

QUESTIONS POSED BY THE IRISH ENVIRONMENTAL PROTECTION AGENCY TO BASF

In connection with the notification for the release into the environment of genetically modified (GM) potatoes with improved resistance to *Phytophthora infestans* (2006-2010); notification number: B/IE/06/01.

1. Please confirm that the GM potato lines that BASF propose to release are tetraploid?

All commercially cultivated potato varieties are tetraploid ($4n = 48$) (OECD, 1997). As the GM potato lines intended for the small-scale release are derived from commercial tetraploid potato varieties, these lines are tetraploid as well.

2. Page 7 of the notification, Point No 3 (b) it is stated that '*Under field conditions the berries do not mature as well as the seeds*'.
Is this not a contradictory statement considering that seed comes from berries? Please clarify.

Revised wording: 'Under field conditions the berries do not mature as well as the tubers. The berry-derived seeds, however can continue to mature when left in the soil.'

3. Under the same paragraph it is stated: '*Plants potentially arising out of those seeds are usually weak, with poor agronomic performance and low competitiveness*'.
Please supply information that supports this statement as in Ireland seed that originates from berries can survive and germinate and produce tubers under field conditions.

True potato seeds (TPS) can survive and germinate in the soil and the resultant potato plants might produce tubers. However, due to genetic segregation, the true seed derived plants differ from their parental plants. Their agronomic performance is poor and they show lower competitiveness. Further seed-derived plants show a lower early vigour as the nutritional resources in the seed are much lower than those in the tubers.

4. Page 11, it is stated '*There are no components of the vectors known to code for harmful substances*'.
Provide evidence to verify this statement.

The coding regions present on the T-DNA are those listed in the Table of genetic elements (see page 11), i.e. the *Rpi-blb2*, *Rpi-blb1* and the *ahas* genes. Based on database comparisons none of these genes are known to code for any harmful substances and none show similarity to known toxins or allergens.

5. Page 14, 2 (a) it is stated: *According to analysis with real-time PCR all genes of the T-DNA; the Rpi-blb1, Rpi-blb2 as well as ahas gene are confirmed to be present in all of the transgenic lines intended for the field trial.*

Provide relevant molecular data to verify this statement.

For all GM potato lines data confirming the presence of the *ahas* gene integrated into the potato genome are presented in *Tables 1* and *2* of *Annex 1*. As, during the integration process, the *ahas* gene is the last genetic element of the T-DNA to be inserted into the potato genome, these data serve as prove for the complete integration of the T-DNA. Additionally, all GM potato lines listed in *Tables 1* and *2* were analysed for presence of the *Rpi-blb2* and *Rpi-blb1* genetic elements and their presence was confirmed positive. The Table below illustrates those data based on a random selection of GM potato lines transformed with construct VCPMA16 and analysed for the presence of *Rpi-blb2* and *Rpi-blb1* genes.

	Line no	Insert analysis - Rpi-blb2				Insert analysis - Rpi-blb1			
		Ct blb2	Ct end ctrl	dCt	Result	Ct blb1	Ct end ctrl	dCt	Result
14	TS-PH05-009-0001	26,92	26,02	0,90	positive	26,95	26,02	0,92	positive
16	TS-PH05-009-0051	24,69	23,98	0,71	positive	24,49	23,92	0,56	positive
38	TS-PH05-010-0041	23,86	22,94	0,92	positive	23,96	23,27	0,70	positive
42	TS-PH05-010-0070	27,21	26,70	0,51	positive	27,41	26,99	0,42	positive
57	TS-PH05-010-0156	25,40	24,52	0,88	positive	25,82	25,28	0,54	positive
60	TS-PH05-010-0178	24,62	23,81	0,81	positive	24,70	24,18	0,52	positive
61	TS-PH05-010-0186	25,26	24,48	0,78	positive	26,06	25,56	0,51	positive
71	TS-PH05-010-0303	23,46	22,55	0,91	positive	25,23	24,44	0,78	positive
80	TS-PH05-010-0466	26,78	26,00	0,78	positive	27,19	26,68	0,51	positive
81	TS-PH05-010-0471	27,63	26,58	1,05	positive	27,85	26,80	1,05	positive
82	TS-PH05-010-0472	27,25	26,24	1,01	positive	26,95	26,42	0,52	positive
93	TS-PH05-011-0026	26,46	25,64	0,82	positive	26,67	26,03	0,64	positive
94	TS-PH05-011-0027	23,73	22,50	1,23	positive	24,38	23,58	0,80	positive
95	TS-PH05-011-0029	23,99	23,28	0,71	positive	23,11	22,44	0,67	positive
96	TS-PH05-011-0048	25,66	24,66	1,00	positive	26,76	26,11	0,65	positive
100	TS-PH05-011-0088	26,77	25,78	0,99	positive	27,46	26,99	0,46	positive
107	TS-PH05-011-0124	26,04	24,74	1,30	positive	26,75	25,79	0,96	positive
125	TS-PH05-015-0001	25,75	24,82	0,94	positive	27,28	26,65	0,63	positive
129	TS-PH05-015-0018	25,30	24,33	0,97	positive	25,04	24,58	0,46	positive
166	TS-PH05-017-0004	26,08	25,23	0,84	positive	26,96	26,41	0,55	positive
177	TS-PH05-018-0011	26,53	25,62	0,91	positive	26,59	25,86	0,74	positive
218	TS-PH05-020-0032	24,16	23,22	0,95	positive	24,87	24,44	0,43	positive
236	TS-PH05-025-0038	25,43	24,51	0,93	positive	25,14	24,63	0,51	positive
240	TS-PH05-025-0067	25,58	24,73	0,85	positive	23,92	23,29	0,63	positive
272	TS-PH05-034-0004	27,39	26,42	0,98	positive	27,50	26,84	0,66	positive
279	TS-PH05-036-0007	26,39	25,14	1,26	positive	26,31	25,52	0,80	positive
	neg ctrl, P835	40,00*	22,84	17,15	positive	40,00*	22,10	17,89	positive
	positive ctrl	26,47	25,85	0,62	positive	28,86	27,99	0,87	positive

