Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

Notification 6786-01-0167

Summary of the risk assessment of the genetically modified potatoe

(*Solanum tuberosum*: Désirée)

within the framework of a proposed deliberate release

carried out by the German Competent Authority

Berlin, 15 March 2006

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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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.

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

(a) The StSDDhpi RNAi construct

The subject of the proposed deliberate release is the study of the role of stromatal density in water balance, growth and tuber formation of genetically modified potato plants under field conditions.

Stomata (microscopic plant pores) maintain the transpiration stream through which water and nutrients are transported in the plant and regulate gas exchange (carbon dioxide, oxygen and water vapour) between cells located in the plant organs and the atmosphere. This gas exchange is essential for the photosynthesis that occurs in the mesophyll cells, thereby determining growth. Under optimal conditions the width of the stomatal opening is regulated to allow a high rate of gas exchange without causing excessive water loss through the transpiration stream. If the water supply is insufficient, narrowing the width of the stomatal aperture can minimize water loss, but can also limit gas exchange and subsequent carbon fixation.

A mutant (*sdd1-1*) exists for *Arabidopsis thaliana*, in which the guard cell density of the leaves is tripled and many guard cell pairs directly border on other guard cell pairs, which is a rare occurrence in the wild type. This *Arabidopsis* mutant facilitated identification of the wild-type gene *sdd1* (stomatal density and distribution). Its gene product (SDD1) is a subtilisin-like serine protease, which evidently functions as a negative regulator of guard cell development. In order to examine the consequences of altered stomatal density on growth, photosynthesis and yield, genetically modified potato plants with elevated stomatal density were generated.

In order to increase guard cell density, the expression of the potato-encoded SDD1 protein was reduced in potato plants with the aid of an RNAi construct (pStSDDhpi). Transcription of the RNAi construct, which contains a subsequence of the *SDD1* gene in sense as well as in antisense orientation – separated by an intron of the pyruvate orthophosphate dikinase (pdk) gene from *Flaveria trinervia* – leads to the formation of an RNA with a so-called “hairpin intron” structure. In the process the sense and antisense fraction of the RNA forms a double strand, the individual strands of which are connected by a hairpin loop originating from the pdk intron. The intron is recognized by cell-specific mechanisms and is correctly spliced, creating a compact RNA double strand. This double strand is recognized by specific enzymes in the plant cell and is broken down into small fragments. These RNA fragments adhere to the mRNA of the relevant endogenous gene (*StSDD1*) and mediate the degradation of the mRNA by the same enzymes. For the purposes of the proposed release four lines were selected in which SDD1 expression was either reduced (line #23, #41 and #45) or completely absent (line #1) in comparison to the parent variety, as demonstrated by quantitative real-time PCR (qRT-PCR). Under greenhouse conditions the genetically modified potato plants exhibited up to 70% higher stomatal density compared with the non-modified parent line. In addition, the genetically modified plants displayed increased heat tolerance: at very high temperatures during the growth phase they also displayed increased tuber yield.

There is a possibility that the genetic modification may have unintended effects on plant metabolism that could result in a change in the toxic or health-damaging properties of the genetically modified potato plants. This has not been investigated to date. However, genetically modified potatoes grown during the proposed deliberate release are not intended for use in the production of fodder or foodstuffs and the trials are to be conducted on a fenced and marked test site, so that no health risks to animals or humans are expected to result.

(b) The *npt*II gene

The *npt*II gene transferred to the genetically modified plants codes for the enzyme neomycin phosphotransferase. It was introduced 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 the ATP-dependent phosphorylation of the 3'-hydroxyl 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 enzyme. However, kanamycin and neomycin are widely used in veterinary medicine.

Given the substrate specificity of the neomycin phosphotransferase, it can be assumed that, in the absence of substrate under field conditions, no new metabolic products can be synthesised in the genetically modified potato plants. Since high concentrations of the relevant antibiotics are not present in soil, the neomycin phosphotransferase does not confer any selection advantage to the genetically modified plants under field conditions. No evidence has been recorded to suggest that this enzyme is toxic to plants, animals, microorganisms or humans.

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

In addition to the StSDDhpi RNAi construct and the expression cassette of the *npt*II gene, the plasmids used to transform these potato plants contain nucleotides of the *lac*I and *lac*Z genes from *E. coli*. These are not functional in plants.

