Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

Notification 6786-01-00048

Summary of the risk assessment of genetically modified aspen

(*Populus tremula L* and *P. tremula L.* x *P. tremuloides Michx.*),

different independent lines,

carried out by the German Competent Authority within

the framework of a proposed deliberate release,

Berlin, 28 May 1996

Explanatory note to this document:

The following text reflects the summary of the risk assessment of (a) genetically modified organism(s) to be used for experimental field trials (deliberate releases) in Germany. The text forms part of the official authorisation regarding applications for the permit of deliberate releases (field trials) of genetically modified organisms in Germany under the legal framework of Directive 2001/18/EC and the German Gene Technology Act (Gentechnikgesetz, GenTG). The authorisation is issued by the Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, BVL [*Federal Office of Consumer Protection and Food Safety*], as the German Competent Authority. It comprises the chapters

I. Consent [to the application]
II. Provisions [to be respected in execution of the field trials]
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III.1. Requirements for approval according to section 16 GenTG [German Gene Technology Act]
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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.

III.1.2.1. Evaluation of changes in the genetically modified plants effected by the transferred nucleic acid sequences

(a) The *rolC* gene

The *rol*C gene is one of 4 *rol* (root locus) genes on the Ri plasmid of *Agrobacterium rhizogenes* which, following natural gene transfer, gives rise to hairy root disease in dicotyledonous plants. Under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter it causes decreased growth and the formation of small, light green leaves in the transgenic plants; expression of the *rol*C gene is equally strong in the leaves and internodes. By contrast, trees in which the *rol*C gene is under the control of the light-inducible *rbc*S promoter exhibit solely a lighter colouring of the leaves. The *rol*C mRNA can be detected only in leaves and not in internodes. The gene product of the *rol*C gene is a cytokinin-beta-glucosidase (22 kDa) which can influence cell division by releasing cytokinin from inactive conjugates, and a number of developmental process by altering the hormone balance (e.g. with abscisic acid).

As a consequence of the genetic modification, the transgenic aspen can be visually distinguished from non-genetically modified trees. The genetic modification therefore has a marker function. The effects of the diverse expression of the *rol*C gene can be followed over the course of the deliberate release trial.

After the trial has ended, the genetically modified plants will be mechanically destroyed and incinerated. They are not intended for human or animal consumption. Even in the event of unintentional consumption by animals or humans, adverse effects on their health would not be expected.

(b) The *nptII* gene

The npt*II* gene transferred to the genetically modified plants encodes the enzyme neomycin phosphotransferase. It was inserted as a marker gene for selecting transformed plant cells.

The neomycin phosphotransferase gene is a type II aminoglycoside 3'-phosphotransferase (APH(3')II) which catalyses the 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 are among the APH(3')II enzyme substrates. The therapeutically important drug gentamicin and other aminoglycosides and aminocyclitoles used in human medicine do not belong to the substrate spectrum of the APH(3')II enzyme. Kanamycin and neomycin are, however, widely used in veterinary medicine. Owing to the substrate specificity of the neomycin phosphotransferase, no new metabolic products are expected to arise in the genetically modified aspen given the lack of substrate under field conditions. Since the relevant antibiotics are not present in the soil in elevated concentrations, the neomycin phosphotransferase confers no selective advantage on 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) Other potentially transferred DNA fragments

The genetically modified aspen were generated by means of transformation with derivatives of the binary vector pPCV002.

In this vector, the multiple cloning site is located within the DNA sequence of the pBR322 plasmid required for bacterial replication and selection. This sequence contains the ColE1 replicon and the gene for the β-lactamase (ampicillin resistance), which is under the control of the bacterial promoter. Expression of the β-lactamase gene in the plants is not to be expected.

The pPCV002 vector and its derivatives also contain the Gen5 promoter from the T_L-DNA of *Agrobacterium tumefaciens* within the T-DNA. This is the first bacterial promoter for which it could be demonstrated that, in plants, it is expressed in a tissue-specific manner and regulated by plant hormones. However, in the present constructs its orientation is opposite to the reading direction, which is why an expression of the bacterial sequence is not to be expected.

