMAB316::pTiB6S3-SE, a so-called cointegrate vector. Cointegrate vectors were developed in the early 1980s for the transformation of plants: The region of the T-DNA of the Ti plasmid that conveys the tumour-inducing properties of *A. tumefaciens* is replaced by a known region that is used for homologous recombination with an intermediate vector. As a result, the Ti plasmid is "disarmed". The target sequences in the intermediate vector are transferred into the transformation vector by homologous recombination and subsequently into the plant genome via *A. tumefaciens*. The structure of the Ti plasmid outside the T-DNA remained largely unchanged.

In the present case, that fragment of the $T_L$-DNA which codes for the phytohormone synthesis and the octopine synthase as well as the right border region and the entire $T_R$-DNA of the wild-type Ti plasmid pTiB6S3 were replaced by a kanamycin resistance gene from the transposon Tn903 by double homologous recombination to create the disarmed vector pTiB6S3-SE (split end vector).

The right T-DNA border region required for the transformation is provided after cointegration of an intermediate vector, in this particular case pMAB316. The vector backbone of pMAB316 consists of the origin of replication (*ori*) from pBR322, the left inside homology (LIH) for homologous recombination with Ti plasmids resident in *Agrobacterium*, the right T-DNA border sequence as well as the nopaline synthase gene of the Ti plasmid pTiT37, the streptomycin/spectinomycin resistance gene from the transposon Tn7 and the *npt*II gene of the transposon Tn5 under the control of the *nos* regulatory elements. As the target gene, the sequence of the phytochrome B gene from *Arabidopsis thaliana* was cloned between the 35S promoter and the *nos* termination signal of the pMON316 in pMAB316. Due to the cointegrate formation, the entire cloning vector, except for the origin of replication, is located within the T-DNA.

(a) The *phy*B gene for phytochrome B

The *phy*B gene from *A. thaliana* codes for a photoreceptor which is converted into its physiologically active form, where it triggers a number of phenotypic changes by absorbing light of 660 nm wavelength. As a result of the genetic modification, the anthocyanin and chlorophyll synthesis is enhanced and the photosynthesis rate is increased in the transgenic plants. Furthermore, a number of morphological and physiological changes were observed in plants of the lines DARA5 and DARA12, such as reduced apical dominance, shorter internodes, dwarfism, delayed senescence, thicker stems, starch deposits and increased specific weight in the stems and leaves, smaller leaves, elongation of the palisade parenchyma cells, enhanced root formation, increased number of tubers and increased tuber yield. In the genetically modified potatoes, the transferred phytochrome B gene from *A. thaliana* is expressed constitutively under the control of the CaMV 35S promoter. Given the complex mode of action of the phytochrome system, the enhanced expression of phytochrome B is expected to have an influence of the secondary metabolism, as demonstrated by the increased anthocyanin (phenol metabolism) and chlorophyll (isoprenoid metabolism) contents.

(b) The *npt*II gene

The *npt*II gene codes for the enzyme neomycin phosphotransferase and was inserted under the control of eukaryotic regulatory sequences as a marker gene for the selection of transformed potato cells. An *npt*II gene under the control of prokaryotic regulatory sequences was used to develop the transformation vector.

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. Clinically relevant gentamicins 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 this substrate specificity of neomycin phosphotransferase, it is not expected that new metabolic products will form in the genetically modified plants in the absence of substrate under field conditions. Since the relevant antibiotics are not present in the soil in elevated concentrations, the neomycin phosphotransferase does not confer any selective advantage to the genetically modified plants under field conditions. There is no evidence to suggest that this enzyme is toxic to plants, animals, microorganisms or humans.

(c) Other DNA fragments located within the T-DNA

- The nopaline synthase gene (*nos* gene)

The T-DNA of the cointegrate vector pMAB316::pTiB6S3-SE contains the *nos* gene from *A. tumefaciens* for the nopaline synthase near the right border region. Nopaline is a non-toxic amino acid conjugate (from $\alpha$-ketoglutarate and arginine or ornithine) that serves as a source of carbon and nitrogen for agrobacteria in plant tumour cells. It is also produced in crown galls of plants infected with phytopathogenic wild-type agrobacteria of the nopaline type. The presence of nopaline synthase activity can be used to verify the insertion of the T-DNA in transformed cells.

- The streptomycin/spectinomycin resistance gene

Within the T-DNA border regions, pMAB316::pTiB6S3-SE contains the bacterial gene *aadA* (enzyme: aminoglycoside-3-adenyltransferase (AAD(3´)(9)) for streptomycin/spectinomycin resistance.

