Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

Notification 6786-01-0095

Summary of the risk assessment of genetically modified potato plants (*Solanum tuberosum* L.) (lines DL10, DL11, DL12, DL13, DC1)

carried out by the German Competent Authority within the framework of a

proposed deliberate release, Berlin, 27 April 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

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 lysozyme gene

The construct from the lysozyme gene of the bacteriophage T4 and the DNA fragment for the signal peptide of the α-amylase from *Hordeum vulgare* (barley) contained in the genetically modified potato plants is constitutively expressed under the control of the 35S promoter and the 35S terminator region of CaMV, flanked by two scaffold attachment regions from the soybean.

The T4 lysozyme is a bactericidal enzyme. As (N-acetyl-)muramidase, it cleaves the glycosidic bond of the murein between the C1 atom of the N-acetylmuramic acid (MuNAc) and the C4 atom of the N-acetylglucosamine (GlcNAc) and breaks down the muropolysaccharide chain into the disaccharide GlcNac-MurNAc. Basically, the upstream location of the signal peptide of the α-amylase causes the co-translational import of the chimeric protein into the endoplasmic reticulum and therewith ultimately its export into the intercellular spaces. It is assumed that the chimeric protein or the processed lysozyme is glycolysed in the endoplasmic reticulum or during passage through the Golgi apparatus; the possibility that the chimeric protein is also exported into the intercellular spaces cannot be ruled out. The gene cassette is flanked by 2 copies of a scaffold attachment region from soybean. These are intended to reduce position effects through the formation of chromosomal loops.

The ability to synthesise lysozyme is widespread among soil microorganisms and plants. In human medicine, lysozyme is used to treat inflammatory diseases of the respiratory tract. Considering the ubiquitous occurrence of lysozyme, in quantitative terms, a possible additional input of lysozyme into the environment as a result of the cultivation of the genetically modified potato plants is considered insignificant. Because, like all other currently known signal peptides, the signal peptide of α-amylase, whether processed or unprocessed, as well as the scaffold attachment region, are not considered potentially harmful to health, it is assumed that the same applies to the signal peptide-enzyme (in this case T4 lysozyme) complex.

(b) The *nptII* gene

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

The neomycin phosphotransferase is a type II aminoglycoside 3'-phosphotransferase (APH(3')II), which catalyses ATP-dependent phosphorylation of the 3'-OH group of the aminohexose ring of specific aminoglycoside antibiotics, causing these to become inactivated. The enzyme is characterised by its high substrate specificity. The antibiotics kanamycin, neomycin, geneticin, butirosin, gentamicin A and B as well as paromomycin belong to the APH(3')II enzyme substrates. Therapeutically relevant gentamicins and other aminoglycosides and aminocyclitoles used in human medicine do not belong to the substrate spectrum of the APH(3')II enzyme. However, kanamycin and neomycin are widely used in veterinary medicine.

Due to the substrate specificity of the neomycin phosphotransferase, no new metabolic products are expected to arise in the genetically modified potato plants in the absence of substrate under field conditions. Since high concentrations of the relevant antibiotics are not present in the soil, 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) Border sequences from Ti plasmids and regulatory sequences

The genetically modified plants contain sequences from the left and the right border region of the T-DNA of the plasmid pSR8-30 or pSR8-40. These sequences, dependent on the gene products of the *vir* region of the helper plasmid present in the *Agrobacterium* strain used for the transformation, which was not transferred to the plants, caused 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 potato plants contain the following regulatory sequences which are functional in plants:

- the 35S promoter of CaMV,
- the promoter of the nopaline synthase gene from *A. tumefaciens*,
- the 35S termination region of CaMV,
- the gene 4 termination region from *A. tumefaciens*.

The promoter and termination sequences regulate the expression of the DNA fragments located between them which, in the genetically modified plants, code for the T4 lysozyme and the neomycin phosphotransferase. Further information on the effects of the formation of these proteins in the plants can be found under III.1.2.1.(a) and (b).

(d) 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. However, the transfer of sequences outside the borders has been reported and, on the basis of the information contained in the notification, this possibility cannot be ruled out. Therefore, the risk assessment also considers those areas of the vectors used for transformation of the potatoes located outside the T-DNA border regions. This concerns, in particular, the following sequences:

[a] the origin of replication of the plasmid pBR 322,

[b] the replicon of the plasmid RK2, consisting of *oriV* and *oriT,*

[c] the β-lactamase gene of the plasmid pBR 322.

