Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

Notification 6786-01-0188

Summary of the risk assessment of genetically modified potato plants (*Solanum tuberosum* L.) (transformation events B33-LegHg-3'OCS 13, 45, 54 and 57) carried out by the German Competent Authority within the framework of a proposed deliberate release,

Berlin, 18 March 2004

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

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 sequence

(a) The cDNA of the leghaemoglobin gene from *Lotus japonicus* (LegHg gene)

In contrast to higher animals, plants do not have a transport system for oxygen. The distribution of oxygen is based on openings in the epidermal tissues (stomata and lenticels) via gas diffusion through intercellular spaces to the sites of consumption. In organs which have a low surface-volume ratio and intercellular spaces with narrow lumen, the supply of oxygen to deeper organ layers can be insufficient. When this happens the supply of oxygen becomes a limiting factor for the intensity of energy-consuming processes including, among others, starch biosynthesis.

In cells of potato tubers, which grow underground and are not capable of photosynthesis, energy-consuming processes are adapted to the availability of energy-rich, reduced compounds via a regulation system. This redox regulation system alters the activity of the enzyme ADP glucose pyrophosphorylase (AGPase), which catalyses the first step in starch biosynthesis and functions as a key control site for starch metabolism.

Therefore, an attempt was made to improve the supply of oxygen in the potato tubers via expression of the gene that encodes the plant protein leghaemoglobin. The gene is derived from the leguminous plant *Lotus japonicus* and encodes a protein which is similar to the proteins responsible for the transport of oxygen in the tissues of vertebrates. In *Lotus japonicus*, this protein is formed in the root nodules, where it ensures the supply of oxygen in the case of minimal partial pressure on free oxygen in tissues.

The LegHg gene used here is derived from a *Lotus japonicus* cDNA library. To generate the cDNA library, poly(A)-mRNA was isolated from root nodules of *Lotus japonicus*. Under the control of the B33 promoter, expression takes place mainly in the tissues of the potato tuber and, sporadically and/or following induction with sucrose, also in the shoot. The transformants proposed for release here were examined on the mRNA level (northern blot) for expression of the gene encoding leghaemoglobin in the tuber tissue. All transformants exhibit leghaemoglobin RNA, which is not detected in the non-transformed parental variety.

In greenhouse trials, formation of the leghaemoglobin RNA led to an increased oxygen content at a depth of 2 mm below the skin of the tuber, enlarged lenticels and an increase of starch content in the potato tubers. Under plastic film greenhouse conditions, in spring/summer 2003, the phenotype of the above-ground part of the shoot did not differ from the parental variety.

Leghaemoglobin is present in the root nodules of wild species of *Lotus*, *Melilotus*, *Medicago* and *Trifolium* and in Fabaceae used in agriculture (lucerne, white clover) and enters the soil during decomposition of the nodules after the plant has died off. The genetically modified po-

tato plants are therefore not expected to have any adverse effects on soil life or underground grub pests.

The leghaemoglobin-containing root nodules of Fabacae are not contained in the harvest products of crop plants. Leghaemoglobin is not known to have direct toxic effects. As a result of the postulated influence of the plant metabolism via the redox regulation systems, the possibility of the genetically modified potatoes having an altered native potato toxic alkaloid content cannot be completely ruled out. There are no data available on this. However, the genetically modified potatoes are not intended for consumption. Moreover, the alkaloid content of the genetically modified tubers is not expected to exceed that of conventional immature or green/partially green tubers. Furthermore, the release site is located on a fenced-in test ground so that access to the release site is restricted for humans and larger animals.

Hence the execution of the proposed trial, resulting in the expression of the leghaemoglobin gene and associated changes in the genetically modified potato plants, does not pose a threat to human or animal health or to the environment.

(b) The *npt*II gene

The *npt*II gene transferred to the genetically modified plants encodes the enzyme neomycin phosphotransferase. It was introduced as a marker gene for selecting transformed plant cells.

The neomycin phosphotransferase is a type II aminoglycoside 3'-phosphotransferase (APH(3')II) which catalyses the 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 aminocyclitoles 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, in the absence of substrate under field conditions, no new metabolic products are expected to be synthesised 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 on the genetically modified plants. There is no evidence to suggest that this enzyme is toxic to plants, animals, micro-organisms or humans.

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

The plasmid used in the transformation of the potato plants is a derivative of the binary vector pART27, which was fully sequenced.