The aminoglycoside-3-adenyltransferase (AAD(3´´)(9)) catalyses the adenylation of the 3´´-OH group of the N-methyl-L-glucosamine ring of streptomycin and the 9-hydroxyl group of spectinomycin, causing these to become inactivated. The enzyme is characterised by its high substrate specificity. Streptomycin and spectinomycin belong to the substrates of the AAD(3´´)(9) enzyme. In human medicine, streptomycin is only used for specific indications (e.g. tuberculosis). Since the *aad*A gene is under the control of its own prokaryotic promoter, it is not assumed to be expressed in the genetically modified potato plants.

- The transcripts 5 and 7 of the LIH

The LIH (left inside homology) of the T-DNA of the plasmid pTiA6 from *A. tumefaciens* comprises 1.8 kb and is sequenced. It offers the homologous region required for the formation of the cointegrate from pTiB6S3-SE and the intermediate vector pMAB316 by homologous recombination. Furthermore, this fragment codes for the transcripts 5 and 7, the functions of which are unknown. As part of the T-DNA of wild-type Ti plasmids, the transcripts of the two transferred genes are involved in the formation of crown galls in plants infected with agrobacteria. In the genetically modified potatoes, crown galls were not observed either in the greenhouse or as part of previous field trials conducted with these plants.

(d) DNA fragments located outside the T-DNA

As a general rule, only DNA located within the border regions is integrated into the plant genome in *Agrobacterium*-mediated transformation events. However, the transfer of DNA fragments outside the border regions has been reported in isolated cases. Since the sequences integrated into the potato plants have not been exactly analysed, the risk assessment is carried out under the assumption that the entire vector has been integrated.

Basically, the following fragments outside the T-DNA border regions are located on a Ti plasmid: the origin of replication for replication in *Agrobacterium*, the virulence region with the *vir* genes – these code for the proteins required for the transfer of T-DNA from the plasmid into the plant genome –, a region for the conjugation functions as well as the genetic information required for opine catabolism. In the present case, this region also contains the bacterial origin of replication ColE1 (ori) from pBR322 as well as an *npt*II gene under the control of prokaryotic regulatory sequences (cf. III.1.2.1 (b)) from the LIH region. The bacterial origin of replication is non-functional in plants.

The use of this plasmid is not known to have any effects on genetically modified plants under field conditions that would suggest any potential risks pursuant to Sec. 1 (1) GenTG.

(e) Border sequences from Ti plasmids and regulatory sequences

The left border region is derived from the plasmid pTiB6S3, the right border region from the $T_L$-DNA from the plasmid pTiT37, both from *A. tumefaciens*. Depending on the gene products of the *vir* region of the helper plasmid 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:

- The 35S promoter and the 35S termination signal of the cauliflower mosaic virus (CaMV),
- The promoter and the termination signal of the nopaline synthase gene from *A. tumefaciens*,
- Prokaryotic regulatory elements of the *aad*A and the *npt*II gene,
- The regulatory elements of the transcripts 5 and 7 from *A. tumefaciens*.

In the genetically modified potato plants, the promoter and termination signals regulate the expression of the genetic elements 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 (a) to (c).

(f) Position effects and context changes; allergenicity

Genes integrated into the plant genome by genetic engineering methods are expressed at different levels, depending on the site of integration on the chromosome and on the neighbouring sequence at the integration site (“position effect”). Under field conditions, the expression level may be influenced by environmental factors, for instance, by temperature. In this particular case, this could mean that the characteristics of the genetically modified potato plants are not altered to the same degree in the field as under climate-controlled or greenhouse conditions. This does not represent a risk to the environment or to human and animal health.

The insertion of foreign genes may influence the expression or regulation of endogenous plant genes at or near the site of insertion. Such processes can affect plant metabolic pathways. In previous work with the genetically modified plants, however, no observations were made that would suggest an adverse effect on the objects of legal protection pursuant to the GenTG.

Mobile genetic elements (transposable elements), which, when transposed within the genome, can exert effects on existing plant genes at the target site, occur naturally in plants and were first identified in maize. The inactivation of genes or alterations in gene regulation also take place in a range of other naturally occurring processes such as point mutations, deletions or translocations and are traditionally used in plant breeding. Therefore, even in non-genetically modified plants, such events can always influence plant metabolic pathways. In this regard, the genetically modified plants to be deliberately released do not differ fundamentally from non-genetically modified plants.

Given the current state of knowledge, it is not possible to make reliable predictions about the potential allergenicity of a protein on the basis of its amino acid sequence. In previous experiments with the genetically modified plants as well as in earlier deliberate release trials with genetically modified plants that express the *npt*II gene under the control of non-tissue-specific promoters, no evidence was found to suggest an increased allergenic potential of the plants.

