Notification 6786-01-0049

Summary of the risk assessment of genetically modified potato plants (*Solanum tuberosum* L.) (lines Dst9-3, Dst9-6, Dst22-6, Dst23-1, Dst23-15, Dst23-17, Dst65-24; Dst4-2, Dst4-5, Dst4-8, Dst4-9, Dst4-12, Dst4-14, Dst4-15, Dst4-17) carried out by the German Competent Authority within the framework of a proposed deliberate release, Berlin, 30 May 1996

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
III.1. Requirements for approval according to section 16 GenTG [German Gene Technology Act]
III.1.1. Requirements for approval according to section 16 (1) Nr. 1 GenTG
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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.

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

a) The *barnase* gene and the *barstar* gene from *Bacillus amyloliquefaciens*

The genetically modified potato clones specified under I.1.1 no. I) contain the *barnase* gene, which is expressed under the control of the *prp1-1* promoter from potato and the terminator region of the nopaline synthase gene from *Agrobacterium tumefaciens*. The *barnase* gene contains an N-terminal signal sequence of the alkaline phosphatase gene from *Escherichia coli*. Furthermore, the genetically modified potatoes also contain the *barstar* gene under the control of the 35S promoter of Cauliflower mosaic virus (CaMV) and the non-coding 3' region of the gene for the transcript 7 of the T-DNA from *A. tumefaciens*.

The *barnase* gene codes for the ribonuclease barnase; the gene product of the *barstar* gene is a specific inhibitor protein for barnase, which forms a 1:1 complex with this protein.

The potato clones were generated by transformation with binary vectors (pTCV15, pTCV17), in which the length of the *prp1-1* promoter in front of the *barnase* gene differs (432 bp in pTCV15 and 273 bp in pTCV17). The *prp1-1* promoter is a promoter which can be selectively induced by various pathogens such as fungi, certain viruses (PVY, PLRV) or nematodes but which does not respond to abiotic stress such as wounding, heat shock or changes in light and darkness.

If a genetically modified potato is infected with a fungal pathogen, e.g. *Phytophtora infestans*, the *barnase* gene is locally induced in the infected cells. The *barnase* gene codes for the ribonuclease barnase, which causes the death of the infected cells. This cell death is supposed to inhibit the spread of the fungal pathogen. Because the *prp1-1* promoter shows weak constitutive expression of the *barnase* gene, the *barstar* gene under the control of the constitutive CaMV promoter was additionally inserted into the genetically modified plants. The *barstar* gene codes for the barnase-specific inhibitor barstar, which prevents cell death in the case of weak expression of the *barnase* gene.

No adverse effect is expected to result from the expression of both genes in the potato plants because ribonucleases with a similar activity to barnase occur naturally in plants. Because of its high specificity for barnase, the inhibitor barstar is unlikely to have the ability to inhibit plant or animal ribonucleases too.

b) The GUS gene (*uidA* gene) from *E.coli*

The GUS gene which is contained in the genetically modified potato clones specified under point I.1.1. Nr. II) is expressed under the control of the *prp1-1* promoter of potato and the nopaline synthase termination region from *Agrobacterium*.

The GUS gene is a reporter gene and makes it possible to examine the activity of the *prp1-1* promoter in various tissues of the genetically modified potatoes by means of an enzymatic test. The enzyme β-glucuronidase cleaves glucuronides and is found in tissues of vertebrates, invertebrates and also in bacteria, but not in plant tissues. After addition of a corresponding substrate, the enzyme activity in the tissues can be demonstrated photometrically. Plants are not expected to gain a selective advantage through the expression of the GUS gene.

c) The *nptII* gene

The *nptII* gene, under the control of the nopaline synthase promoter and the terminator of the octopine synthase gene, was inserted into all of the genetically modified potato clones. The *nptII* gene served as a marker gene for the selection of transformed plant cells and encodes the enzyme neomycin-phosphotransferase.

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. The clinically relevant antibiotic gentamicin and other aminoglycosides and aminocyclitoles used in human medicine do not belong to the substrate spectrum of the APH(3')II enzyme. Kanamycin and neomycin are, however, widely used in veterinary medicine. Owing to the substrate specificity of neomycin phosphotransferase, in the absence of substrate under field conditions no new metabolic products are expected to arise in the genetically modified potatoes. Since the relevant antibiotics are not present in the soil in high concentrations, 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.

d) Border sequences from Ti-plasmids and regulatory sequences

The genetically modified plants contain sequences from the left and the right border region of the TL-DNA of the plasmid pTiB6S3 from *A. tumefaciens*. These sequences, dependent on the gene products of the *vir* region of the helper plasmid pGV2260 present in the *Agrobacterium* strain used in the transformation which was not transferred to the plants, cause the genes located between the border regions to integrate into chromosomes of the potato plants. These border regions of the Ti plasmids 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 plants contain the following regulatory sequences:

- the 35S promoter of Cauliflower mosaic virus (CaMV),
- the promoter region of the *prp1-1* gene from *Solanum tuberosum*,
- the promoter of the nopaline synthase gene from *A. tumefaciens*,
- the terminator region of the nopaline synthase gene from *A. tumefaciens*,
- the terminator region of the octopine synthase gene from *A. tumefaciens*,
- the terminator region of the gene for transcript 7 of the T-DNA from *A. tumefaciens*.