The origin of replication of pBR 322 [a] derives from the plasmid pMB1, which belongs to the group of ColE1-like plasmids. The ColE1 replicon has a narrow host range which is limited to *E. coli* and some other related bacteria. The origin of replication of pBR 322 is non-functional in the cells of the potato plants.

The origins of replication *oriV* and *oriT* [b] of the plasmid RK2 facilitate the replication of the plasmid in a broad host range of gram-negative bacteria or its conjugative transfer, provided the mobilisation functions are supplied by a helper plasmid. There is no evidence to suggest that the origins of replication of RK2 would have a function in higher plants.

Since the ß-lactamase gene [c] is under the control of a bacterial promoter, it is not expected to be expressed in plants. The gene product of the β-lactamase gene is a TEM-1-β-lactamase which has the

ability to inactivate a broad spectrum of β-lactam antibiotics through hydrolysis of the cyclic amide bond in the β-lactam ring. Owing to the specificity of this reaction to such β-lactam antibiotics, even in the presence of small amounts of β-lactamase in the genetically modified potato plants, no new metabolic products would be expected to arise in the plants in the absence of substrate.

TEM-1-β-lactamases are widespread in different enterobacteria, amongst others in *E. coli*. *Enterobacteriaceae* are part of the intestinal flora of animals and humans. There is no evidence to suggest that the enzyme has a toxic effect. It is assumed that a β-lactamase that is ingested with food, like proteins in general, would be degraded and digested in the digestive tract.

(e) 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 genetically modified potato plants might not be resistant to infestation by *Erwinia carotovora* and other microorganisms to the same extent in the field as under climate-chamber or greenhouse conditions. This is not expected to pose a risk to the environment or to human or animal health. Also, with respect the *nptII* gene, an altered level of expression is not expected to pose a threat 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 potato 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 greenhouse experiments with the genetically modified plants, as well as in deliberate release trials with other genetically modified plants which express the relevant genes under the control of non-tissue-specific promoters, no evidence of increased allergenicity of the plants was found.

In any case, the pollen of potato plants is dispersed only to a limited extent by wind and in general does not play a noteworthy role in triggering pollen allergies.

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

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

sometimes found outside cultivated areas, but only on non-natural sites such as waysides and other ruderal areas. Since potatoes are not frost resistant, they do not establish permanently in these areas either.

As a result of potato cultivation "volunteer potatoes" can emerge from tubers which remained in the ground after harvesting on surfaces used for agriculture. Potato tubers are sensitive to frost. Their survival is therefore influenced primarily by winter temperatures.

The harvested tubers, some of which remain in the ground beyond the usual harvesting period until the end of the respective year, will be transferred to a genetic engineering facility for further processing as planned. The leaves and stalks are to be tilled into the soil on the release sites. The release sites are not to be ploughed after the tuber harvest. In the subsequent cultivation periods, crop rotation with genetically modified oilseed rape and *Lolium multiflorum* (Italian rye-grass) is to be practiced on the release sites so that post-trial monitoring for potentially emerging potato plants can be carried out. Any potatoes that might re-emerge in the following year will be identified and destroyed.

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 pursuant to supplementary provision II.8. To remove any tubers that might have been left in the ground, after the tuber harvest as well as in the spring of the following year, the release site is to be loosened to a depth of approx. 15 cm. Any tubers found are to be destroyed such that they no longer have the capacity to germinate.

Plants of the potato variety "Désirée" flower and produce seeds. Under Central European climate conditions potato seeds are unlikely to overwinter and give rise to plants.

Should tubers or seeds remain in the soil despite these measures, any plants that might emerge from these would be detected within the scope of the post-trial monitoring measures planned by the applicant and/or stipulated in supplementary provision II.9. A possible alteration of the frost sensitivity of the tubers as a consequence of the genetic modification cannot be completely ruled out. However, this possibility is adequately taken into account by the post-trial monitoring programme. During the post-trial monitoring period after the end of the trial, no plants or only plants that do not impede post-trial monitoring are to be cultivated on the control sites. In this way volunteer potatoes can be easily identified.

For the reasons stated above, no establishment or uncontrolled persistence of the genetically modified plants is to be expected.