(d) The genetically modified plants contain the following regulatory sequences integrated into the genome:

- the light-inducible promoter of the small subunit of the ribulose-1,5-bisphosphate carboxylase (rbcS) from *Solanum tuberosum,*
- the CaMV 35S promotor and terminator of CaMV,
- the promotors of the nopaline synthase gene from *Agrobacterium tumefaciens*,
- the terminator region of the octopine synthase gene from *Agrobacterium tumefaciens*.

The promoter and termination sequences regulate the expression of the *rolC* and *nptII* genes in the genetically modified plants. Statements on the effects of the expression of these sequences in the plants can be found under point III.1.2.1.(a) to (b).

(e) DNA fragments located outside the T-DNA

As a general rule, only DNA sequences located within the border regions are integrated into the plant genome in *Agrobacterium*-mediated transformations. However, individual cases of the transfer of DNA sequences from outside the border regions have been reported. The pPCV002 vector also contains the ori and parts of the transfer system of the RK2 plasmid which enable replication and mobilisation of the vector in gram-negative bacteria. To transfer the vector into the plant genome, the *tra* and *vir* functions of a helper plasmid (e.g. pMP90RK) are required. Additional DNA sequences which could give rise to gene products in the plants are not present outside the T-DNA in the pPCV002 vector.

(f) Position effects and context changes; allergenicity

The level of expression, also of genes that have been integrated into the plant genome by genetic engineering methods, is dependent on the integration site on the chromosome and/or on the nucleotide sequences neighbouring the integration site (“position effect”). Under field conditions the level of expression may be additionally influenced by environmental factors, for instance by temperature. In the present case, this could mean that the characteristics of the genetically modified aspen plants would not be modified to the same degree in the field as under greenhouse conditions. This is not expected to pose risks to the environment or to human or animal health.

The insertion of the foreign genes may influence the expression or regulation of the plant's own genes at or near the site of insertion. Such processes may alter plant metabolic pathways. In previous greenhouse and field trials with these genetically modified plants, no observations were made that would indicate 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, e.g. point mutations, deletions or translocations, and are traditionally used in plant breeding. Therefore, there is always a possibility that such events may influence plant metabolic pathways, even in non-genetically modified plants. Therefore, with respect to these characteristics, the genetically modified plants to be deliberately released here do not differ fundamentally from non-genetically modified plants.

With the current state of knowledge it is not possible to predict the potential allergenic effect of a protein based on its amino acid sequence. However, in previous trials conducted in and outside Germany with these genetically modified plants and with plants that express the *npt*II gene under the control of non-tissue-specific promoters, no evidence of increased allergenicity of these plants was found.

Pollen from aspen will not be produced in this trial because the applicant and the provisions provide for the implementation of measures to prevent this.

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

In preliminary trials with transgenic potatoes and tobacco plants which expressed the *rolC* gene from *A. rhizogenes* an increased susceptibility to certain fungal pathogens or male sterility of the plants, respectively, was demonstrated. Since the genetically modified aspen exhibit decreased growth in the greenhouse, this is also expected to be exhibited in the deliberate release trial. There is no evidence for an increased viability and fertility that would promote persistence or invasiveness of the genetically modified plants.

The intention is to incinerate the genetically modified aspen after the end of the trial. The approval authority assumes that this will be done in an environmentally compatible manner. Any potentially remaining material which is capable of re-growth should be inactivated by applying a herbicide. During the two years after the end of the trial the field should be inspected and treated with a herbicide. According to the systematic experience of the applicant, the likelihood of the genetically modified plants persisting as a result of root parts potentially remaining in the ground after the end of the trial is very small. Consequently, the possibility that plants might establish by this means is negligible.

For the reasons mentioned above, neither establishment nor uncontrolled persistence of the 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

The pollen of *Populus* species is dispersed by wind. If the trial plants were to reach the flowering stage, the possibility of outcrossing to plants outside the deliberate release area cannot be excluded. The applicant plans to complete the experiment before the plants reach the

generative phase and intends to inspect the area for the formation of flower buds in order to prevent any sexual exchange with plants in the surrounding area.

Trees found to exhibit premature flowering will be removed. Furthermore, if 5% of the trees enter the generative phase, the experiment will be discontinued. Therefore, the probability that the transgenes will outcross is considered negligible.

