Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

Notification 6786-01-0195

Summary of the risk assessment of the genetically modified organisms

wheat (*Triticum aestivum*) KP4 Greina 16 and KP4 Golin 5

within the framework of a proposed deliberate release

carried out by the German competent authority

Berlin, 13 Mai 2008

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.

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III.1.2.1. Evaluation of changes in the genetically modified plants effected by the transferred nucleic acid sequence transfer

(a) The *kp4* gene

The *kp4* gene is derived from the genome of a double-stranded RNA virus, which is present in the tissue of certain fungal strains of corn smut (*Ustilago maydis*). The effect of the *kp4* gene was first detected in yeasts, where its expression kills off competing yeast strains. This phenotype is mediated by the gene product KP4 (killer protein 4). *Ustilago* strains that harbour the RNA virus were therefore also referred to as killer strains. In the meantime it has been established that fungal cells inactivated by KP4 are able to continue growing following extra-cellular application of calcium. Hence in *Ustilago* KP4 evidently leads to a reversible inhibition of hyphal growth, but does not kill off competing strains. The KP4 toxin's mode of action is based on blocking voltage-dependent L-type calcium channels. KP4 binds competitively to the calcium binding sites of this channel type, thereby blocking the transport of calcium. In fungi, calcium plays a role in the development of the fruiting body, in the growth of the hyphae and in cAMP regulation. The effect of KP4 toxins on the permeability of fungal, and also mammalian, calcium channels was demonstrated in "whole cell patch clamp" experiments. Nonetheless, to date a toxic effect has only been verified *in vivo* in the case of *Ustilago* strains. Attempts to demonstrate the influence of KP4 on other fungi, e.g. of the genera *Aspergillus*, *Fusarium* or *Penicillium*, or on different bacteria have not yet been successful. In addition, KP4 has no effect on the viability of plant cells, for instance tobacco mesophyll cell cultures, or on animal-derived cells such as cells of the hamster cell line CHO-K1 or cells derived from human kidney tissue. Incubation of neonatal heart cells with KP4 did not give rise to morphological changes in the organelle structures. Furthermore, previous experiments show that the digestion of KP4 in simulated gastric fluid is associated with a loss of fungicidal activity. Exposure to heat leads to rapid inactivation of the protein. Moreover, KP4 does not exhibit any amino acid motives that are known from established allergens.

Southern blot analyses carried out to determine the number of copies transferred revealed the presence of two copies of the *kp4* gene in the KP4 Greina16 line, and more than two copies of the *kp4* gene in the KP4 Golin 5 line.

Whether the expression of KP4 in the genetically modified wheat also gives rise to additional unintended effects in the plant metabolism has not yet been investigated. The genetically modified wheat referred to in the proposed deliberate release is not intended for human or animal consumption. The proposed release site covers a very small area.

Against the background of existing studies, the proposed security measures and the size of the release site, no adverse effects on human health or on the environment are anticipated.

(b) The *bar* gene

The *bar* gene derived from *Streptomyces hygroscopicus* encodes the enzyme phosphinothricin-N-acetyltransferase (PAT) and is driven by the maize actin promoter and the 35S termination sequence of the cauliflower mosaic virus. This marker gene confers tolerance to phosphinothricin (glufosinate), the active ingredient in the herbicide Basta®, and was transferred for selection purposes in the production of the genetically modified plants.

Several copies of the bar gene were inserted into the KP4 Greina 16 line, the number of copies present in the KP4 Golin 5 line was not determined.

L-phosphinothricin is a glutamine acid analogon that inhibits glutamine synthetase in plants. Inhibition of glutamine synthetase causes accumulation of ammonium ions, thus leading to cell death. For this reason phosphinothricin is used as the active ingredient in the non-selective herbicide Basta®. Basta® contains the enantiomers D- and L-phosphinothricin at a ratio of 1 : 1. D-phosphinothricin does not act as a glutamine synthetase inhibitor.

Unlike non-genetically modified plants treated with Basta®, in genetically modified plants treated with Basta® L-phosphinothricin is acetylated by phosphinothricin acetyltransferase, leading to the production of N-acetyl-L-phosphinothricin, which has no herbicide effect. As a result the genetically modified plants are tolerant to the herbicide Basta®. Phosphinothricin acetyltransferase is a highly substrate specific enzyme. Even the phosphinothricin analogon glutamate is barely converted. D-phosphinothricin is not metabolised by phosphinothricin acetyltransferase.

N-acetyl-phosphinothricin present in the genetically modified plant material remaining in the field following completion of the trials may enter the soil during decomposition and here it may be converted back into L-phosphinothricin by soil micro-organisms. D/L-phosphinothricin is likewise broken down in the soil by micro-organisms.

