Notification 6786-01-0107 / 42010.0107

Summary of the risk assessment of the genetically modified sugar beet

(*Beta vulgaris* L. ssp. *vulgaris*) T120-7 und T252

within the framework of a proposed deliberate release

carried out by the German Competent Authority

Berlin, 13 July 1999

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 sugar beets effected by the transferred nucleic acid sequences

(a) The synthetic *pat* gene

In the genetically modified sugar beets, the synthetic *pat* gene codes for a phosphinothricin acetyltransferase (PAT).

L-phosphinothricin is a glutamic acid analogue and inhibits glutamine synthetase in plants. The inhibition of glutamine synthetase leads to apoptosis resulting from accumulated ammonium. This is why phosphinothricin is used as the active ingredient in the non-selective herbicide Basta® (Liberty®). Basta® contains the enantiomers D- and L-phosphinothricin in a 1:1 ratio. D-phosphinothricin does not act as a glutamine synthetase inhibitor.

Unlike in non-genetically modified plants treated with Basta®, the use of Basta® in genetically modified plants causes L-phosphinothricin to be acetylated by the phosphinothricin acetyltransferase (PAT), thereby creating N-acetyl-L-phosphinothricin, which has no herbicidal effect. This makes the genetically modified plants tolerant to the herbicide Basta®. The substrate specificity of phosphinothricin acetyltransferase is high. Even the phosphinothricin analogue glutamate is hardly acetylated. D-phosphinothricin is not metabolised by phosphinothricin acetyltransferase.

Due to its good water solubility, N-acetyl-L-phosphinothricin formed in the genetically modified plants after treatment with Basta® is distributed in the plants during further plant growth, while its concentration is reduced with increasing biomass. There are no indications of N-acetyl-phosphinothricin being further metabolised in the genetically modified plants.

Any N-acetyl-phosphinothricin still present in those parts of the genetically modified plants that remain on the field enters, as the case may be, the soil during decomposition, where it is converted back into L-phosphinothricin by microorganisms. D/L-phosphinothricin is degraded in the soil, also by microorganisms.

According to the available data, N-acetyl-L-phosphinothricin has a significantly lower toxicity than phosphinothricin (= active ingredient in the herbicide Basta®). Basta® is approved by the Federal Biological Research Centre (Biologische Bundesanstalt) under the German Plant Protection Act (Pflanzenschutzgesetz). As part of the authorisation process, the herbicide and its metabolites were assessed for toxicity and ecotoxicity. Based on the toxicological and ecotoxicological data on phosphinothricin and N-acetyl-L-phosphinothricin, the residues or metabolites of the herbicide Basta® contained in the genetically modified sugar beet plants are not expected to pose a risk to human and animal health or the environment.

The coding region of the *pat* gene was derived from the amino acid sequence of the PAT enzyme of the soil bacterium *Streptomyces viridochromogenes* Tü494 and chemically synthesised. The original gene exhibits a high GC content (70%) typical of this group of bacteria. In order to ensure effective expression of the gene in plants, codons that are typical of plant genes were selected for gene synthesis. This alteration of the nucleic acid sequence is not expected to pose any risk, since the amino acid sequence of the gene product, the PAT enzyme, is not altered.

No adverse effects are expected to result from the consumption of parts of the genetically modified plants containing phosphinothricin acetyltransferase by animals or 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 proteins in food (heat stability, stability in the digestive tract) and no sequence homology with known allergens.

(b) The *npt*II gene

The *nptII* gene codes for the enzyme neomycin phosphotransferase and was inserted as a marker gene for the selection of transformed sugar beet 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) The plasmid πAN7 and the *cos* site of the bacteriophage λ

The *ori* of pBR322 and the supF t-RNA gene from *Escherichia coli* are located on the plasmid πAN7. The supF t-RNA gene codes for a bacterial tyrosine t-RNA and suppresses the stop codon UAG. The bacterial supF t-RNA gene is not expected to be functional in plants. The gene has bacterial regulatory sequences. Moreover, the production of a loaded, functional t-RNA is a highly specific process. The *ori* of pBR322 and the *cos* site of the bacteriophage λ are also not expected to be functional in plants.

