Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

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Summary of the risk assessment of genetically modified tobacco plants (*Nicotiana tabacum*) carried out by the German Competent Authority within the framework of a proposed deliberate release

Berlin, 25 May 2012

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

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Only the original German document is legally binding. The following passage is a courtesy translation of the chapter III.1.3. and was prepared for the Biosafety Clearing-House.

III.1.3. Authorisation requirements according to § 16 (1) No. 3 GenTG

The condition for approval required under § 16 (1) No. 3 GenTG that, according to the current state of scientific knowledge in relation to its purpose, the deliberate release is not likely

to have any unacceptable harmful effects on the legal interests named in § 1 No. 1 GenTG is met, as will be justified in the following:

III.1.3.1. Evaluation of changes in the genetically modified plants effected by the transferred nucleic acid sequences

(a) The *gfp* gene

Fluorescent proteins are stable proteins which are suitable for use as reporters for detecting gene expression and for protein localisation in living cells after stimulation with UV light. Reporter genes have a variety of uses in organisms and cell cultures. Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is produced as a reporter gene in genetically modified (GM) plants, (phytopathogenic) fungi, nematodes, mice and zebra fish, among others. There is no evidence that the vitality of these organisms is impaired. In a feeding study with rodents it was shown that GFPs do not pose any health risks. A comparison of the amino acid sequence with known allergens as well as the stability test of GFP in simulated gastric fluid did not show any evidence of allergenic potential of GFP (Richards et al., 2003).

Given the existing studies, the safety measures already applied in the GM plant as well as those planned within the context of the execution of the field trial, and the size of the proposed project, adverse effects on human health and the environment as protected assets under § 1 No. 1 GenTG are not to be expected. Therefore, according to the state of science and technology, no additional safety measures are required.

(b) The *aad*A gene

According to § 6 (1) sentence 2 GenTG, which was inserted in 2005 to comply with Article 4 of the Deliberate Release Directive 2001/18, the risk assessment of deliberate releases of GMOs must take into special consideration the use of antibiotic resistance markers in GMOs that confer resistance to antibiotics in use for medical or veterinary treatment in view of the identification and phasing out of the use of antibiotic resistance markers in GMOs which can have harmful effects on human health and the environment by 31 December 2008. Accordingly, the use of such antibiotic resistance markers is no longer permitted after 1 January 2009. Against this background, the *aad*A gene which was transferred to the tobacco plastids is assessed as follows:

The *aadA* gene [*ant*(3´´)-Ia; $Strep^R$/$Spec^R$] which was transferred to the plastids of the GM plants originates from the plasmid R538-1 from *E. coli*. This gene codes for the enzyme aminoglycoside-3 adenyltransferase which modifies the 3”-hydroxyl position of the N-

methyl-L-glucosamine ring of streptomycin and the 9-hydroxyl position of spectinomycin. The transferred *aadA* gene is controlled by the promoter and the terminator of the plastidial *psbA* gene from *N. tabacum*.

The *aadA* gene confers resistance to streptomycin and spectinomycin. The presence of the *aadA* gene has been demonstrated in numerous bacteria in different media such as soil, waste water, seawater, foodstuffs, clinical samples and faeces. Bacteria which are resistant to streptomycin/spectinomycin are widespread in the environment. Therefore, resistance to these antibiotics can also be spread through horizontal gene transfer from non-GM microorganisms.

These antibiotics have only limited uses in human medicine, but they are still therapeutically relevant in the treatment of tuberculosis (streptomycin) and gonorrhoea (spectinomycin) when drugs with less toxic potential cannot be applied.

The GM tobacco plants are only to be released on a limited area for a restricted period. These plants are not permitted in the production of feed or food. Given the very low probability of horizontal gene transfer from plant DNA to microorganisms and the absence of selection pressure on the release sites, the presence of the *aadA* gene in the GM tobacco plants is not expected to lead to a measurable increase in the overall frequency of this resistance mechanism in microorganisms.

