Department for Environment, Food and Rural Affairs

Application for consent to release a GMO – Higher plants

Part A1: Information require under Schedule 1 of the Genetically Modified Organisms (Deliberate Release) Regulations 2002

Part 1

General information

1. The name and address of the applicant and the name, qualifications and experience of the scientist and of every other person who will be responsible for planning and carrying out the release of the organisms and for the supervision, monitoring and safety of the release.

The Sainsbury Laboratory

Norwich Research Park

Norwich NR4 7UH

The responsible scientist has over 35 years experience in molecular biology, plant pathology and working with transgenic plants.

2. The title of the project.

Improving late blight (*Phytophthora infestans*) resistance in potato using resistance genes from wild potato relatives.

Part II

Information relating to the parental or recipient plant

3. The full name of the plant -

(a) family name, **Solanaceae**

(b) genus, ***Solanum***

(c) species, ***Solanum tuberosum* L**.

(d) subspecies, ***tuberosum***

(e) cultivar/breeding line, **Maris Piper**

(f) common name. **Potato**

4. Information concerning -

(a) the reproduction of the plant:

(i) the mode or modes of reproduction,

For agricultural purposes, vegetative reproduction via tubers is the primary mode of reproduction. Sexual reproduction resulting in seed production is also possible. Selfing is more likely than cross-pollination; estimates of the rates of cross-pollination under field conditions range from 0 to about 20% (Plaisted, 1980). Other studies have shown that the cross-pollination rates are 2% at a distance of 3 metres from the crop, reducing to 0.017% at a distance of 10 metres (McPartlan and Dale, 1994).

(ii) any specific factors affecting reproduction,

Tubers are frost-sensitive and are rendered non-viable if exposed to temperatures of -3 °C or lower. During the winter period, wet soils also reduce tuber viability.

(iii) generation time; and

Tuber to tuber or seed to tuber generation time is one year or growing season under European conditions.

(b) the sexual compatibility of the plant with other cultivated or wild plant species, including the distribution in Europe of the compatible species.

Solanum tuberosum subsp. *tuberosum* is sexually compatible with other cultivated genotypes of the same species. It is not sexually compatible with other UK crops or with either of the only two known wild *Solanum* species that grow in the UK, *Solanum dulcamara* (woody nightshade) and *Solanum nigrum* (black nightshade) (Eijlander and Stiekema, 1994; Raybould and Gray, 1993; McPartlan and Dale, 1994).

5. Information concerning the survivability of the plant:

(a) its ability to form structures for survival or dormancy,

Potatoes can survive as tubers or seed.

(b) any specific factors affecting survivability.

Potato tubers are sensitive to frosts and generally cannot survive temperatures of -3 °C and below. Generally, temperatures below zero impact on survivability with tubers being rendered non-viable after 2 hours of exposure to temperatures of -1.9 °C (Boydston et al, 2006). Tubers rarely survive winters in European soils due to the cool, wet conditions and the use of agricultural practices such as ploughing and the application of herbicides to clear land following potatoes. Potatoes are more often than not rotated and crops grown on land previously sown to potatoes often out-compete any surviving groundkeepers. Tubers that are not harvested (groundkeepers) and survive periods of ground frost may persist and produce plants in subsequent growing seasons. Careful management of the site will minimise such occurrences. Any volunteer plants that do form will be removed to prevent further survival through tuber production.

Although potato seed can survive winter temperatures, berries do not typically mature under UK field conditions and thus seed is rare. The heterozygous tetraploid genetic nature of cultivated potatoes means that seeds arising from sexual reproduction are often weak, have much lower agronomic performance than the parent plants and suffer competitively. Although potato seeds may survive in the soil for up to 8 years (Bock et al, 2002), plants arising from any seed that does germinate in the ground are unlikely to survive the winter conditions in the UK. In any case, any such volunteers that grow on the trial site will be identified and destroyed.

6. Information concerning the dissemination of the plant:

(a) the means and extent (such as an estimation of how viable pollen and/or seeds decline with distance where applicable) of dissemination; and

Potato can be spread as tubers, botanical seeds and pollen. Dissemination of tubers and botanical seed is normally limited to the area of cultivation. Dissemination of tubers and botanical seed is mainly caused by man while carrying out transports, handling and cultural practices. Animals, especially large birds, may also cause a limited amount of dissemination. Such dissemination of botanical seed, however, is practically excluded, as the seeds are contained in very poisonous berries.