(d) Border sequences from Ti plasmids

The genetically modified sugar beet plants contain sequences of the left and right border region of the T-DNA of the plasmids pTiT37 and pTiAch5. Both plasmids are derived from *Agrobacterium tumefaciens*. Depending on the gene products of the *vir* region of the helper plasmids pMP90RK and pEHA101 that are contained in the C58C1 *Agrobacterium* strains used for transformation and are not transferred into the plants, these sequences cause the genes located between the border regions to integrate into the nuclear genome of the sugar beet plants. The border regions of the Ti plasmids transferred into the plants are non-functional in the genetically modified sugar beet plants and are not expected to cause any changes in the plants.

(e) Regulatory sequences functional in plants

Integrated into the genome, the genetically modified sugar beet plants contain the 35S promoter and terminator of the cauliflower mosaic virus (CaMV) as well as the promoter of the

nopaline synthase gene and the terminator of the octopine synthase gene from *A. tumefaciens*. In the genetically modified plants, the 35S promoter and terminator sequences as well as the promoter sequences of the nopaline synthase gene and the terminator sequences of the octopine synthase gene regulate the expression of the coding sequence of the phosphinothricin acetyltransferase and neomycin phosphotransferase located between them. Further information on the effects associated with the formation of these enzymes in the plants can be found in III.1.2.1 (a)-(b).

(f) Sequences located outside the T-DNA

As a general rule, only DNA located within the border regions is integrated into the plant genome through *Agrobacterium*-mediated transformation events. By means of molecular biological testing, southern blot analysis and PCR analysis, the applicant was able to prove that one copy of the T-DNA was transferred into each of the recipient plants during the transformation of the two transgenic sugar beet lines T120-7 and T252. PCR analyses with DNA of the genetically modified sugar beets demonstrate that when using primers that hybridise outside the T-DNA no PCR products are synthesised, which indicates that only the T-DNA with its T-DNA border sequences was transferred. Furthermore, the tetracycline and streptomycin resistance genes located outside the T-DNA on the transformation vectors pOCA18/Ac and pHoe6/Ac were not detected in the DNA of the genetically modified sugar beets using PCR.

Based on the test results presented by the applicant, it has been adequately proven that no transformation vector sequences located outside the T-DNA were transferred into the sugar beets.

(g) 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 expression level may be influenced by environmental factors, for instance, by temperature. In this particular case, this could mean that the genetically modified plants do not tolerate glufosinate ammonium to the same degree in the field as under climatic-controlled or greenhouse conditions. The application of Basta® (Liberty®) could result in damage to the genetically modified plants. This does not represent a risk to the environment or to human and animal health. In terms of the other traits transferred, a change in the expression level also does not pose a risk to the environment or to human and animal health.

The insertion of foreign genes may influence the expression or regulation of endogenous plant genes at or near the site of insertion. Such processes can affect plant metabolic pathways. Based on the information provided by the applicant, during greenhouse experiments 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. 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 under greenhouse and field conditions as well as in earlier deliberate release trials with other genetically modified plants that express the corresponding genes under the control of non-tissue-specific promoters, no evidence was found to suggest an increased allergenic potential of the plants.

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

If required, samples of vegetatively propagated sugar beets will be taken and analysed in a laboratory (e.g. to ascertain quality parameters). Towards the end of the vegetation period, the remaining vegetatively propagated sugar beet plants will be harvested mechanically and inactivated by topping. A portion of the beet harvest will be transferred to laboratories for analysis. If the yield intended for analysis is found to contain plant material still capable of propagation, it is deemed adequate in terms of safety if it is inactivated during the course of the analysis, e.g. by subsequent topping of the beets in the beet laboratory. Inactivation is, in any case, an inherent part of the pulp production for the analysis process.