Concerning the *aadA* gene, in its statement of December 2008 the *Zentrale Kommission für die Biologische Sicherheit*, ZKBS [Central Commission for Biological Safety] determined that in view of the improbability of horizontal gene transfer between plants and microorganisms and the already existing distribution of the *aadA* gene in the environment, the presence of the *aadA* gene in the genome of the GM plants will not have any effect on the distribution of this antibiotic resistance gene in the environment.

In its opinion of 26 March 2009 on the use of antibiotic resistance genes as marker genes in GM plants, the GMO Panel of the European Food Safety Authority (EFSA) found that, based on the current state of knowledge, the transfer of the *aadA* gene from plants to bacteria is not expected to have adverse effects on human health or on the environment.

Expression of the *aadA* gene from *E. coli* in plants is not expected to result in a selective advantage, since the plants are unlikely to be exposed to streptomycin or spectinomycin in agricultural or natural ecosystems.

Therefore, in line with the opinions of the ZKBS and the GMO Panel of the EFSA, harmful effects on human health and the environment or the other protected assets referred to in § 1 No. 1 GenTG are not anticipated.

(c) Additional DNA fragments of the inserted transformation plasmids

Transformation of the tobacco plants *N. tabacum* of the “Petit Havana” variety took place by particle bombardment with DNA-loaded gold particles. This transformation involved the insertion of the plasmid pDK53 derived from the plasmid pUC119. The plasmid pDK53 contains the following genetic elements of the original plasmid pUC119:

- the ampicillin resistance gene *bla*$_{TEM-1}$
- the bacterial origin of replication *ori*

To facilitate the integration of the desired fragment into the plastid DNA by homologous recombination, plastidial sequences were inserted into pUC119 in front of and behind the cloning site of the transgene. These flanking sequences correspond on the one hand to a partial sequence of the 16S RNA subunit (*rrn*16) including neighbouring sequences for the plastid tRNA Valine (*trnV*) and on the other hand to the 3’ untranslated region of the ribosomal protein S12 (*rps12*) from tobacco. The *rrn16* and *rps12/7* sequences served to integrate the foreign genes into the plastid DNA of tobacco by two crossovers in the corresponding homologous plastidial regions.

The possible transfer of parts of the vector backbone, in particular of the bacterial ampicillin resistance gene *bla*$_{TEM-1}$, to the plastid or the nuclear genome of the tobacco plants was investigated by the applicant by means of PCR analysis, for which in each case a *bla* gene-specific and a backbone-specific primer was used. With these primer sets no specific PCR fragment from the DNA of *N. tabacum* could be amplified. Although the transfer of the functional ampicillin resistance gene *bla*$_{TEM-1}$ from the vector backbone is thus unlikely, no reliable statement can be made about the presence of the ampicillin resistance gene *bla*$_{TEM-1}$ and other sequences of the vector backbone from this investigation. Consequently, the risk assessment is carried out under the assumption that these sequences are contained in the plants.

The aforementioned segments located outside the region that is suitable for homologous recombination regulate expression in bacteria and have no function in plants. The formation of significant amounts of functional gene products based on these sequences is not anticipated in the GM plants, since they are not driven by plant-specific promoters nor are they adapted to codon usage in plants.

Therefore, the additional DNA segments of the applied transformation plasmids are also not expected to have any harmful effects on human health and the environment or on the other legal interests referred to in § 1 No. 1 GenTG. Consequently, according to the state of science and technology, no additional safety precautions are required.

(d) Position effects and context changes; allergenicity

The expression level of genes which have been integrated into the plant genome by genetic engineering operations depends 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 this particular case this could mean that the traits of the GM plants are not modified to the same degree in the field as under the conditions found in a climate chamber or greenhouse. This does not indicate a risk to the environment or to human or animal health.

The insertion of foreign genes may influence the expression or regulation of endogenous plant genes at or near the site of insertion. Such processes may alter plant metabolic pathways. However, no evidence of such effects has been found to date. According to the information provided by the applicant, in greenhouse trials the GM tobacco plants did not exhibit any phenotypic differences to the isogenic parent plants.

