Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

Notification 6786-01-0157

Summary of the risk assessment of the genetically modified potato

(*Solanum tuberosum*; Desireé) 3 lines of B33-Apy1-RNAi 1331

within the framework of a proposed deliberate release

carried out by the German Competent Authority

Berlin, 22 March 2005

Explanatory note to this document:

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

I. Consent [to the application]
II. Provisions [to be respected in execution of the field trials]
III. Justification
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III.1.4. Formal requirements according to section 16 (4, 5) GenTG
III.2 Appraisal of and reply to objections
IV. Costs
V. Legal instruction

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

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

(a) The RNAi construct of the apyrase gene from *Solanum tuberosum*

The activity of the potato-encoded enzyme apyrase was reduced in the genetically modified potato plants with the aid of an RNAi construct.

Apyrases are enzymes that transform ATP via ADP to AMP without triggering an endergonic reaction in the process. These enzymes have been identified in the tissues of animals, plants (among others in *Arabidopsis thaliana*, legumes and potatoes) and fungi and apparently have predominantly regulatory functions.

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Double knockout mutations of both apyrase genes from *A. thaliana* inhibit pollen germination and give rise to male sterile plants. Apyrases play a role in the formation of nodules in leguminous plants, and are also thought to be involved in phosphate uptake.

Regulation of transporters that, amongst other things, facilitate the transport of xenobiotics out of the plant cell has been demonstrated for plant apyrases. The blocking of apyrase by specific inhibitors increases the sensitivity of the plants to different herbicides, as well as the concentration of the applied herbicides in the plants. Over-expression of the apyrase psNTP9 from *Pisum sativum* in *A. thaliana* increases the resistance of the plants to herbicides and phytohormones.

Apyrase activity in the potato tubers is very high and is probably localised in the area of the cell wall. Together with other enzymes that influence the ATP/ADP/AMP ratio, apyrase activity is suspected to have a regulatory effect on starch biosynthesis in the potato tubers.

To test this hypothesis the apyrase activity in the tubers of the genetically modified potato plants was reduced with the aid of an RNAi construct. Using the RNAi construct, the RNA of a part of the apyrase gene is coded in sense and antisense orientation, so that the sense and antisense RNA are separated by a sufficiently long spacing sequence (RNA of the Pdk intron from *Flaveria trinervia*). In the present case RNAi synthesis takes place under the control of the B33 promoter specifically in the potato tubers. The sense and antisense RNAi fractions form a double strand, the individual strands of which are linked by a hairpin loop. This double-stranded RNA is recognised by specific enzymes in the plant cell and is fractionated into small fragments. These bind to the mRNA of the respective gene (the apyrase gene) and mediate the decomposition of the RNA by the same enzymes.

A decrease of about 25% in apyrase activity in the tubers of the transformants selected for release has been demonstrated. Under greenhouse conditions the tuber yield of two of the three transformants was significantly higher in comparison with the parent varieties.

A change in the genetically modified potato plants in relation to possible toxic or health-damaging constituents is possible due to the known effects of apyrase activity on a range of different metabolic processes. Studies relating to this aspect were not carried out. However, the genetically modified potatoes proposed for release in this application are not intended for use in the production of foodstuffs or animal feed, and the release will be carried out within a defined and marked trial area, so that the proposed experimental trials are not expected to result in any risks to animal or human health.

(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 gene 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 paramycin 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, both kanamycin and neomycin are widely used in veterinary medicine.

Given the substrate specificity of neomycin phosphotransferase, it is expected that in the absence of substrate under field conditions no new metabolic products will 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, microorganisms or humans.

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

The plasmid inserted for transformation of the potato plants is a derivative of the binary vector pART27, which has been fully sequenced.

In addition to the apyrase RNAi construct and the expression cassette of the *npt*II gene, the plasmid contains nucleotides of the T7 promoter, the SP6 promoter and the *lac* operon and/or the *lac*Z gene from *E. coli* within the T-DNA. These are non-functional in plants.

(d) Sequences located outside the T-DNA

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

The transformation plasmid B33-Apy1-RNAi is a derivative of the pART27 vector. This contains the following outside the border regions:

- the *ori*T 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 *trf*A gene of the RK2 plasmid for the replication in *E.coli* and in *A. tumefaciens*;
- a sequence homologous to pBIN19 (EMBL PPU09365), positions 4561 to 5601;
- the ColE1 origin of replication of the RK2 plasmid from *E. coli*;
- the Tn7 transposon with the *aad*A gene, which confers resistance to the antibiotics streptomycin and spectinomycin;
- the origin of replication *ori*V of the RK2 plasmid from *E. coli*.