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 hybridisation took place between the genetically modified potatoes and *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 aid of artificial methods (embryo rescue) was it possible to regenerate a small number of hybrids which, however, turned out to be sterile. Potato and *Solanum dulcamara* (bittersweet or woody nightshade) proved to be strictly bilaterally incompatible species; in crossbreeding experiments pollination of the ovule was not achieved. Therefore, the following passage deals only with a possible transfer of pollen 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 an isolation distance of 20 m to neighbouring potato cultivation plots which are not part of the field trial, and this is considered adequate. If, despite this measure, pollen is transferred to potato plants cultivated to produce table potatoes, no adverse effects would be expected. Planting material for the agricultural cultivation of potatoes is propagated vegetatively, i.e. not via seeds. As already explained above, the probability that potentially generated seeds would give rise to plants under the given climatic conditions is very low. 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.

A transfer of the T4 lysozyme gene to microorganisms would not create a special situation, because soil microorganisms, and in particular bacteriophages with the ability to synthesise lysozyme, are widespread. Even in the event of a transfer of the T4 lysozyme gene from the genetically modified potato plants to microorganisms, a significant increase in the amount of lysozyme in the environment would not be expected nor would the bacteria, if they were to survive, have a growth advantage.

As already elaborated under point III.1.2.1.(b), the antibiotics inactivated by the neomycin-phosphotransferase are of little relevance in human medicine but are widely used in veterinary medicine. Therefore, it was necessary to examine whether a potential horizontal gene transfer of the *nptII* gene might compromise the therapeutic use of the relevant antibiotics.

The inactivation of aminoglycoside antibiotics by phosphorylation is a naturally occurring resistance mechanism in soil microorganisms. 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 localised on plasmids, thus enabling effective transfer between microorganisms by conjugation. Even in the event of a horizontal gene transfer from the genetically modified potato plants to microorganisms, the overall frequency of this resistance mechanism would not noticeably increase.

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 a transfer from the genetically modified plants. The same applies to the signal peptide of the α-amylase from barley and the scaffold attachment regions from soybean, which have no coding function. The theoretical possibility of a transfer of the CaMV sequences from the genetically modified plants would not constitute a new situation compared to the existing situation in nature 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 during *Agrobacterium*-mediated transformation. 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:

(a) the origin of replication of the plasmid pBR322,

(b) the replicon of the plasmid RK2, comprising *oriV* and *oriT*,

(c) the β-lactamase gene of the plasmid pBR 322.

The origin of replication of pBR322 (a) derives from the plasmid pMB1 which belongs to the ColE1-type plasmids whose host range is limited to a small 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 quite 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 the horizontal gene transfer.

RK2 belongs to a group of broad host-range plasmids (incl. RP1, RP4, R18, R68) which are capable of replication in numerous gram-negative bacteria. For the RK2-derived DNA fragments (b), therefore, 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.

TEM-1-β-lactamases are widespread in various enterobacteria, amongst others in *E. coli*. *Enterobacteriaceae* are part of the intestinal flora of animals and humans. There is no evidence for a toxic effect of the enzyme. A gene transfer from the 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 β-lactamase gene in the plant chromosome is not expected to contribute to an increase in the overall frequency of the horizontal gene transfer.

III.1.2.5. Agrobacteria used to generate the genetically modified plants

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

In contrast to the ubiquitous wild forms of *A. tumefaciens*, the *Agrobacterium* strain used for the transformation is disarmed, i.e. it no longer has the capacity to induce tumours. In the unlikely but theoreti-

cally conceivable event of an *Agrobacterium*-mediated transfer of the inserted foreign genes to a cell of another plant, that cell would have to spontaneously regenerate into a whole, fertile plant for the foreign genes to enter the germ cells and thereby be passed on the progeny of the plant. Such an event is not expected to occur under natural conditions.

Assuming that the presence of small amounts of recombinant agrobacteria in the genetically modified plants cannot be ruled out, a potential transfer by conjugation of the binary vector plasmids contained in the agrobacteria to wild-type agrobacteria present in the environment (*A. tumefaciens* or *A. rhizogenes*) must also be considered, since these could, in turn, pass on the foreign genes to individual cells of other plants. In the case of infection and subsequent transformation by wild-type *A. tumefaciens* or *A. rhizogenes,* a crown gall or hairy root tumour would develop from the transformed plant cell. A tumour of this type would not be expected to give rise to a plant under natural conditions.

A possible transfer of the inserted genes from agrobacteria to other soil bacteria must also be considered. The potential impact of such a transfer has already been addressed under point III.1.2.4..