Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

Notification 6786-01-0145

Summary of the risk assessment of genetically modified poplars (*Populus x canescens* (Aiton) Sm. = *Populus tremula* L. x *Populus alba* L. - grey poplar) ggs 11
carried out by the German Competent Authority
within the framework of a proposed deliberate release
Berlin, 28 April 2003

Explanatory note to this document:

The following text is a summary of the risk assessment of genetically modified (GM) organisms intended for use in an experimental field trial (deliberate release) in Germany. The text forms part of the official authorisation issued in response to an application for the deliberate release of GM organisms in Germany in accordance with Directive 2001/18/EC and the German Genetic Engineering Act (Gentechnikgesetz, GenTG). The authorisation was issued by the Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, BVL [Federal Office of Consumer Protection and Food Safety], as the German Competent Authority under the law on genetic engineering, and comprises the chapters:

I. Authorisation

II. Provisions

III. Justification

III.1. Authorisation requirements according to § 16 GenTG [German Genetic Engineering Act]

III.1.1. Authorisation requirements according to § 16 (1) No. 1 GenTG

III.1.2. Authorisation requirements according to § 16 (1) No. 3 GenTG

III.1.3. Authorisation requirements according to § 16 (1) No. 2 GenTG

III.1.4. Authorisation requirements according to § 16 (4, 5) GenTG

III.2 Appraisal of and response to objections

IV. Costs

V. Legal instruction

Only the original German authorisation document is legally binding. The following extract is a courtesy translation of chapter III.1.2., prepared for the Biosafety Clearing House.

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

(a) The *gsh*I gene

The *gsh*I gene from *Escherichia coli* codes for a γ-glutamylcysteine synthetase. γ-glutamylcysteine synthase plays an important role in the synthesis of glutathione (glutamyl-cysteinyl-glycine, GSH) insofar as it specifically catalyses the ligation of glutamic acid and cysteine to form glutamylcysteine under energy consumption. Other γ-ECS substrates are not known. Glutathione is formed in a second step, in which glycine is bound to the dipeptide by glutathione synthetase.

Glutathione performs several functions in plant cells. As an antioxidant, it protects plant cells against oxidation, acts as a reserve for organic sulphur and as a precursor for phytochelatins [$(\gamma\text{-glutamylcysteine})_n$-glycine; n=2-11] it contributes to the detoxification of xenobiotics and heavy metals. This is brought about by the formation of stable complexes with metal ions from the thiol group of the cysteine residues of phytochelatins, which are pumped under ATP consumption into the plant cell vacuoles where they are stored.

To transform the grey poplar the start codon of the endogenous *gsh*I gene from *E. coli* was modified from TTG to ATG in the p70*gsh*I construct. This causes an amino acid exchange from leucine to methionine. Expression of the *gsh*I gene takes place under the control of the 35S promoter from the cauliflower mosaic virus (CaMV) with a double enhancer region and the CaMV 35S termination signal. Southern blot analysis demonstrated that two copies of the *gsh*I gene were transferred into the genome of the ggs11 transformant.

Results of studies on the ggs11 transformant showed that the transfer of the *gsh*I gene leads to increased expression of active γ-ECS in the cytosol and that increased levels of glutathione are synthesised in these plants compared with control plants. In contrast, in comparison to the wild type there was no increase in either the total protein content of the examined leaves from the transgenic plant or in the level of glutathione reductase activity recorded. Phenotypic differences between the GM grey poplar and the wild type were not observed.

γ-glutamylcysteine synthetase (γ-ECS) is found in all plants. Therefore the enzyme is not expected to have toxic properties.

An increased production of glutathione is considered essential if the plant to perform its intended function, i.e. the improved uptake of heavy metals. The formation of phytochelatins necessary for this may alter the *source-sink* distribution of the individual components (in particular cysteine and glutamic acid). This process will be the focus of studies to be conducted within the scope of the deliberate release (field trial). This is not expected to have harmful effects on human health or the environment.

The ggs11 plants produce more glutathione in the stem and in old and young leaves than the non-transformed wild type. In the roots of both plant groups no differences in glutathione synthesis were found. Results of studies on absorption of cadmium from the soil solution revealed that in comparison to the non-transgenic control plants, only the young leaves of the ggs11 plants exhibited a significantly higher Cd content. In the roots, stem and old leaves, as well as in the whole plants, the Cd concentrations did not differ between the GM plants and the non-GM control plants. To date, it is not known to what extent the transgenic plants differ from non-transgenic grey poplars and other plants that grow in the area of the release site with regard to their capacity to absorb additional pollutants and to store these in different plant organs. This question is to be examined within the scope of the proposed deliberate release (field trial).

(b) The *nptII* gene

The *nptII* gene transferred to the GM 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. 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. Kanamycin and neomycin are, however, widely used in veterinary medicine.

Due to the substrate specificity of the neomycin phosphotransferase, no new metabolic products are expected to arise in the GM plants in the absence of substrate under field conditions. Since high concentrations of the relevant antibiotics are not present in soil, the neomycin phosphotransferase does not confer any selection advantage to the GM 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 transformations. However, the transfer of DNA sequences located outside the border regions has been reported and based on the information contained in the application this possibility cannot be ruled out.

