



Notification 6786-01-0188

Summary of the risk assessment of genetically modified black nightshade

(*Solanum nigrum* L.) (transformation events S03/71-13 and S03/82-3)

carried out by the German Competent Authority within

the framework of a proposed deliberate release,

Berlin, 20 July 2007

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
 - III.1.2. Requirements for approval according to section 16 (1) Nr. 3 GenTG
 - III.1.3. Requirements for approval according to section 16 (1) Nr. 2 GenTG
 - 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 construct for silencing the *nigpro* gene

The prosystemin gene (*nigpro*) codes for the peptide prosystemin (200 amino acids), from which the active 18 amino acid long peptide systemic is cleaved by proteolysis. Systemin is a signaling molecule which plays an important role in mediating the systemic wound response in plant tissues by transmitting information from the wound site to more distant plant tissues, where additional defence responses are induced.

Studies on genetically modified tomatoes expressing a prosystemin cDNA in antisense orientation demonstrated that plants which were only able to produce small amounts of the prosystemin peptide had a significantly reduced capacity to form type I and type II protease inhibitors as a systemic response to wounding. In addition, the plants were significantly less well able to respond to attack by chewing larvae of *Manduca sexta*.

In the genetically modified plants proposed for release, synthesis of the plant's native prosystemin or active systemin is to be reduced in order to examine the function of the peptide in other interactive processes between the plant and the environment. For this purpose, a construct was developed in which two complementary internal fragments of the prosystemin gene from *S. nigrum*, separated by a spacer, were arranged in sense- and in antisense orientation. The sense and the antisense region of the transcript of the *nigpro* gene are expected to hybridise and the production of prosystemin is expected to be reduced as a consequence of the triggering of so-called post-transcriptional gene silencing. In this case, too, the third intron of the pyruvate orthophosphate dikinase gene from *Flaveria trinervia* was used as a spacer, which triggers splicing activity. The expression is likewise controlled by the promoter and the termination signal of the 35S gene of cauliflower mosaic virus (CaMV).

As a consequence of the genetic modification, targeted reduction of the production of the prosystemin peptide lowers the defences of the genetically modified plant against herbivores/pathogen infestation. If and in what form this will affect the further development of the plant is to be investigated in the course of the proposed field trials. Previous trials carried out under greenhouse and field conditions (Notification 6786-01-0156) were able to show that a reduction of the endogenous systemin content in *S. nigrum* had no effect on the weight gain of larvae of the tobacco hornworm (*Manduca sexta*), the number of aphids (*Myzus persicae nicotianae*) or the extent of the feeding sites of the flea beetle. From this, the applicant concludes that the prosystemin peptide does not mediate direct defence mechanisms in *S. nigrum*.

S. nigrum is not cultivated as a crop or food plant in Central Europe. Human consumption of the plant is neither planned nor expected. In the opinion of the Central Commission for Biological Safety (ZKBS), the described changes in these genetically modified plants are not expected to pose any threat to human or animal health or to the environment.

(b) The hygromycin phosphotransferase gene *hptII*

The T-DNA of the transformation plasmid used contains the hygromycin phosphotransferase gene (*hptII*) from *E. coli*, which functions as a selectable marker for selecting genetically modified plant tissue after transformation. The hygromycin phosphotransferase encoded by the *hptII* gene specifically inactivates the antibiotic hygromycin by phosphorylation. This sub-

strate specificity justifies the expectation that in the absence of substrate no new metabolic products can arise in the genetically modified plants under field conditions. Moreover, this gene does not confer any selective advantage to the genetically modified plants under field conditions, since hygromycin is not present in the soil in higher concentrations.

Since hygromycin is highly toxic to eukaryotic organisms, it is not used in human medicine and it is only applied in specific areas of veterinary medicine. Hygromycin-resistant *Enterobacteriaceae* containing a gene that codes for hygromycin phosphotransferase have been found in sample material of human and animal origin (faeces, urine, blood) and are released into the environment by those humans/animals.

