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Summary of the risk assessment of the genetically modified grapevines

(*Vitis vinifera* L. ssp. *sativa*) pGJ40 and pGJ42

within the framework of a proposed deliberate release

carried out by the German Competent Authority

Berlin, 15 June 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

I. Consent [to the application]
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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 grapevines effected by the transferred nucleic acid sequences

(a) The chitinase gene *chi*26 from *Hordeum vulgare*

Chitinases catalyse the hydrolysis of chitin, a linear homopolymer of β-1.4 glycosidically linked N-acetylglucosamine residues. Together with glucanases, they belong, mainly as PR (p*athogenesis*-r*elated*) proteins, to a complex defence system used by plants to protect themselves against pest infestation. Chitinases are found in many plant species, including vine, and in all plant parts, including grapes.

Chitinases are classified into five groups based on their primary structure; *chi*26 belongs to group II. They predominantly hydrolyse the substrate chitin, which is, however, not formed in plants. In addition, the hydrolysis of bacterial lipooligosaccharides is subject to discussion. This function also seems to be associated with the plant's defences.

Chitin is a component of fungal cell walls and the exoskeleton of e.g. insects. According to a generally accepted model dealing with the formation of fungal cell walls, chitin (poly-N-acetyl-D glucosamine) and β-1,3-glucan are synthesised by fungal hyphae in a turgor-deformable, plastic apex and are continuously deposited in the apex tip. These primary homopolymers are transformed into a turgor-resistant, rigid tube within a period of a few minutes, accompanied by chitin crystallisation and formation of microfibrils, beta 1,6 branches and triple helix conformation of glucan, covalent bonding of both polymers, modification (e.g. deacetylation) and protein deposition. The entire process is designed in such a way to ensure flow equilibrium between apex and tube to keep the growth tip accessible to substrate molecules and enzymes.

Only the unbranched and homopolymer chitin in the apex tip seems to be accessible to chitinases and hydrolysable. In conjunction with the β-1,3-glucanase described below, this interferes with the formation of the cell wall, which can lead to bursting of the apex due to turgor. The specificity of the plant chitinase for the unmodified growing primary chitin chain is therefore expected to be so high that finished elements of exocuticle or endocuticle are not accessible to insects. Attempts to generate resistance to insects using genetically inserted chitinases failed when the chitinase was of plant origin, and were only successful with chitinases from insects.

(b) The glucanase gene *glu*32 from *H. vulgare*

The *glu*32 gene codes for a β-1,3-glucanase. β-1,3-glucanases catalyse the hydrolysis of β-1,3-glucan, a linear homopolymer of β-1,3 glycosidically linked glucose residues. Together with chitinases, the β-1,3-glucanase belongs to the above-mentioned complex defence system used by plants to protect themselves against pest infestation. Glucanases are also found in many plant species, including vine, and in all plant parts, including grapes.

β-1,3-glucan is a component of fungal cell walls; referred to as callose, it serves in plants as a sealing material after tissue injuries. In fungi, only the unbranched and homopolymer β-1,3-glucan in the apex tip seems to be accessible to β-1,3-glucanases and hydrolysable. In conjunction with the chitinase described above, this interferes with the formation of the cell wall, which can lead to bursting of the apex due to turgor. The β-1,3-1,4-glucan, which is also formed in plants, is a component of plant cell walls and is not hydrolysed by β-1,3-glucanases.

(c) The gene for a ribosome-inactivating protein (RIP) from *H. vulgare*

Under the name “barley toxin”, the ribosome-inactivating protein RIP from *H. vulgare* belongs to a number of substances that are commonly found in plants and inactivate foreign ribosomes by modifying a highly conserved region of the large rRNA of the large subunit to defend the plant against pathogens. Some ribosomes remain intact; the sensitivity increases with the evolutionary distance and is found to be highest in fungi. The differences are attributed to the ribosomal proteins. A distinction is made between two types: Like all RIP from grain, barley toxin belongs to type I. It is an RNA N-glycosidase and consists of a chain with a molecular weight of approx. 30 KD. It depurinates *in vitro* the 28S rRNA of the 60S subunit of rat liver ribosomes in the 5´-AGUACGAGAGGA-3´ sequence at the A4324 position. This modification inhibits the bonding of the elongation factor EF-2 and thus the translocation of the chain formed. In this system, fungal ribosomes are 10 times as sensitive as mammal ribosomes.