* no signal detected after 40 cycles

- On page 14, it is stated that the lines intended for release contain 1 or 2 copies of the AHAS gene. This statement would appear to be inconsistent with the RTPCR data given in pages 37 to 45, where there are several delta Ct values approaching and greater than 2. Information on the PCR efficiencies is not provided and the copy number of the endogenous gene has not been given to allow for interpretation. Assuming the PCR reactions were

both highly efficient, a delta Ct of 2 would indicate 4 copies of an insert if the endogenous gene was present in single copy.

You are requested to explain this apparent inconsistency and provide support for your statement on the number of inserts.

Please note, that the insert copy numbers are not determined from the delta Ct values as this requires a known copy number for the endogenous control and identical Ct values for both reactions at a given copy number and identical efficiencies. This is not always the case. Instead, the copy numbers are calculated as compared to a calibrator sample with known copy number analysed in parallel with the sample to be determined, as this is a more robust method. In the following the method used to determine the copy number is explained in detail:

Transgene copy number determination, potato

Transgene copy numbers were determined by real-time PCR on the instrument ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The application of the fluorogenic 5' nuclease (TaqMan) assay for quantitative determination of transgene copy number was published by Ingham et al, 2001. For the transgene copy number determination in the potato plants primers and probes for the endogenous gene, used for normalisation, as well as for the *ahas* gene were designed using the Primer Express software (Applied Biosystems). PCR reactions were multiplexed, using the endogenous genomic gene for normalisation. Calibrator samples with known copy numbers are analysed in parallel. The software SDS 2.0 (Applied Biosystems) was used for the calculations of C_T values and calculations of transgene copy number were performed using the so-called comparative C_T method ("User Bulletin #2:ABI PRISM 7700 sequence Detection System", Applied Biosystems.). The method uses arithmetic formulas to achieve the result for relative quantification eliminating the need for a standard curve and the adverse effect of any dilution errors made in creating the standard curve samples.

With the comparative C_T method the amount of target, normalized to an endogenous control and relative to a calibrator is given by:

$$2^{-\Delta\Delta CT}$$

where

C_T = the fractional cycle number at which the amount of amplified target reaches a fixed threshold

ΔC_T = C_{T,X} – C_{T,R}, the difference in threshold cycles for target and reference

ΔΔC_T = ΔC_{T,q} – ΔC_{T,cb}, where q is any sample and cb is the calibrator

7. Page 15, 3 (a), it is stated: *'In the genetically modified lines a low level of expression of both Rpi-blb1 and Rpi-blb2 has been demonstrated by real-time PCR analysis in leaves, stems, tubers and roots.'*

**Do low levels of gene expression give tolerance to the blight fungus?
Please clarify.**

Yes, only low levels of gene expression of the inserted *Rpi-blb1* and *Rpi-blb2* genes are needed to confer resistance against *Phytophthora infestans*. The R-gene products exert no direct activity towards *Phytophthora infestans*. Instead, they are acting as “guards” that recognize pathogen-derived molecules and upon recognition initiate the resistance response including a localized cell death trapping the pathogen. For this recognition to take place only small amounts of R-gene products are required. NBS-LRR genes are usually expressed at low levels (Michelmore, 2000). Massively parallel signature sequencing (MPSS; Meyers et al., 2004) data of NBS-LRR proteins in Arabidopsis and rice obtained from Plant MPSS Databases (<http://mpss.udel.edu/>) further confirms low expression levels for many more such genes.

Further, the GM potato plants that express the inserted *Rpi-blb1* and *Rpi-blb2* genes at low levels are able to resist infection by *Phytophthora infestans* when challenged in the greenhouse.

8. Page 15, under Survivability it is stated: ‘*potatoes expressing the AHAS protein have been evaluated for frost tolerance in field, but no alterations compared to the parental varieties were observed*’.

Please provide information in support of this statement.

GM potato lines expressing the AHAS protein, though not harbouring the *Rpi-blb1* and *Rpi-blb2* genes, and non-GM comparator potato lines were exposed to conditions of frost in the field. During those so-called overwintering trials in the season 2004-2005 potato tubers were left in the ground from autumn to spring the following year at field trial locations in Sweden, the Netherlands and Germany. Whereas at locations in Sweden and Germany no sprouting potatoes (GM and non-GM comparator) could be detected in the spring, the estimated survival rate for the GM potatoes in the Netherlands (under conditions of a milder winter) ranged between 25 and 99 % and did not significantly differ from the survival rates of the non-GM comparator varieties.