Pollen is produced in low quantities and can be disseminated either by wind or insects. In the case of potatoes, dissemination of pollen is almost exclusively by insects, with the contribution of wind being very limited (Eastham and Sweet, 2002; White, 1983). Dissemination is usually restricted to less than 10 metres (Conner and Dale, 1996; McPartlan and Dale, 1994; Tynan et al, 1990) so the transgenic trial crop can be easily isolated reproductively from other potato crops. Selfing is the most frequently observed form of reproduction (Plaisted, 1980); cross-pollination rates have been shown to be just 2% at a distance of 3 metres from the crop, reducing to 0.017% at a distance of 10 metres (McPartlan and Dale, 1994).

(b) any specific factors affecting dissemination.

Tubers are dispersed by activities of man in crop husbandry and transport. Fruits are not often consumed by animals as they are highly poisonous and hence seed is not dispersed by this means. Pollen is produced by some cultivars. It can be dispersed by insects such as bumblebees although, as potato flowers lack nectar, pollen dissemination by honeybees is unlikely and they tend not to forage in potato crops (Sanford and Hanneman, 1981). Wind dissemination is considered to be marginal (Eastham and Sweet, 2002; White, 1983). Overall, pollen dissemination is minimal at distances of 5 to 10 metres from a potato crop (Bock et al, 2002).

7. The geographical distribution of the plant.

The potato originates from South America (the Andes). Potatoes are widely cultivated throughout the world and rank as the 4th most important food crop. In the UK potatoes are grown solely as agricultural produce, there are no ornamental or wild potato varieties.

8. Where the application relates to a plant species which is not normally grown in the United Kingdom, a description of the natural habitat of the plant,

including information on natural predators, parasites, competitors and symbionts.

Not applicable.

9. Any other potential interactions, relevant to the genetically modified organism, of the plant with organisms in the ecosystem where it is usually grown, or elsewhere, including information on toxic effects on humans, animals and other organisms.

Potatoes in the UK are hosts to a number of pests and disease-causing organisms, including slugs, insects, nematodes, viruses, bacteria, oomycetes and fungi. A number of beneficial organisms, such as bees, parasitoids and insects that feed upon aphids for example, also associate with potato crops.

Above ground parts of potato plants, including berries, contain significant levels of glycoalkaloids which are toxic to mammals and birds and nitrates which are anti-nutritional. Glycoalkaloid levels in tubers of cultivated potatoes are generally less than 100 mg/kg fresh weight which is below the maximum acceptable level of 200 mg/kg fresh weight established by OECD. The modifications made to the transgenic potatoes referred to in this application are not expected to affect any of these characteristics.

Part III

Information relating to the genetic modification

10. A description of the methods used for the genetic modification.

Transgenic potato plants were generated using *Agrobacterium tumefaciens* strain AGL1 (Hellens et al, 2000) or *Agrobacterium tumefaciens* strain GALLS. The GALLS strain was provided by Walt Ream at Oregon State University, Corvallis. It comprises a *virE2* mutant of *Agrobacterium tumefaciens* strain EHA105, complemented with the *GALLS* gene from *Agrobacterium rhizogenes* (Ream, 2009). For transformation, a standard protocol similar to that of Kumar et al (1996) was used. Stem internode sections of the potato cultivar Maris Piper were co-cultivated with AGL1 or GALLS and incubated in conditions that favour the development of callous tissue. Shoots that regenerated from callous tissue were excised and incubated in conditions that promote root development. Internode sections and shoots were exposed to the selectable agent chlorsulfuron during tissue culture to favour regeneration of lines carrying the T-DNA of interest. In addition, all transgenic plants were treated with the antibiotics timentin and cefotaxime to kill any remaining *Agrobacterium*.

11. The nature and source of the vector used.

Vector pAGM31195 was used to assemble plasmids SLJ24895, SLJ24896 (S/L) and SLJ24897. Vector pAGM32305 was used to assemble plasmids SLJ24904, SLJ24909 (S/L), SLJ24918 (S/L) and SLJ24933 (S/L).

These plant transformation vectors belong to the Level P class of Golden Gate vectors (Werner et al, 2012; Engler et al, 2014). Both pAGM31195 and pAGM32305 carry on its backbone the *nptII* gene (for bacterial selection only) and the *ipt* gene (for counter-selection of plants where the backbone has been integrated) (Richael et al, 2008). They also carry sequences that function as bacterial origins of replication. pAGM31195 carries the pMB1 replicon (same pMB1 variant as in the pUC19 vector) and the RiA4 replicon while pAGM32305 carries the pBR322 and the RiA4 replicons.