A PCR analysis performed by the applicant demonstrated the presence of the pBIN19 sequence (EMBL PPU09365) in all three transformants. This sequence “shows similarity to tetA GenBank Accession Number L13842” according to the annotation. The part of the L13842 sequence that contains the homology to the *tetA* gene comprises 42 nucleotides (nucleotide positions 1-42) and is not contained in the pART27 vector. Therefore it can not confer tetracycline resistance. For positions 233 to 1327 of the L13842 sequence, which are homologous to pART27 positions 6511 to 7606 (antisense strand), the function is largely unknown and no open reading frame is annotated. The genetically modified potatoes, therefore, do not contain the gene for tetracycline resistance.

The applicant also performed a polymerase chain reaction with *aadA* gene-specific primers using the potato lines proposed for release, whereby a positive result was shown for all three transformants.

In the case of all the remaining sequences outside the border regions no confirmation of presence or absence in the genetically modified plants was provided. Therefore the risk assessment is performed under the assumption that these sequences may be present in the plants.

The generation of functional gene products based on these sequences is, however, not expected in the genetically modified plants, since they are not driven by plant-specific promoters.

(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 integration on the chromosome, and on the 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 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 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 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. From 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. In any case the 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. <u>Evaluation of the ability of the genetically modified plants to persist or establish in the environment</u>

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. 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 the cultivated areas, but only on non-natural sites such as verges and other ruderal areas. Owing to the lack of frost hardiness the cultivated potato does not establish in these areas either.

After harvesting, the tubers of the genetically modified potatoes will be weighed and brought to a genetic engineering facility for further examination or for use as reference samples. Surplus potato tubers will be inactivated by steaming. The leaves and stalks of the potato plants are left to decompose on the release site, or they are destroyed by steaming.

The intention after harvesting is to level out the surface of the experimental area by harrowing. Crop rotation in the experimental area is designed in such a way that after the deliberate release of genetically modified potatoes no potatoes will be cultivated on the site for a minimum of two vegetation periods. In the year following the release the area will be monitored for potato volunteers. On sites where genetically modified potatoes were cultivated monitoring will continue until such time as no potato plants are found over an entire vegetation period. Thereafter no potatoes will be cultivated on the site for at least one further vegetation period.

Potato plants can blossom and bear fruit. Under Central European climate conditions potato seeds are not likely to 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.

Under greenhouse conditions two of the three transformants exhibited increased tuber yield in comparison to the parental variety. However, also in the case of conventional potato plants tuber yield is subject to considerable variation, depending on the particular variety and environmental conditions, without this resulting in increased invasiveness or altered competitiveness against wild plants.

Even taking into account a possible increase in tuber yield, there is no reason to assume that the genetically modified potato plants will exhibit different plant sociological traits compared to conventionally cultivated potatoes, nor are they expected to colonise natural ecosystems. Moreover, in the unlikely event that fruit, seeds or tubers of the genetically modified plants were to be dispersed by animals, these GM plants would not be expected to 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 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. The potato and *Solanum dulcamara* (bittersweet or woody nightshade) proved to be bilaterally incompatible; in crossbreeding experiments pollination of the ovule was not achieved. Similarly, the potato can not be crossbred 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, pollen transfer by wind only takes place over

short distances. Potatoes are largely self-pollinating; even in a flowering potato field cross-pollination is rare, the likelihood being greatest between neighbouring plants.

The planned minimum isolation distance of 20 m to neighbouring potato fields, as described in the field trial application, is considered sufficient. If despite these measures pollen is transferred to other potato plants cultivated for the production of table potatoes, no adverse effects are to be expected, since in an agricultural environment potato plants are propagated vegetatively, i.e. not by seed.

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 possible in principle, although it is assumed that a gene transfer of this type would constitute an extremely rare event.

Assuming that an exchange of genetic material between organisms as 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 RNAi construct of the apyrase gene from potato

The apyrase gene sequences present in the construct are derived from the potato; they are thus already widespread in the environment. Horizontal gene transfer to microorganisms is therefore much more likely to occur from non-genetically modified organisms. The same applies to the sequence of the Pdk intron from *Flaveria trinervia*. Furthermore, in the event of a horizontal gene transfer to microorganisms, the RNAi construct would not be expected to have a function in the recipient organisms.

