Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

Notification 6786-01-0193

Summary of the risk assessment of the genetically modified

black nightshades (*Solanum nigrum*) S04-74, S04-84 and S04-156

carried out by the German competent authority

within the framework of a proposed deliberate release

Berlin, 11 April 2008

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 construct for silencing the lipoxygenase 3 gene

The native *S. nigrum* gene *SnLOX3* (*S. nigrum* lipoxygenase 3) codes for a jasmonic acid biosynthesis enzyme. Jasmonates are known plant signalling substances. They play an important role in development processes (maturation of pollen, fruit and seed) and also in plant reactions to biotic and abiotic stress factors, including the production of antibodies for the direct and indirect defence of plants against chewing insects. Constitutive reduction of the lipoxygenase 3 contents can influence one of the insect-induced plant defence cascades, it can alter the defence response of the plant to abiotic and other biotic stress factors, and it can affect the development processes of the plant in general.

In the genetically modified plants intended for release lipoxygenase 3 synthesis is to be reduced with the aim of examining the effects of lowering the jasmonate content in the plants in this way on further interaction processes between the plant and the environment. For this purpose a construct was developed in which two complementary, internal fragments of the lipoxygenase 3 gene derived from *S. nigrum* were arranged in sense and in antisense orientation, separated by a spacer. The transcription products of the two fragments are expected to hybridise and the resulting so-called post-transcriptional gene silencing is expected to limit the formation of endogenous lipoxygenase 3 and, therewith, the formation of jasmonate. 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 terminator signals of the 35S gene of the cauliflower mosaic virus (CaMV).

Regarding the functions of the gene under examination, the results of greenhouse experiments on the phenotype of the genetically modified SOL3LOX plants confirm the functions described in the literature. Damage inflicted by the tobacco hornworm (*Manduca sexta*) on SOL3LOX plants is significantly greater than that inflicted on wild-type and “empty vector” plants. These observations are now to be corroborated under field conditions in a natural environment.

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 *hpt*II

The T-DNA of the transformation plasmid used contains the hygromycin phosphotransferase gene (*hpt*II) from *E. coli*, which functions as a selectable marker for selecting genetically modified plant tissue after transformation. The hygromycin phosphotransferase encoded by the *hpt*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 Enterobacteriacae 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 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 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 *hpt*II gene was developed from the *hph* gene by targeted mutagenesis. Both genes 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 *hpt*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 Agrobacteria-mediated transformation events. However, the transfer of DNA fragments outside the border regions has been reported. The plasmids used in the transforma-

tion, pSOL3LOX and pSOL3NC, can be traced back to the binary plasmid pSOL1, which was developed from functional elements of the plasmids pCAMBIA-1301, pBI121 and pUC19. The sequences of these plasmids are known and can be accessed via databases. The transformation vector contains the following outside the border regions:

- the *aph*AIII (= *npt*III) gene from *Streptococcus faecalis* (= *Enterococcus faecalis*), under the control of its own promoter, which is only functional in bacteria;
- the ColE1 replication origin 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 studies 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 genetically 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 methods are expressed at different levels, depending on the site of integration on the chromosome and 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 represent 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 in these genetically modified plants, only the hygromycin phophotransferase 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. 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 post 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 establish in the environment

S. 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. These 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. Seeds 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 at least every two days for the possible appearance of flower buds, which would then be eliminated. After the plants and their roots have been removed from the release site, the area will 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 will 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 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 at maximum intervals of two days. 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 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. Provision II.9 of the decision on this application stipulates the permanent labelling of the genetically modified plants, thereby 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 types with *S. nigrum* has been observed. Spontaneous hyrbids 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 foreign pollination. Currently there are no findings on the distances over which outcrossing can 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 the 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 could be concluded that the occurrence of an exchange of heterologous genetic material does not in itself represent a safety criterion, since such an exchange could always result in the uptake of all forms of heterologous genetic material, including all forms of plant DNA.

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 the lipoxygenase 3 gene

Both lipoxygenase 3-specific internal fragments of the construct were isolated from *S. nigrum*; the spacer between the two complementary fragments is derived from intron 3 of the pyruvate orthophosphate dikinase gene from *Flaceria 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 regulation sequences used in the constructs are derived from CaMV and *Agrobacterium tumefaciens*. *A. tumefaciens* is widespread in the environment. In wild-type Agrobacteria the sequences referred to above 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 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 *hpt*II

The *hpt*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 Enterobacteriacae 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 greater than the probability of horizontal gene transfer from the genetically modified plants to micro-organisms.

(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 plasmids used, pSOL3LOX and pSOL3NC (*npt*III gene), 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 genetically modified 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. CoIE1 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 presence of the origin of replication in the plant chromosome can not 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 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 the common 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 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 have already been discussed under III.1.2.4.