Notification 6786-01-00075

Summary of the risk assessment of genetically modified sugar beet plants

(*Beta vulgaris* L.*)* (Transformants T 210-3 and T 219-5)

carried out by the German Competent Authority within

the framework of a proposed deliberate release,

Berlin, 05 March 1998

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

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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 beet plants effected by the transferred nucleic acid sequences

(a) The envelope protein gene of BNYVV

The genetically modified sugar beets contain the cDNA of the envelope protein gene with the beginning of the read-through part of BNYVV. The cDNA is supposed to confer a BNYVV resistance to the genetically modified plants.

The genome of most European isolates of BNYVV consists of four single-stranded positive-strand RNA molecules. The functions required for infectivity are distributed over the four RNAs. Thus, the gene for replication is found on RNA1; RNA2 carries information for the envelope protein, an important domain for encapsidation, vector transmissibility and cell-to-cell transmission; RNA3 is involved in the expression of foliar symptoms and proliferation in roots; and RNA4 plays an important role in vector transmissibility under natural conditions. So, pathogenicity of the envelope protein or of the truncated read-through protein alone can be excluded.

The envelope protein gene expressed under the control of the CaMV promotor gives rise to a concentration of envelope protein in the genetically modified plant tissue which is far below the concentration found in plants infected with BNYVV. However, there is a difference in the situation between cases of natural infection and the genetically modified plants concerning the distribution of the envelope protein in the plants. In the genetically modified plants, the envelope protein is produced in every cell, whereas in cases of natural, non-systemic infection of sugar beet, the protein occurs in the side roots and taproots as well as in the beet body. However, systemically diseased plants are also found under natural conditions, where the virus, and with it the virus envelope protein, are then also found in the green plant parts. So, qualitatively speaking, the genetic modification does not give rise to a fundamentally new situation as compared to the situation following natural infection.

(b) The *nptII* gene

The *nptII* gene transferred to the genetically modified plants encodes the neomycin phosphotransferase enzyme and was inserted as a marker gene for the selection of 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 certain aminoglycoside antibiotics causing these to become inactivated. The enzyme displays 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. Therapeutically important 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, no new metabolic products are expected to arise in the genetically modified plants in the absence of substrate under field conditions. Since high concentrations of the relevant antibiotics are not present in soil, the neomycin phosphotransferase confers no selective advantage on the genetically modified plants under field conditions. There is no evidence indicating that this enzyme is toxic to plants, animals, microorganisms or humans.

(c) The *uidA* gene (*gus* gene)

The *gus* gene from *E. coli,* which is contained in the T 219-5 transgenic plant, is under the control of the 35S promotor of CaMV and the NOS terminator sequence from *Agrobacterium tumefaciens.*

The *gus* gene was inserted as a reporter gene for histochemical evidence of successful transformation in the sugar beet genome. In order to prevent its expression in bacteria, the gene was equipped with an intron from the *ST-LS1* gene from potato. The enzyme β-glucuronidase cleaves glucuronides and is found in tissues of vertebrates and invertebrates and in bacteria. Plants also have low levels of endogenous β-glucuronidase activity which, however, can be suppressed through appropriate methods. Following addition of a corresponding substrate, the activity of the enzyme in transgenic tissue can be demonstrated. Plants are not expected to gain any selective advantage through expression of the *gus* gene.

In the event that parts of the plants are consumed by animals, no adverse effects would be expected as one can assume that the GUS enzyme would be degraded in the digestive tract.

(d) The coding sequence of the α part of the β-galactosidase, *lacI* sequences

In order to generate the genetically modified plants, vectors (pBIN19, pBI121) were utilised in which the multiple cloning site is located within the coding sequence of the α-fragment of the β-galactosidase from *E. coli*.

The native enzyme β-galactosidase cleaves β-D-galactoside into galactose and the corresponding alcohol compound. The most important physiological substrate is lactose, which is hydrolysed into galactose and glucose. The first 146 amino-terminal amino acids of the β-galactosidase are termed the α part. The α part is not enzymatically active by itself, but complementation is possible in suitable hosts.