(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 transformations. However, the transfer of DNA fragments outside the border regions has been reported.

The transformation plasmid ppStSDDhpi is a derivative of the binary vector pART27 and contains the following genetic elements outside the border regions:

- the *ori*T of the plasmid RP4 from *E. coli*;
- the transposable insertion sequence IS1 from *E. coli*;
- nucleotides of the *traJ* gene of the plasmid RP4 from *E. coli*;
- the replication origin *ori*V of the plasmid RK2 from *E. coli*;
- the *trfA* gene of the plasmid RK2 from *E. coli*;
- nucleotides of the *pecM*-like gene of the plasmid RK2 from *E. coli*;
- the replication origin of the plasmid pBR322 (ColE1 *ori*) from *E. coli*;
- nucleotides of the *bla* gene of the plasmid pBR322 from *E. coli*;
- the *sat1* gene of the Tn7 transposon from *E. coli*;
- the *aadA* gene of the Tn7 transposon from *E. coli*.

PCR analysis performed by the applicant demonstrated the presence of the *aadA* gene in three (#1, #41 and #45) of the four transformants proposed for release. Therefore the risk assessment is carried out under the assumption that all genes contained in the plasmid pStSDDhpi outside the border regions may be integrated in the plant DNA.

However, these sequences are not expected to lead to the formation of functional gene products in the genetically modified plants since they are not controlled by plant-specific promoters.

(e) Position effects and context changes; allergenicity

Genes that have been 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 nucleotide sequences neighbouring the integration site ("position effect"). Under field conditions the level of expression may be influenced by environmental factors, for instance, by temperature. In the present case, this could result in the characteristics of the genetically modified potato plants not being modified to the same degree 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.

The integration of foreign genes may influence the expression or regulation of the plant's own genes at or near the integration site. Such processes may alter plant metabolic pathways. However, during the trials carried out to date on these genetically modified plants, 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. 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 there is a possibility that such events may influence plant metabolic pathways. Therefore, with regard to these characteristics the genetically modified plants planned for release 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 possible allergenic effect of a protein based on its amino acid sequence. However, in numerous deliberate releases of plants that express the *npt*II gene under the control of non-tissue-specific promoters, no evidence was found that would indicate an increased allergenicity of these plants. In any event, pollen from potato plants is only marginally dispersed by wind and generally plays a negligible 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 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 the following year. 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. 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 the cultivated potato does not establish in these areas either.

Tubers of the trial plants will be harvested, graded, weighed and transferred to a genetic engineering plant for further analysis or for storage as reference samples. Surplus tubers will be inactivated by steam treatment. The leaves and stalks of the potato plants will be left to decompose on the release site.

After harvesting, the release site will be tilled in order to force any residual tubers to the surface and to level the area. Crop rotation is designed in such a way that no potatoes will be cultivated on the trial site for a minimum of two years following the release of genetically modified potatoes. In the year after the release, the site will be monitored for volunteers. The monitoring period will be extended until the site on which the genetically modified potatoes were cultivated has been declared free of volunteers for one whole vegetation period. After that, potato plants may not be cultivated on the site for at least one further vegetation period.

Potato plants can flower and bear fruit. However, under Central European climate conditions it is unlikely that potato seeds will overwinter and produce plants. In the event that tubers or seeds remain in the soil, the resulting plant growth would be detected during post-trial monitoring.

In greenhouse trials the genetically modified potato plants exhibited increased tuber yield under very high temperature conditions during the growth phase. Owing to the higher number of tubers in comparison to the untransformed parent variety, this could lead to a higher rate of post-emergence following hot vegetation periods in combination with mild winters. However, even taking this factor into account, there is no reason to assume that the genetically modified potato plants possess different plant ecological traits to conventionally cultivated potatoes or that they can colonise natural ecosystems. Therefore, even if the fruit, seeds or tubers of the genetically modified plants were to be dispersed by animals, which is unlikely, the GM potato plants would not be expected to establish in the environment. Within the framework of the project, the possible post-emergence of tubers will be adequately monitored by post-trial measures.

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 not successful. 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, potato does not crossbreed with 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 potatoes. 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 primarily self-pollinating and cross-pollination, even within a flowering potato field, is rare. It is most likely to occur between neighbouring plants.

