Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

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Summary of the risk assessment of the genetically modified black nightshade

(*Solanum nigrum* L.) S03/140-5 and S03/144-2

within the framework of a proposed deliberate release

carried out by the German Competent Authority

Berlin, 2nd of June 2005

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
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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 construct for silencing the *pr*-1S gene

The *pr*-1S gene derived from *Solanum nigrum* codes for a *pathogenesis-related protein*. PR proteins are proteins encoded by the host plant which are specifically formed in response to pathogen attacks or comparable stress factors. They do not only accumulate in the leaf inoculated by the pathogen. In connection with what is referred to as systemic acquired resistance (SAR), the formation of PR proteins is also induced systemically. As a consequence of SAR, the plants are largely or fully protected against later infections with the same or even another pathogen.

Based on their primary amino acid structure, their serological relation and/or their enzymatic or biological activities, PR proteins are classified into 14 groups. Only the group of PR-1 proteins has so far not been assigned a specific function. Their mode of action and their relation to other proteins is unknown.

In the transgenic plants intended for release, the synthesis of the plant's PR protein 1S is to be reduced with the aim of examining the function of the protein during further interaction processes between the plant and the environment. For this purpose, a construct was developed in which two complementary internal fragments of the *pr*-1S gene derived from *Solanum nigrum* were arranged in sense and antisense orientation, separated by a spacer. The two transcribed fragments are expected to hybridise and the resulting so-called post-transcriptional gene silencing is expected to limit the formation of the endogenous PR-1S protein. The basic functionality of this system is verified by corresponding tests with *Nicotiana attenuata*. The third intron of the pyruvate orthophosphate dikinase gene, of which splicing activity is suspected, was used as a spacer. Expression is controlled by the promoter and the terminator signals of the 35S gene of the cauliflower mosaic virus (CaMV).

As a result of genetic modification, the resistance of the transgenic plant to phytopathogenic microorganisms is reduced by targeted reduction of the formation of the PR-1S protein. The applicant's observations of these genetically modified plants in the greenhouse demonstrate that they also exhibit increased susceptibility to herbivorous insects. Whether and how this will have an influence on the plant's further development is to be examined in the course of the proposed deliberate release.

Solanum 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 Committee on Biological Safety (ZKBS), the described changes in these genetically modified plants are not expected to pose any risk to human or animal health or to the environment.

(b) The hygromycin phosphotransferase gene *hpt*II

The T-DNA of the transformation plasmid used contains the hygromycin phosphotransferase gene (*hph*II) from *E. coli*, which functions as a selectable marker for the enrichment of transgenic plant tissue after transformation. The hygromycin phosphotransferase encoded by the *hph*II gene specifically inactivates the antibiotic hygromycin by phosphorylation. This substrate 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 animal and human-derived specimens (faeces, urine, blood) and are thereby released into the environment.

The European Food Safety Authority's (EFSA) *Scientific Panel on Genetically Modified Organisms* has evaluated the possible use of antibiotic resistance genes as selection 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 applied 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 transgenic 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 Central Committee on Biological Safety. The use of the hygromycin resistance gene in genetically modified plants is not expected to pose a risk to human health or to the environment.

The *hph*II gene was developed from the *hph* gene by targeted mutagenesis. Both genes and/or gene products are practically identical (on the nucleic acid level 99%, on the amino acid level 99.4%). There is no evidence to suggest that the *hph*II gene used in the genetically modified plants should be evaluated 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 *Agrobacterium*-mediated transformation events. However, the transfer of DNA fragments outside the border regions has been reported. The plasmid used for transformation, pSOL3PRIS, 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. Outside the border regions, the transformation vector contains:

- the *aphA*III (= *npt*III) gene from *Streptococcus faecalis* (= *Enterococcus faecalis*), under the control of its own promoter, which is only functional in bacteria;

- the ColE1 origin of replication for replication in *E. coli*;

- the replication region of the plasmid pVS1 derived from *Pseudomonas aeruginosa* with the genetic information for replication and stability in *A. tumefaciens*.

The results of PCR analyses submitted together with the application indicate that there is no whole *npt*III 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 transgenic 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 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 neighbouring sequence at 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 modified to the same degree in the field as under climate-controlled or greenhouse conditions. This does not represent a risk to the environment or to human or animal health.

The insertion of foreign genes may influence the expression or regulation of native plant 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 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 influence plant metabolic pathways. In this regard, the genetically modified plants do not differ fundamentally from non-genetically modified plants.

Of the genetic changes in these genetically modified plants, only the hygromycin phosphotransferase gene leads to the formation of a protein. Given the current state of knowledge, it is not possible to make reliable predictions about the potential allergenicity of a protein on the basis of its amino acid sequence. However, in previous experiments with genetically modified plants and in numerous deliberate release trials with plants which express the *hph* gene under the control of non-tissue-specific promoters, no evidence was found to suggest an increased allergenic potential of the plants.