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

The inserted sequences are stably integrated into chromosomes of the recipient organisms. There is no evidence 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.

If it is assumed that an exchange of genetic material between organisms which are as distantly related in terms of taxonomy as plants and bacteria does actually take place, it would have to 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.

The marker gene was derived from *A. rhizogenes* and is commonly found in the environment. Hence it could – with far greater probability – also be introduced into other microorganisms by means of bacterial gene transfer.

As already described under point III.1.2.1.(b), antibiotics that are inactivated by the neomycin phosphotransferase are of little relevance in human medicine, but they are widely used in veterinary medicine. Thus, it was necessary to examine whether the therapeutic use of the relevant antibiotics would be compromised by a potential horizontal gene transfer of the *npt*II gene.

The inactivation of aminoglycoside antibiotics by phosphorylation is a naturally occurring resistance mechanism in soil microorganisms. Furthermore, APH(3')II enzymes have 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 the fact that these genes are often located on plasmids, thus enabling effective transfer by conjugation. Even in the event of a horizontal gene transfer from the genetically modified aspen to microorganisms, the overall frequency of this resistance mechanism would not be noticeably increased.

The genetically modified aspen contain an ampicillin resistance and the origin of replication of the plasmid ColE1 as a recombinant DNA sequence. Because these sequences are widespread in *Enterobacteriaceae*, the likelihood of a transfer between bacteria is far greater than the likelihood of spreading by horizontal gene transfer from the genetically modified plants to microorganisms.

Even in the event of a transfer of the other regulatory sequences used in the construct, an increase in the overall frequency of the corresponding DNA fragments is not to be expected. These regulatory sequences were derived from *A. tumefaciens* and CaMV.

A. tumefaciens is widespread in soils and the sequences mentioned above are found in wild-type *Agrobacteria* on Ti plasmids, which can be exchanged by conjugation. Therefore, with respect to a horizontal gene transfer of these sequences to microorganisms, a transmission of the relevant sequences from *Agrobacterium* is expected to be far more likely than a transmission from the genetically modified plants.

The theoretical possibility of a transfer of the CaMV sequences from the genetically modified plants would not represent a new situation as compared to the current natural situation because CaMV, a double-stranded plant-infecting DNA virus, is already found in plants.

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

To generate the genetically modified plants, aspen leaves were inoculated with Agrobacteria which contained the genes intended for transfer between the border regions of a binary vector plasmid. After transformation had occurred, antibiotic treatment was used to eliminate the Agrobacteria. In order to demonstrate that the propagating material of the plants intended for deliberate release are free of agrobacteria, tissue homogenates were spread on appropriate culture media. No Agrobacteria were detected in the process.

The possibility that the plants intended for deliberate release might contain minute quantities of the Agrobacteria used for the transformation cannot be ruled out. Unlike the commonly found wild-type strains of *A. tumefaciens*, the *Agrobacterium* strain used for the transformation is disarmed, i.e. it no longer has the capacity to induce tumours. In the unlikely but theoretically conceivable event of a transfer of the inserted foreign genes through Agrobacteria originating from the deliberately released genetically modified aspen plants into a cell of another plant, that cell would have to spontaneously regenerate into a whole, fertile plant for the foreign genes to enter germ cells and thereby allow them to be passed on to offspring of the plant. This is not expected to occur under natural conditions.

Assuming that the presence of small quantities of recombinant Agrobacteria in the genetically modified plants cannot be ruled out, a possible transfer of the binary vector plasmids contained in the Agrobacteria through conjugation to wild-type Agrobacteria present in the environment (*A. tumefaciens* or *A. rhizogenes*) which, in turn, could potentially transfer the foreign genes to individual cells of other plants also has to be taken into consideration.

In the event of infection and subsequent transformation by wild-type strains of *A. tumefaciens* or *A. rhizogenes*, the transformed plant cell gives rise to a tumour (crown gall and hairy root disease, respectively). The development of such tumours is not expected under natural conditions.

Furthermore, a transfer of the inserted genes from Agrobacteria into other soil bacteria also has to be taken into consideration. The potential effects of such a transfer have already been explained under point III.1.2.4..