Through insertion of the cp gene, or of the cp gene and the *gus* gene, into the multiple cloning site, the coding sequence for the α part of the β-galactosidase was interrupted, so that in this form, in *E. coli* bacteria, among others, it no longer has the capacity to code for an α part that is capable of complementation. The interrupted sequence of the α part of the β-galactosidase is under the control of a bacterial promotor. This sequence does not code for any functional gene product. Changes in the genetically modified sugar beet plants are not expected due to the presence of this sequence.

Furthermore, 5' and 3' sequences of the *lacI* repressor gene are also probably present in the genetically modified plants. However, these 5' and 3' sequences are separated from each other by the *lacZ* and M13 *ori* sequences. The *lacI* sequences are not expected to have any functional capacity in the genetically modified plants.

(e) M13 sequences

The genetically modified plants which were generated by transformation with derivatives of the pBIN19 and pBI121 vectors probably contain two fragments from M13mp19, i.e. a 440 bp fragment that includes a part of an open reading frame of a structural protein of M13, as well as a 433 bp fragment containing the origin of replication of the M13 phage.

If a transcription of the fragment of the open reading frame of the structural protein were to occur in the genetically modified sugar beet, this would not result in a functional protein as

the fragment only codes for 167 of a total of 423 amino acids in the complete phage protein. Thus, the presence of this fragment is not expected to have any effects on the metabolism of the plants.

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

(f) The fragment of the *ocd* genes

The plants, which were generated by transformation with derivatives of the pBIN19 and pBI121 vectors, 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 terminator sequence. As this sequence is transcribed as part of the mRNA of the *nptII* gene, but is located behind the termination codon of the *nptII* gene, the sequence is not expected to be translated.

(g) Border sequences from Ti-plasmids and regulation sequences

The genetically modified plants contain sequences from the left and right border regions of the TL-DNA of the pTiT37 plasmid from *Agrobacterium tumefaciens*. Depending on the gene products of the *vir* region of the helper plasmid pEHA101 present in the *Agrobacterium* strain used for the transformation, which was not transferred to the plants, these sequences effected the integration of the genes located between the border region into chromosomes of the sugar beet plants. These border regions of the Ti-plasmid have no function in the genetically modified plants and do not give reason to expect any changes in the plants.

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

- the 35S promotor of cauliflower mosaic virus (CaMV) (not in offspring of the T210-3 transformants),
- the double 35S promotor (35SS) of CaMV,
- the promotor of the nopaline synthase gene from *Agrobacterium tumefaciens*,
- the 35S terminator region of CaMV,
- the terminator region of the nopaline synthase gene from *Agrobacterium tumefaciens*.

The promotor and termination sequences regulate the expression in the genetically modified plants of the coding sequences of the BNYVV envelope protein located between them, the neomycin phosphotransferase and the β-glucuronidase. Statements on the effects of the formation of these proteins in the plants are provided under point III.1.2.1.(a) to (c).

(h) Sequences located outside the T-DNA

As a rule, only DNA sequences located within the border regions are integrated into the plant genome in *Agrobacterium*-mediated transformations. However, the transfer of DNA sequences from outside the border regions has been reported in individual cases and, based on the information contained in the application, this possibility cannot be ruled out. As a re-

sult, the risk assessment also considers those regions which are located beyond the T-DNA border regions on the vector used for transformation of the sugar beets. These include, in particular, the following sequences:

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

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

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

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

(v) a non-functional fragment of the *klaC* gene from the RK2 plasmid;

(vi) the *tetA* gene of the RK2 plasmid (interruptedby the insertion of the T-DNA region);

(vii) the IS1 transposon within the *nptIII* gene;

(viii) the origin of replication of the pMB1 plasmid.

As the *nptIII* gene (i) is under the control of a bacterial promotor, it is assumed that it would not be expressed in plants. Even in the event of expression of the gene, it would not be expected to have any effects on the plant metabolism.