Solanum nigrum is not cultivated as a crop or food plant in Central Europe. Human consumption of the plant is neither planned nor expected. As a rule, the pollen of black nightshade does not play a noteworthy role in triggering pollen allergies. The genetic modifications described are not expected to pose any risk 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 establish in the environment

Solanum nigrum is an annual herbaceous plant. *S. nigrum* plants die off after completion of the generative phase. 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. The plants are sensitive to frost; they do not survive the low winter temperatures of Central European latitudes.

In the proposed field trials, greenhouse-cultivated plants are to be released. Seed will not be sown in the field. To prevent the dispersal of pollen and the formation of seed, the application includes plans to control the release site on a daily basis for the possible appearance of flower buds, which would then be eliminated. After the plants have been removed from the

release site by hand, the release site will be ploughed, kept free of vegetation and will be controlled for the emergence of genetically modified *S. nigrum* plants at regular intervals up to the end of December of the respective experimental year. Any emerging plants will be removed and destroyed. Moreover, at the end of the proposed release, the release area will be subject to a post-trial monitoring period which, in the case of the appearance of transgenic 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 transgenic plants are not expected to persist.

Solanum 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 transgenic plants from this deliberate release are not 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

In the field, the plants will be controlled for the appearance of flower buds on a daily basis. Any flower buds found will be removed prior to anthesis, i.e. before dispersing pollen. As a result, the transgenic plants are not expected to form or disperse pollen.

Non-genetically modified black nightshade control plants that may also reach the flowering stage will be planted in close vicinity to the genetically modified plants. The supplementary provision II.7 of the notification on this application stipulates the permanent labelling of the transgenic plants, thereby ensuring that the transgenic plants can be easily differentiated from the control plants.

Although species of the genus *Solanum* are considered predominantly self-pollinating species, outcrossing and cross-pollination are possible, and the appearance of hybrid species and types with *S. nigrum* has been observed. Spontaneous hybrids derived from the hexaploid *S. nigrum* and *S. physalifolium var. nitidibaccatum* (diploid) and *S. villosum* (tetraploid) have been reported, which are also present in Germany. It is known that in some *Solanum* species flower-visiting bees and hover flies are, to a small extent, responsible for cross-pollination. Currently, there are no findings on the distances over which outcrossing can take place.

To prevent the emergence of *S. nigrum* plants, a clover/grass mix will be planted around the 1 hectare release site. The applicant also plans to carry out inspections and take measures around the release site. Within a radius of 35 m around the release area, sexually compatible plant species are to be removed before reaching maturity.

III.1.2.4. Assessment of the possibility of horizontal gene transfer of the inserted foreign genes from the genetically modified plants to microorganisms

The inserted sequences are firmly 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 microorganisms takes place under natural conditions. However, studies on the transformation ability of soil bacteria under natural conditions suggest that the transfer of plant genetic material to soil bacteria is theoretically

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

Insofar as we assume that an exchange of genetic material between organisms that are as distantly related in terms of taxonomy as plants and bacteria is actually possible, it can 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.

Ecologically relevant effects of gene transfer would only be expected 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 the *pr-1S* gene

Both *pr-1S*-specific internal fragments of the construct were isolated from *Solanum nigrum*; the spacer between the two complementary fragments is derived from intron 3 of the pyruvate orthophosphate dikinase gene from *Flaveria trinervia*. *S. nigrum* is widespread in Central Europe. Pyruvate orthophosphate dikinase belongs to the primary enzymatic structure of plants with C4 physiology, such as maize.

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

The *hph*II gene, which codes for the hygromycin phosphotransferase enzyme, is derived from *E. coli*. 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 animal and human-derived specimens (faeces, urine, blood) and are thereby released into the environment. The probability of genetic spread by transfer between bacteria is therefore far higher than the probability of horizontal gene transfer from the genetically modified plants to microorganisms.

(c) DNA fragments located outside the T-DNA

On the basis of PCR analysis, it was shown that the bacterial selection marker of the transformation plasmid used, pSOL3PRIS (*npt*III gene for kanamycin resistance), was not transferred into the genome of the transgenic plants proposed for release. However, whether the remaining plasmid fragments (*ori* ColE1, *ori* pVS1, *repA*, *staA*) were transferred to the transgenic plants was not investigated.

The host range of the *origin of replication* (*ori*) ColE1 is limited to a few gram-negative bacteria. Essentially, the replicon can be replicated 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. The probability of enterobacteria-mediated gene transfer to other bacteria should be regarded as far more likely than a 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 excluded, this is not expected to contribute to an increase in the overall frequency of horizontal gene transfer.

The replication region of the plasmid pVS1 derived 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 microorganisms.

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.
Following transformation, antibiotic treatment was carried out to eliminate the agrobacteria.

In contrast to the common wild-types of *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 cell 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 case 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, the transfer of the inserted genes from agrobacteria to other soil bacteria should be considered. Possible effects were already discussed under III.1.2.4.