In addition to the expression cassettes of the LegHg gene and the *npt*II gene, the plasmid contains within the T-DNA nucleotides of the T7 promoter, the SP6 promoter and the *lac* operon or the *lac*Z gene from *E. coli*. These are not functional in plants.

(d) Sequences located outside the T-DNA

As a rule, only DNA located within the border regions is integrated into the plant genome during *Agrobacterium*-mediated transformation. However, the transfer of DNA fragments outside the border regions has been reported.

Because there is no detailed analysis of the sequences integrated into the potato plants available, the risk assessment is performed under the assumption that the entire plasmid may have been integrated.

The transformation plasmid contains the following outside the border regions:

- the oriT of the RP4 plasmid from *E. coli*, which is required for triparental mating;
- the insertion element IS1 from *E. coli*;
- the *tra*J gene from the RK2 plasmid which, as a regulation factor, influences the expression of mobilisation genes for bacterial conjugation;
- a DNA fragment with sequence homologies to the trfA gene of the RK2 plasmid for the replication in *E.coli* and in *A. tumefaciens*;
- the *tetA* gene of the RK2 plasmid (codes for a membrane protein which causes the excretion of tetracyclines thus conferring to cells resistance to the tetracycline group of antibiotics);
- the origin of replication ColE1 of the RK2 plasmid from *E. coli*;
- the Tn7 transposon with the *aadA* gene, which confers resistance to the antibiotics streptomycin and spectinomycin;
- the origin of replication *ori*V of the RK2 plasmid from *E. coli*.

These sequences are not expected to lead to the formation of functional gene products in the genetically modified plants, because they are not under the control of plant-specific promoters.

(e) Position effects and context changes; allergenicity

Genes which have been integrated into the plant genome by genetic engineering are expressed at different levels, depending on the integration site on the chromosome or rather on the sequences neighbouring the integration site (“position effect”). Under field conditions the level of expression may also 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 modified to the same degree in the field as under climate-controlled or greenhouse conditions. 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 on these genetically modified plants, no observations indicating the occurrence of such an event were made.

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. 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 have an effect on plant metabolic pathways. In this respect the genetically modified plants proposed for release here do not differ fundamentally in their 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 the amino acid sequence. However, in numerous releases of plants that express the *npt*II gene under the control of non-tissue-specific promoters no evidence has been found to indicate an increased allergenicity of the plants. The leghaemoglobin gene is derived from *Lotus japonicus* (Fabacea). Leghaemoglobin genes occur naturally in a range of plant species, including crop plants. In any case, pollen of potato plants is only dispersed to a small extent by wind and does not generally 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. In areas where potatoes have been cultivated, tubers or seeds may remain in the soil after harvesting. Depending on temperatures in the winter following cultivation, these may give rise to volunteer potato plants in the following year. However, in Europe, the establishment of potatoes in natural ecosystems has not been observed, since potatoes compete poorly against wild plants and they are not frost resistant. Potato plants are occasionally found beyond cultivated areas, but only on non-natural sites such as verges and other ruderal areas. Because potatoes are not frost hardy they do not form persistent populations in these areas either.

After harvesting, the tubers of the genetically modified potatoes will be analysed or stored for replanting in the following year. Surplus tubers are to be inactivated. Transgenic plant residues that remain on the release site are to be left there to decompose. Potatoes will not be cultivated during the post-trial monitoring period. During this period, volunteer potato plants will be identified and destroyed.

Potato plants of the "Désirée" variety can flower and produce fruit. Potato seeds are unlikely to overwinter and give rise to plants under Central European climate conditions. Should tu-

bers or seeds remain in the soil, the resulting plants would be detected during the planned post-trial monitoring period.

According to information provided by the applicant, in greenhouse trials, the genetically modified plants differed phenotypically in that they exhibited enlarged lenticels and an increased starch content in the potato tubers. Under plastic film greenhouse conditions in spring/summer 2003 the phenotype of the above-ground part of the shoot did not differ from the initial variety. Conventional potato varieties with an increased starch content in the tubers already have a long history of cultivation.

Redox regulation has an influence on numerous process in plants. Some publications have postulated that redox regulation is involved in the manifestation of frost tolerance in the context of frost hardening, inter alia, in frost-tolerant wild potato species. However, in contrast to the wild potato species, the genes which contribute to the acquisition of frost tolerance in the context of hardening are less strongly expressed in cultivated potatoes of the species *Solanum tuberosum* ssp. *tuberosum.* Therefore, influencing the redox regulation system is unlikely to lead to a change in the frost tolerance of the cultivated potatoes used for the field trials.