9. Page 15, *Potato already contains many other R-genes of the NBS-LRR class. Some NBS-LRR genes are introgressed from Solanum demissum and confer resistance to some races of P. infestans.*

- **Do the 3 potato breeding lines that you transformed have any of these R- genes from *S. demissum* using traditional breeding methods?**

Crosses of *Solanum tuberosum* with *Solanum demissum* were first introduced in the early 20th century as a source for resistance against *P. infestans* and have been widely used in potato breeding programmes in Europe (Scotland, Germany) and the US. Thus, many potato cultivars contain *S. demissum* R-genes. Disappointingly, the resistance introgressed from *S. demissum* turned out to be rather short-lived and the broad breeding programmes based on *S. demissum* genes were discontinued (Wastie, 1991). However, many older potato varieties contain *S. demissum* genetic material and therefore also more recent potato varieties derived from crosses with these may still contain *S.*

demissum genes. The three breeding varieties that were used for the transformations may very well contain genetic material from *S. demissum*, though due to the low efficiency against late blight those R-genes are not always detectable in resistance screenings.

- **Please indicate whether R-genes from *S. demissum* or from *S. bulbocastanum* have been sequenced?**

The two R-genes, *Rpi-blb1* and *Rpi-blb2*, from *S. bulbocastanum* that are described in the notification have undergone DNA sequence analysis. From *S. demissum*, two genes conferring resistance against *P. infestans*, *R1* and *R3a*, have been cloned and sequenced (Ballvora et al., 2002; Huang et al., 2005).

- **Where sequencing has been carried out indicate the level of homology (DNA) between the different R-genes from the different potato species?**

The upper part of the *Rpi-blb1* gene has 91% sequence identity (at DNA level) to an EST from potato and 81-90% sequence identity to partial NBS fragments of tomato (van der Vossen et al., 2003). The *Rpi-blb2* gene is 90% identical to the *Mi-1* R-gene from tomato (van der Vossen et al., 2005).

DNA sequence homology (introns excluded) comparing four R-genes from *S. demissum* and *S. bulbocastanum* using AlignX function in Vector NTI 9.0 (InforMax, MD, USA) are indicated in the table below:

	R1 demissum	R3a demissum	Rpi-blb1 bulbocastanum	Rpi-blb2 bulbocastanum
R1 demissum	100%	31%	29%	48%
R3a demissum		100%	43%	40%
Rpi-blb1 bulbocastanum			100%	36%
Rpi-blb2 bulbocastanum				100%

10. Page 16, No 7, *Information on any toxic, allergenic or harmful effects on human health and the environment arising from the genetic modification.*
Please indicate if the following studies have been addressed/ examined/taken into consideration. Where the studies have not been undertaken please explain why.

- **comparison of homology (bioinformatic database search) between amino acid sequence of all the inserted genes (blight and herbicide tolerance genes) and amino acid sequence of known toxic/allergenic proteins?**

The AHAS, *Rpi-blb1* and *Rpi-blb2* protein sequences were compared *in silico* to all proteins in a downloaded Food Allergy Research and Resource Program (FARRP) Allergen Protein Database (version 6.00) via a BlastP (version 2.2.6) analysis. None of the submitted proteins showed 35% or greater identity over

80 amino acids to a known allergen. Additionally, no submitted protein shared a sequence of eight consecutive identical amino acids with a known allergen. The AHAS, Rpi-blb1 and Rpi-blb2 proteins were also analyzed for sequence homology to known toxins via a BlastP search of the downloaded June 2005 GenBank non-redundant peptide sequence database. This BlastP analysis did not identify any known toxins with significant homology to the submitted proteins.

- **possible epigenetic or pleiotropic effects pertaining to the introduced DNA?**

Potential epigenetic or pleiotropic effects in plant development generally relate to heritable changes in gene expression that are not associated with alterations in DNA sequence. These effects can be exerted at transcription level or posttranscriptionally. Many epigenetic changes depend on the recognition of sequence homology at the DNA or RNA level. This recognition can lead to transcriptional or posttranscriptional gene silencing. None of the genetic elements present on the T-DNA to be integrated into the potato genome show sufficient DNA sequence homology to respective endogenous potato genes in order to be able to trigger any epigenetic or pleiotropic effects. Therefore the likelihood of the occurrence of possible epigenetic or pleiotropic effects in the GM potato lines pertaining to the introduced DNA is remote.

- **have any of the proposed 340 GM potato lines been measured for the main toxic or anti-nutritional substances (glycoalkaloids and nitrates) found in potatoes?**

None of the GM potato lines intended for the release have been analysed for tuber composition, i.e. glycoalkaloids or nitrates. The genetically modified potatoes differ from conventional potato varieties in their resistance to *Phytophthora infestans* conferred by the introduced R-genes. Potato already contains a large number of resistance genes conferring resistance against other plant diseases where the majority of those genes belong to the NBS-LRR class. Included are also genes introgressed from wild potato species. None of the genes are known to exert any toxic or allergenic effects to human health. The mode of action is a hypersensitive response upon infection by the fungus leading to plant cell necrosis. The introduced genes are expressed by their endogenous promoters at very low levels that are comparable to those from other endogenous resistance genes.

The introduced selection marker gene is expressed as the enzyme AHAS, which is an enzyme found in all plant species and not known to confer any toxic or allergenic properties. The safety of different crop species with AHAS-mediated tolerance to imidazolinones has been assessed by the Canadian Food Inspection Agency.

The potato plants are intended to be released within the scope of a small-scale field trial, are not for human consumption and measures taken with regard to planting, harvest, storage and transportation will minimize any

contact to humans. Therefore the overall impact on human health is negligible.