According to the supplementary conditions, any genetically modified plant material remaining on the release site after harvesting will be shredded or inactivated by appropriate chemical measures and is subsequently worked into superficial soil layers. Sites where genetically modified sugar beets are only propagated vegetatively will not be cultivated with sugar beets in the following year and will be monitored for the emergence of voluntary beets.

The genetically modified beets will be sown using drilling machines or by hand. Vernalised beets will be planted out. Since the plants will not reach the flowering stage, no new seeds will be produced on the vegetatively propagated plants. Under certain circumstances, particularly when incorporated into deeper soil layers, sugar beet seeds can remain germinable for many years. However, based on general farming experience, planted seed which does not germinate is considered inactive and will therefore be incapable of germinating in subsequent years.

In addition to the seeds placed, seeds on the vernalised plants will be produced on the selfing, hybridisation and propagation sites in the course of the trial at the location of Wetze and at potential further locations to be registered later under the simplified procedure. The genetically modified seed will be harvested mechanically or by hand and subsequently transferred to a genetic engineering facility. After harvesting, the remainder of the generative plant parts will be inactivated by autoclaving or steam sterilisation.

It is assumed that the seeds produced on the generative plants may enter the soil. The requirement proposed in the supplementary conditions to carry out non-turning soil cultivation in the first year of the post-trial monitoring period is intended to ensure that any seeds that enter the soil do not get buried in deeper soil layers where they could persist for a long time.

Any seeds that persist in the soil would result in the emergence of genetically modified plants in the following years. These plants would be detected and removed before flowering in the course of the proposed post-trial monitoring period. If genetically modified sugar beet plants emerge in the last year of the post-trial monitoring period, the post-trial monitoring period of the seed production sites will be extended for a further year.

Considering the above, the measures proposed in the application in conjunction with the supplementary conditions to this notification are suitable to prevent the establishment of the released genetically modified plants themselves and to detect and eliminate any potentially emerging genetically modified volunteer plants. Even if individual genetically modified seeds were to be dispersed as a result of the trial, the uncontrolled spread of the genetically modified plants is not anticipated, since they could be destroyed by mechanical methods (hoeing) or by using other herbicides.

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

Sugar beets are crossable with all species of the beta section. Possible crossing partners in Germany include the cultivated *Beta* species (e.g. chard, fodder beet, beetroot) as well as sea beets (*Beta vulgaris* ssp. *maritima*). In Germany, sea beets are only found on Heligoland and along the Baltic coast. Furthermore, the emergence of annual “weed beets” is observed in beet cultivation areas, which have been found in recent molecular genetic tests to be the product of cross-breeding between sugar beets and wild beets as a result of pollen dispersal from the seed production regions (e.g. South France, Italy).

Sugar beets are cross-pollinating; however, self-pollination also occurs. The pollen is mainly dispersed by wind; pollination by insects only plays a subordinate role. The pollen can be dispersed by wind over several kilometres. However, trials investigating the dispersal of sugar beet pollen in Denmark and Belgium have shown that the frequency of pollen transfer dropped considerably within the first 100 m around the pollen source (for example, from 32% to 3% in one trial) and that the likelihood of successful pollination was significantly reduced if the recipient plants had a sufficient amount of fertile pollen themselves.

At the locations of Klein Wanzleben and Prosselsheim, as well as at potential further locations to be registered later under the simplified procedure, where only vegetative beets are intended to be cultivated, the genetically modified plants will not reach the flowering stage.

At the location of Wetze, as well as at potential further locations to be registered later under the simplified procedure, the genetically modified plants on the selfing, hybridisation and propagation sites will reach the flowering stage and produce seeds. At the same time, non-transgenic reference plants are planned to be selfed and hybridised. At these locations, a maximum of 10,000 genetically modified beet plants per year and location will reach the flowering stage.

