Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

Notification 6786-01-0041

Summary of the risk assessment of genetically modified tobacco plants (*Nicotiana tabacum L.)* (transformants XynZ No. 34 and XynZ No. 46) carried out by the German Competent Authority within the framework of a proposed deliberate release, Berlin, 04 April 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

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 xylanase gene

The genetically modified tobacco plants contain a truncated form of the xylanase gene *xynZ* from *Clostridium thermocellum* under the control of the 35S promoter of the constitutive cauliflower mosaic virus (CaMV) and the octopine synthase termination region of *Agrobacterium*. The recombinant *xynZ* gene was fused with the nucleic acid sequence of the signal peptide of potato proteinase inhibitor II, resulting in the transport of the gene product into the apoplast.

Xylan is a component of the hemicellulose of plant cell walls. It is found mainly in the secondary walls as well as in the primary walls of monocotyledons. In the primary walls of dicotyledons, xylan is quantatively less significant. Xylan is composed of 1,4-ß-polyxylose, branched and substituted with arabinose, glucuronic acid and methylglucuronic acid. Xylan is broken down initially by the activity of xylanases which hydrolyse the glycosidic linkages between non-substituted xylose residues. The resulting branched oligosaccharides are then broken down by other enzymes into the corresponding monomers. Xylanases are found in bacteria, fungi and also in plants. They are grouped into nine families (A to H) on the basis of sequence homologies of the catalytic domains. Family F, to which the xylanase from *C. thermocellum* belongs, includes both bacterial and fungal xylanases. The xylanase from *C. thermocellum* is distinguished by the fact that it has a temperature optimum of approximately 70 °C. Furthermore, it has high substrate specificity for xylan because it does not hydrolyse cellulose as observed in the case of other xylanases.

The complete *xynZ* gene from *C. thermocellum* has a nucleotide length of 2511 nucleotides. However, deletion analyses have shown that the C-terminal region is sufficient for xylanase activity. For this reason, to generate the recombinant xylanase gene, the 3'-terminal 1025 bp of the gene was combined with the regulatory sequences mentioned above and the nucleic acid sequence for the signal peptide, and was transferred into tobacco via *Agrobacterium*. Through transport into the apoplast, the xylanase is removed from the protease-rich intracellular space and is thus protected from degradation.

According to the applicant, the proportion of the recombinant xylanase in the genetically modified tobacco plants amounts to approximately 4% of the total protein. The enzyme activity and the temperature optimum of the recombinant enzyme correspond with the enzyme produced by *C. thermocellum*. In greenhouse trials, despite the presence of the xylanase in the genetically modified plants, no change in the plant growth, susceptibility to pathogens or cell wall composition could be detected.

Nevertheless, the possibility that effects of the recombinant xylanase on the plants could become noticeable under field conditions cannot be ruled out. On the one hand, xylanase XynZ is distinguished by its high temperature optimum and its substrate specificity and, in addition, the proportion of xylan in dicotyledons is relatively low; furthermore, xylanases also occur naturally in plants (family E xylanases). On the other hand, even at 25 °C, the xylanase XynZ still exhibits approx. 20% of its maximum activity, and the xylanase fraction in the genetically modified tobacco plants is particularly high. Thus the possibility that a slight change could occur in the cell wall composition of the genetically modified tobacco plants cannot be ruled out, especially when outdoor temperatures and solar radiation are high. However, the result, especially under field conditions, would be a reduced fitness of the genet-

ically modified plants. This is not expected to have harmful effects on the legally protected interests specified under § 1(1) of the German Gene Technology Act (GenTG).

(b) The *nptII* gene

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

The neomycin phosphotransferase is a type II aminoglycoside 3'-phosphotransferase (APH(3')II), which 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 as well as 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. Kanamycin and neomycin are, however, widely used in veterinary medicine. Due to the substrate specificity of neomycin phosphotransferase, in the absence of substrate under field conditions no new metabolic products are expected to arise in the genetically modified tobacco plants. Since high concentrations of the relevant antibiotics are not present in the soil, the neomycin phosphotransferase does not confer any selection 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) The coding sequence of the α-fragment of the ß-galactosidase, the *lacI* sequences

The vector pBIN19, in which the multiple cloning site is located within the coding sequence of the α-fragment of the ß-galctosidase from *E. coli,* was used to generate the genetically modified plants.

The native enzyme ß-galactosidase cleaves ß-D-galactoside into galactose and the corresponding alcohol compound. The most physiologically important substrate is lactose, which is hydrolysed into galactose and glucose. The α-fragment refers to the first 146 amino terminal amino acids of ß-galactosidase. The α-fragment by itself is not enzymatically active, but complementation may occur in suitable hosts.