Moreover we would like to point out that the release, if consent is granted, will be a small-scale experimental trial at one location to be conducted in two phases (A) screening of events (proof of concept; duration 1 to 2 years) and (B) development (stability of trait, safety studies; duration 2 to 3 years). During the course of the trial the following will be observed and recorded: Phase (A): agronomic performance (e.g. plant vigour, yield, susceptibility to climatic factors), altered agronomic properties (e.g. disease susceptibility), event screening (susceptibility to *Phytophthora infestans*), plant characteristics (e.g. emergence, leaf shape and colour, flowering, ground coverage, maturation), management of volunteers and Phase (B): stability of expression (e.g. sampling of plant tissue at various developmental stages in to order to conduct gene expression studies), potential effects on non-target organisms (e.g. soil flora, potato-related insects), altered qualitative properties (e.g. tuber composition, nutrients, anti-nutrients, feeding studies). Therefore the field trial will enable the generation of GM potato material grown under Irish field conditions for compositional analyses.

- **Have animal feeding trials been carried out on any of these GM potato lines?**

No animal feeding trials have been carried out on any of the GM potato lines intended for the release. However, the field trial will enable the generation of sufficient GM potato material grown under field conditions in Ireland that may be used for animal feeding studies within the frame of safety studies (see point above). During the course of the trial measures taken under current release practice will protect the trial against damage by wild animals (e.g. fences) and also ensure that seed stock and plant material are harvested, stored, transported or disposed of (e.g. cleaning of machinery, packaging) in such a way to minimise contact to animals. Measures will also be taken in order to prevent that GM potatoes enter the food or feed chain.

11. Page 17 No 7, it is stated: *The expression of the introduced R-genes (Rpi-blb1 and Rpi-blb2) in the genetically modified potatoes, under the control of the respective endogenous promoters is at very low levels and comparable to those from other resistance genes.*

What other resistance genes are you referring to? Please clarify this statement.

We are referring to R-genes in general and especially those of the NBS-LRR group, which is the most common group of R-genes. NBS-LRR genes are generally found to be constitutively expressed at low levels, based on the data available (Michelmore, 2000). As an example, Grant et al. (1995) have in detail studied one NBS-LRR gene, the *Rpmi* gene, and noticed a very low constitutive expression.

12. *Detection methodology:*

Please provide a protocol that fully describes the detection method (including DNA extraction) in tuber and leaf tissue matrices. It is essential that the method for the endogenous gene be included and that positive controls are also supplied.

As indicated in the notification (Section D.12) a qualitative detection method is provided in Annex I, section 3: *Detection of genetically modified potato lines (ahas-gene)*. The described method allows for detection and identification of the GM potato lines intended for the release and distinction from non-GM potato lines. Since the method is a qualitative detection method (standard PCR) it does not rely on an endogenous gene as reference. In case of inspections of the experimental release by the authority at time of planting or at any later stage, leaf or tuber tissue may be collected by the authority in order to verify that potato lines released carry the T-DNA described in the notification. If required, the notifier will provide positive control samples at that point in time. Please also find an alternative detection method outlined in the response to question 15.

13. The method that you have supplied targets the AHAS gene. This method could give a positive response for other GM events and conceivably for non-potato plant tissue, given that the AHAS enzyme 'is an enzyme found in all plant species'? **Please clarify.**

The use of the *ahas*-gene as selection marker in potato is developed in-house (patent application no WO 2004/005516 A1) and to our knowledge not used by any other company.

The method given has been tested carefully on non-GM potato lines showing no cross-reaction with endogenous *ahas* genes. To our knowledge no potato *ahas* gene has so far been cloned and sequenced, but at the protein level the identity between the *A. thaliana* AHAS protein and potato AHAS is around 80%. Generally, dicot AHAS proteins share a high degree of similarity (>70%) at the amino acid level. The method provided has not been tested on other plant species, since as described in the notification, the method serves the purpose to detect and identify GM-potato lines to be released in the environment for experimental purposes only and according to the specific handling conditions described in the notification.

14. Page 17, No 7, it is stated that: '*Due to the specificity of the response reaction no effects on other organisms than P. infestans are expected other than those that also apply to the interaction of non-genetically modified potatoes with non-target organisms under conventional agricultural practice*'. Also, on page 25, H (v) you conclude that: '*The overall impact on non-target organisms is considered negligible*'.

- **Please supply information on what is known about the potential effects of GM potatoes on beneficial soil microbes e.g., *Mycorrhizal fungi*?**

No changes in *Mycorrhizal* interactions are anticipated as the R-genes function only in living plant cells, and can only affect potato-penetrating

organisms. NBS-LRR gene products are localized in the cytoplasm of the cells. Further the triggering of the plant's defence response requires an organism to introduce elicitor proteins into the cytoplasm. This injection of elicitor proteins into the cytoplasm of potato cells can only be accomplished by potato pathogens, not by loosely associated microorganisms. In addition there is no evidence suggesting soil-dwelling or mycorrhizal fungi to transfer proteins to the cytoplasm of plants they are interacting with.

- **Outline the studies, if any, that have been carried out with these GM potato lines on soil biodiversity?**

If such studies have not been carried out heretofore and taking account of the precautionary principle, you are advised to strongly consider such studies be carried out under Part B notifications submitted in different Member States, in particular, to include one for this notification as it would be pertinent for Irish soil conditions, pending consent from the Agency.

As you are probably aware the information gleaned from such a study will be a requirement for a Part C product application under Directive 2001/18/EC that your company might consider placing on the market in the EU in the future.