The *oriV* (ii) or *oriT* (iii) origins of replication of the RK2 plasmid enable the replication of the plasmid in a broad host range of gram-negative bacteria, or its conjugative transfer, as long as the mobilisation functions are provided by a helper plasmid. There is no indication 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) would have a function in higher plants. Moreover, several of the DNA fragments are incomplete (v) or interrupted (vi).

(i) Position effects and context changes; allergenic potential

The level of expression of genes that have been integrated into the plant genome by genetic engineering methods is dependent on the site of insertion on the chromosome and/or on the environment around the insertion 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, in the field, the genetically modified plants are not as resistant to BNYVV as they are under climate-chamber or greenhouse conditions. Also with regard to the other transferred traits, risks to the environment or to human or animal health cannot be deduced from altered levels of expression.

The insertion of foreign genes can influence the expression or regulation of the native plant genes at and/or near the site of insertion. Such processes can affect plant metabolic pathways. However, during the work to date with the genetically modified plants in greenhouses and in open fields, no observations were reported that would suggest such an event.

Mobile genetic elements (transposable elements), which when transposed within the genome can exert effects on existing plant genes at the target site, occur naturally in plants and were first 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, such events can always influence plant metabolic pathways, even in non-genetically modified plants. In this regard the genetically modified plants intended for release here do not differ fundamentally from non-genetically modified plants.

With the current state of knowledge, it is not possible to predict the potential allergenicity of a protein on the basis of its amino acid sequence. However, in previous greenhouse and field trials with the genetically modified plants, as well as in deliberate releases of other genetically modified plants that express the relevant genes under the control of non-tissue-specific promoters, no evidence for an increased allergenic potential of the plants was found.

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

At the Oberviehhausen site, only vegetative plants are to be cultivated. At the Wetze site, in addition to the planted seed, seeds will also develop in the vernalised plants in the selfing, cross-breeding and propagating plots over the course of the trial (see below).

Towards the end of the vegetation period, the vegetatively cultivated sugar beet plants are to be harvested mechanically. A portion of the harvested beets will be transferred to laboratories for analysis (determination of quality parameters). If the yield intended for laboratory analysis is found to contain plant material still capable of propagation, it is deemed adequate from a safety perspective if its propagation capacity is removed during the course of the analysis. For example, subsequent topping of the beet bodies in the beet laboratory represents a suitable measure in this regard. Inactivation is, in any case, an inherent part of the analysis process.

Surplus harvest material (beets) and other excess vegetative plant material from the genetically modified beets are to be shredded and worked into the trial field. With this approach, genetically modified plants are not expected to regenerate from this material.

The genetically modified beet seeds are to be sown using drilling machines or by hand. Vernalised beets are to be planted out. Since the plants will not reach the flowering stage, seeds will not develop in the vegetatively cultivated plants. Under certain circumstances, particularly when worked into deeper soil layers, sugar beet seeds can remain germinable for many years. However, according to general crop-farming experience, planted seed which does not germinate is considered inactive and is therefore incapable of germinating in subsequent years.

At the "Wetze" site, in addition to the planted seed, over the course of the trial seeds will form in the vernalised plants in the selfing, cross-breeding and propagating plots. The genetically modified trial seeds are to be harvested by machine or by hand and then transferred to a genetic engineering facility. The rest of the generative plant parts are to be autoclaved or steamed after harvesting.

This form of disposal ensures that the cultivation of the vegetative beets will not lead to persistence of the genetically modified plants in the field. However, it must be assumed that the seeds which develop in the generative plants will enter the soil. The requirement contained in provision II.8. is intended to ensure that seeds which enter the soil do not, through subsequent soil cultivation, reach depths which would allow them to survive for a longer period.

Any seeds still surviving in the soil would lead to the emergence of genetically modified plants in subsequent years. These plants would be detected during the post-trial monitoring period proposed by the applicant and removed before flowering. Post-trial monitoring of the seed production plots is to be extended by a further year if any genetically modified sugar beet plants emerge in the final year of the respective monitoring period.