The European Food Safety Authority's (EFSA) *Scientific Panel on Genetically Modified Organisms* has evaluated the possible use of antibiotic resistance genes as selectable markers in genetically modified plants for deliberate release and placing on the market. As a result, antibiotic-resistance genes commonly used in the transformation of plants were classified into three groups. The *hph* gene was assigned to Group I, since the antibiotic hygromycin is not used in human medicine and its use is seldom indicated in veterinary medicine. In the case of the antibiotic resistance genes assigned to Group I, the scientific panel believes that it is highly unlikely that the presence of these genes in the genome of genetically modified plants will lead to perceptible changes in the spread of these genes in the environment or have any appreciable impact on human or animal health. This evaluation is in line with the position of the ZKBS (Central Commission on Biological Safety). The use of the hygromycin resistance gene in genetically modified plants is not expected to pose a threat to human health or to the environment.

The *hptII* gene was developed from the *hph* gene by targeted mutagenesis. Both genes and their gene products are practically identical (99% on the nucleic acid level, 99.4% on the amino acid level). There is no evidence to suggest that the *hptII* gene used in the genetically modified plants should be assessed any differently to the *hph* gene.

(c) Sequences located outside the T-DNA

As a general rule, only DNA located within the border regions is integrated into the plant genome in *Agrobacteria*-mediated transformation events. However, the transfer of DNA fragments outside the border regions has been reported. The plasmid used in the transformation, pSOL3SYS, can be traced back to the binary plasmid pSOL1, which was developed from functional elements of the plasmids pCAMBIA-1301, pBI121 and pUC. The sequences of these plasmids are known and can be accessed via databases. The transformation vector contains the following outside the border regions:

- the *aphAIII* (= *nptIII*) gene from *Streptococcus faecalis* (= *Enterococcus faecalis*), under the control of its native promoter, is only functional in bacteria;
- the ColE1 origin of replication for replication in *E. coli*;
- the origin of replication of the pVS1 plasmid from *Pseudomonas aeruginosa* with the genetic information for replication and stability in *A. tumefaciens*.

The results of PCR studies submitted together with the application indicate that there is no whole *nptIII* gene present in the genetically modified lines intended for release. Since there are no known results from additional analyses on the sequences integrated into the genet-

ically modified plants, for the purposes of the risk assessment, a possible integration of other vector fragments located outside the T-DNA is assumed. There is no evidence to suggest that the replication regions of ColE1 and pVS1 have any function in higher plants.

(d) Position effects and context changes; allergenicity

Genes which have been integrated into the plant genome by genetic engineering are expressed at different levels, depending on the integration site on the chromosome or rather on the sequence 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 black nightshade plants are not altered to the same degree in the open field as under climate-chamber or greenhouse conditions. This does not 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 can affect plant metabolic pathways. However, in greenhouse studies on the genetically modified plants no observations were reported 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 influence plant metabolic pathways. In this regard the genetically modified plants do not differ fundamentally from non-genetically modified plants.

Of the genetic changes present in the genetically modified plants in question here, only the hygromycin phosphotransferase gene leads to the formation of a protein. According to current knowledge, it is not possible to predict the potential allergenicity of a protein on the basis of the amino acid sequence. However, in previous experiments with genetically modified plants and in numerous deliberate releases with plants which express the *hph* gene under the control of non-tissue-specific promoters, no evidence for an increased allergenic potential was found.

S. nigrum is not cultivated as a crop or food plant in Central Europe. Human consumption of the plant is neither planned nor expected. The pollen of black nightshade does not generally play a noteworthy role in triggering pollen allergies. The genetic modifications described are not expected to pose any threat to human or animal health or to the environment.

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

S. nigrum is an annual herbaceous plant. *S. nigrum* plants die off after the generative phase has come to an end. New plants can only emerge from seeds that mature in berries; vegetative propagation does not occur. After entering secondary dormancy the seeds can survive under favourable conditions for many years without losing their ability to germinate. These plants are sensitive to frost; they do not survive the low winter temperatures of Central Europe.