According to the present data, the level of toxicity exhibited by N-acetyl-L-phosphinothricin is significantly lower than that of phosphinothricin (= the active agent in the herbicide Basta®). Basta® has been authorised for use under the German Plant Protection Act by the Federal Biological Research Centre for Agriculture and Forestry (BBA) and the Federal Office of Consumer Protection and Food Safety (BVL). An assessment of the toxicological and ecological impact of the herbicide was conducted within the scope of the approval process.

Furthermore, if parts of the genetically modified plants are consumed by humans or animals the phosphinothricin-acetyltransferase contained in these plants is not expected to cause any harmful effects. If ingested orally it can be assumed that the enzyme, like proteins in general, would be degraded in the digestive tract. The phosphinothricin-acetyltransferase does not possess any of the typical properties (heat stability, stability in the digestive tract) of aller-

genic proteins from food, nor does it have any sequence homology with known allergens. The use of Basta® as a herbicide within the scope of the experimental release is not planned.

(c) Additional DNA fragments in the inserted transformation plasmids

To achieve transformation the intact transformation plasmids were inserted by microprojectile bombardment. The pAct::*bar* and pUbi::*kp4* plasmids used in the transformation of these wheat plants are derived from the plasmid pUC19. Based on the information available, it can not be ruled out that parts of the other DNA fragments of the pUC19 plasmid were transferred to this line. This includes:

- The *bla* gene, which encodes β–lactamase,
- nucleotides of the *lacZ* gene derived from *E. coli,*
- the origin of replication ColE1 *(ori).*

Southern blot analysis verified the presence of the *bla* gene in the genome of the KP4 Greina 16 line. The KP4 Golin line was not examined. However, the presence of the above-mentioned elements must also be assumed in this case. These elements are dedicated to gene expression in bacteria, and have no function in plants. If these DNA fragments were to be transferred into the genome of the genetically modified wheat, they would not be expected to be expressed. Therefore, harmful effects on human health and the environment are not anticipated.

(d) 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 integration on the chromosome and on the neighbouring sequence at the integration site (“position effect”). Under field conditions the level of expression may be influenced by environmental factors, for instance, by temperature. In this particular case this could mean that the characteristics of the genetically modified plants are not modified to the same degree in the field as under climate-chamber or greenhouse conditions. This does not represent 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 and can potentially lead to alterations in plant metabolic pathways. Such processes may influence plant metabolism pathways. During previous trials on the genetically modified wheat plants, both in the greenhouse and under open field conditions, no alteration in phenotype was observed in comparison to the control plants. The mode and rate of propagation, dispersal and persistence all remained unchanged.

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 respect the genetically modified plants 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 possible allergenic action of a protein on the basis of its amino acid sequence. The KP4 protein produced in the genetically modified wheat belongs to a group of proteins generated in the maize fungus *Ustilago maydis*. No allergenic effects have been reported. For this group of proteins cytostatic properties could only be demonstrated in other *Ustilago* strains. The products of this trial are not intended for use in foodstuffs or animal feed.

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

Wheat has a long history of cultivation; hexaploid wheat is not known to exist as wild-type wheat. It only appears as a wild plant on waysides and ruderal sites in the vicinity of existing cultivated areas. As a weakly competitive plant, wheat is not known to establish in natural, intact plant communities. Experience gained from greenhouse and open field trials did not produce any evidence to suggest that the genetically modified wheat differs from non-genetically modified wheat in its ability to establish in the environment as a result of the genetic modification.

On completion of the generative phase the wheat plants die off. From the produced seed new plants can emerge. During harvesting the seeds (grains) are separated from the wheat ears by threshing. After entering secondary dormancy they can survive under favourable conditions for up to two years without losing the ability to germinate. Given the right conditions these seeds may germinate in subsequent stocks of cultivated plants. There are no indications that this genetic modification leads to altered persistence in comparison with non-genetically modified wheat.

The applicant proposes to dispose of harvested plant material not needed for analysis in a biogas plant. After harvesting, the remaining plant residue will be chopped into pieces and worked into the soil using a disc harrow. Any grain that emerges following this procedure will be treated with a herbicide in the same year.

Subsequent to completion of the proposed trial only plant species that allow the identification of potential volunteer wheat should be sown on the experimental release site. During post-trial monitoring in the planned cultivation interval, any emerging wheat plants should be de-

stroyed before flowering. A three-metre wide strip around the border rows is to be included in the two-year post-trial monitoring required by provision II.12. [of the decision on this application]. Both the cultivation interval and the post-trial monitoring period are to be extended if volunteer wheat plants are observed in the final year of the release (provision II.12).