Pollen of potato plants is dispersed by wind only to a little extent and generally does not play a noteworthy role in triggering pollen allergies.

III.1.2.2 Evaluation of the ability of the genetically modified plants to persist or establish in the environment

The cultivation of potatoes in Central Europe goes back more than 200 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 that have overwintered in the soil.

The tubers are planned to be harvested and analysed or stored for replanting in the following year. Any remaining tubers will be inactivated, for example by shredding, and subsequently composted. The remaining transgenic plant parts will be left on the field to decompose. During the two vegetation periods following the completion of the experiments, a plant species will be cultivated that allows for monitoring the release site and controlling any emerging potato plants. Potatoes will not be cultivated during the two-year post-trial monitoring period. Volunteer potatoes that emerge during this period will be destroyed.

The probability of persistence of genetically modified plants due to any tubers remaining in the ground after harvest is minimised by the measures pursuant to the supplementary provision II.8. To remove any tubers remaining in the ground, the soil on the trial site will be tilled to a depth of about 15 cm after harvesting the tubers. Any tubers found will be inactivated.

Potato plants can flower and produce seeds. It is conceivable that the genetically modified potatoes may be fertilised by the introduction of foreign potato pollen, which is why the formation of seeds cannot be ruled out completely, but is unlikely. Under Central European climate conditions, it is unlikely that potato seeds will overwinter and produce plants. The applicant plans to destroy the potato

plants prior to reaching maturity by means of a haulm topper or chemical methods. This measure serves to prevent seed maturation.

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 post-trial monitoring proposed by the applicant in accordance with the supplementary provision II.9. There is no evidence to suggest a change in the frost sensitivity of the tubers as a result of the genetic modification. In addition, this possibility is adequately addressed by the proposed two-year cultivation gap and post-trial monitoring. 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 *S. 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 20 m between the release site and the nearest 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.

The inserted genes, including the regulatory sequences, are derived from *Arabidopsis thaliana*, *Escherichia coli*, the cauliflower mosaic virus and *Agrobacterium tumefaciens*, 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.

(a) The phytochrome B gene

The gene for phytochrome B is derived from *A. thaliana*. The phytochrome B gene is already widespread in non-genetically modified plants and the environment and is only used here for the purpose of overexpression. Horizontal gene transfer from non-genetically modified organisms to microorganisms in the environment is thus also possible.

(b) The kanamycin resistance gene

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

(c) The streptomycin/spectinomycin resistance gene

In soil microorganisms, the inactivation of aminoglycoside antibiotics by phosphorylation is a naturally occurring process. Bacteria with resistance to streptomycin are also commonly found in the environment. The resistance to these antibiotics can therefore also be spread by horizontal gene transfer from non-genetically modified microorganisms. Even if the specified resistance genes were to be transferred from the genetically modified plants to microorganisms, the overall frequency of this resistance is unlikely to increase significantly in the environment.

(d) Regulatory sequences

The sequences inserted into the potatoes to regulate the transferred genes are derived from microorganisms such as *A. tumefaciens, E. coli* and CaMV. Regarding the horizontal gene transfer of these sequences to microorganisms, it should be noted that *A. tumefaciens* is widespread in the environment and the transfer of the corresponding sequences from *Agrobacterium* is far more likely than their transfer from the genetically modified plants. The same applies to *E. coli*, which occurs naturally in the intestinal flora of humans. 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.

(e) Sequences located outside the border regions

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. In this particular case, the following DNA fragments may have been integrated into the genetically modified plants by the transfer of sequences located outside the border regions:

(i) The *npt*II gene (see above)

(ii) The origin of replication of the plasmid pBR322 from pMB1

(iii) The "disarmed" Ti plasmid pTiB6S3-SE

pBR322 is a prototype for safety vectors commonly used in genetic engineering. Its pMB1-derived replicon (ii) 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 *Escherichia 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.

The Ti plasmid pTiB6S3-SE was created – mainly by deletions – from the wild-type octopine plasmid pTiB6S3, which is commonly found in agrobacteria.

III.1.2.5 Agrobacteria used to generate the genetically modified plants

Following transformation, all sterile crop plants were examined for the presence of agrobacteria before placement for tuber production. Only potato plants that were free of agrobacteria were used further.

In contrast to the common wild types of *Agrobacterium tumefaciens*, the *Agrobacterium* strain used 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 were transferred to a cell of another plant by agrobacteria from the released genetically modified potato plants, this cell would have to spontaneously regenerate into a whole, fertile plant for the foreign genes to enter the germ cells and 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 (*Agrobacterium 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.