The p70*gsh*I transformation vector used was developed from pBIN19. The backbone of this vector contains, among others, the *nptIII* gene. The GM grey poplar ggs11 was tested for the presence of components of the *nptIII* gene using PCR analysis. The results show that ggs11 does not contain any complete *nptIII* gene.

Since further analyses of the sequences integrated into the grey poplar were not carried out, the risk assessment is performed under the assumption that all of the remaining vector has been integrated. In addition to the *nptIII* gene, the pBIN19 vector also contains the following outside the border regions:

(1.) the replication origin *ori*V of the plasmid RK2 from *E. coli*;
(2.) a fragment of the *klaC* gene from *Klebsiella aerogenes*;
(3.) the transposon *IS*1 from *Bacillus subtilis;*
(4.) the *trfA* gene of the plasmid pRK2 for replication in *E.coli* and in *A. tumefaciens*;
(5.) the *tetA* gene of the plasmid pRK2 from *E. coli*, interrupted by the T-DNA;
(6.) the origin of replication of the plasmid pUC (ColE1 *ori*) from *E. coli*;
(7.) a *traF* fragment, containing the *ori*T of the plasmid RP4 from *E. coli*.

(1) and (7): The origin of replication *oriV* of the plasmid RK2 (1) and the *oriT* (7) of the plasmid RP4 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.

(2), (3), (4), (5) and (6): There is no evidence to suggest that the *oriV* of RK2 or the *oriT* of RP4, the replication origin of ColE1 (6) or the remaining DNA fragments of bacterial origin (2, 3, 4, 5) have a function in higher plants. Moreover, some of the DNA fragments are incomplete (2) or interrupted (5).

(d) Position effects and context changes; allergenicity

Genes that 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 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 the present case, this could mean that the characteristics of the GM plants would not be modified to the same degree in the field as under climate chamber or greenhouse conditions. This is not expected to pose a risk to the environment or to human or animal health.

The integration of foreign genes may influence the expression or regulation of the plant's own genes at or near the integration site. Such processes may alter plant metabolic pathways. However, in trials carried out to date on these GM 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 and were first demonstrated 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-GM plants there is a possibility that such events may influence plant metabolic pathways. Therefore, with respect to these characteristics the GM plants planned for release do not differ fundamentally from non-GM plants.

Given the present state of knowledge, it is not possible to make predictions about the potential allergenic effect of a protein based on its amino acid sequence. No parts of the GM grey poplars are expected to be used for human consumption. Furthermore, in previous trials with GM plants and in numerous deliberate releases of 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. Due to its key function in metabolism γ-glutamylcysteine synthetase is also not expected to have any increased allergenic potential. The GM trees are not to come into flower during the course of the proposed deliberate release.

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

At the end of the trial, the GM grey poplars are to be removed from the soil and burned in an incinerator equipped with the appropriate filters, together with the leaves that have accumulated and been collected over the period of the deliberate release, 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.

Grey poplar has the capacity to form adventitious shoots (stolons, root suckers) from its roots. Any residual material with the potential to re-sprout shall be inactivated by applying herbicide. During the release period, the trial sites are to be monitored for the emergence of stolons. 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 killed off with herbicides. During and after the trials, monitoring is to include a 15 m area surrounding the release sites. The possibility that the grey poplar root system might also grow beyond the area of the release site during the course of the trial and that suckers could develop from these roots is thus taken into account.

In studies and observations carried out to date on the morphological characteristics of the GM grey poplar plants in question, both under greenhouse conditions and within the framework of a currently ongoing field trial, the applicant reports that no differences between the transgenic and the non-transgenic plants were found. Evidence of increased vitality or fertility of the transgenic poplars which would promote the persistence or invasiveness of the GM plants has not been found. Root parts that may remain in the ground following trial completion and post-trial monitoring are not expected to result in the persistence of the GM plants. Therefore, the risk of the GM grey poplar persisting in the environment or establishing new plants in this manner is extremely low.

The genetic modification transferred to the plants basically confers a selective advantage under the conditions of heavy metal contamination expected at the release sites. However, taking into account the measures planned during the trial (trial duration, prevention of flowering) and following completion of the project (post-trial monitoring), the GM grey poplars are not expected to have the ability to establish on these sites.

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

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

Grey poplar is dioecious. The GM plants intended for release originate from the female grey poplar clone INRA 717 1-B4. Pollen formation and dispersal can thus be ruled out. The pollen of *Populus* species is dispersed by wind. However, if the trial plants were to reach the flowering stage, the possibility of in-crossing and the development of fruit on the GM grey poplars could not be excluded.

According to the applicant, the GM poplars for the proposed deliberate release have been propagated vegetatively since the summer of 2002. By the time they are to be planted out (spring 2003) they will have reached a stage of development that roughly corresponds to one-year-old trees. Generally speaking, grey poplars reach the generative stage after about 7 to 15 years; under conditions of stress it appears that flowering may occur earlier. The traits transferred give no reason to expect a significant reduction in the time taken for the transgenic plants to reach sexual maturity.