In barley, large amounts of CHI26 and RIP30 are deposited in the aleurone layer during the late seed development phase. The glucanase gene *glu*32 is expressed in the aleurone layer and the barley seedling in small amounts during seed development and in somewhat larger amounts during germination. Consumption is not known to be associated with any harmful effects, e.g. for storage insect pests. Long-term experience with grain seed of any dosage form as part of human or animal nutrition also provides no indication of any harmful effects. Based on all existing knowledge, any transgenic products present in grapes, must or wine are expected to be degraded in the digestive tract of humans and animals.

In the genetically modified grapevines, the transferred target genes *chi*26, *bgl*32 and *rip*30 are expressed in the plant under the control of the constitutive 35S promoter and termination signal from CaMV. The applicant provides no information regarding the concentration of the two hydrolases and the RIP in grapes, must, wine or seeds. However, based on existing experience with transgenic maize and oilseed rape, the above-described high accumulation of chitinase and RIP in the seeds of the donor organism barley is not expected under the control of the constitutive promoter.

Studies have shown that the expression of the genes named in (a) to (c) in corresponding genetically modified plants results in increased resistance to fungi. None of the studies performed provided an indication that the inserted genes cause changes in the transgenic plants other than those planned.

The genetically modified grapevines are intended to be released ungrafted or grafted. Studies on the effects of various chitinase and glucanase genes that have been expressed in genetically modified tobacco plants under the control of the 35S promoter demonstrate that the development of the mycorrhizal organism *Glomus mossae* is not adversely affected. The presence of these proteins is therefore not expected to have any harmful effects on the natural mycorrhizal organisms of grapevine.

(d) The *gus* gene (*uidA* gene) from *E.coli*

The *gus* gene codes for a β-glucuronidase and was introduced into the genome of some of the genetically modified grapevines with the plasmid p35Sgus-int in order to be able to examine the outcrossing rate in vines by histochemical verification of the GUS activity. The *gus* gene is expressed under the control of the 35S promoter and 35S termination signal of the

CaMV. The gene was provided with an intron to prevent expression in bacteria.

The enzyme β-glucuronidase cleaves glucuronides and is found in the tissue of vertebrates and invertebrates as well as in bacteria. Plants also exhibit minor endogenous β-glucuronidase activity, which can, however, be suppressed using appropriate methods. After adding a corresponding substrate, the enzyme activity can be verified in transgenic tissue. The expression of the *gus* gene from *E. coli* is not expected to confer a selective advantage to plants.

The consumption of plant parts by animals or humans is not expected to have any harmful effects, since the GUS enzyme is assumed to be degraded in the digestive tract.

(e) The *nptII* gene

The *nptII* gene codes for the enzyme neomycin phosphotransferase and was inserted as a marker gene for the selection of transformed grapevine 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.

(f) The coding sequence of the α-fragment of the ß-galactosidase, *lac*I sequences

The genetically modified plants were created by using derivatives of the vector pBIN19, the multiple cloning site of which is located within the sequence coding for the α fragment of the ß-galactosidase from *E. coli*.

The native enzyme β-galactosidase splits β-D-galactosides into galactose and the related alcohol compound. The physiologically most important substrate is lactose, which is hydrolysed into galactose and glucose. The first 146 amino-terminal amino acids of the β-galactosidase are referred to as the α fragment. The α fragment by itself is not enzymatically active; however, complementation in suitable hosts is possible.

The sequence coding for the α fragment of the β-galactosidase was interrupted by the insertion of the various expression cassettes into the multiple cloning site, preventing it from coding for an α fragment capable of complementation in *E. coli* bacteria. The interrupted sequence of the α fragment of the β-galactosidase is under the control of a bacterial promoter.

This sequence does not code for a functional gene product. The presence of this sequence is not expected to cause any changes in the genetically modified grapevines.

The genetically modified plants additionally contain 5' and 3' sequences of the repressor gene lacI. 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 be functional in the genetically modified plants.

(g) M13 sequences

The genetically modified plants contain two fragments from M13mp19, namely a 440-bp fragment, which encompasses one part of an open reading frame of a structural protein of M13, and a 433-bp fragment, which contains the origin of replication of phage M13.