Mobile genetic elements (transposable elements), which can exert effects on existing plant genes at the target site when transposed within the genome, occur naturally in plants and were first demonstrated in maize. The inactivation of genes or changes 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-GM plants such events can always influence plant metabolic pathways. In this respect the GM plants proposed for release here do not differ basically from non-GM plants.

Given the current state of knowledge, it is impossible to make reliable predictions about the possible allergenic action of a protein on the basis of its amino acid sequence. Since these GM plants are not intended for use in food or feed and are to be cultivated on a limited area only, risks to human health and life within the meaning of § 16 (1) No. 3 GenTG as a result of a potential allergenic effect are not anticipated. Therefore, according to the state of science and technology, no additional safety precautions are required in this case.

III.1.3.2. Evaluation of the ability of the GM plants to persist or establish in the environment

N. tabacum belongs to the nightshade family (*Solanaceae*) and is an annually cultivated plant which grows to a height of up to 3 m. A single plant can bear up to 150 flowers and produce up to several 100,000 very small seeds (thousand-seed weight 0.06 to 0.08 g).

Tobacco seeds have a minimum germination temperature of 10°C. The seeds require light for germination. High levels of moisture and dry spells have a negative impact on emergence. Even cold-adapted seedlings can only tolerate temperatures below -3°C for a few hours. Mature tobacco plants are definitely sensitive to frost. For these reasons, normally it is

not the seeds but plants pre-grown under controlled greenhouse conditions that are planted in the field for cultivation.

Tobacco seeds remain germinable over many years. Under favourable conditions tobacco seeds can persist in the ground for several years.

The appearance of volunteer *N. tabacum* plants is not reported in the literature. Feral tobacco plants have only rarely been observed outside agricultural areas, e.g. on newly overturned soil on construction sites. Long-term establishment has not been observed to date.

Since the tobacco plants intended for planting in the proposed trials are to be pre-grown in the greenhouse, the likelihood of the presence of ungerminated seeds is greatly reduced compared to direct seeding. The transplastomic donor plants are to be cut immediately after the pollen has been collected (before the seed ripens) so that seed losses from the GM tobacco plants themselves can be prevented. Of the seeds of the non-GM recipient plants, which might be lost during harvesting of the seed capsules, only a very small proportion are expected to contain genetically modified plastids (see chapter III.1.3.3.). If these seeds were to emerge on the release sites after the trial they would be identified and destroyed during the course of post-trial monitoring.

Therefore, also in this regard no harmful effect on the legal interests within the meaning of § 16 (1) No. 3 is to be expected. According to the state of science and technology, it is not necessary to take any further safety precautions beyond those planned.

III.1.3.3. Assessment of the possibility of pollen-mediated transfer of the inserted genes from the GM plants to other plants

According to the applicant, the purpose of the deliberate release proposed here is to demonstrate and quantify the pollen-mediated transfer of the genetic modification from transplastomic tobacco plants to neighbouring non-GM tobacco plants under field conditions. For this purpose it is necessary that the GM plants reach the flowering stage. As a result, pollen from the GM plants may be transferred to other plants.

N. tabacum is a facultatively self-pollinating plant. Inside the flower the anthers often release the pollen before or during the opening of the corolla so that a large proportion of the ovules may already be pollinated when the flowers open. Pollen transfer is mainly accomplished by insects such as honey bees (*Apis mellifera* L.)*,* bumble bees (*Bombus* species) as well as hoverfly species (*Syrphidae)* and various moth species (*Sphingidae*).

The cross-pollination rates of up to 4% mentioned in the literature are mean values. Individual flowers can demonstrate cross-pollination rates of up to 50%. In principle, it can be as-

sumed that the cross-pollination rate increases with increasing pollinator density, while it is greatly reduced with increasing distance between the donor and the recipient plants. While mean outcrossing rates of up to 10% are observed between directly neighbouring tobacco plants, the outcrossing rate in recipient plants located at a distance of 10 m is already less than 1%.