As described in Section F.1. of the notification the purpose of the experimental release is to evaluate and to screen the genetically modified potato lines for improved resistance to *P. infestans*. Additionally, the release is aiming at, in the context of safety studies, collecting necessary data in comparison to unmodified recipient varieties and to conventional potato varieties. Data on agronomic properties and also on environmental interactions will be generated. Further within the frame of the monitoring plan any changes in susceptibility to insects and pests, effects of soil and climate will be observed according to conventional agricultural practice and recorded. The intended release therefore will provide the unique opportunity to conduct studies relating to soil biodiversity under Irish soil conditions and the notifier intends to conduct those studies as part of the safety studies that will be carried out during the course of the planned release period from 2006 to 2010.

15. Page 18, No 12, *Description of detection and identification techniques for the genetically modified plant.*

Mention is made of assays based on real-time PCR that have been developed for the *Rpi-blb1*, the *Rpi-blb2* as well as the *ahas* gene. However, only a qualitative method based on the *ahas* gene is given.

- **Please explain this omission?**

The conventional PCR method described can be used universally in any laboratory for detection of our transgenic material. The real-time PCR assays that we have used in our laboratory are developed specifically for and have only been tested on our ABI Prism 7900HT Sequence Detection

System. A description of the real-time PCR assays can only be handed over under confidentiality.

- **It is noted that the qualitative method given for the *ahas* gene as outlined in Annex 1 contains only an element within the construct as opposed to a construct specific method. The point at issue is, can the GM potatoes BASF propose to use be identified using this method, in particular, if other companies use a similiar *ahas* gene insert? Please clarify.**

A reason for targeting the *ahas* gene is that both *Rpi-blb1* and *Rpi-blb2* have to some extent been used in breeding programmes and may also be used by other parties in developing late blight resistant potatoes via applying plant biotechnology approaches. The *ahas*-gene as selection marker in potato is a development of the notifier (patent application no WO 2004/005516 A1) and to our knowledge not used by any other company. At the described field trial location in Arodstown only BASF Plant Science intends to release genetically modified potato lines. We are not aware of any other entity conducting field releases with similar constructs or traits in genetically modified potatoes close to the intended site of release or in Ireland at all. The conditions of the release as described in Section G 1. to 6. of the notification and measures taken with regard to control, monitoring and treatment of the release are outlined in order to assure that at all times GM potatoes are handled separately from non-GM potatoes. The qualitative detection method (as described below and in Annex 1 of the notification) will be able to serve as tool of control for the respective authorities to detect and identify the GM potato lines in the framework of the release experiment intended to be performed by BASF Plant Science between 2006 and 2010.

DNA Extraction:

DNA from leaf tissue was extracted using the Wizard Magnetic 96 DNA Plant System (Promega) according to protocol from manufacturer.
 DNA extraction from tubers is generally difficult due to the presence of starch. For extraction of DNA from tubers, the protocol above can be used if the first steps are held cool and a larger volume of lysis buffer is used.

Real-time PCR :

1) Conditions:

Real-time PCR ABI 7900 HT

	Stage 1	Stage 2	
Temperature	95°C	95°C	60°C
Time	5 min	15 s	1 min
		40 cycles	

2) Assays:

A) AHAS gene:

Forward primer: GATCCTCAGGTAAACCAGGTATCTGT

Reverse primer: ATCGGCTAATCCGCTAACGA

Probe: CCACTTCAGGTCCCGGA

B) Rpi-blb2 gene

Forward primer: TTCAAACCCCAAATAAGTTTCAAC

Reverse primer: CCATGCTTGCTGTACTTTGCA

Probe: CGTTACCCAGTCCTTCGGCG

C) Endogenous control (reference):

Eukaryotic 18S rRNA Endogenous Control (Applied Biosystems, Part Number 4310893E)

(Suitable for qualitative analysis, but not for copy number determinations.)

16. Page 18, No 13, *Genetically modified potato lines carrying the Rpi-blb2 gene were released in the field in Sweden in 2005 (consent B/SE/05/450). During these trials no unforeseen effects as compared to conventional potato varieties have been observed.*

Please provide the results and any additional information relating to this trial. What if any monitoring studies were carried out?

Three genetically modified potato lines carrying the Rpi-blb2 gene were released in the field in Sweden in 2005. Throughout the trial the performance of the GM lines in comparison to the non-GM parental potato variety and the variety Bintje with regard to resistance against *Phytophthora infestans* was followed. Growth and development of the GM lines was recorded as normal and comparable to the non-GM comparator lines and no significant differences were observed till the early flowering stage. Upon early flowering the plants became infected with *Phytophthora infestans*. While the non-GM comparator plants suffered substantial defoliation by the disease, the three GM potato lines tested showed the intended phenotype of late blight resistance. Apart from the late blight infection no other pests or diseases were recorded for the duration of the field trial. Monitoring for volunteers as part of the monitoring plan is ongoing.

17. Page 19. under E, No 3, it is stated: *No potatoes will be cultivated in close vicinity (< 20 m) to trial.*

It should be noted that at least 20 m (if not greater) would be required to limit gene dispersal, primarily, mechanical admixture.

Revised wording: 'No potatoes will be cultivated in close vicinity (at least or > 20 m) to the trial.'

18. Page 20, No 4, *In addition Phytophthora infestans inoculation might be carried out. Seed potatoes will be planted directly into the field.*

Provide written confirmation that regulatory approval is not required to release an indigenous fungal pathogen into an Irish soil from the relevant authority in Ireland (Department of Agriculture and Food).

Revised wording: 'Seed potatoes will be planted directly into the field.'