If transcription of the fragment of the open reading frame of the structural protein were to occur in the genetically modified grapevines, no functional protein would result, since the fragment only codes for 167 of the total 423 amino acids of the complete phage protein. The presence of this fragment is thus not expected to affect plant metabolism.

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

(h) The fragment of the *ocd* gene

The plants created by transformation using derivatives of the vector pBIN19 contain a fragment of the *ocd* gene (ornithine cyclodeaminase), which is located between the 3' terminal end of the translated sequence of the *nptII* gene and the NOS terminator sequence. Since this sequence is transcribed as part of the mRNA of the *nptII* gene, but is located downstream of the termination codon of the *nptII* gene, this sequence is not expected to be translated.

(i) Border sequences of the Ti plasmid pTiT37; regulatory sequences

The genetically modified plants contain sequences of the left and right border region of the TL-DNA of the plasmid pTiT37 from *A. tumefaciens*. Depending on the gene products of the *vir* region of the helper plasmid pAL4404 that is contained in the *Agrobacterium* strain LBA4404 used for transformation and is not transferred into the plants, these sequences cause the genes located between the border regions to integrate into the chromosomes of the grapevines. These border regions of the Ti plasmid 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:

- The 35S promoter and the 35S termination signal of the cauliflower mosaic virus (CaMV),

- The promoter and terminator of the nopaline synthase gene (*nos* gene) from *A. tumefaciens*.

In the genetically modified plants, the promoter and terminator sequences regulate the expression of the genes located between them. Further information on the effects associated with the expression of these sequences in the plants can be found in III.1.2.1 (a) – (e).

(j) DNA fragments 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. However, the transfer of DNA fragments outside the border regions has been reported. Based on the information provided in the application, the following functional units may have been transferred into the genetically modified grapevines in this particular case as a result of the integration of DNA fragments located outside the border regions:

(1) The origin of replication *oriV* of the plasmid RK2;

(2) The *traF* region containing the *oriT* of the plasmid RK2;

(3) The *trfA* locus of the plasmid RK2 (codes for two proteins required for the replication of the plasmid);

(4) A non-functional fragment of the *klaC* gene from the plasmid RK2;

(5) The *tetA* gene of the plasmid RK2 (interrupted by insertion of the T-DNA region);

(6) The origin of replication of the plasmid pMB1.

(1) and (2): The origins of replication *ori*V (1) and *ori*T (2) of the plasmid RK2 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.

(3) - (6): There is no evidence to suggest that *ori*V or *ori*T of RK2, the origin of replication of pMB1 (6) or the remaining DNA fragments of bacterial origin (3 - 5) have a function in higher plants. Moreover, some of the DNA fragments are incomplete (4) or interrupted (5).

Outside the T-DNA border sequences, the vector backbone of the vector pBIN19 also contains the *npt*III gene, which confers resistance to the second-line antibiotic amikacin and others. The genetically modified grapevines were examined for the presence of the *npt*III gene by means of a PCR analysis. None of the lines intended for release was found to contain the complete gene. Even if the complete *npt*III gene were present, a functional gene product would not be expected to be expressed in the genetically modified plants, since this gene is under the control of prokaryotic regulatory sequences.

(k) 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 grapevines are not resistant to

fungi or do not express GUS to the same degree in the field as under climate-controlled or greenhouse conditions. This does not represent 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. In previous work 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 and were first identified 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-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 as well as in earlier deliberate release trials with genetically modified plants that express the *npt*II gene under the control of non-tissue specific promoters, no evidence was found to suggest an increased allergenic potential of the plants.

Grapevine pollen is dispersed by wind. It does not play a noteworthy role in triggering pollen allergies. Based on the available information concerning the traits transferred, changes in the pollen's allergenic potential are not expected.

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

As a result of the proposed measures, the genetically modified grapevines are not expected to spread to areas outside the release site, nor are they expected to persist or establish in the environment.

Unless samples are to be taken, the released grapevines will be uprooted, shredded and worked into the soil. In view of these precautions, the regeneration of genetically modified plants from material remaining on the vineyards is not expected.