The proposed minimum isolation distance of 20 m to neighbouring potato plantings is considered sufficient. However, in the event that pollen is actually transferred to potato plants for the production of table potatoes, no adverse effects would be expected, since potatoes for cultivation 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.

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 stably integrated into the chromosomes of the recipient organisms. There is no evidence 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 which are so distantly related in terms of taxonomy as plants and bacteria is actually possible, it could 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 StSDDhpi RNAi construct

The fragments of the *SDD1* gene present in the construct - separated by the pdk intron from *Flaveria trinervia* and controlled by the 35S promoter from cauliflower mosaic virus as well as the *ocs* terminator from *Agrobacterium tumefaciens* – are derived from potato *(Solanum tuberosum).* All sequences contained in the construct are already widespread in the environment, so that for each part of the construct there is a far higher probability of horizontal gene transfer to microorganisms from non-genetically modified organisms. Moreover, a higher frequency of transfer of the RNAi construct is not to be expected. Even in the event of a horizontal gene transfer, this construct would not be functional in microorganisms.

(b) The *npt*II gene

In these genetically modified plants the *npt*II gene is under the control of the *nos* promoter. This gene codes for the enzyme aminoglycoside 3'-phosphotransferase II (APH(3')II), which catalyses the ATP-dependent phosphorylation of certain aminoglycoside antibiotics (kanamycin, neomycin, geneticin), causing their inactivation.

As already elaborated in III.1.2.1. (b), the antibiotics inactivated by the aminoglycoside 3'-phosphotransferase II 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.

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

The Scientific Panel on Genetically Modified Organisms (GMO Panel) of the European Food Safety Authority (EFSA) has allocated the *npt*II gene to the group of genes which, in terms of safety, provide no grounds to restrict or ban their usage, either for field trials or for the purpose of placing on the market. In its statement of 6.7.1999 on the biological safety of antibiotic resistance genes in the genome of genetically modified plants, the Central Committee on Biosafety (ZKBS) allocated the *npt*II gene to the group of antibiotic resistant genes which “(a) are already widespread in soil and enterobacteria and (b) whose relevant antibiotics have no, or only little significance in human and veterinary medicine, so that

one can assume that the presence – if any - of these antibiotic resistance genes in the genome of transgenic plants will have no effect on the spread of these antibiotic resistance genes in the environment".

(c) The antibiotic resistance gene *aadA*

The *aadA* (Strep/SpecR) gene is derived from the transposon Tn7 from *E. coli* and codes for an aminoglycoside adenyltransferase. Although the *aadA* gene is located outside the T-DNA on the transformation plasmid, it was transferred to three (#1, #41 and #45) of the four transformants proposed for release. Streptomycin and spectinomycin have limited uses in human medicine, but they are by all means still therapeutically relevant for the treatment of tuberculosis (streptomycin) and gonorrhea (spectinomycin). Streptomycin-resistant bacteria are widespread in the environment. Therefore resistance to this antibiotic can also be spread by horizontal gene transfer from non-genetically modified microorganisms. For this reason the Central Committee on Biosafety (ZKBS) allocated the *aadA* gene to the group of antibiotic resistance genes which are "a) already widespread in soil and enterobacteria and (b) whose relevant antibiotics have no, or only little significance in human and veterinary medicine, so that one can assume that the presence – if any - of these antibiotic resistance genes in the genome of transgenic plants will have no effect on the spread of these antibiotic resistance genes in the environment".

In its report of 2 April 2004 on the use of antibiotic resistance genes as marker genes in genetically modified plants, the GMO Panel of the European Food Safety Authority allocated the *aadA* gene to the group of genes which should only be used in experimental field trials and which should not be present in genetically modified plants intended for placing on the market. The genetically modified potato plants are only to be released on a limited area for a specified period. These plants may not be used in the production of feed or food. Given the very low probability of horizontal gene transfer from plant DNA to microorganisms and the absence of selection pressure on the release sites, the presence of the *aadA* gene in the genetically modified potato plants is not expected to lead to a significant increase in the overall frequency of this resistance mechanism in microorganisms.