In the proposed field trial, plants which have been raised in the greenhouse are to be released. Seeds will not be sown in the field. The application describes plans to control the release site at most every three days and to remove any flower buds that may develop in order to prevent the dispersal of pollen and the formation of seed. In view of the fact that the flowering phase lasts a minimum of only five days, provision II.10 (to the present application) stipulates that the genetically modified plants are to be controlled for the emergence of flower buds at least every two days. After the plants and their roots have been removed from the release site, the area is to be controlled for the emergence of genetically modified *S. nigrum* plants at regular intervals up to the end of November of the respective experimental year. Any emerging plants shall be removed and destroyed. Moreover, at the end of the proposed release the experimental area will be subject to a post-trial monitoring period which, in the case of the appearance of genetically modified black nightshade plants, will be extended for a further year. These measures are adequate to ensure that no genetically modified plants remain in the field; the formation of seed can also be ruled out. Hence, the genetically modified plants are not expected to persist.

S. nigrum preferentially colonises open and disturbed habitats such as gardens, crop land, waysides, hedgerows, railway tracks, landfill sites, etc. The presence of the herb in the vicinity of the release site is documented for that region. Due to frost-sensitivity the plants die off at the beginning of the frost period; to date there have been no reports of overwintering in our climate. In view of the precautionary measures described above the genetically modified plants from this deliberate release are not expected to become established 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 the field, the plants are to be controlled at maximum intervals of two days for the appearance of flower buds. Any flower buds found will be removed prior to anthesis, i.e. before dispersing pollen. As a result, the genetically modified plants are not expected to produce or disperse pollen.

Non-genetically modified black nightshade control plants which can also reach the flowering stage will be planted in close proximity to the genetically modified plants. Provision II.8 to the decision on this application requires the permanent labelling of the genetically modified plants, thus ensuring that the genetically modified plants can be easily differentiated from the control plants.

Although *Solanaceae* are considered predominantly self-pollinating species, outcrossing and foreign pollination are possible, and the appearance of hybrid species and genera with *S. nigrum* has been observed. For example, spontaneous hybrids derived from the hexaploid *S. nigrum* and *S. physalifolium* var. *nitidibaccatum* (diploid) and *S. villosum* (tetraploid), which also occur in Germany, have been reported. In some *Solanum* species, flower-visiting bees and hover flies are known to be responsible to a small extent for foreign pollination. Currently, there is no information available regarding the distances over which outcrossing can be expected to take place.

To prevent the emergence of *S. nigrum* plants outside the 1.5 hectare release site, a 3 m wide strip of clover/grass mix will be planted along its inner border. The applicant also plans to carry out inspections of a 35 m area around the release site, during which sexually compatible plant species are to be removed before reaching maturity.

III.1.2.4. Assessment of the possibility of transfer of the inserted foreign genes from the genetically modified plants to micro-organisms by horizontal gene transfer

The inserted sequences are stably integrated into the chromosomes of the recipient organisms. No evidence exists to suggest 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.

If we assume that an exchange of genetic material between organisms which are so distantly related in terms of taxonomy as plants and bacteria actually takes place, it must be concluded that the occurrence of an exchange of heterologous genetic material cannot by 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 gene transfer would only be expected to have ecologically relevant effects if selection pressure were to favour the transferred gene. Furthermore, the evaluation would have to consider whether the gene in question already exists in corresponding populations or whether we are dealing with a new gene. In the absence of selection pressure for the traits transferred with the constructs, however, no ecological consequences are anticipated.

(a) The construct for silencing of the *nigpro* gene

Both *nigpro*-specific internal fragments of the construct were isolated from *S. nigrum*; the "spacer" between the two complementary fragments originates from the third intron of the pyruvate orthophosphate dikinase gene from *Flaveria trinervia*. *S. nigrum* is widespread in Central Europe. Pyruvate orthophosphate dikinase belongs to the primary enzyme structure of plants with C4 physiology, for example, maize.

