Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

Notification 6786-01-0159

Summary of the risk assessment of the genetically modified potatoes

(*Solanum tuberosum*; Désirée), BinARHyg-AtSDD1 lines 1, 2, 7, 9, 11 and

BinAR-StSDD1 lines 2, 6, 12

carried out by the German Competent Authority

within the framework of a proposed deliberate release

Berlin, 01 April 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]
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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 AtSDD1 overexpression construct and the StSDD1 RNAi construct

The subject of the proposed deliberate release is the physiological characterisation of water use efficiency in potato plants with altered stomatal density 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 too much 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. The overexpression of SDD1 in transgenic *Arabidopsis thaliana* causes a reduction of guard cell density to approximately 40% of the wild type. In order to examine the consequences of altered stomatal density on growth, photosynthesis and yield, genetically modified potato plants that featured either an increase or a decrease in stomatal density were generated.

In order to decrease guard cell density, a construct (pBinARHyg-AtSDD1) was transferred that causes the potato plants to overexpress the wild-type SDD1 protein from *Arabidopsis thaliana*. The stomatal density of the transgenic potato lines is reduced to approximately 65% of the stromatal density of lines from non-transformed parent variety. In greenhouse experiments conducted in 2002 and 2003, the plants showed a 50% reduction in tuber yield. At low light intensities, the shoot phenotype of the plants did not differ from that of the parent variety. At higher light intensities the plants displayed severe leaf roll.

In order to increase guard cell density, the expression of the potato's own SDD1 protein was reduced in other potato plants with the aid of an RNAi construct (pBinAR-StSDD1). Using the RNAi construct, the RNA of a part of the SDD1 gene is transcribed in sense and antisense orientation so that sense and antisense RNA are separated by a sufficiently long spacing sequence. The sense and antisense sections of the RNAi form a double strand, the individual strands of which are connected by a hairpin loop. The RNA double strand is recognized by specific enzymes in the plant cell and broken down into small fragments. These RNA frag-

ments adhere to the mRNA of the relevant gene (SDD1 gene) and activate the degradation of the mRNA by the same enzymes. Four lines were selected for the proposed release. For each of these lines Western Blot analysis showed a reduction of the SDD1 protein level compared to the parent variety. Under greenhouse conditions these plants have a stomatal density of 115%. In 2002, the tuber yield from the greenhouse plants did not differ from the yield from plants of the parent variety. In the particularly hot summer of 2003, on the other hand, the yield was significantly (15-22%) higher than the yield from the control plants. At high light intensities, the transgenic plants showed a lower incidence of leaf roll than the non-transformed parent variety.

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 an isolated, designated test site, so that no health risks to animals or humans are to be expected in the context of the experimental release.

(b) The *hph* gene

Plants transformed using the construct pBinARHyg-AtSDD1 contain a *hph* gene from *Streptomyces hygroscopicus* as a selection marker. The hygromycin phosphotransferase encoded by the *hph* gene specifically inactivates the antibiotic hygromycin by phosphorylation. This substrate specificity supports the assumption that, in the absence of substrate, no new metabolic products can be synthesised in genetically modified plants under field conditions. Moreover, this gene does not confer any selection advantage to genetically modified plants under field conditions, since the soil does not contain higher concentrations of hygromycin.

(c) 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.

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

In addition to the constructs described above, the plasmids used to transform the potato plants contain nucleotides of the *lac*I and *lac*Z genes from *E. coli*, the replication origin and the gene III of the *E. coli phage* M13, as well as nucleotides of the Tn5 transposon from *E. coli* within the T-DNA. These fragments are not functional in plants.

(e) 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 DNA fragments outside the borders has been documented.

The transformation plasmids pBinARHyg-AtSDD1 and pBinAR-StSDD1 are derivates of the binary vector pBin19 and contain the following genetic elements outside the border regions:

- the *aphAIII* (= *nptIII*) gene from *Streptococcus faecalis* (= *Enterococcus faecalis*), which is interrupted by the transposon *IS1*, but is functional in prokaryotic systems;
- a sequence with homologous subsequences of the *tetA* gene of the plasmid pRK2, interrupted by the T-DNA;
- the *trfA* gene of the plasmid pRK2 for replication in *E. coli* and *A. tumefaciens*;
- a fragment of the *klaC* gene from *Klebsiella aerogenes*;
- a *traF* fragment, comprising the *ori*T of the plasmid RP4, from *E. coli*;
- the replication origin *ori*V of the plasmid RK2 from *E. coli*;
- the replication origin of the plasmid pUC (ColE1 *ori*) from *E. coli*.

A PCR analysis conducted by the project applicant showed that the *nptIII* gene is not contained in any of the transformants proposed for deliberate release.

In the case of the other sequences located outside the border regions, no evidence was produced to demonstrate either their presence or absence in the genetically modified plants. For the purposes of the risk assessment it is therefore assumed that they may be present in the plants.

However, an accumulation of functional genetic products based on these sequences is unlikely to occur in genetically modified plants, as they are not controlled by plant-specific promoters.

(f) Positional effects and contextual 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 *hph* gene or 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 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.

The 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 at least two vegetation periods after 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, no potato plants may be cultivated on the site for a further vegetation period.

Potato plants can flower and bear fruit. However, under Central European climate conditions there is little likelihood 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.