With respect to the deliberate release proposed here it also has to be taken into account that a pollen-mediated transfer of plastid-localised genetic information is in principle highly unlikely. Having originally assumed exclusive maternal inheritance of plastids for *N. tabacum*, paternal transmission of plastids was demonstrated for the first time ever in 1986. Under greenhouse conditions with the parental lines proposed here pollen-mediated transfer of the genetically modified plastids was demonstrated at a frequency of approx. 2×10^{-5} in cotelydons (not relevant for transmission to successive generations) and of approx. 3×10^{-6} in shoot meristems (relevant for transmission to successive generations through the germline) of the F1 generation. In other experiments with *N. tabacum* higher transmission rates of plastid-coded markers of up to 2×10^{-2} in coteyledons and 5×10^{-3} in shoot meristems of the F1 generation were found.

Male sterile recipient plants are used in these experiments in order to increase the rates of transmission. Due to the existing pollen competition the probability of a transmission of the plastid-localised genetic modifications to male fertile tobacco plants would be expected to be even lower than the values previously reported. However, in corresponding experiments with *N. tabacum* similar rates were found for parental inheritance of plastids to the F1 generation in the case of pollen transfer to male sterile recipient plants (1×10^{-4} to 8×10^{-4}) as for pollen transfer to male fertile plants (3×10^{-5} to 6×10^{-4}). Summarising the information currently available, for *N. tabacum* a pollen-mediated transfer of plastid-localised genetic information to the shoot meristem of plants of the F1 generation at a (low) rate in the range 5×10^{-3} to 3×10^{-6} is to be expected.

When assessing the pollen-mediated transfer of plastid-localised genetic information it must also be taken into account that in plants plastid DNA can be transferred to the nuclear genome to a very relevant extent. For the risk assessment it is also relevant that such genes that may have been transferred can only become functional under certain conditions. According to current findings, such an event (plastid DNA is transferred to the nuclear genome and is functional) is expected to occur at an overall very low rate of 10^{-14} to 10^{-15}.

The presence of crossing partners of *N. tabacum* outside the release sites cannot be completely ruled out. Cultivated tobacco may be found in gardens or window boxes in the locality; at both proposed release sites the minimum separation distance is 100 m. Provision II.11. stipulates that cross-compatible tobacco plants which come into flower within a distance of

100 m around the release sites are to be treated as GM tobacco plants. This measure serves to ensure the spatial delimitation of the release.

The main tobacco growing areas in Germany are in Baden-Württemberg and Rhineland-Palatinate. In Saxony-Anhalt, 16 different growers cultivated 49.74 ha of tobacco in 2008. For Mecklenburg-Vorpommern, no mention of any larger cultivation areas was found in the literature. Since the flower heads are often removed from commercially grown tobacco to improve the quality of the tobacco, no fertilisation of these plants with the pollen of the GM plants can occur under these conditions.

Apart from Virginian tobacco (*N. tabacum*), other species of *Nicotiana* such as Aztec tobacco (*N. rustica*), woodland tobacco (*N. sylvestris*), flowering tobacco (*N. alata*), and Sanders tobaco (*N. x sanderae*) are also found in Germany. However, natural cross-products between *N. tabacum* and *N. rustica*, if they occur at all, lead only to a very reduced extent to viable progeny. For the other species mentioned, hybridization with *N. tabacum* cannot be ruled out.

Overall the likelihood of a transfer of the genetic modification to these plants is very low because, as explained above, on the one hand the outcrossing rate for paternally inherited transplastomic characteristics is very low and on the other hand this rate decreases sharply with increasing distance to the donor plants.

A harmful effect on the protected assets referred to in § 1 No. 1 GenTG – for instance in the form of a negative impact on the marketability of tobacco seeds – is not anticipated due to the factors mentioned. Therefore, according to the state of science and technology in this regard, safety precautions that go beyond those listed in Provision II.11. are not required.