(d) The antibiotic resistance gene *sat1*

The *sat1* gene, like the *aadA* gene, is derived from the Tn7 transposon from *E. coli* and codes for a streptothricin acetyl transferase. It is located outside the T-DNA on the transformation plasmid. Although the presence of the *sat1* gene in the genome of the genetically modified plants was not investigated, the close proximity of this gene to the *aadA* gene shown to be contained in the transformants #1, #41 and #45, suggests that the *sat1* gene may also be integrated in the plant DNA. The *sat1* gene confers resistance to the streptothricin group of antibiotics. Streptothricins are secondary metabolites produced by a range of streptomyces; they comprise a streptolidine ring, gulosamine and a polylysine side chain which varies in length. The antibacterial effect of streptothricins is based on interaction with the 30S ribosomal subunit, resulting in the termination of transcription or a transcription error. Due to the acteylisation of the β amino group of the lysine residue of a polylysine side chain, streptothricin acetyltransferases have the capacity to inactivate streptothricin group antibiotics. Streptothricins are not used for therapeutic purposes in human or veterinary medicine. Resistance to streptothricins is widespread in enterobacteria in Central Europe, probably due to the fact that during the period 1983 to 1990 streptothricins were used intensively as growth promoters in pig production in the former German Democratic Republic. In addition, the spread of streptothricin resistance genes by horizontal gene transfer is promoted by the fact that the genes are found on different transposons. In the meantime resistance genes of this type have also been found in *Shigella*. The probability of horizontal gene transfer from genetically modified plants to microorganisms is, therefore, very small in comparison to the probability of a naturally occurring gene transfer between microorganisms. To date the EFSA has not carried out an assessment of the streptothricin resistance gene *sat1*. In its decision on this application the ZKBS does not object to the use of the *sat1* gene in deliberate release trials, since in Central

Europe streptothricin resistance is widespread in intestinal bacteria and streptothricins are not used for therapeutic purposes in human or veterinary medicine.

(e) Nucleotides of the *lac*I gene and the *lac*Z gene from *E. coli*

The genes *lacI* and *lacZ* derive from *E. coli* and are thus prevalent in the environment. The presence of fragments of these genes in the genetically modified potato plants is not expected to pose any potential risk.

(f) Additional 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 pART27 derivatives used:

- the *trfA* gene of the plasmid RK2 from *E.coli*;
- the replication origin *ori*V of the plasmid RK2 from *E. coli*;
- nucleotides of the *pecM*-like gene of the plasmid RK2 from *E. coli*;
- the *ori*T of the plasmid RP4 from *E. coli*;
- nucleotides of the *traJ* gene of the plasmid des RP4 from *E. coli;*
- the replication origin of the plasmid pBR322 (ColE1 *ori*) from *E. coli*;
- nucleotides of the *bla* gene from the plasmid pBR322 from *E. coli*;
- the transposable insertion sequence IS1 from *E. coli*.

RK2 and RP4 belong to a group of broad host range plasmids (incl. RP1, R18, R68) that can be replicated in numerous gram-negative bacteria. RK2- and RP4-derived DNA fragments are more likely to spread by transfer between bacteria than by horizontal gene transfer from the genetically modified plants to microorganisms. Moreover, some of the DNA fragments are incomplete (*traJ*, *pecM*).

The pBR322 replicon belongs to the ColE1-type plasmids, having a host range that is limited to a small number of gram-negative bacteria. Essentially it has the ability to replicate in *E. coli* and other related species of bacteria. In most gram-negative soil bacteria replication does not occur. ColE1 plasmids are relatively common in enterobacteria. The likelihood of gene transfer from enterobacteria to other bacteria is far greater than the likelihood of horizontal gene transfer from the genetically modified plants to bacteria. Therefore it is not anticipated that the potential presence of the replication origin of pBR322 in the plant chromosome would contribute to an increase in the overall frequency of horizontal gene transfer. Furthermore the *bla* gene is incomplete.

The transposable insertion element IS1 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 IS1 the number of copies per bacterial genome can amount to more than 40. Copies of IS1 may be localized on the chromosome or the plasmid and were also found in prophages. It is assumed that these insertion elements may be easily spread by horizontal gene transfer between bacteria. Therefore, although theoretically conceivable, the risk of spreading by horizontal gene transfer from the genetically modified plants to microorganisms is negligible in comparison.