Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

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Summary of the risk assessment of the genetically modified hybrid aspens

(*Populus tremula* L. x *P. tremuloides* Michx.) T89-35S-Bar

within the framework of a proposed deliberate release

carried out by the German Competent Authority

Berlin, 23 May 2000

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.1.2. 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 native *bar* gene

The *bar* gene from *S. hygroscopicus* codes for a phosphinothricin acetyltransferase (PAT) that catalyses the acetylation of L-phosphinothricin. L-phosphinothricin, the active component of the herbicidal agent glufosinate-ammonium (=ammonium-D,L-phosphinothricin), is a glutamic acid analogue and inhibits glutamine synthetase. In non-transgenic plants, the application of herbicides containing glufosinate-ammonium (e.g. trade name Basta®) causes an accumulation of ammonium in green plant tissue, resulting in the death of these plants. In genetically modified plants expressing the *bar* gene, L-Phosphinothricin is converted by acetylation into its derivative N-acetyl-phosphinothricin, which has no herbicidal effect. The formation of other metabolic products is unlikely, since only amino acids that are structurally related to phosphinothricin, such as glutamate, are suitable substrates for the PAT enzyme. Moreover, relevant studies have shown that even glutamate and other structurally related amino acids are hardly converted.

The *bar* gene (or the closely related *pat* gene from *S. viridochromogenes*) is usually used to genetically engineer herbicide-resistant cultivated plants. In the proposed trial of the Federal Research Centre for Forestry and Forest Products (BFH), on the other hand, the *bar* gene is to be used to prove horizontal gene transfer from genetically modified hybrid aspens to ectomycorrhizal fungi. This does not require the development of herbicide tolerance in the hybrid aspens or the application of the complimentary herbicide in the plant population. In ectomycorrhizal fungi, however, the *bar* gene should be expressed following successful horizontal gene transfer and the PAT enzyme should be formed.

For this reason, a construct was developed which expresses the native *bar* gene under the control of two different promoters: The promoter of the glyceraldehydes-3-phosphate dehydrogenase gene (GPD promoter) from the fungus *Cochliobolus heterostrophus* is located at the 5’ end of the *bar* gene, and is meant to allow the selection of transgenic ectomycorrhizal fungi as proof of completed horizontal gene transfer. The 35S promoter of the cauliflower mosaic virus (CaMV) was used as the second promoter. Positioning the 35S promoter in the opposite direction of normal transcription at the 3’ end of the *bar* gene causes the gene to be expressed in antisense orientation. This approach is meant to prevent the development of herbicide tolerance in the genetically modified hybrid aspens in the event that the GPD promoter may also be active in plants. There is no available data on a possible phosphinothricin resistance of the transgenic hybrid aspens. No herbicides containing phosphinothricin are planned to be used on the genetically modified plans during the trial.

Even if the PAT protein were to be expressed in the genetically modified hybrid aspens, no adverse effects are expected to result from the consumption of plant parts by animals – or in the unusual event of consumption by humans. In the event of oral intake, it can be assumed that this enzyme would be fully degraded in the digestive tract, as is generally the case with proteins. The PAT protein does not possess any properties typical of allergenic food proteins (heat stability, stability in the digestive tract) and no sequence homology with known allergens.

(b) The *nptII* gene

The *nptII* gene transferred into the genetically modified plants codes for the enzyme neomycin phosphotransferase. It was inserted as a marker gene for the selection of transformed plan cells. Neomycin phosphotransferase is a type-II aminoglycoside-3'-phosphotransferase

(APH(3')II) that catalyses ATP-dependent phosphorylation of the 3'-OH group of the aminohexose ring of specific aminoglycoside antibiotics, causing these to become inactivated. The enzyme is characterised by its high substrate specificity. The antibiotics kanamycin, neomycin, geneticin, butirosin, gentamicin A and B, and paromomycin belong to the APH(3')II enzyme substrates. Clinically relevant gentamicins and other aminoglycosides and aminocyclitoles used in human medicine do not belong to the substrate spectrum of the APH(3')II enzyme. However, kanamycin and neomycin are widely used in veterinary medicine.

Given the substrate specificity of neomycin phosphotransferase, it is expected that in the absence of substrate under field conditions no new metabolic products will form in the genetically modified plants. Since the relevant antibiotics are not present in the soil in high concentrations, the neomycin phosphotransferase does not confer any selective advantage to the genetically modified plants under field conditions. There is no evidence to suggest that this enzyme is toxic to plants, animals, microorganisms or humans.

(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 and cannot be ruled out based on the information provided in the application.

The employed transformation vector pBI121-bar was developed from pBIN19 via pBI121. The backbone of this vector contains the *npt*III gen. The genetically modified hybrid aspens were tested for the presence of the *npt*III gene using PCR analysis. The results show that none of the five primary transformants proposed for release contain a complete *npt*III gene.