(b) The *npt*II gene

As already described under III.1.2.1., antibiotics that are inactivated by neomycin phosphotransferase do not play a significant role in human medicine, but they do have manifold uses 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 microorganisms. 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 horizontal gene transfer from the genetically modified potatoes to microorganisms, the overall frequency of this resistance mechanism would not increase noticeably.

The GMO Panel of the European Food Safety Authority (EFSA) has classified the *npt*II gene among the group of genes for which, with respect to safety, there are no grounds for prohibiting or limiting use - either for field trials or for the purpose of placing on the market. In its position statement of 6.7.1999 on the biological safety of antibiotic resistance genes in the genome of genetically modified plants, the German Central Commission on Biological Safety (ZKBS) assigned the *npt*II gene to the group of antibiotic resistance genes "that (a) are already widespread in soil microorganisms and enterobacteria and (b) their relevant antibiotics have no, or very little, therapeutic significance in human or veterinary medicine, so that it can be assumed that, if at all, the presence of these antibiotic-resistance genes in the genome of transgenic plants would have no effect on the distribution of these antibiotic resistance genes in the environment".

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

The *lac*Z gene is derived from *E. coli* and is therefore widespread in the environment. Therefore, potential risks are not expected to result from the presence of parts of the *lac*Z gene in the genetically modified potato plants.

(d) Sequences located outside the T-DNA

The genetically modified potatoes may contain the following genetic elements, which are found outside the border regions of the applied transformation plasmid:

- the oriT RP4 plasmid from *E. coli*, which is required for triparental mating;
- the insertion element IS1 from *E. coli*;
- the traJ gene from the RK2 plasmid that, as a regulation factor, influences the expression of the mobilisation gene 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*;
- a sequence homologous to pBIN19 (EMBL PPU09365), positions 4561 to 5601;
- 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 origin of replication *ori*V of the RK2 plasmid from *E. coli*.

The *aadA* (strep/specR) gene is derived from the R538-1 plasmid from *E. coli* and encodes a streptomycin adenyltransferase. Streptomycin and spectinomycin have only limited uses in human medicine, but they are by all means still clinically relevant for the treatment of tuberculosis (streptomycin) and gonorrhoea (spectinomycin). Streptomycin-resistant bacteria are widespread in the environment. The ZKBS classified the *aadA* gene among the Group II antibiotic resistance genes that are (a) widespread in microorganisms and (b) the relevant antibiotics of which are now only used for therapy in certain areas of human and veterinary medicine, so that it can be assumed that, if at all, the presence of these antibiotic resistance genes in the genome of transgenic plants would only have a very small impact on the distribution 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

Scientific Panel on Genetically Modified Organisms (GMO Panel) of the European Food Safety Authority (EFSA) classified the *aadA* gene among the group of genes that are restricted to experimental field trials. These genes 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 limited period of time. The use of these plants as animal fodder or for human consumption is excluded. In view of the very low risk of horizontal gene transfer of 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 result in any significant increase in the overall distribution of this resistance mechanism in microorganisms.

The insertion element IS1 occurs naturally in several species of Enterobacteriaceae. For example, it has been found in species of the genera *Escherichia*, *Shigella*, *Klebsiella*, *Serratia* and *Salmonella.* In the case of IS1, the number of copies per bacterial genome can amount to more than 40. Copies of IS1 may be localised on chromosomes as well as on plasmids, and they have also been identified in prophages. It is assumed that the spread of this insertion element by horizontal gene transfer between bacteria is easily possible. In contrast, the risk of spreading by horizontal gene transfer from the genetically modified plants to microorganisms, although theoretically possible, is negligible.

RK2 belongs to a group of broad host range plasmids (among others, RP1, RP4, R18, R68) that can be replicated in numerous gram-negative bacteria. In the case of DNA fragments derived from RK2, therefore, the probability of a spread by transfer between bacteria is far greater than the probability of horizontal gene transfer from the genetically modified plants to microorganisms.

It was demonstrated that the homologous sequence to pBIN19 (EMBL PPU09365), positions 4561 to 5601, does not contain a tetracycline resistance gene.