Furthermore, as a consequence of the expression of the leghaemoglobin and the enlarged lenticels leading to improved oxygen supply in the tubers of the genetically modified potato plants, it is theoretically possible that they will be better able to grow on sites with low oxygen than the initial “Désirée” variety.

These possibilities are adequately addressed by the planned cultivation gap of two years, by the post-trial monitoring measures stipulated in provision II.11 (to the present application) and by the planned isolation measures. During the post-trial monitoring period after completion of the release, no plants or only plants that do not interfere with monitoring may be planted on the trial site.

Taking all these factors into account there are still no grounds to assume that the genetically modified potato plants have different ecological traits compared to conventionally cultivated potatoes, nor are they expected to have the ability to colonise natural ecosystems. Even if the fruit, seeds or tubers of the genetically modified plants were to be dispersed by animals, which is unlikely to happen, the genetically modified potato plants would not be expected to establish in the environment.

III.1.2.3. Assessment of the possibility of a 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 outcrossing 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. Potato and *Solanum dulcamara* (bittersweet or woody nightshade) proved to be strictly bilaterally incompatible species; in cross-breeding experiments pollination of the ovule was not achieved. Similarly, potato does not cross-breed with tomato (*Lycopersicon esculentum*). In agricultural practice, potatoes are propagated vegetatively via tubers.

Hence the following passage deals solely with a possible pollen transfer from the genetically modified potato plants to other potato plants. Pollen of potato plants can be transferred by insects or by wind. However, wind dispersal only takes place over short distances. Potatoes are primarily self-fertilizing. Cross-fertilization, even within a field of flowering potato plants, is rare. It is most likely to occur between neighbouring plants.

The above-ground parts of the genetically modified potato plants proposed for release do not differ phenotypically from the parental variety under plastic greenhouse conditions. There is no evidence to suggest that the fertility of the genetically modified potato plants is altered compared to the parental variety. The proposed separation distance of at least 20 metres between the release sites and the border of the test ground described in the application is considered sufficient. Should pollen nevertheless be transferred to potato plants being cultivated for the production of table potatoes, no adverse effects would be expected since planting material for the agricultural cultivation of potatoes is propagated vegetatively, i.e. not via seeds.

As already elaborated above, the probability that potentially generated seeds could give rise to plants under the given climate conditions is very slight. In the course of crop rotation, such plants would be eliminated by conventional soil preparation practices.

III.1.2.4. Assessment of the possibility of a transfer of the inserted foreign genes from the genetically modified plants to micro-organisms by horizontal gene transfer

The inserted sequences are stably integrated into the chromosomes of the recipient organisms. There is no evidence that a transfer of genetic information from plants or its expression in micro-organisms 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 also theoretically possible, although it is assumed that a gene transfer of this type would constitute an extremely rare event.

If we assume that an exchange of genetic material between organisms which are so distantly related in terms of taxonomy is actually possible, it must 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 all forms of heterologous genetic material, including all forms of plant DNA.

(a) The cDNA of the leghaemoglobin gene from *Lotus japonicus*

Leghaemoglobin genes are naturally present in root-nodule-forming leguminous plants. The regulatory sequences are derived from *S. tuberosum* and *A. tumefaciens*. Therefore, these

sequences are already widespread in the environment. A horizontal gene transfer in micro-organisms is therefore far more likely to occur via non-genetically modified organisms.

(b) The *npt*II gene

As already stated under III.1.2.1. antibiotics that are inactivated by neomycin phosphotransferase do not play a significant role in human medicine, but they are widely used in veterinary medicine. Therefore, an examination of whether the therapeutic application of the respective antibiotics would be impaired by the possible horizontal gene transfer of the *npt*II gene was required.

The resistance mechanism for inactivation of aminoglycoside antibiotics through phosphorylation occurs naturally in soil micro-organisms. APH(3')II enzymes have also been found in human clinical isolates. The widespread distribution of genes that confer resistance to aminoglycoside antibiotics can be explained by the frequent use of these antibiotics, and also by the fact that these genes are often localised on plasmids, enabling effective transfer by conjugation. Even in the case of a horizontal gene transfer from the genetically modified potatoes to micro-organisms, the overall frequency of this resistance mechanism would not increase noticeably.