The promoter and termination sequences regulate the expression of the genes located between them; these are the *barnase* gene, the *barstar* gene, the GUS gene and the *nptII* gene. Further information on the effects of the expression of these sequences in the plants are found under III.1.2.1.a) to c).

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, individual cases of the transfer of sequenc-

es outside the borders have been reported and, on the basis of the information contained in the notification, this possibility cannot be ruled out.

According to the information gleaned from the notification, in the present case, the following functional units could have been transferred to the genetically modified potato plants through the integration of DNA fragments located outside the border regions: (i) a spectinomycin/streptomycin resistance gene for selection in bacteria, (ii) the origin of replication of the vector pBR322, (iii) the origin of replication of the pseudomonas plasmid pVS1, as well as (iv) in the case of the vectors pTCV15 and pTCV17, a second copy of the *barstar* gene with regulatory elements for expression in *E.coli.*

(i) Because the spectinomycin/streptomycin resistance gene derives from a plasmid of gram-negative bacteria and is expressed under the control of a bacterial promoter, it is not expected to be expressed in the plants. An effect of the gene on the plant metabolism is therefore not expected.

(ii) and (iii) The origins of replication of pBR322 and pVS1 enable the replication of the original vectors pTCV1, pTCV15 and pTCV17 in a broad host range of gram-negative bacteria. The vectors are only mobilisable between bacteria in the presence of the appropriate helper plasmids; there is no evidence that the origins of replication have any function in plants.

(iv) In the case of the vectors pTCV15 and pTCV17, a second copy of the *barstar* gene was inserted into the coding region of the ampicillin resistance gene of pBR322. It is controlled by expression signals for *E. coli* in order to guarantee inhibition of the ribonuclease barnase in case of replication in bacteria. The ampicillin resistance gene is inactivated by the insertion. Expression of the *barstar* gene in plants is not expected to result from this construct.

f) 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 could mean that the properties of the genetically modified potato plants in relation to resistance behaviour to fungal pathogens might not be altered to the same extent under field conditions as under climate-chamber or greenhouse conditions, i.e. the level of resistance in the field could be increased or reduced. This does not imply any risks 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 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 experiments with the genetically modified plants no evidence of increased allergenicity of these plants was found.

Under normal cultivation conditions in the greenhouse and in the field, the potato variety “Bintje” is male sterile. This means that pollen does not play a role as a potential trigger for pollen allergies. In general, potato pollen does not play a significant role as an allergen.

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 occasionally found outside cultivated areas on non-natural sites such as waysides and other ruderal areas. However, because potato is not frost resistant, no permanent establishment occurs in in those areas either.

As a result of potato cultivation, in the following growing season "volunteer potatoes" can emerge on land used for agriculture from tubers left in the soil after harvesting. Potato tubers are sensitive to frost. Their survival is therefore primarily affected by winter temperatures.

The plan is to harvest the tubers of the genetically modified potatoes by hand and, following evaluation, to inactivate them by autoclaving, provided they are not required for further scientific investigation.

The potato leaves and stalks are to be left to decompose on the release plots. After harvesting, only shallow harrowing of the soil is to be carried out in order to level the surfaces. During the growing seasons of the two years following the trial, the plots are to remain fallow or, alternatively, only plants that do not impede post-trial monitoring for potentially emerging potato plants are to be cultivated. During the two-year cultivation gap any potentially emerging potato plants are to be removed.

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 required by supplementary provision II.8. In order to remove any tubers that may have been left in the soil, the soil of the release plot is to be loosened to a depth of approx. 15 to 20 cm after the tuber harvest as well as in the spring of the following year. Any tubers found are to be destroyed.

Plants of the “Bintje” potato variety are male sterile. Because potato is primarily self-fertilising, under normal growing conditions in the field and in the greenhouse, the “Bintje” variety does not form any berries and so does not produce seeds. However, it is conceivable that the genetically modified potatoes could be fertilised as a result of the spread of foreign potato pollen. Therefore, although it is highly unlikely, the possibility that seeds might develop cannot be ruled out completely.