For the purpose of evaluating the GM potato lines for late blight resistance non-GM potato lines sensitive to *Phytophthora infestans* infection (variety Bintje) will be planted next to the GM potato lines in order to attract and spread the Irish indigenous strains present in the field to the GM lines. No

artificial inoculation is planned to be carried out, due to the presence of sufficient *Phytophthora infestans* spores in the Irish agriculture environment and the favourable climatic conditions. The notifier does not intend to import *Phytophthora infestans* strains from outside of Ireland.

19. Page 20, F 1, *additionally, the release is aiming at, in the context of safety studies, collecting necessary data in comparison to unmodified recipient varieties and to conventional potato varieties. Data on agronomic properties and also on environmental interactions will be generated.*

Specify the safety studies and studies on environmental interactions you propose to carry out, if consent is granted?

If consent is granted the following studies are proposed to be carried out within the timeframe of 5 years (2006 to 2010) for selected GM potato lines. We would like to point out that the release, if consent is granted, will be a small-scale experimental trial at one location to be conducted in two phases (A) screening of events (proof of concept; duration 1 to 2 years) and (B) development (stability of trait, safety studies; duration 2 to 3 years). During the course of the trial the following will be observed and recorded: Phase (A): agronomic performance (e.g. plant vigour, yield, susceptibility to climatic factors), altered agronomic properties (e.g. disease susceptibility), event screening (susceptibility to *Phytophthora infestans*), plant characteristics (e.g. emergence, leaf shape and colour, flowering, ground coverage, maturation), management of volunteers and Phase (B): stability of expression (e.g. sampling of plant tissue at various developmental stages in to order to conduct gene expression studies), potential effects on non-target organisms (e.g. soil flora, potato-related insects), altered qualitative properties (e.g. tuber composition, nutrients, anti-nutrients, feeding studies).

20. Page 21, G. 1 (a), *the year directly following the release the field plot will either remain fallow or will be cultivated with a species that facilitates weed management of the area.*

- **Is one year sufficient for post release monitoring considering tubers and seed can survive in the soil for a number of years?**
- **how do you propose to control any fruit that might occur at the proposed trial site?**

It should be noted that there would be little or no control of groundkeepers with frost under Irish soil conditions having regard to the fact that Irish winters have been very mild over the last number of years. In view of the above outline in detail an amended proposal for post release monitoring.

Section G 2. outlines: *The release site will be managed according to conventional agricultural practice. The first year following the release the volunteer monitoring programme starts and the field plot will either remain fallow or will be cultivated with a species that facilitates weed management of the area that year. For the duration of the volunteer monitoring programme no potatoes will be planted on the field plot, however other crops*

(except for the first year) can be planted as long as they allow volunteer monitoring. Emerging volunteers will be destroyed by herbicide treatment (systemic herbicide e.g. glyphosate) prior to flower setting. The monitoring for volunteers will continue till no volunteers emerge. The cultivation of the release site in the years after the monitoring programme has concluded will be according to local crop rotation practice for potatoes.

From the above it should be understood that in the case that volunteer plants appear the year after the trial has been carried out, those plants will be destroyed and the monitoring period will be prolonged by another year. In that way the monitoring will continue until no volunteers appear in the respective year.

21. Page 21, G. 2. (b), *any excess potato material (tubers after planting, after harvest) will be inactivated (e.g. via heat or via chopping).*

- **What type of heat will be used to inactivate the tubers?**
- **At what location will heat inactivation of the tubers be carried out?**
- **How will heat inactivated tubers be disposed of?**
- **Are you referring to the inactivation of both GM lines and non-GM controls that you propose to use in the experimental site as well?**

It should be noted that the chopping of potato material is not considered an appropriate method of inactivation as this could lead to the propagation of tubers. Please clarify.

Section G. 3. outlines: *Harvested tubers will be transported from the release site to certified contained facilities in and outside Ireland. Any leftover tubers identified on the release area after harvest, will be collected and inactivated e.g. via heat, via chopping. Above ground green parts will be inactivated prior to harvest either chemically or mechanically. Any remaining dead leaves and stems will be left at the release site for decomposition.*

Revised wording: ‘Harvested tubers will be transported from the release site to certified contained facilities outside of Ireland.’

All potatoes grown within the release trial will be handled as GM potato lines irrespective of being GM potato lines or comparator lines. Therefore no distinction with regard to handling and destruction is made between GM and non-GM potato lines. Any excess tubers (remainders from planting, after harvest) will be transported from the release site outside of Ireland and steamed or autoclaved at respective facilities approved for the purpose of handling GM material. The inactivated tubers will be placed for decomposition at approved contained facilities.

22. Page 21, G. 3, *Harvested tubers will be transported from the release site to certified contained facilities in and outside Ireland.*

- **Please indicate whether this refers to both GM and non-GM potatoes within the experimental site?**

- **What method(s) will be used to differentiate between the GM and the non-GM lines before planting during planting and at/post harvest?**

Please note that the storage of the GM lines in a contained facility in Ireland requires a consent from the EPA in accordance with Part III of the GMO (Contained Use) Regulations, S.I. No 73 of 2001.

Revised wording: ‘Harvested tubers will be transported from the release site to certified contained facilities outside of Ireland.’ All potatoes grown within the release trial will be handled as GM potato lines irrespective of being GM potato lines or comparator lines. Therefore no distinction with regard to handling and destruction is made between GM and non-GM potato lines. Any excess tubers (remainders from planting) and also the complete harvest will be transported from the release site, either processed for analytical purposes or steamed/autoclaved at respective contained facilities approved for the purpose of handling GM material outside of Ireland. The inactivated tubers will be placed for decomposition at approved contained facilities.