In studies carried out to date with the genetically modified wheat and in observations of the morphological traits of the plants under greenhouse conditions, the applicant reports that no differences between the genetically modified and the non-genetically modified plants were found. The genetically modified wheat was not found to exhibit any increased vitality or fertility which would promote the persistence or invasiveness of the genetically modified plants in the environment. Accordingly, the possibility that the genetically modified wheat might persist in the open field or that plants might establish in this way is extremely slight.

One of the aims of the experiment is to test whether the wheat exhibits increased resistance to the loose smut fungus *Ustilago tritici* as a result of the genetic modification. There are no indications that such a trait would alter the general inability of the cultivated plants to compete against wild plant species. For these reasons neither the establishment nor the uncontrolled persistence of the genetically modified plants is to be expected.

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

Wheat (*Triticum aestivum*) is the most important crop cultivated in the temperate zones. It is an annual cereal grass with both summer and winter varieties. The upright stem (rachis) comprises a distichous arrangement of alternating spikelets each containing 3-6 androgenous florets, of which only about 3 produce seeds. The flowering period of each individual floret is extremely short, lasting approximately one hour. Due to the staggered onset of flowering in the individual florets of the spikelet, in the entire head and in the different heads on the main stem and on the auxiliary stems of the same plant, it can take over a week for all the florets of an entire wheat plant to flower. As a rule self-pollination takes place before the flower opens, although to a certain extent foreign pollination is possible, depending on genotype and on climatic conditions during flowering. The rate of cross-pollination is said to be around 1-3%, but under warm and dry weather conditions the rate for some genotypes may be higher. On enquiry, the Federal Office for Plant Varieties (Bundessortenamt) stated a cross-pollination rate of 1-3% for varieties cultivated in Germany.

Although wheat pollen is dispersed by wind, the possibility of spreading is limited because of the weight of the pollen grains. The possibility of cross-pollination is similarly limited, since only a relatively small amount of pollen is produced. Furthermore, wheat pollen is only fertile over a very short interval. Under optimal conditions the fertile period lasts for about three

hours; under field conditions, less than 30 minutes. The out-crossing distance is always determined as a function of the size of the field of pollen receiver and donor plants, since this influences the available pollen mix and, therefore, the relationship between competing native and foreign pollen. If the donor plants are cultivated on a large area, the probability that pollen from this source will be found at a certain distance is higher than if the donor plants are cultivated on a small area.

Numerous studies have been conducted on out-crossing in wheat, in which the out-crossing characteristics of a range of summer wheat varieties were examined. However, even in varieties with high out-crossing rates, out-crossing distances of over 33 m were not recorded. In a study on the effects of a disjunctive method of propagation, 8 different wheat accessions were examined over 24 reproduction cycles. For this method of cultivation, the propagation plots for the different wheat accessions are placed just a few metres apart. In these studies DNA material from current stocks was compared with 50-year-old reference samples using microsatellite analysis. Despite the cultivation methods described above, even after 24 reproduction cycles, no molecular evidence of any out-crossing that might influence the integrity or genetic stability of the accessions was found. According to the information available to the BVL, the furthest out-crossing distance demonstrated in non-commercial cultivars was 300 m, with an out-crossing rate of 0,005%. In that study the donor area was 2500 sq m - an area almost 35 times larger than that foreseen for the proposed trial (72 sq m per location and year). An aspect of this study that is more interesting than the maximum distance detected is the determination of out-crossing values at various distances from the pollen donor field. Here it is noticeable that from a distance of 40 m the gene flow rate in most directions investigated was 0 and in isolated cases did not exceed 0.03%. Therefore, if the pollen donor area is downscaled by a factor of 35, it can be assumed that these values would not be exceeded. Accordingly, minimisation of out-crossing would be adequately addressed by ensuring a separation distance of 50 m. Although out-crossing has been reported at distances of 2.7 km from the pollen donor, these results can not be applied to the proposed small-area release experiment. These out-crossing events were detected on the basis of pollen donor areas of 22 and 33 hectares. The pollen donor areas referred to in these studies are therefore approximately 3000 – 4500 times larger than the proposed release areas.

As a measure to protect against unwanted incrossing in wheat propagation areas, seed legislation requires that these areas be separated from neighbouring cereal stocks by a separation strip (the width of which is not given). Other minimum separation distances must not be observed.