III.1.3.4. Assessment of the possibility of horizontal gene transfer of the inserted foreign genes from the GM plants to microorganisms

The inserted sequences are stably integrated in the plastids of the recipient organisms. There is no evidence to suggest that a transfer of genetic information from plants and its expression in microorganisms takes place under natural conditions. Studies on the transformation capacity of soil bacteria under natural conditions do however suggest that a transfer of plant genetic material to soil microorganisms is in principle possible, although it is assumed that such a gene transfer would constitute an extremely rare event.

Insofar as it is assumed that a genetic exchange between organisms that are so distantly related in terms of taxonomy as plants and microorganisms 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.

Therefore the mere presence of foreign DNA does not represent a harmful effect on plants or microorganisms. A harmful effect would be e. g. if outcrossing were to lead to a toxicologically relevant change in plants intended for human consumption (see Dederer in Eberbach/Lange/Ronellenfitsch, GenTR/BioMedR, volume 1, § 16 marginal no. 100). However – as will be outlined below - this is not to be expected.

(a) The *gfp* gene

The *gfp* gene derives from the genome of the jellyfish *Aequorea victoria* and has long been used as a reporter gene in gene expression studies in prokaryotes and eukaryotes. In the unlikely event of a horizontal gene transfer from the transplastomic plants to microorganisms, no selection advantage has been identified that would be conferred through the transferred *gfp* gene.

(b) The *aadA* gene

The *aadA* gene is already widespread in soil and enterobacteria and it is also commonly found in clinical isolates as well as in probiotic bacteria used as starter cultures in the food industry. Due to the widespread distribution of the *aadA* gene in microorganisms, even if horizontal gene transfer were to occur, this would not be expected to result in a discernible increase in the overall frequency of the gene.

Accordingly, in its opinion of 2009 the EFSA comes to the conclusion that the current state of knowledge suggests that no adverse effects on human health or the environment are to be expected as a result of the transfer of the *aadA* gene from plants to bacteria.

(c) Additional DNA fragments located on the transformation plasmids

The GM tobacco plants may contain partial sequences of the following genetic elements which are localised on the plasmids used:

- the ampicillin resistance gene bla_{TEM-1} and
- a bacterial origin of replication *ori*.

The bla_{TEM-1} gene confers resistance to ampicillin, a semi-synthetic antibiotically active drug from the ß-lactam group of antibiotics. The bla_{TEM-1} gene is widespread in microorganisms. Approx. 35% of all clinical *E. coli* isolates from humans are resistant to ampicillin, 90% of which are due to a ß-lactam-mediated mechanism of action. Similarly, 74% of all *E. coli* isolates from cattle and pigs show resistance to ampicillin. The latest studies on antibiotic resistance in microorganisms in the environment show that a high percentage of soil bacteria

are naturally resistant to a wide range of ß-lactam antibiotics, which is partly explained by the polymorphism of the *bla* gene in these microorganisms. Furthermore, the latest findings from field studies show that e. g. a 10-year monoculture of GM maize containing the *bla* gene does not alter the distribution of naturally occurring soil-bacterial antibiotic resistance in comparison with conventional cultivation.

In its statement of December 2008, the ZKBS included antibiotic resistance marker genes in GM plants in the safety assessment of GM plants in a uniform manner (without giving consideration to the groups established in 1999). At the same time, the latest scientific insights have been incorporated into the present safety assessment of horizontal gene transfer (HGT) of such marker genes from GM plants to bacteria. This has led to the conclusion that such HGT events, if they occur, are of negligible importance compared with the natural transfer and recombination processes and the natural presence of the resistance genes in question in the global community of microorganisms.

Concerning the origin of replication *ori* for plasmid replication, the likelihood of spreading by exchange between bacteria is far greater than the likelihood of spreading by horizontal gene transfer between GM plants and microorganisms.