For the reasons mentioned above, the measures proposed in the application in conjunction with the provisions attached to this notification are sufficient to prevent establishment of deliberately released genetically modified plants and to detect and remove any potentially emerging genetically modified volunteer plants. Even if individual genetically modified seeds were to be dispersed as a result of the trial, this would not give cause for concern that the genetically modified plants could spread uncontrollably as these could be destroyed by mechanical measures (hoeing) or by applying 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 beet is cross-compatible with all species of the *Beta* section. Cultivated *Beta* species (including, among others, chard, mangold and beetroot) as well as sea beet (*Beta vulgaris* ssp. *maritima*) are the potential cross-breeding partners found in Germany. Sea beet only grows in Germany on the island of Helgoland and, occasionally, in coastal regions of Schleswig-Holstein. Furthermore, in beet cultivation regions, one-year "weed beets" emerge which, according to new molecular genetic methods of analysis, can be traced back to crosses between sugar beet and sea beet which can occur in seed-producing regions (e.g. South France, Italy) as a result of cross-pollination events.

Although sugar beet is an allogamous species, self-pollination also occurs. Pollination is accomplished mainly by wind, while pollination by insects is of only minor importance. Pollen can be dispersed over several kilometres by wind. However, trials carried out in Denmark and Belgium on the dispersal of sugar beet pollen already demonstrated a marked decrease in the frequency of pollen transfer within the first 100 metres around the pollen source (for instance from 32% to 3% in one trial), and that the probability of successful fertilisation was significantly reduced even when there was sufficient availability of the respective recipient plants' own fertilisable pollen.

At the Oberviehhausen site, the genetically modified plants are not supposed to reach the flowering stage, so that the question of a pollen transfer to other plants at this site does not arise. At the Wetze site, the plants cultivated in the selfing, cross-breeding and propagating plots are supposed to flower and set seeds. Overall at this site, a maximum of 10,000 genetically modified plants are to reach the flowering stage each year.

Selfing, cross-breeding and propagations will be conducted. At the same time, non-transgenic reference plants are to be selfed and crossed. If selfings and cross-breeding are conducted at the same site, the selfing block is to be isolated from the cross-breeding block by means of hemp sheeting.

Propagations, e.g. for the production of seeds for variety registrations, should always take place at separate sites. These are to be isolated in such a way as to ensure that no production of basic seed or certified seed of fodder beet or sugar beet occurs within a radius of 1,000 metres.

Precursor proliferations of lines will take place in 2 x 8 metre plastic-sheeted greenhouses. A maximum of 45 plants are to be cultivated per greenhouse. Alternatively, propagations for the production of basic seed can be conducted in large plastic-sheeted greenhouses with approx. 300 square metres of floor space. A maximum of 800 plants are to be cultivated per greenhouse.

In accordance with the regulation on the marketing of seed from agricultural plant and vegetable species (*Verordnung über den Verkehr mit Saatgut landwirtschaftlicher Arten und von Gemüsearten, SaatV*), a minimum distance of 1,000 metres to the pollination source of the genus *Beta* is to be maintained for the production of basic seed for beet. This isolation distance of 1,000 metres to the seed-production fields is proposed in the application for the Wetze site. Provision II.6. stipulates that areas in which genetically modified plants are allowed to reach the open flowering stage are to be shielded with a 5-metre wide hemp crop, or additionally with the erection of fibre mats or dividing walls, as required. Furthermore, it is laid down in provision II.6. that during the flowering of genetically modified sugar beet, bolters of all varieties of *Beta vulgaris* that are cross-compatible with sugar beet are to be removed within a radius of 1,000 metres around the release areas. This measure serves the purpose of reducing any transfer of pollen from the genetically modified plants to other plants. It goes beyond the requirements stipulated in the Seed Regulation.

Through the intended and/or required isolation and shielding measures, any spreading of pollen and in turn any potential pollen transfer from the genetically modified plants to plants outside the trials are effectively minimised. However, such a transfer cannot be completely excluded.