The seed for hybrid wheat varieties permitted in Germany is produced abroad. Studies on pollen spread in emasculated wheat, as required for hybrid seed, showed about 10% budding on pollen-sterile wheat plants which had been planted approximately 30 m from the pol-

len source. By contrast, in field studies it was found that the bud on pollen-sterile wheat plants declined to 10% at distances as short as 1-3 m. The necessary pollen sterility is not genetically induced, but is achieved by the application of gametocides that are not permitted in Germany. Therefore, the cultivation of hybrid wheat is not expected to increase the potential for out-crossing.

The most commonly grown wheat in our region (*Triticum aestivum* or bread wheat) is hexaploid. Additional types grown with a regional focus include durum wheat (*Triticum durum*, tetraploid, for pasta products) and occasionally spelt (*Triticum spelta*, hexaploid, e.g. for pearl barley or semolina). Other species of wheat such as poulard (*Triticum turgidum*, tetraploid), emmer (*Triticum dicoccum*, tetraploid) and einkorn (*Triticum monococcum*, diploid), however, are only found very occasionally in agricultural areas. Pollen sterile wheat is not used for cultivation purposes.

As an important crop, wheat has long been the subject of crossing experiments with crossing partners both within the genus Triticum and from other species. Hexaploid wheat types and species can be crossed with one another to produce fertile progeny. In contrast, the fertility of the F1 from crossings between hexaploid and tetraploid species is often extremely limited, and progeny resulting from crossings between hexaploid and diploid species are usually sterile. One exception is the cross between *T. aestivum* and *T. turgidum* (tetraploid), which produces fertile F1.

Of the possible crossing partners for intergeneric hybrids of *T. aestivum* mentioned in the consensus document of the OECD, species of *Agropyron, Elymus, Hordeum*, *Leymus*, *Setaria* and *Sorghum* as well as *Secale cereale* (rye) and triticale, and also *Aegilops* are found in Germany. Crossings of the mentioned wheat species and types with other species are often only achieved by applying special techniques (pollination by hand, male sterile lines, embryo rescue methods) and produce mostly sterile offspring. The risk of spontaneous hybridisation occurring under field conditions is considered to be extremely low. Apart from the genetic incompatibility of the crossing partners, other requirements, such as the synchronous flowering of both partners, must be met before successful hybridisation can be achieved under natural conditions. Agrotriticum, an intergeneric hybrid from *Triticum aestivum* and *Agropyron* sp., which is said to be back-crossable with both parent species, is not cultivated in Germany. The appearance of spontaneous hybrids from rye and wheat (triticale) has only been reported in older publications. This probably has to do with the open flowering mode of the cultivars used at that time. In the cases described, rye was the pollen donor and wheat the pollen acceptor. The spontaneous appearance of triticale in neighbouring rye fields is therefore not expected. Naturally occurring hybrids between wheat cultivars and barley or oat cultivars are not known.

In conjunction with the provisions of the decision on this notification, the measures proposed in the application documentation ensure that a minimum separation distance of 50 m to other areas on which soft or hard wheat, rye, or triticale is sown will be observed. The described measures will adequately minimize the risk of out-crossing to neighbouring crop cultivations.

Should these genetic modifications spread into a species of the aforementioned plant genera intended for consumption (e.g. rye), despite the proposed measures and under consideration of the biological properties of wheat, the evaluation carried out under III.1.2.1. (a)-(d) of the characteristics transferred indicates that no harmful effects on human health or the environment would be expected to result. Any isolated hybridisation events that might occur between the genetically modified plants and wild plants in spite of the described measures would most likely not lead to the spread of the transferred genes to wild plant populations, since this would require the subsequent backcrossing of the hybrid with the wild plant species.

III.1.2.4. Assessment of the possibility of transfer of the inserted foreign genes from the genetically modified plants to micro-organisms

The inserted sequences are stably integrated into the chromosomes of the recipient organisms. There is no evidence that the transfer of genetic information from plants or its expression in micro-organisms takes place under natural conditions. However, studies on the transformation ability of soil bacteria under natural conditions suggest that the transfer of plant genetic material to soil bacteria is theoretically possible, although it is assumed that a gene transfer of this type would constitute an extremely rare event.

Insofar as we assume that an exchange of genetic material between organisms which are so distantly related in terms of taxonomy as plants and micro-organisms is actually possible, it could be concluded that the occurrence of an exchange of heterologous genetic material does not in itself represent a safety criterion, since such an exchange could always result in the uptake of all forms of heterologous genetic material, including all forms of plant DNA.

(a) The *kp4* gene

The gene transferred into the genetically modified plants originates from a double-stranded RNA virus that exists in symbiosis with the host fungus (corn smut) and is commonly found in maize cultivations. Therefore, the possibility exists that this gene may also be spread by horizontal gene transfer from non-genetically modified organisms.