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 down in provision II.6 of the decision on this notification.

Experience shows that in trees which flower prior to leaf development the formation of flower buds can be reliably identified before the flowers open. As a result, the pollen-mediated transfer of genetic information from other plants to the GM poplars is not expected to occur.

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

The inserted sequences are stably integrated into the chromosomes of the recipient organism. 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, 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 GM poplars to bacteria and mycorrhizal fungi.

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

(a) The *gsh*I gene

γ-glutamylcysteine synthetase (γ-ECS) is found in all plants. The gene which was transferred to the transgenic plant ggs11 derives from *E. coli*. Consequently, this gene may also be spread by horizontal gene transfer from non-GM organisms. The release sites are located in areas where metals have

been extracted and processed for centuries. Organisms living on these sites have established themselves under existing selection pressure from metal contamination. Even in the highly unlikely event of a transfer of the *gsh*I gene from the GM plants to microorganisms, a selective advantage is not expected to result.

(b) The *nptII* gene

As already 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.

The inactivation of aminoglycoside antibiotics by phosphorylation is a naturally occurring resistance mechanism in soil microorganisms. 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 a horizontal gene transfer from the GM poplars to microorganisms, the overall frequency of this resistance mechanism would not be noticeably increased.

(c) Regulation sequences

Even in the case of a transfer of the regulation sequences used for the construct there is no reason to expect an increase in the overall frequency of the respective DNA sequences. These regulation sequences originate from the cauliflower mosaic virus (CaMV), a double-stranded DNA virus that infects plants, and from the ubiquitous soil bacterium *Agrobacterium tumefaciens*.

(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 GM plants:

(1.) parts of the *npt*III gene from *Streptococcus faecalis* (encodes an aminoglycoside-3' phosphotransferase type III) for resistance to aminoglycoside antibiotics;
(2.) the replication origin *ori*V of the plasmid RK2;
(3.) the *tra*F region, containing the *ori*T of the plasmid RP4;
(4.) the *trf*A locus of the plasmid RK2 (encodes two proteins that are needed for 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 from *Bacillus subtilis*;
(8.) the replication origin of the plasmid pUC (pMB1; ColE1 *ori*) from *E. coli*.

According to a PCR analysis performed by the applicant, the ggs11 line proposed for release does not contain a complete nptIII gene. 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 nptIII gene in the GM poplars 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 GM plants to microorganisms. Moreover, some of the DNA fragments are incomplete (5) or interrupted (6).

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 transfer between bacteria. In comparison, the probability of distribution by horizontal gene transfer from the GM 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 probable than a horizontal gene transfer from the GM plants to bacteria. Therefore, the potential presence of the replication origin 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 GM plants

To generate these GM plants small sterile pieces of grey poplar trunk were incubated with Agrobacteria which harbour the genes to be transferred between the border regions of the binary plasmid. After transformation had occurred the plant parts were washed thoroughly and treated with antibiotics to eliminate the Agrobacteria.

In contrast to the common wild-type *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 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 offspring. Such an event is not expected to occur under natural conditions.

Assuming that the presence of small amounts of recombinant Agrobacteria in the GM 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 *A. tumefaciens* or *A. rhizogenes*, a crown gall or hairy root tumour would develop from the transformed plant cell. A tumour of this type would not be expected to give rise to a plant under natural conditions.

The transfer of the inserted genes from Agrobacteria to other soil bacteria also has to be considered. The potential impact of such a transfer was already addressed in III.1.2.4.

III.1.2.6. Contaminated leaves of the transgenic trees

Based on the existing data it cannot be ruled out that the leaves of the transgenic poplars contain higher concentrations of contaminants compared to the control plants. As a result, the potential consequences of an accumulation of contaminants in the leaves of the transgenic trees were assessed within the scope of the approval procedure.

Fallen leaves that accumulate over the course of the trial are to be collected and stored on the release sites and destroyed together with the cleared trees at the end of the trial period. Provision II.7. [of the decision on this application] defines measures that should limit the chances of transfer of GM poplar plant parts, in particular the leaves, to other sites. Nevertheless, the possibility of leaves or other parts of the transgenic plants being transferred from the release sites to other sites cannot be completely ruled out. This should be considered against the background of heavy contamination of the region as a result of the processing of nonferrous heavy metals, which has gone on over many years. Contaminated materials may also be displaced to other sites by erosion movements (e.g. wind, water). Therefore, although the transfer of plant parts from the proposed deliberate release site to other sites is possible in isolated cases, an increased risk to resources protected under the German Genetic Engineering Act (GenTG) is not indicated.

There are plans to cover the soil beneath the area intended for storage of the leaves accumulated during the trial period, for example, with plastic sheeting. This should reduce the risk of pollutants that may have accumulated in the leaves re-entering the soil. However, it can be assumed that parts of the collected plant material will rot during storage. Resulting from this decay, substances which the plant had previously absorbed from the soil solution can re-enter the soil solution, where they are subject to site-specific chemical and biological processes. From the viewpoint of the law on genetic engineering, this cycle is not expected to result in any adverse effects.