The regulation sequences used in the constructs are derived from CaMV and *Agrobacterium tumefaciens*. *A. tumefaciens* is widespread in the environment. In wild-type *Agrobacterium* those sequences are found on the Ti-plasmids, which can be exchanged between different strains of Rhizobiaceae by conjugation. CaMV is a plant-infecting, double-stranded DNA virus which is widespread in plants.

Therefore, all of these genetic elements can be spread by horizontal gene transfer from non-genetically modified organisms.

(b) The antibiotic resistance gene *hptII*

The *hptII* gene, which codes for the hygromycin phosphotransferase enzyme, originates from *E. coli*. Since hygromycin is highly toxic to eukaryotic organisms, it is not used in human medicine and it is only used for specific indications in veterinary medicine. Hygromycin-resistant Enterobacteriaceae containing a gene that codes for hygromycin phosphotransferase have been found in sample material of animal and human origin (faeces, urine, blood) and are released into the environment by those animals/humans. The probability of this gene being spread by transfer between bacteria is therefore far greater than the probability of being spread by horizontal gene transfer from the genetically modified plants to micro-organisms.

(c) DNA fragments located outside the T-DNA

Using PCR analysis it was possible to demonstrate that the bacterial selection marker of the transformation plasmid pSOL3SYS (*nptIII* gene for kanamycin resistance) was not transferred into the genome of the transgenic plants intended for release. However, whether the remaining plasmid fragments (*ori* ColE1, *ori* pVS1, *repA*, *staA*) were transferred to the genetically modified plants was not investigated.

The host range of the *origin of replication* (*ori*) ColE1 is limited to a small number of gram-negative bacteria. Essentially, the replicon can replicate in *E. coli* and in closely related species of bacteria, such as *Serratia* or *Salmonella*. Replication does not take place in the majority of gram-negative soil bacteria. ColE1 plasmids occur quite frequently in enterobacteria. Genetic material is far more likely to be transferred from enterobacteria to other bacteria than by horizontal gene transfer from the genetically modified plants to bacteria. Therefore, although the presence of the origin of replication in the plant chromosome cannot be ruled out, it is not expected to contribute to an increase in the overall frequency of horizontal gene transfer.

The replication region of the pVS1 plasmid from *Pseudomonas aeruginosa* holds the genetic information for replication and stability and enables replication of the plasmid in *Agrobacterium tumefaciens*. In the case of these DNA fragments, too, the probability of genetic spread by transfer between bacteria is far higher than the probability of spread by horizontal gene transfer from the genetically modified plants to micro-organisms.

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

In order to generate the genetically modified plants, hypocotyl leaves from in vitro-cultivated, one-week-old seedlings of the *S. nigrum* inbred line Sn30 were inoculated with Agrobacteria containing the transformation constructs between the border regions of the binary plasmids. After transformation, antibiotic treatment was carried out to eliminate the Agrobacteria.

In contrast to widespread wild-type *A. tumefaciens*, the applied Agrobacterium strain LBA 4404 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 via these Agrobacteria, the plant would have to spontaneously regenerate into a whole, fertile plant to enable the foreign genes to enter the germ cells. This is the only way that these genes could be passed on to the plant progeny. 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, the potential transfer by conjugation of the binary plasmids contained in the Agrobacteria to wild-type Agrobacteria (*A. tumefaciens* or *A. rhizogenes*) present in the environment would also have to be considered, since these could, in turn, pass on the foreign genes to individual cells of other plants.

In the event of infection and subsequent transformation via wild-type bacteria (*A. tumefaciens* or *A. rhizogenes*) a crown gall or hairy root tumour would develop from the transformed plant cell. Under natural conditions such a tumour would not be expected to give rise to a plant.

Furthermore, a possible transfer of the inserted genes from Agrobacteria to other soil bacteria should be considered. Possible effects have already been discussed under III.1.2.4.