Due to the decreased stomatal density, the pBinAR-Hyg-AtSDD1 plants reacted more sensitively to high light intensities under greenhouse conditions. It is also conceivable that the decrease in the number of stomata in these plants reduces their susceptibility to phytopathogenic fungus infections which penetrate the leaves via the stomata. However, no risk is expected due to the lack of frost hardiness in potato tubers.

The increased tuber yield of the pBinAR-StSDD1 plants could, due to the increased number of tubers and buds compared to the untransformed parent variety, result in a higher post-emergence after a mild winter. Even taking these factors into account, it is still unlikely that genetically modified potato plants have different plant ecological traits to conventionally cultivated potatoes or that they can colonise natural ecosystems. Therefore, even in the unlikely event that the fruit, seeds or tubers of the genetically modified plants were to be dispersed by animals, 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, the potato does not crossbreed with the tomato (*Lycopersicon esculentum*). In agricultural practice, potatoes are propagated vegetatively via tubers.

The following passage, therefore, deals only with a possible pollen transfer from the genetically modified potato plants to other 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 minimum isolation distance of 20 m between the release sites and other potato plantings is considered sufficient. However, should pollen be transferred to potato plants producing table potatoes in spite of these measures, no adverse effects are to be expected, since in an agricultural environment potato plants are propagated vegetatively, i.e. not via seeds.

As elaborated above, the probability that potentially generated seeds could give rise to plants under the given climatic conditions is very slight. In agricultural areas such plants would be eliminated in the course of conventional soil preparation practices.

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 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 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 is actually possible, it could be concluded that the oc-

currence 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 AtSDD1 overexpression construct and the StSDD1 RNAi construct

The sequences of the SDD1 gene in the construct derive from *Arabidopsis thaliana* or from the potato, so they are commonly found in the environment. As a result, there is a far greater probability of horizontal gene transfer from non-GM organisms to micro-organisms. Furthermore, in the event of a horizontal gene transfer to microorganisms, it is unlikely that either the AtSDD1 overexpression construct or the RNAi construct would be functional in microorganisms.

(b) The *hph* gene and the *npt*II gene

The *hph* gene, which encodes the enzyme hygromycin phosphotransferase, was isolated from *E. coli*. Due to its high toxicity for eukaryotic organisms, hygromycin is not used in human medicine and only for a specific range of applications in veterinary medicine. Hygromycin-resistant Enterobacteriaceae, which contain a gene for a hygromycin phosphotransferase, were found in human and animal sample material (faeces, urine, blood) and are released into the environment by animals and humans.

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. (c), 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 occurs naturally 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 GMO Panel of the European Food Safety Authority (EFSA) has allocated the genes *hph* and *npt*II 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 state-

ment 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 *hph* gene and 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) Nucleotides of the *lac*I gene and the *lac*Z gene from *E. coli*

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

(d) M13 sequences

pBin19 and its derivates contain two fragments from M13mp19 within the T-DNA, namely a 440 bp fragment, which encompasses one part of an open reading frame of a structural protein of M13, and a 433 bp fragment, which contains the replication origin of phage M13. Phage M13 is one of the F-specific *E. coli* phages. Therefore, in the case of these nucleic acid fragments, the probability of proliferation via transfer between bacteria is considerably higher than the probability of proliferation by horizontal gene transfer from genetically modified plants to microorganisms. If expression of the gene fragment for the structural protein were to occur, no functioning protein would result, since the fragment only codes for 167 of the total 423 amino acids in the complete phage protein. This fragment of the structural protein is not expected to be functional in bacteria.

(e) Sequences located outside the T-DNA

Genetically modified potatoes can contain the following genetic elements, which are located on the transformation plasmid outside the border regions:

- a sequence with homologous subsequences of the *tetA* gene of the plasmid pRK2, interrupted by the T-DNA,
- the *trf*A gene of the plasmid pRK2 for replication in *E. coli* and *A. tumefaciens*,
- a fragment of the *kla*C gene from *Klebsiella aerogenes,*
- a *tra*F fragment, which comprises the *ori*T of the plasmid RP4, from *E. coli,*
- the replication origin *ori*V of the plasmid RK2 from *E. coli,*
- the replication origin of the plasmid pUC (ColE1 *ori*) from *E. coli*.

RK2 belongs to a group of broad host range plasmids (incl. RP1, RP4, R18, R68), which can be replicated in a variety of gram negative bacteria. For DNA fragments deriving from RK2, the probability of proliferation via transfer between bacteria is far higher than the probability

of proliferation by horizontal gene transfer from genetically modified plants to microorganisms. Some of the DNA fragments are also interrupted or incomplete (*klaC*, *tetA*).

The pUC replicon belongs to the type of ColE1 plasmids, whose host range is restricted to certain gram negative bacteria. Basically, the replicon can replicate in *E. coli* and closely related species of bacteria, such as, for example, *Serratia* or *Salmonella*. Replication does not occur in most gram negative soil bacteria. ColE1 plasmids occur very frequently in enterobacteria. A gene transfer from enterobacteria to other bacteria is far more likely to occur than a horizontal gene transfer from genetically modified plants to bacteria. The potential presence of the replication origin of pUC in the plant chromosome is thus not expected to contribute to an increase in the total frequency of horizontal gene transfer.