Through the insertion of the xylanase gene into the multiple cloning site, the coding sequence for the α-fragment of the ß-galactosidase was interrupted so that in this form, inter alia in *E. coli* bacteria, it no longer has the ability to code for an α-fragment that is capable of complementation. The interrupted sequence of the α-fragment of ß-galactosidase is expressed under the control of a bacterial promoter. A functional gene product is not encoded by this sequence. Changes in the genetically modified tobacco plants are not expected to result from the presence of this sequence.

In addition, the genetically modified plants probably also contain 5’ and 3’ sequences of the repressor gene *lacI*. However, these 5’ and 3’ sequences are separated from one another by the *lacZ* and M13 *ori* sequences. The *lacI* sequences are unlikely to have any functional capacity in the genetically modified plants.

(d) M13 sequences

The genetically modified plants were generated by transformation with a derivative of the vector pBIN19 and probably contain two fragments from M13mp19, namely, a 440-bp fragment comprising a component of an open reading frame of a structural protein from M13 as well as a 433-bp fragment containing the origin of replication of the M13 phage.

If transcription of the fragment of the open reading frame of the structural protein were to occur in the genetically modified tobacco plants, a functional protein not would not result because the fragment encodes only 167 amino acids of a total of 423 amino acids of the complete phage protein. Effects of the metabolism of the plants due to the presence of this fragment are therefore not expected.

The origin of replication of M13 causes replication of the phage in *E. coli* when *E. coli* is infected with M13, f1 or fd phages. The origin of replication is not expected to be functional in plants.

(e) The fragment of the *ocd* gene

Furthermore, the plants probably contain a fragment of the *ocd* gene (ornithine cyclodeaminase), which is located between the 3' end of the translated sequence of the *nptII* gene and the NOS termination sequence. Because this sequence is transcribed as part of the mRNA of the *npt*II gene but is located behind the termination codon of the *npt*II gene, the sequence is not expected to be translated.

(f) Border sequences from Ti plasmids and regulatory sequences

The genetically modified plants contain sequences from the left and the right border region of the T-DNA of the plasmid pTiT37 from *Agrobacterium tumefaciens*. These sequences, dependent on the gene products of the *vir* regions of the helper plasmid pGV2260 present in the *Agrobacterium* strain used in the transformation, which was not transferred to the plants, caused the genes located between the border regions to integrate into chromosomes of the tobacco plants. These border regions of the Ti plasmids are non-functional in the genetically modified plants and are not expected to cause any changes in the plants.

Integrated into the genome, the genetically modified plants contain the following regulatory sequences which are functional in plants:

- the 35S promoter of Cauliflower mosaic virus (CaMV),
- the promoter of the nopaline synthase gene from *Agrobacterium tumefaciens*,
- the termination regions of the nopaline synthase gene and octopine synthase gene from *Agrobacterium tumefaciens*.

The promoter and termination sequences regulate the expression of the coding sequences of the xylanase gene and the *nptII* gene located between them in the genetically modified plants. The effects of the production of these proteins in the plants are described in detail under III.1.2.1.(a) and (b).

(g) Sequences located outside the T-DNA

As a general rule, only sequences located within the borders are integrated into the plant genome in *Agrobacterium*-mediated transformation events. However, individual cases of the transfer of sequences outside the borders have been reported and, on the basis of the information contained in the notification, this possibility cannot be ruled out. According to the information given in the notification, in the present case, the following DNA fragments could have been integrated into the genetically modified plants through the integration of sequences located outside the borders:

(i) the *nptIII* gene (codes for a type III aminoglycoside-3'-phosphotransferase) for resistance to aminoglycoside antibiotics;

(ii) the origin of replication *oriV* of the plasmid RK2;

(iii) the *traF* region, containing the *oriT* of the plasmid RK2;

(iv) the *trfA* locus of the plasmid RK2 (codes for two proteins which are required for replication of the plasmid);

(v) a non-functional fragment of the *klaC* gene from the plasmid RK2;
(vi) the *tetA* gene of the plasmid RK (interrupted by insertion of the T-DNA region);
(vii) the insertion element IS1 within the *nptIII* gene;
(viii) the origin of replication of the plasmid pMB1.

Because the *nptIII* gene (i) is under the control of a bacterial promoter, it is not expected to be expressed in plants. The gene is therefore not expected to affect the metabolism of the plant.

The origins of replication *oriV* (ii) and *oriT* (iii) of the RK2 plasmid facilitate the replication of the plasmid in a broad host range of gram-negative bacteria or its transfer by conjugation, provided the mobilisation functions are supplied by a helper plasmid. There is no evidence to suggest that the origins of replication of RK2, the origin of replication of pMB1 (viii) or the other DNA fragments of bacterial origin (iv, v, vi, vii) have any function in higher plants. Moreover, some of the DNA fragments are incomplete (v) or interrupted (vi).