(d) Regulatory sequences

Also in the case of the transfer of the regulatory sequences used in the construct there is no reason to expect an increase in the overall frequency of the corresponding DNA segments. The regulatory sequences derive from plastids of *N. tabacum*, which is cultivated in many agricultural regions around the world.

Owing to the circumstances described above, according to the state of science and technology, no further safety precautions are required.

III.1.3.5 Assessment of the possibility of pollen-mediated introduction of characteristics of the GM tobacco plants into bee products

As already outlined in chapter III.1.3.3., for the purpose of the deliberate release proposed here it is necessary that the GM plants reach the flowering stage. In the case of the present deliberate release trial, therefore, due to the systematic foraging activity of bees it cannot be ruled out that pollen in which the genetic modification is detectable may be introduced into bee products. As a result, following the decision of the European Court of Justice of 06/09/2011 (case C-442/09) these products would not be marketable because the GMO used in the present deliberate release trial is not generally authorised as a food in the European Union. If one takes a radius of 5000 m (estimation of the maximum distance bees cover during regular foraging activity) around the release site as a basis, roughly 12 beekeepers

around the Thulendorf site and roughly 4 beekeepers around the Ausleben site would be affected, whereby the data for Thulendorf derives from the place of residence of registered beekeepers and for Ausleben from the beehive location register.

In relation to the proposed release trial, it must be taken into account that the extent to which pollen seeds contain plastid-localised genetic information is most likely very small compared to vegetative cells. However, information on whether and to what extent the detectability of the genetic modification in pollen corresponds with the experimentally determined frequencies of paternal transmission of plastid-localised genetic information (see III.1.3.3.) is not available. The formation and development of pollen seeds suggest that plastid-localised genetic information is more frequently detected in pollen seeds than is actually transmitted to the next generation.

In the flower, pollen can fall into the nectar and be imbibed by the bees along with the nectar. In terms of quantity, this primary dispersal is the most significant introduction of pollen into honey. Secondary dispersal occurs in the beehive. Pollen brought into the hive deliberately or pollen which adheres to the bees' bodies ends up in the stored honey adventitiously.

There is a possibility that bees specifically target tobacco plants in order to collect pollen and store it separately from honey in specific combs. Hence tobacco pollen could also occur in pollen products.

Depending on the structure of the landscape and the availability of food, average foraging distances of between 0.5 and 5.5 km are reported. Melliferous plants at significantly greater distances are also visited. Foraging distances of over 10 km are reported. Large foraging distances have only been observed in very intensively cultivated farming areas without melliferous plants that are attractive to bees. When honeybees visit melliferous plants far away from the hive, then these plants are visited and foraged intensively. That bees visit tobacco flowers is reported in the literature, although there is no evidence that tobacco plants are particularly valuable or attractive melliferous plants.

An exact appraisal of separation distances by which the entry of GM pollen into the honey of individual beehives can be avoided is not possible due to the complex interrelations. Current scientific knowledge demonstrates that the foraging behaviour of bees is affected by different factors such as landscape structure, availability of food or weather conditions. This suggests that there is no monocausal connection between foraging radii and the proportion of pollen in honey. A precise evaluation of the effectiveness of separation distances is therefore not possible. Even though the entry of GM pollen is close to zero at distances of greater than 10 km, it still cannot be completely ruled out.

Because of this complex interdependency, planting shelter crops (trap plants) with more attractive melliferous plants is also not a suitable measure to minimise or rule out the entry of pollen into honey. Notably, the installation of bee-proof netting is not an option either because the purpose of the experiment demands normal field conditions (e. g. light conditions, microclimate).

According to the state of science and technology it is therefore reasonable to assume that the deliberate release proposed here could potentially expose bee products, as material assets within the meaning of § 1 No. 1 GenTG, to harmful effects. This potential harmful effect does not however involve a potential threat to human health, but merely the limited marketability of products containing GM pollen. However, for the deliberate release proposed here, according to the state of science and technology – as already stated – no adequate safety precautions exist which would minimise the entry of pollen into bee products on the one hand and not compromise the purpose of the experiment on the other.