23. Page 21, *Description of post-release treatment methods for the genetically modified plant material including wastes.*

What Standard Operating Procedures do you propose to use for the transportation of the GM material on/off site. Provide details.

A compliance field notebook in use at the trial location will contain instructions, forms and guidance documents for each step of the process from packaging and labelling, to transport, receiving and handling, to planting, harvesting and shipping (including cleaning of equipment) as well as monitoring. All personnel handling GM material will receive compliance training prior to entering the release site. During transport on and off site the genetically modified potatoes are packaged in secure closed containment in order to prevent any spillage or unintentional release. The containment will be clearly labelled as containing GMO. For planting the potatoes are unpacked at the release site. Transport vehicles are inspected and cleaned on site. GM material intended for the release is always to be transported clearly separated from any conventional non-GM potatoes. The transportation is accompanied by shipping documentation including the consent for the release. Harvested potatoes are packaged on site in secure closed containment, labelled and shipped in transport vehicles outside of Ireland again accompanied by the appropriate shipping documentation.

24. Page 22, *under Emergencies Plan.*

Please note that the ‘ploughing in’ would not be an acceptable method of inactivation as this could lead to propagation of the tubers.

“Ploughing in” in this context applies to the green parts of the plants. As far as tubers are already build at this moment in time, they would be removed and shipped off for disposal or alternatively remain in the soil and be treated as volunteers the years after.

25. Page 22, No 6, *Methods and procedures to protect the site.*

Please provide a proposal to provide a more secure fence rather than that proposed in the notification. Electric fences will not adequately protect the proposed field trial from intrusion by animals especially small animals.

Revised wording: The release site may be fenced (non-electric fence) – if required – to protect against damage by animals.

26. Page 22, *Methods and procedures to protect the site.*

Please provide a method to protect the tubers (planting, during the growing season, at harvest) from possible bird intrusion (e.g., crows) to reduce the possibility of dissemination of the tubers to neighbouring fields by birds and small mammals.

At planting as well as at harvesting a number of employees will be present/working at the release site, which will make it unlikely that birds (e.g. crows) will enter the place at this time. After planting seed tubers will be covered with soil and after harvest tubers will be removed from the site in secure closed containment. It is not planned to apply special bird protection measures during the course of the growing season. Up to our knowledge there is no report and no observation from other release trials that potatoes have been disseminated by birds or small animals or constitute a preferred food thereof.

27. Page 26, H (vi), *None of the genes are known to exert any toxic or allergenic effects to human health.*

Also page 30, ERA, it is stated that *the potential effect on human or animal health due to introduced R-genes is negligible. NBS-LRR genes not known to confer toxic or allergenic properties and that the overall impact is negligible.*

Provide data to support these statements and explain how you concluded in your determination of the risk of the GMOs (in the ERA) that the overall impacts are negligible, if you cannot support these statements with relevant data/studies? See also question 10 above.

The Rpi-blb1 and Rpi-blb2 protein sequences were compared *in silico* to all proteins in a downloaded Food Allergy Research and Resource Program (FARRP) Allergen Protein Database (version 6.00) via a BlastP (version 2.2.6) analysis. None of the submitted proteins showed 35% or greater identity over 80 amino acids to a known allergen. Additionally, no submitted protein shared a sequence of eight consecutive identical amino acids with a known allergen. The Rpi-blb1 and Rpi-blb2 proteins were also analyzed for sequence homology to known toxins via a BlastP search of the downloaded June 2005 GenBank non-redundant peptide sequence database. This BlastP analysis did not identify any known toxins with significant homology to the submitted proteins.

As NBS-LRR proteins in general are not known to be toxic or allergenic and as none of our introduced R-proteins shows any homology to known allergens or toxins we concluded in the e.r.a. that the overall impact to human or animal health is negligible.

28. Page 26, H (vii), *Measures taken under current release practice will protect the trial against damage by wild animals (e.g. fences) and also ensure that seed stock and plant material are harvested, stored, transported or disposed of (e.g. cleaning of machinery, packaging) in such a way to minimise contact to animals. Therefore the overall impact on animal health is negligible.*

Describe precisely these measures.

The following measures will be taken: Transport to and from the site of release will be in secure close containment in order to prevent any unintentional release and thereby exposure to animals. Any equipment used for transport, planting and harvesting will be inspected and cleaned on site after use. Any remaining tuber material will be destructed via heat. The release site may be fenced if required. Transport of GM material will always be clearly separated from non-GM conventional potatoes in order to prevent any mixing. The material will be packaged and labelled appropriately in order to prevent it from entering the food or feed chain.

29. On page 27, *The overall impact on biogeochemical processes is negligible.*

Provide data to support this statement?

Effects on biogeochemical processes have not been measured previously with the genetically modified late blight resistant potato lines intended for the release. Therefore the small-scale field trial, if consent is granted, will provide the unique opportunity to verify the trait of increased late blight resistance conferred to the GM potatoes under Irish agricultural conditions and to perform studies that can reveal any potential effects on biogeochemical processes via measuring parameters such as e.g. soil fertility.

30. Page 27, *Therefore the overall impact on the environment is negligible and comparable to the effect of the cultivation of non-genetically modified potatoes with a potentially positive impact on soil microflora.*

Please indicate if these parameters have been measured previously and the results thereof.

Environmental impacts due to specific cultivation and management practices have not been measured previously, since it will be for the first time that the genetically modified potato lines can be tested under field conditions in Ireland. Therefore the small-scale experimental field trial, if consent is granted, will provide the unique opportunity to verify the trait of increased resistance to late blight under Irish agricultural conditions and to perform studies that explore potential environmental effects. Only under those conditions will it be possible to determine if specific cultivation and

management practices need to be applied and if so, what the potential effects might be on the soil and its flora.