Since no further analyses of the sequences integrated into the hybrid aspens were carried out, the risk assessment is performed under the assumption that the entire remaining vector has been integrated. In addition to the *npt*III gene, the pBIN19 vector also contains the following outside the border regions:

(1.) The origin of replication *ori*V of the plasmid RK2;

(2.) The *traF* region containing the *ori*T of the plasmid RP2;

(3.) The *trfA* locus of the plasmid RK2 (codes for two proteins required for the replication of the plasmid);

(4.) A non-functional fragment of the *klaC* gene from the plasmid RK2;

(5.) The *tetA* gene of the plasmid RK2 (interrupted by insertion of the T-DNA region);

(6.) The origin of replication of the plasmid pMB1.

(1) and (2): The origins of replication *oriV* (1) and *oriT* (2) of the plasmid RK2 allow replication of the plasmid in a broad host range of gram-negative bacteria and/or its conjugative transfer, as long as the mobilisation functions are provided by a helper plasmid.

(3), (4), (5) and (6): There is no evidence to suggest that *oriV* or *oriT* of RK2, the origin of replication of pMB1 (6) or the remaining DNA fragments of bacterial origin (3, 4, 5) have a function in higher plants. Moreover, some of the DNA fragments are incomplete (4) or interrupted (5).

(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 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 and 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 previous work with 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 and were first identified in maize. 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 to be deliberately released do not differ fundamentally from non-genetically modified plants.

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. In previous experiments with the genetically modified plants, and also in earlier deliberate release trials with genetically modified plants that express the *npt*II gene or the *bar* gene under the control of non-tissue specific promoters, no evidence was found to suggest an increased allergenic potential of the plants. In the proposed field trials, the genetically modified trees do not reach the flowering stage.

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

At the end of the trial, the genetically modified hybrid aspens are to be removed from the soil and burned in an incinerator, except in the case that parts of these trees are needed for further analysis. The approval authority expects this to be done in an environmentally sound manner. Any residual material with the potential to re-sprout shall be inactivated by applying herbicide. The fields are to be monitored and treated with herbicide for a period of two years after completion of the trials. Any emerging root suckers are to be removed from the soil and destroyed in a controlled manner (e.g. by incineration). During and after the trials, monitoring is to include a 15 m area surrounding the release site. This takes into account the possibility

that the root system of the hybrid aspens in this release and of those in the ongoing release (file ref. 6786-01-0048), which is taking place on an area in the immediate vicinity to the preferred release site for this trial, might also grow beyond the area of the release site during the course of the trial and that suckers could develop from these roots.

The applicant's experiences suggest that root parts which may remain in the ground following trial completion and post-trial monitoring are not expected to result in the persistence of the genetically modified plants. Therefore, the risk of the genetically modified hybrid aspens persisting in the environment or establishing new plants in this manner is extremely low. There is no evidence indicating increased viability or fertility that would promote persistence or long-term establishment of these genetically modified plants.

For these reasons, neither the establishment nor the uncontrolled persistence of these genetically modified plants is to be expected.

III.1.2.3. Assessment of the possibility of pollen-mediated transfer of the inserted genes from the genetically modified plants to other plants

Hybrid aspens are dioecious. The genetically modified plants intended for release originate from the hybrid aspen clone T89, which produces only male flowers. The pollen of *Populus* species is dispersed by wind. Outcrossing with plants outside the release site could not be ruled out if the trial plants were to reach the flowering stage.

The genetically modified hybrid aspens are to be planted out at an age between 6 months and 1 year. Hybrid aspens generally reach the generative stage after about seven to 15 years. The applicant plans to conclude the trial after three years, i.e. before the generative stage has been reached. Furthermore, the applicant plans to monitor the trees for emerging flower buds prior to leaf development in the spring, and to remove any occurring flower buds before anthesis in order to prevent any sexual exchange with other plants in the environment. Corresponding measures are described in the regulations set out in the supplementary provision II.6 of this notification.

The applicant's extensive experience in handling hybrid aspens, including genetically modified ones, shows that the formation of flower buds on hybrid aspens can be reliably identified before the flowers open. As a result, the pollen-mediated transfer of genetic information from these genetically modified hybrid aspens to other plants is not expected to occur.

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 organism. No evidence exists to suggest that the transfer of genetic information from plants and 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. The aim of the proposed deliberate release is to collect scientifically sound data on the possibility and, if applicable,

the frequency of horizontal gene transfer from the genetically modified hybrid aspens to mycorrhizal fungi, a specific group of microorganisms living in close association with plant roots.

Insofar as we assume that an exchange of genetic material between organisms that are so distantly related in terms of taxonomy as plants and bacteria or mycorrhizal fungi 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.

(a) The *bar* gene

In soil microorganisms, the inactivation of phosphinothricin by acetylation is a naturally occurring process. Bacteria with a corresponding resistance are commonly found in the environment. This resistance can therefore also be spread by horizontal gene transfer from non-genetically modified microorganisms. Even if the *bar* gene were to be transferred from the genetically modified plants to microorganisms, the overall frequency of this resistance in the environment would not be significantly increased.

(b) The *nptII* gene

As described in III.1.2.1. (b), the antibiotics which are inactivated by neomycin phosphotransferase are of little relevance in human medicine, but they are widely used in veterinary medicine. It was therefore necessary to examine whether a potential horizontal gene transfer of the *npt*II gene might affect the clinical use of the relevant antibiotics.