The cultivated grapevine will be propagated vegetatively and requires extensive care. It has an extremely low potential to establish and spread. In Germany, grapevines are found on the northern border of their cultivation area, which is located between 30 and 50 degrees north latitude. The emergence of individual plants outside vineyards is only reported in exceptional cases. As a result of grapes falling down and the common use of pomace as fertiliser in normal cultivation, many seeds are dispersed to the vineyards. This procedure will also be used in the proposed deliberate release trial. Based on general experience, occasionally emerging seedlings are easily recognised in the far-spaced rows of older woody plants and will be re-

moved in the course of regular vineyard maintenance. The applicant plans to monitor the release site for emergence of grapevines in the year following conclusion of the trial. Any emerging or budding vines will be inactivated.

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

Grapevine is self-fertile. Grapevine pollen can be dispersed by insects and by wind. The release sites are surrounded by vineyards. One goal of the field trial is to measure the outcrossing rate to neighbouring non-transgenic grapevines by means of the *gus* gene. A separation distance to non-genetically modified grapevines is not planned.

The successful pollination of flowers of non-transgenic grapevines by the transgenic grapevines would result in the development of genetically modified seeds in the grapes. As a result of the measures described in III.1.2.2, even if these seeds were to be dispersed to the vineyards and germinate, the genetically modified grapevines are not expected to spread, persist or establish outside the release site.

The vineyards surrounding the release site serve to produce must and wine. Dessert grapes are generally not produced in these regions. Since only the seeds are genetically modified – the cells of the fruit pulp derive from somatic tissue of the pollen-receiving plant – the gene products are expected to be found only in small amounts in the whole grapes. Based on all existing knowledge, any transgenic products present in grapes, must or wine are expected to be degraded in the digestive tract of humans and animals. This is not expected to have any adverse effect on human life and health.

Cross-breeding between the genetically modified grapevines and native wild plants is not expected to occur. Although it is not established that the cultivated grapevine is related to the rarely occurring dioecious wild vine *Vitis vinifera silvestris* GMELIN found in the riverside forests along the Upper Rhine, cross-breeding is considered possible. Wild vines are not found near the two release sites.

III.1.2.4. Assessment of the possibility of horizontal gene transfer of the inserted foreign genes from the genetically modified grapevines 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 the absence of selection pressure for the traits transferred along with the genes, such gene transfer is not expected to have any ecological consequences.

(a) The genes for resistance to fungi

The genes used to confer resistance to fungi are derived from barley, i.e. are already widespread in the environment. Horizontal gene transfer from non-genetically modified organisms to microorganisms is thus far more likely to occur.

(b) The *gus* gene

The *gus* gene derived from *E. coli* was inserted for control purposes and codes for a β-glucuronidase. Glucuronidase is commonly found in microorganisms, which is why a noticeable increase in overall frequency is not expected, even if horizontal gene transfer were to occur.

(c) The *npt*II gene

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

(d) Additional DNA fragments located outside the T-DNA

Outside the border regions, the genetically modified plants intended for release may contain the origins of replication RK2 *ori*V and *tra*F for replication in *E. coli* and *A. tumefaciens*, the element IS1 and other bacterial fragments (see above). The genetically modified grapevines were examined for the presence of the *npt*III gene located on the used transformation vectors outside the border regions by means of a PCR analysis. None of the lines intended for release was found to contain the complete gene.

In the case of all these nucleic acid fragments, the probability of genetic spread by transfer between bacteria is far higher than the probability of horizontal gene transfer from the genetically modified plants to microorganisms. Furthermore, the transfer of an incomplete *npt*III gene to microorganisms is not expected to confer antibiotic resistance to them.

(e) Regulatory sequences

Even if regulatory sequences used in the constructs 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 grapevines

In order to generate the genetically modified plants, wounded plant explants were incubated in suspension with agrobacteria which harbour the genes to be transferred between the border regions of the binary vector plasmid. Following transformation, antibiotic treatment was carried out to eliminate the agrobacteria.

In contrast to the common wild types of *A. tumefaciens*, the *Agrobacterium* strain used 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 progeny. Such an event is not expected to occur under natural conditions.

Assuming that the presence of small amounts of recombinant agrobacteria in the genetically modified 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 event of infection and subsequent transformation by wild-type *A. tumefaciens* or *A. rhizogenes*, a crown gall or hairy root would develop from the transformed plant cell. Under natural conditions, such a tumour would not be expected to give rise to a plant.

Furthermore, the transfer of the inserted genes from agrobacteria to other soil bacteria would have to be considered. The potential effects of such a transfer were already addressed in III.1.2.4.