(b) Nucleotides of the *lac*Z gene from *E. coli*

The *lac*Z gene derives from *E. coli* and is therefore already widespread in the environment. The presence of parts of the *lac*Z gene in the genetically modified potato plants is therefore not expected to pose any potential risk.

(d) Sequences located outside the T-DNA

The genetically modified potatoes may contain the following genetic elements, which are located outside the border regions on the plasmid used for the transformation:

- the oriT of the RP4 plasmid from *E. coli*, which is required for triparental mating;
- the insertion element IS1 from *E. coli*;
- the *tra*J gene from the RK2 plasmid which, as a regulation factor, influences the expression of mobilisation genes for bacterial conjugation;
- a DNA fragment with sequence homologies to the trfA gene of the RK2 plasmid for the replication in *E.coli* and in *A. tumefaciens*;
- the *tetA* gene of the RK2 plasmid (codes for a membrane protein which causes the excretion of tetracyclines thus conferring to cells resistance to the tetracycline group of antibiotics);
- the ColE1 origin of replication of the RK2 plasmid from *E. coli*;
- the Tn7 transposon with the *aadA* gene, which confers resistance to the antibiotics streptomycin and spectinomycin;
- the *ori*V origin of replication of the RK2 plasmid from *E. coli*.

The *tetA* gene from the transposon Tn10 codes for a membrane protein which exports antibiotics of the tetracycline group out of the cell, thus making it resistant to tetracyclines. Tetracyclines are characterised by their broad spectrum of activity and continue to be of clinical relevance in human medicine; they are used, among other things, to treat infections caused by *Brucella*, *Chlamydia*, *Mycoplasma*, *Rickettsia* and *Vibrio*.

The *aadA* (strep/specR) gene originates from the R538-1 plasmid from E. coli and encodes a streptomycin adenyltransferase. Like the *tetA* gene, the *aadA* gene is located outside the T-DNA of the transformation plasmid, but its transfer to the genetically modified plants has not been ruled out. Streptomycin and spectinomycin have only limited uses in human medicine, but they are still clinically relevant for the treatment of tuberculosis (streptomycin) and gonorrhoea (spectinomycin). Streptomycin-resistant bacteria are widespread in the environment. Resistance to these antibiotics can therefore also be spread through horizontal gene transfer from non-genetically modified micro-organisms.

The genetically modified potato plants are only to be released on a limited area for a limited period of time. These plants may not be used for animal or human consumption. In view of the very low risk of a horizontal gene transfer of plant DNA to micro-organisms and the absence of selection pressure on the release sites, the presence of the genes *tetA* and *aadA* in the genetically modified potato plants is not expected to lead to any significant increase in the overall distribution of this resistance mechanism in micro-organisms.

The insertion element *IS*1 occurs naturally in different species of Enterobacteriaceae. It has, for example, been found in species of the genera *Escherichia, Shigella, Klebsiella, Serratia* and *Salmonella*. In *IS*1 the number of copies per bacterial genome can amount to more than 40. Copies of *IS*1 may be localised on the chromosome or the plasmid and were also found in prophages. These insertion elements are likely to be easily spread by horizontal gene transfer between bacteria. Therefore, in comparison, the theoretically conceivable risk of a spread by horizontal gene transfer from the genetically modified plants to micro-organisms is negligible.

RK2 belongs to a group of broad host-range plasmids (including RP1, RP4, R18 and R68) which are capable of replication in numerous gram-negative bacteria. Therefore, for DNA fragments derived from RK2, the likelihood of spreading by transfer between bacteria is far greater than the likelihood of spreading by horizontal gene transfer from the genetically modified plants to micro-organisms.

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 containing the genes to be transferred between the border regions of the binary vector plasmid. After transformation had occurred, the plant parts were treated with antibiotics to eliminate the Agrobacteria.

In contrast to the ubiquitous wild-type *A. tumefaciens,* the *Agrobacterium* strain used for the transformation is disarmed, i.e. it no longer has the capacity to induce tumours. In the unlikely but theoretically conceivable event that the inserted foreign genes are transferred to a cell of another plant by these Agrobacteria, 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 plasmids contained in the Agrobacteria to wild-type Agrobacteria (*A. tumefaciens* or *A. rhizogenes*) present in the environment 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 via 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 in III.1.2.4.