In the event of a pollen transfer from the genetically modified sugar beet to sea beet, the corresponding crossbred offspring would be expected to demonstrate resistance to BNYVV and certain aminoglycoside antibiotics. Neither sugar beet nor sea beet are expected to develop altered phytosociological characteristics or populate other biotopes due to their resistance to BNYVV or the antibiotics in question. As resistances to BNYVV can occur naturally in sea beet, this characteristic would not confer any fundamentally new selective advantage on sea beet. Resistance to aminoglycoside antibiotics does not represent a selective advantage under field conditions.

With regard to any potential crossing into other cultivated forms of *Beta vulgaris*, it should be taken into consideration that this would not lead to any numerically significant spreading of the foreign gene as such plants are generally only cultivated vegetatively. Production of sugar beet seed for commercial purposes occurs in maritime climatic regions (the South of France, the Po Valley, the South of England). Cultivation of other forms of *Beta vulgaris* (chard, beetroot) for the purpose of private seed propagation is not usual, however it cannot be excluded. Even if the foreign genes were to cross, e.g. into chard or beetroot, for the reasons presented under point III.1.2.1. any consumption of such plants would not be expected to represent a health risk.

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

The inserted sequences are integrated into chromosomes of the recipient organisms. Studies on the transformation capacity of soil bacteria under natural conditions suggest that a transfer of plant genetic material to soil microorganisms is in principle possible, although it is assumed that such a gene transfer would constitute an extremely rare event.

Insofar as we assume that an exchange of genetic material between organisms which are so distantly related in terms of taxonomy as seed plants and bacteria actually does take place, it would have to be concluded that the occurrence of such 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 transfer of the BNYVV envelope protein gene to microorganisms would not confer any apparent selective advantage on them. In the absence of selective pressure, a spread of this gene among microorganisms is unlikely. However, in the event that it is retained in the microorganisms, no ecological consequences are anticipated due to the lack of a selective advantage.

The β-glucuronidase gene (*gus* gene) from *E. coli* which was transferred into the sugar beet plants was equipped with an intron from the *ST-LS1* gene from potato in order to prevent expression of the gene in bacteria. β-glucuronidase genes code for β-D-glucuronoside gluconohydrolases, which cleave the glucuronide hydrolytically. They are widespread in nature and are found, for example, in vertebrates, invertebrates, bacteria and even in plants. Thus, any gene transfer of the *gus* gene from the genetically modified sugar beet plants to microorganisms would not be expected to noticeably increase the prevalence of this gene. Furthermore, the gene could only be expressed in bacteria after a recombination event which removes the intron.

As already described in section 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. It was therefore 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. 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 by the fact that these genes are often localised on plasmids, thus enabling effective transfer by conjugation. Even in the event of a horizontal gene transfer from the genetically modified sugar beet to microorganisms, the overall frequency of this resistance mechanism would not be noticeably increased.

The gene for the α fragment of the β-galactosidase is interrupted, so that no gene products that are capable of functioning can be formed. This would also be the case for bacteria which have received the gene through horizontal transfer. The same applies to the 3' and 5' sequences of the *lacI* gene.

A similar situation occurs with the fragment of the gene for a structural protein of the M13 phage, as well as with the fragment of the *ocd* gene. These fragments are not expected to be capable of functioning in bacteria. Moreover, it is likely that the fragment of the *ocd* gene would not be translated, as explained under point III.1.2.1.(f).

The genetically modified sugar beets probably contain the replication origin of M13. M13 belongs to the F-specific *E. coli* phages. Thus, for this origin of replication, the probability of

spreading by transfer between bacteria is far greater than the probability of spreading by means of horizontal gene transfer from the genetically modified plants to microorganisms.