31. In the ERA part of the notification you are requested to comment on the following potential effect:

Please comment on whether the introduction of your GM blight resistant potatoes might alter the pathogenicity of the potato blight fungus under Irish soil conditions thus facilitating the dissemination of infectious diseases and/or creating new reservoirs or vectors.

The mechanism of response conferred to the genetically modified potatoes by the R-genes is one of hypersensitivity and very specific to the interaction of the host and the pathogen. R-genes encode receptors that will recognize specific elicitors injected by the pathogen into the plant cell. This recognition will through a signalling network trigger both local and systemic defense responses. The local response aims at trapping the pathogen in the cells by localized cell death thus stopping further penetration and spreading.

Further the durability of the conferred resistance and therefore the lack of the pathogen to alter its pathogenicity is also supported by a large body of evidence. Schilde-Rentschler et al. (2002) produced somatic hybrids between *S. tuberosum* and *S. bulbocastanum*. The hybrid material containing the *blb1* gene has been tested in several countries (Germany, Spain, Bolivia, Columbia, Costa Rica and Ecuador). Furthermore, Helgeson et al. (1998) reported the production of somatic hybrids containing *blb1*. This material was tested at Hancock, Wisconsin and in Mexico for several years, whereas the progeny showed remarkable high levels of late blight resistance. Breeding clones containing *blb2* have shown high levels of resistance in experimental organically grown fields in the Netherlands from 1999 to 2002 (not published). The basic material from which those breeding clones were derived has been tested already in the eighties for resistance in Mexico (Hermsen, 1983) and proven highly resistant.

In addition to the evidence for the durability of the resistance, the genetically modified late-blight resistant potatoes are intended to be released into the environment within the frame of a small experimental field trial. Due to the scale (size, timing) of the trial as well as the presence of conventional potato varieties with a susceptibility to *P. infestans* the selection pressure exerted onto the oomycete will be negligible and the likelihood of a change in its pathogenicity is considered negligible.

32. **Please confirm if the potato donor of the R-genes (*S. bulbocastanum*) is a table potato consumed in some countries or is it a wild potato species not consumed by any humans or animals?**

S. bulbocastanum (Ornamental nightshade) is a wild potato species and based on our current knowledge generally not consumed by humans or animals.

References

- Ballvora A., Ercolano M.R., Weiss J., Meksem K., Bormann C.A., Oberhagemann P., Salamini F. and Bebhardt, C. (2002).** The *R1* gene for potato resistance to late blight (*Phytophthora infestans*) belongs to the leucine zipper/NBS/LRR class of plant resistance genes. - Plant J. 30:361-371.
- Grant M.R., Godiard L., Straube F., Ashfield T., Lewald J., Sattler A., Innes R.W. and Dangl J.L. (1995).** Structure of the Arabidopsis RPM1 gene enabling dual specificity disease resistance. – Science 269:843-846
- Helgeson, J.P., J.D. Pohlman, S. Austin, G.T. Haberlach, S.M. Wielgus, D. Ronis, L. Zambolim, P.Tooley, J.M. McGrath, R.V. James, and W.R. Stevenson (1998).** Somatic hybrids between *Solanum bulbocastanum* and potato: a new source of resistance to late blight. Theor. Appl. Genet. 96, 738-742.
- Hermsen, J.G. (1983).** Report of the XXVI Planning conference. Dec. 12-14, 1983. International Potato Center, Lima (CIP), Peru, pp 115-132.)
- Huang S., van der Vossen E.A.G., Kuang H., Vleeshouwers V.G.A.A., Zhang N., Borm T.J.A., van Eck H.J., Baker B., Jacobsen E. and Visser R.G.F. (2005).** Comparative genomics enabled the isolation of the *R3a* late blight resistance gene in potato. Plant J. 42:251-261.
- Ingham D.J., Beer S., Money S. and Hansen G. (2001).** Quantitative real-time PCR assay for determining transgene copy number in transformed plants. - BioTechniques 31:132-140
- Meyers B.C., Vu T.H., Tej S.S., Ghazal H., Matvienko M., Agrawal V., Ning J. and Haudenschild C.D. (2004).** Analysis of the transcriptional complexity of *Arabidopsis thaliana* by massively parallel signature sequencing. Nature Biotechnology 8:1006-1011.
- Michelmore R. (2000).** Genomics approaches to plant disease resistance. – Curr. Opin. Plant Biol. 3:125-131.
- Schilde-Rentschler et al. (2002).** EAPR Conference on Potato Research, Hamburg, Germany 2002, paper 102.
- Van der Vossen, E., Sikkema, A., te Lintel Hekkert, B., Gros, J., Stevens, P., Muskens, M., Wouters, D., Pereira, A., Stiekema, W. and Allefs, S. (2003).** An ancient R gene from the wild potato species *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans* in cultivated potato and tomato. – Plant J. 36:867-882
- van der Vossen E.A., Gros J., Sikkema A., Muskens M., Wouters D., Wolters P., Pereira A. and Allefs S. (2005).** The Rpi-blb2 gene from *Solanum bulbocastanum* is an Mi-1 gene homolog conferring broad-spectrum late blight resistance in potato.- Plant J. 44:208-222
- Wastie, R.L. (1991).** Breeding for resistance. - Advances in Plant Pathology 7: 193-224.