(h) Position effects and context changes; allergenicity

Genes which have been integrated into the plant genome by genetic engineering methods are expressed at different levels, depending on the site of insertion on the chromosome, and on the environment at the site of insertion (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 xylanase yield might deviate from expectations derived from tests carried out under greenhouse conditions. This is not expected to pose a risk to the environment or to human or animal health. Also with regard to the other characteristics transferred, an altered expression level is not expected to pose a risk to the environment or to human or animal health.

The insertion of foreign genes may influence the expression or regulation of the plant's own genes at or near the site of insertion. Such processes may alter plant metabolic pathways. However, during the course of the work carried out to date 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, even in non-genetically modified plants such events can always have an effect on plant metabolic pathways. In this respect the genetically modified plants proposed for release here do not differ fundamentally in those characteristics from non-genetically modified plants.

Given the current state of knowledge, it is not possible to make reliable predictions about the possible allergenic action of a protein on the basis of its amino acid sequence. However, in previous experiments with the genetically modified plants, no evidence of increased allergenicity of the plants was found.

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

N. tabacum is an annual plant. Tobacco is sensitive to frost and can only withstand temperatures below -3 °C for a short time. Because the vegetative plant parts are destroyed by frost, in our latitudes *N.*

tabacum reproduces exclusively by seed. In Germany, *N. tabacum* does not become weedy, and its establishment outside agricultural areas does not occur.

According to the trial plan, the genetically modified organisms are not to be sown as seeds but as plants, and the inflorescences are to be removed before flowering begins so that no pollen is released and no seeds will develop on the genetically modified tobacco plants. Harvesting of the genetically modified tobacco plants is to be done by hand. Some of the harvested plant material will be transported to a genetic engineering facility for further tests. The remaining material is to be shredded and composted on a marked area on the grounds of the institute.

Since no genetically modified seeds are to be brought onto the deliberate release areas or produced there during the course of the trial, new growth is not anticipated in the years following the trial owing to the frost sensitivity of the vegetative plant parts. Nevertheless, tobacco shall not be cultivated on the deliberate release areas in the season following the trial and these areas are to be controlled for emerging tobacco plants during the two years following completion of the deliberate release. If volunteer plants appear, they are to be removed.

The genetically modified tobacco plants are therefore not expected to establish or persist in the environment.

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

N. tabacum is primarily self-pollinating. Cross-pollination accounts for approx. 8% to 10% of all pollination. Cross-pollination is achieved mainly through insects. The areas of natural distribution of *Nicotiana* include America, Australia, the Pacific Islands and – represented with one species - Africa. However, *N. tabacum* and other species of *Nicotiana* are also grown in Germany as crop plants and ornamental plants. *N. rustica* sometimes appears as a weed, but has rarely been observed in the area around Gatersleben. Furthermore, fertile hybrids between *N. tabacum* and *N. rustica* are not produced under natural conditions. Also, with respect to the distantly related wild species *Solanum nigrum* and *Solanum dulcamara*, which are found in Europe, no cross-breeding with *N. tabacum* has been observed.

The applicant intends to remove the inflorescences before flowering begins. This is to be guaranteed by daily monitoring of the trial areas by specialist personnel. Since there is a gap of several days between the appearance of the visible flower buds and the maturing of the flowers, and a release of pollen is only expected after the mature flowers have opened, daily monitoring is sufficient in order to be able to identify and remove any emerging flowers. Hence a transfer of pollen from the genetically modified plants to other plants is unlikely.

To take into account the possibility that flowers might not be removed in a timely manner, in its opinion statement, the Central Committee on Biological Safety (ZKBS) has recommended a minimum isolation distance of 200 m to flowering tobacco plants. Therefore, supplementary provision II.6 stipulates that if the timely removal of the inflorescences is not guaranteed, a minimum isolation distance of 200 m to flowering tobacco plants is to be maintained. This measure ensures that in any event the spread of pollen is sufficiently limited.

Even if, against expectations, pollen is transferred to other plants, due to the characteristics of the transferred genes (see section III.1.2.1.) this would not represent a threat to the natural resources protected under § 1(1) of the German Gene Technology Act (GenTG).

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

The inserted sequences are stably integrated into the chromosomes of the recipient organisms. There is no proof that a 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 a transfer of plant genetic material to soil bacteria might, in principle, be possible.

Insofar as we assume that an exchange of genetic material between organisms as distantly related in terms of taxonomy as plants and bacteria actually occurs, it follows 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.

Xylanases are widespread among microorganisms. They are found especially in bacteria and fungi which use plant material as a food source. A transfer of the recombinant xylanase to these microorganisms would not confer any novel traits to them. As a result of its temperature optimum of approx. 70 °C, the xylanase XynZ has only low activity under our climate conditions and therefore does not confer any significant selective advantage to the microorganisms in question. In addition, an exchange of xylanases between microorganisms is far more likely than a horizontal gene transfer from the genetically modified tobacco plants. The DNA fragment for the signal peptide of the proteinase inhibitor II from potato is also not expected to be functional in microorganisms.