The sequences inserted into the sugar beet for the regulation of the transferred genes are derived from *Agrobacterium tumefaciens* and CaMV. With regard to horizontal gene transfer of these sequences to microorganisms, it should be noted that *Agrobacterium tumefaciens* is widespread in soils so that a transfer of the corresponding sequences from *Agrobacterium* is far more probable than a transfer from the genetically modified plants. The theoretical possibility of a transfer of the CaMV sequences to these plants would not represent a new situation compared to the naturally occurring one, because CaMV, as a double-stranded, plant-infecting DNA virus, is found in plants anyway.

As a general rule, only DNA sequences located within the border regions are integrated into the plant genome in *Agrobacterium*-mediated transformations. However, in view of the details provided in the application, a transfer of sequences beyond the borders cannot be excluded. In the present case, the following DNA fragments could become integrated into the genetically modified plants through a transfer of sequences located outside the border regions:

(i) the *nptIII* gene from *Streptococcus faecalis* (which encodes a type III aminoglycoside-3'-phosphotransferase) for resistance to aminoglycoside antibiotics;

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

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

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

(v) a non-functional fragment of the *klaC* gene from the RK2 plasmid;

(vi) the *tetA* gene of the RK2 plasmid (interrupted through insertion of the T-DNA region);

(vii) the IS1 transposon within the *nptIII* gene;

(viii) the origin of replication of the pMB1 plasmid.

According to literature, the *nptIII* gene (i), which could be contained in the genetically modified plants with its own promotor, confers resistance not only to kanamycin and neomycin, but also to the antibiotic amikacin. Amikacin is not authorised for use as a veterinary medicinal product in Germany, but it is used in human medicine where it represents an important reserve antibiotic. Amikacin resistance is not widespread to date. Due to the low probability of horizontal gene transfer from plant DNA to microorganisms and the absence of selection pressure on the release sites, the presence of this gene in the genetically modified sugar beet is not expected to 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 (including, among others, RP1, RP4, R18, R68) which are replicable in a wide range of gram-negative bacteria. Thus, for the DNA fragments originating from RK2 (ii to vi), the probability of spreading by transfer between bacteria is far greater than the probability of spreading by means of horizontal gene transfer from

the genetically modified plants to microorganisms. Moreover, several of the DNA fragments are incomplete (v) or interrupted (vi).

The IS1 insertion element (vii) occurs naturally in various species of *Enterobacteriaceae*. For instance, it has been found in species of the genera *Escherichia*, *Shigella*, *Klebsiella*, *Serratia* and *Salmonella*. With IS1, more than 40 copies can be present per bacterial gnome. Copies of IS1 can be localised on chromosomes as well as on plasmids and they have also been verified in prophages. It is to be assumed that spreading of this insertion element via horizontal gene transfer between bacteria is readily possible. Compared to this, the theoretically conceivable spreading of the insertion element through horizontal gene transfer from the genetically modified plants to microorganisms is negligible.

The pMB1 replicon (viii) belongs to the ColE1 plasmid types, which have a host range limited to a small number of gram-negative bacteria. Essentially, the replicon can be replicated in *E. coli* and in closely related species of bacteria such as *Serratia* or *Salmonella*. Replication does not take place in the majority of gram-negative soil bacteria. ColE1 plasmids occur quite frequently in enterobacteria. The probability of an enterobacteria-mediated gene transfer to other bacteria should be regarded as far more likely than the probability of a horizontal gene transfer from the genetically modified plants to bacteria. Therefore, the potential presence of the origin of replication from pMB1 in the plant chromosome cannot be 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 sugar beet plants

To generate the original transformants from which the genetically modified sugar beet plants proposed for release were derived, sterile cotyledons were inoculated with Agrobacteria, which contained the genes intended for transfer between the border regions of the corresponding binary vector plasmids. Unlike the common wild-type A. *tumefaciens*, the *Agrobacterium* strains used here are "disarmed", i.e. they no longer have the capacity to induce tumours. After transformation had occurred, antibiotic treatment was carried out to eliminate the Agrobacteria. Furthermore, the plants intended for release were propagated by seed. Through this generative propagation, any Agrobacteria that remained after the antibiotic treatment were removed from the genetically modified sugar beet lines.