Should tubers or seeds remain in the soil, the resulting plant growth would be detected during the post-trial monitoring period planned by the applicant and required by supplementary provision II.9. The frost sensitivity of the tubers is unlikely to change as a result of the genetic modification. During the monitoring period following completion of the deliberate release, no plants or only plants that do not impede monitoring may be cultivated on the trial sites. This means that volunteer potatoes can be detected easily.

For these reasons, the genetically modified plants are not expected to establish or persist 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

In principle, the possibility of a pollen-mediated transfer of genetic material to other plants is very limited. Attempts to crossbreed potatoes with wild relatives found in Central Europe (*Solanum nigrum, Solanum dulcamara*) were unsuccessful. A successful transfer of pollen could only take place to other potato plants, whereby the probability that potato plants would grow from seeds is low. Such plants would be eliminated in the course of the usual cultivation measures during crop rotation. Planting material for the cultivation of potatoes is propagated vegetatively, i.e. not via seeds.

In the case of the potato variety “Bintje”, the possibility of a pollen-mediated transfer of the inserted genes to other potato plants can be excluded because, under normal growing conditions in the greenhouse and in the field, the variety is male sterile, i.e. pollen production does not occur. Histological analyses of the pollen sacks show that “Bintje” does not produce intact, viable pollen. For this reason, an isolation distance to neighbouring potato fields is not required. The applicant plans to establish a control strip of 3 m around the trial site and this is considered adequate to take into account any potential emergence of genetically modified potatoes on adjacent areas due to possible inaccuracies during planting, cultivation or harvesting.

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 to suggest that genetic information is transferred from plants and expressed in microorganisms 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 might, in principle, be possible.

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 such 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.

The *barnase* gene and the *barstar* gene derive from the ubiquitously distributed microorganism *Bacillus amyloliquefaciens.* Hence these genes could also – with a far higher probability – enter into other organisms in the environment by horizontal gene transfer from this non-genetically modified organism. The GUS gene codes for a glucuronidase which does not occur in plants. However, glucuronidases are widespread in microorganisms so that here too – even in the case of a horizontal gene transfer – no noticeable increase in the overall frequency would result.

As already mentioned under III.1.2.1.(c), the antibiotics inactivated by the neomycin-phosphotransferase are of little relevance in human medicine but are widely used in veterinary medicine. Whether a potential horizontal gene transfer of the *nptII* gene would affect the therapeutic use of the relevant antibiotics was examined as a precautionary measure.

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 between microorganisms 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.

Also, in the case of a transfer of the other regulatory sequences used in the constructs, there are no grounds for concern that the overall frequency of the relevant DNA sequences would be increased. The *prp1-1* promoter is naturally present in potato. The other regulatory sequences derive from *A. tumefaciens* and CaMV. *A. tumefaciens* is widespread in soils and the sequences in question are found on Ti-plasmids in wild-type agrobacteria which can be exchanged between various *Rhizobiaceae* by conjugation. The theoretical possibility of a transfer of the CaMV sequences from the genetically modified plants would not constitute a new situation compared to the naturally occurring situation because CaMV, as a double-stranded plant-infecting DNA virus, is already present in plants.

The genetically modified potato plants may also contain nucleic acid fragments which originate from the regions of the binary plasmids outside the T-DNA (see III.1.2.1.e). It should be noted that the origins of replication of the plasmids pBR322 and pVS1 are present in gram-negative bacteria. Therefore, the likelihood of these nucleic acid fragments being spread through transfer between bacteria is far higher than the likelihood of their being spread through horizontal gene transfer from the genetically modified plants to microorganisms.

The same evaluation applies to the spectinomycin/streptomycin resistance gene, which originally derives from a gram-negative plasmid of the incompatibility group P. The ß-lactamase gene, which is inactivated through the insertion of a second copy of the *barstar* gene, is present in a range of *Enterobacteriaceae* as well as in a number of other gram-negative bacteria. Even if it were present in its intact form, no noteworthy increase in the overall frequency of this resistance mechanism in the environment would be expected.

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

To generate the genetically modified plants, sterile potato leaves were inoculated with agrobacteria which contained the genes to be transferred between the border regions of binary vector plasmids. Following transformation, antibiotic treatment was carried out to eliminate the agrobacteria. In order to demonstrate that the propagation material of the plants proposed for release was free from agrobacteria, tissue homogenates were spread onto appropriate culture media. No agrobacteria were detected in the cultures.

The possibility that the plants proposed for release might contain very small amounts of the agrobacteria used for the transformation cannot be ruled out. In contrast to the ubiquitous wild-type *Agrobacterium*, the *Agrobacterium* strain used for the transformation is disarmed, i.e. it no longer has the capacity to induce tumours. In the unlikely but theoretically conceivable event that the inserted foreign genes are transferred to a cell of another plant, that cell would have to spontaneously regenerate into a whole, fertile plant for the foreign genes to enter the germ cells. This is the only way that these genes could be passed on to the 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 plasmid 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..