As already elaborated under point III.1.2.1.(b), the antibiotics inactivated by the neomycin-phosphotransferase are of little relevance in human medicine but are widely used in veterinary medicine. It was thus necessary to examine whether the therapeutic use of the relevant antibiotics would be compromised by a potential horizontal gene transfer of the *nptII* gene.

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 use of these antibiotics, and by the fact that these genes are often located on plasmids, enabling their effective transfer between micro-organisms by conjugation. Even if a horizontal gene transfer from the genetically modified tobacco plants to microorganisms were to occur, the overall frequency of this resistance mechanism would not noticeably increase.

The gene for the α-fragment of the ß-galactosidase is interrupted so that no functional gene product can be produced. This would also be the case in bacteria that receive the gene through horizontal gene transfer. The same applies to the 3' and 5' sequences of the *lacI* gene.

A similar situation applies to the fragment of the gene for a structural protein of the M13 phage and the fragment of the *ocd* gene. These fragments are not expected to be functional in bacteria. In addition, the fragment of the *ocd* gene is unlikely to be translated, as explained in III.1.2.1.(e).

The genetically modified tobacco plants probably contain the origin of replication from M13. M13 belongs to the F-specific *E. coli* phages. Therefore, this origin of replication is far more likely to be spread by transfer between bacteria than by horizontal gene transfer from the genetically modified plants to microorganisms.

The sequences inserted into the tobacco plants to regulate the transferred genes derive from *A. tumefaciens* and CaMV. Regarding a horizontal gene transfer of these sequences to microorganisms, it should be noted that *A. tumefaciens* is widespread in soils and that a transfer of the relevant sequences from *Agrobacterium* is far more likely than their transfer from the genetically modified plants. The theoretical possibility of a transfer of the CaMV sequences from these plants would not constitute a new situation compared to the naturally occurring situation because CaMV, as a double-stranded plant-infecting DNA virus, is already present in plants.

As a general rule, only sequences located within the T-DNA borders are integrated into the plant genome in *Agrobacterium*-mediated transformation events. However, on the basis of the information contained in the notification, a transfer of sequences outside the borders cannot be ruled out. In the present case, the following DNA fragments could have been integrated into the genetically modified plants through the transfer of sequences located outside the borders:

(i) the *nptIII* gene from *Streptococcus faecalis* (codes for a type III aminoglycoside-3'-phosphotransferase) for resistance to aminoglycoside antibiotics;
(ii) the origin of replication *oriV* of the plasmid RK2;
(iii) the *traF* region, containing the *oriT* of the plasmid RK2;
(iv) the *trfA* locus of the plasmid RK2 (codes for two proteins which are required for replication of the plasmid);
(v) a non-functional fragment of the *klaC* gene from the plasmid RK2;
(vi) the *tetA* gene of the plasmid RK (interrupted by insertion of the T-DNA region);
(vii) the insertion element IS1 within the *nptIII* gene;
(viii) the origin of replication of the plasmid pMB1.

For the *nptIII* gene (i), as for the *nptII* gene (see above), the corresponding resistance mechanism is widespread in bacteria.

The insertion element IS1 (vii) is naturally present in various species of *Enterobacteriaceae*. For example, it has been found in species of the genera *Escherichia*, *Shigella*, *Klebsiella*, *Serratia* and *Salmonella*. In the case of IS1, the number of copies per bacterial genome can amount to > 40 copies. Copies of IS1 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 between bacteria via horizontal gene transfer. In comparison, the probability of a theoretically conceivable spread from the genetically modified plants to microorganisms via horizontal gene transfer is negligible.

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 (ii to vi), the probability of a spread by transfer between bacteria is far higher than the probability of a spread via horizontal gene transfer from the genetically modified plants to microorganisms. Moreover, some of the DNA fragments are incomplete (v) or interrupted (vi).

The pMB1 replicon (viii) belongs to the ColE1-type plasmids whose host range is limited to a number of gram-negative bacteria. Basically, this replicon can replicate 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

To generate the original transformants from which the genetically modified plants proposed for deliberate release are derived, injured leaves from a sterile culture of *N. tabacum* cv. Samsun were inoculated with agrobacteria containing the genes to be transferred between the border regions of a binary vector plasmid. In contrast to the ubiquitous wild-type *A. tumefaciens*, the *Agrobacterium* strain used for the transformation is disarmed, i.e. it no longer has the capacity to induce tumours. After transformation had occurred, antibiotic treatment was carried out to eliminate the agrobacteria. The plants intended for deliberate release have been propagated by seed. As a result of this propagation by seed, the agrobacteria used for the transformation would also have been removed from the genetically modified plants.