In soil microorganisms, the inactivation of aminoglycoside antibiotics by phosphorylation is a naturally occurring resistance mechanism. APH(3')II enzymes have also been found in human clinical isolates. The prevalence of genes which confer resistance to aminoglycoside antibiotics can be explained by the frequent application of these antibiotics, and by the fact that these genes are often located on plasmids, enabling effective transfer by conjugation. Even in the event of horizontal gene transfer from the genetically modified hybrid aspens to microorganisms, the overall frequency of this resistance mechanism would not be noticeably increased.

(c) Regulatory sequences

Even if regulatory sequences used in the constructs were to be transferred, there is no reason to fear that the overall frequencies of the respective DNA sequences will increase. These regulatory sequences are derived from the cauliflower mosaic virus (CaMV), a plant-infecting, double-stranded DNA virus, and from the ubiquitous soil bacterium *Agrobacterium tumefaciens* as well as the widespread fungus *Cochliobolus heterostrophus*.

(d) Sequences located outside the T-DNA

Through the transfer of sequences located outside the border regions, the following DNA fragments may have been integrated into the genetically modified plants:

(1.) Parts of the *npt*III gene from *Streptococcus faecalis* (codes for an aminoglycoside-3'-phosphotransferase type III) for resistance to aminoglycoside antibiotics;

(2.) The origin of replication *ori*V of the plasmid RK2;

(3.) The *tra*F region, containing the *ori*T of the plasmid RK2;

(4.) The *trf*A locus of the plasmid RK2 (codes for two proteins required for the replication of the plasmid);

(5.) A non-functional fragment of the *kla*C gene from the plasmid RK2;

(6.) The *tet*A gene of the plasmid RK2 (interrupted by insertion of the T-DNA region);

(7.) The transposon IS1 within the *npt*III gene;

(8.) The origin of replication of the plasmid pMB1.

According to a PCR analysis performed by the applicant, none of the lines proposed for release contain the complete *npt*III gene. The literature states that the nptIII gene, which is driven by its own promoter, confers resistance not only to kanamycin and neomycin, but also to the antibiotic amikacin. In Germany, amikacin is not authorised for use as a veterinary medicinal product but it may be employed in human medicine as a so-called reserve antibiotic. Because of its status as a reserve antibiotic and its attendant infrequent use, amikacin resistance is so far not widespread. Given the low probability of a horizontal gene transfer from plant DNA to microorganisms and the absence of selection pressure at the release sites, it can also be assumed that the presence of the complete *npt*III gene in the genetically modified hybrid aspens would not lead to a significant increase in the overall frequency of this resistance mechanism in microorganisms.

RK2 belongs to a group of broad host-range plasmids (incl. RP1, RP4, R18, R68), which are replicable in numerous gram-negative bacteria. Hence, in the case of the RK2-derived DNA fragments (2 – 6), the likelihood of genetic spread by transfer between bacteria is far greater than the likelihood of spreading by horizontal gene transfer from the genetically modified plants to microorganisms. Moreover, some of the DNA fragments are incomplete (5) or interrupted (6).

Based on the presented result of a PCR analysis, the complete insertion element IS1 is not expected to have been transferred to the genetically modified hybrid aspens. The insertion element IS1 (7) occurs naturally in various species of *Enterobacteriaceae*. It has been found, for example, in species of the genera *Escherichia*, *Shigella*, *Klebsiella*, *Serratia* and *Salmonella*. In the case of IS1, the number of copies per bacterial genome can be up to > 40. IS1 copies can have either a chromosomal or a plasmid location and have also been detected in prophages. It can be assumed that this insertion element would be easily spread by horizontal gene transfer between bacteria. In comparison, the probability of spread by horizontal gene transfer from the genetically modified plants to microorganisms, although theoretically conceivable, would be negligibly low.

The pMB1 replicon (8) belongs to the ColE1-type plasmids, whose host range is limited to a number of gram-negative bacteria. Basically, this replicon can be replicated in *E. coli* and closely related species of bacteria such as *Serratia* or *Salmonella*. In most gram-negative soil bacteria, replication does not take place. ColE1 plasmids occur frequently in enterobacteria. Gene transfer from enterobacteria to other bacteria is considered far more likely than a horizontal gene transfer from the genetically modified plants to bacteria. Therefore, the potential presence of the origin of replication of pMB1 in the plant chromosome is not expected to contribute to an increase in the overall frequency of horizontal gene transfer.

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

In order to generate the genetically modified plants, sterile hybrid aspen leaves were incubated with agrobacteria which harbour the genes to be transferred between the border regions of the binary vector plasmid. Following transformation, antibiotic treatment was carried out to eliminate the agrobacteria.

In contrast to the common wild-types of *A. tumefaciens*, the *Agrobacterium* strain used 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 were transferred to a cell of another plant by these agrobacteria, this cell would have to spontaneously regenerate into a whole, fertile plant for the foreign genes to enter the germ cells. This is the only way that these genes could be passed on to the 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 by 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 would have to be considered. The potential effects of such a transfer were already addressed in III.1.2.4.