According to the regulation on the marketing of seed of agricultural and vegetable crops (Seed Marketing Regulation – Saatgutverordnung), a minimum separation distance of 1,000 m must be kept to pollen sources of the *Beta* genus while producing basic seed of fodder beets or sugar beets. This separation distance of 1,000 m to seed production fields for basic

seed or certified seed of fodder beets or sugar beets is proposed in the application for all trials with generative genetically modified sugar beets.

The supplementary conditions stipulate that all *Beta vulgaris* species that are crossable with sugar beets will be removed within a radius of 1,000 m around the release sites during the flowering stage of genetically modified sugar beet bolters. By placing a 5-m-wide border strip of hemp and by installing bast mats or parting walls in addition or as an alternative, this measure can be limited to a radius of 500 m. These measures serve to reduce the transfer of pollen from the genetically modified plants to other plants.

The proposed and any additional separation and protective measures effectively minimise, but cannot rule out, pollen dispersal and thus potential pollen transfer from the genetically modified plants to plants outside the release sites.

If pollen were to be transferred from the genetically modified sugar beets to weed beets or wild beets, the corresponding crossbred progeny is anticipated to be resistant to glufosinate ammonium and potentially to certain aminoglycoside antibiotics. Weed beets or wild beets are not expected to develop altered plant sociological traits and to populate other biotopes as a result of resistance to glufosinate ammonium or the corresponding antibiotics. Since herbicides containing glufosinate ammonium are not used in the natural habitats of wild beets, this trait would not confer a new selective advantage to the wild beets. Resistance to aminoglycoside antibiotics also does not represent a selective advantage under field conditions.

It should be considered that potential crossing with sugar beets or other cultivated species of *Beta vulgaris* would not result in a significant increase in the spread of foreign genes, since such plants are usually only propagated vegetatively. Sugar beet seeds are produced for commercial purposes in maritime climatic regions (Southern France, Po basin, Southern England). The cultivation of other *Beta vulgaris* species for the purpose of private seed propagation is uncommon, but cannot be excluded. For the reasons stated in III.1.2.1, it is assumed that even the cross-breeding of foreign genes in chard or beetroot and the consumption of the resulting plants are not expected to have any adverse health effects.

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

The inserted sequences are integrated into the chromosomes of the recipient organisms. 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.

Insofar as we assume that an exchange of genetic material between organisms that are as distantly related in terms of taxonomy as seed plants and bacteria 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 any heterologous genetic material, including all forms of plant DNA.

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 *pat* 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.

As described in III.1.2.1 (e), 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 sugar beets to microorganisms, the overall frequency of this resistance mechanism in the environment would not be noticeably increased.

The *cos* region is derived from the bacteriophage λ. λ is a phage that infects *E. coli*. Hence, the *cos* region is already present in bacteria. The same applies to the nucleic acid sequences located on the plasmid πAN7 as well as to the DNA fragments located outside the T-DNA border, all of which are of bacterial origin.

Even if regulatory sequences used in the construct were to be transferred, there is no reason to fear that the overall frequency of the respective DNA sequences will increase. These regulatory sequences are derived from *A. tumefaciens* and CaMV. *A. tumefaciens* are widespread in the environment. In wild-type agrobacteria, the specified sequences are located on Ti plasmids, which can be exchanged between different strains of Rhizobiaceae by conjugation. CaMV is a plant-infecting, double-stranded DNA virus commonly found in plants.

III.1.2.5. Agrobacteria used to generate the genetically modified sugar beets

In order to generate the original transformants from which the genetically modified sugar beet plants intended for release originate, cotyledons were inoculated with agrobacteria containing the genes to be transferred between the border regions of the corresponding binary transformation vector plasmids. In contrast to the common wild types of *A. tumefaciens*, the *Agrobacterium* strains used are disarmed, i.e. they no longer have the capacity to induce tumours. Following transformation, antibiotic treatment was carried out to eliminate the agrobacteria. Furthermore, the plants intended for release were propagated by seed. As a result of this generative propagation, any agrobacteria that survived the antibiotic treatment were removed from the genetically modified sugar beet lines.