(b) The *bar* gene

In soil micro-organisms the inactivation of phosphinothricin by acetylation is a naturally occurring process. Bacteria with a corresponding resistance are commonly found in the envi-

ronment. The *bar* gene, therefore, can also be spread by horizontal gene transfer from non-genetically modified micro-organisms. Even if the *bar* gene were to be transferred from the genetically modified plants to micro-organisms, the overall frequency of this resistance in the environment would not be significantly increased.

(c) Additional DNA fragments located on the transformation plasmids

The genetically modified wheat plants may contain the following genetic elements which are located on the transformation plasmids:

- The ß-lactamase gene for resistance to the antibiotic ampicillin,
- the gene for the α-fragment of β-galactosidase from *E. coli*,
- the origin of replication ColE1 for replication in *E. coli*,
- additional fragments of the pUC19 plasmid.

The ß-lactamase gene is commonly found in micro-organisms. About 35% of all clinical *E. coli* isolates from humans are resistant to ampicillin, 90% of which can be attributed to a ß-lactamase-mediated mechanism of action. Similarly, 74% of all cattle- and pig-derived *E. coli* isolates display resistance to ampicillin. Recent studies on antibiotic resistance in micro-organisms in the environment show that a high percentage of soil bacteria are resistant to a broad range of ß-lactam antibiotics due, among other things, to the polymorphism of *bla* genes in these microorganisms. Furthermore, recent field studies showed that, for example, a 10-year monoculture of genetically modified maize containing the *bla* gene had no impact on the distribution of antibiotic resistance in naturally occurring soil bacteria when compared to conventional cultivation.

In its statement of 6.7.1999 on the biological safety of antibiotic-resistance genes in the genome of genetically modified plants, the Central Commission for Biological Safety (*ZKBS*) assigned the ß-lactamase gene to the Group II antibiotic-resistance genes. These genes are (a) widespread in microorganisms and (b) their relevant antibiotics are currently only applied in specific areas of human and veterinary medicine, so that it can be assumed that the presence of these antibiotic-resistance genes in the genome of genetically modified plants would have only minimal, if any, impact on the distribution of these antibiotic-resistance genes in the environment. This appraisal was confirmed in 2007.

In the opinion of the Scientific Panel on Genetically Modified Organisms of the European Food Safety Authority (EFSA) on the use of antibiotic resistance genes as marker genes in genetically modified plants, adopted on 2 April 2004, the ß-lactamase gene is classed among the group of genes only permitted for use in experimental release trials; these genes should not be present in genetically modified plants intended for the market. It should be borne in

mind that the proposed deliberate release experiment is to be conducted on a limited area and for a limited period of time.

The α-peptide of the *lacZ* gene for β–galactosidase contains the multiple cloning site pUC18/19. The native β–galactosidase enzyme splits β–D-galactosidase into galactose and the corresponding alcohol complex. On its own, the α-fragment is not active. Besides, the α-fragment in the genetically modified wheat would be interrupted by the insertion of the gene on the multiple cloning site, so that no functional gene product can be formed. The same would also apply to bacteria that receive the gene by horizontal gene transfer.

The pUC replicon is one of the ColE1-type plasmids with a host range that is restricted to a few gram-negative bacteria. Essentially, the replicon can be replicated in *E. coli* and in other closely related species of bacteria such as, for example, *Serratia* or *Salmonella*. In most gram-negative soil bacteria no replication occurs. ColE1 plasmids are quite common in enterobacteria. The likelihood of enterobacterium-mediated gene transfer to other bacteria is considered far greater than the likelihood of horizontal gene transfer from the genetically modified plants to bacteria. Therefore, the possible presence of the pUC origin of replication in the plant chromosome is not expected to contribute to an increase in the overall frequency of horizontal gene transfer.

(d) Regulation sequences

Also in the case that regulation sequences used in the construct are transferred, there is no reason to fear that the overall frequency of the respective DNA fragments will increase. These regulation sequences are derived from *Zea mays*, *Oryza sativa* and the cauliflower mosaic virus. CaMV is a plant-infesting, double-stranded DNA virus commonly found in plants. Maize and rice are common crops in agricultural regions around the world.

(e) Additional DNA sequences

As a result of the use of microprojectile bombardment technology for transformation additional fragments of the transformation plasmids may have been integrated into the genome of the genetically modified wheat plants. These fragments do not encode and have no regulatory function; a horizontal gene transfer of these fragments to micro-organisms would therefore not have any impact.