Located between the two border sequences of the T-DNA region of the Level P transformation vectors is the Golden Gate cassette sequence. This enables the insertion of the genes to be transferred to plant hosts by the Golden Gate cloning technique (Engler et al, 2008). This cloning cassette includes recognition sites for Type IIS restriction endonucleases and 4-nucleotide-overhang sequences to determine the polarity of the insert. The Golden Gate cassette includes the *LacZ* gene which is replaced by the genes of interest upon cloning. The orientation of the T-DNA boundary sequences in these vectors means that any new DNA integrated in the Golden Gate cassette site is transferred to the plant DNA.

Details of the vectors' constituent fragments that remain in the plasmids after cloning the genes of interest are listed in the following table. As mentioned above, the *LacZ* gene is removed during the cloning procedure. In addition, 'silent' nucleotide changes (i.e., changes that do not affect the protein sequence) have been introduced into the *ipt* gene to make it amenable to cloning.

Table of genetic elements in the vector backbone:

Abbreviation	Name & Function	Size (bp)	Origin
pAGM31195			
LB	Left border of T-DNA from a nopaline-type Ti plasmid.	151	*Agrobacterium tumefaciens*
RBO	Right border of T-DNA from a nopaline-type Ti plasmid (with full overdrive sequence).	155	*Agrobacterium tumefaciens*
p-*ipt*	Promoter region of *isopentenyl transferase (ipt)* gene.	526	*Agrobacterium tumefaciens*

ipt	Coding region of *isopentenyl transferase (ipt)* gene.	723	*Agrobacterium tumefaciens*
t-*ipt*	Terminator region of *isopentenyl transferase (ipt)* gene.	391	*Agrobacterium tumefaciens*
p-*bla*	Promoter region of the *beta-lactamase (bla)* gene. Drives the expression of the bacterial selectable marker gene (*nptII)*. Cloned from the pUC19 vector.	108	*Escherichia coli*
nptII	Coding region of the *neomycin phosphotransferase II* (*nptII*) gene.	795	*Escherichia coli*
pMB1 Replicon	Fragment of the pUC19 cloning vector containing a high-copy-number variant of the pMB1 replicon. Functions as origin of replication in *Escherichia coli*.	792	*Escherichia coli*
RiA4 Replicon	Plasmid pRiA4 replicator region. Functions as origin of replication in *Agrobacterium tumefaciens*.	4604	*Agrobacterium rhizogenes*
pAGM32305			
LB	Left border of T-DNA from a nopaline-type Ti plasmid.	151	*Agrobacterium tumefaciens*
RBO	Right border of T-DNA from a nopaline-type Ti plasmid (with full overdrive sequence).	155	*Agrobacterium tumefaciens*
p-*ipt*	Promoter region of *isopentenyl transferase (ipt)* gene.	526	*Agrobacterium tumefaciens*
ipt	Coding region of *isopentenyl transferase (ipt)* gene.	723	*Agrobacterium tumefaciens*
t-*ipt*	Terminator region of *isopentenyl transferase (ipt)* gene.	391	*Agrobacterium tumefaciens*
p-*bla*	Promoter region of the *beta-lactamase (bla)* gene. Drives the expression of the bacterial selectable marker gene (*nptII)*. Cloned from the pUC19 vector.	108	*Escherichia coli*

nptII	Coding region of the *neomycin phosphotransferase II* (*nptII*) gene.	795	*Escherichia coli*
pBR322 Replicon	Fragment of the pBR325 cloning vector containing the moderate-copy-number replicon pBR322. Functions as origin of replication in *Escherichia coli*.	1863	*Escherichia coli*
RiA4 Replicon	Plasmid pRiA4 replicator region. Functions as origin of replication in *Agrobacterium tumefaciens*.	4604	*Agrobacterium rhizogenes*

12. The size, intended function and name of the donor organism or organisms of each constituent fragment of the region intended for insertion.

The T-DNA of plasmids SLJ24895, SLJ24896 (S/L), SLJ24897, SLJ24904, SLJ24909 (S/L), SLJ24918 (S/L) and SLJ24933 (S/L) contain as plant selectable marker an allele of the tomato acetolactate synthase (*ALS/CSR*) that is resistant to inhibition by the herbicide chlorsulfuron (CS). This resistance is due to 2 mutations that have been described in *Nicotiana tabacum* (Lee et al, 1988). The *ALS/CSR* gene is required only for the *in vitro*-selection of transgenic lines. It is under the control of its endogenous regulatory sequences and is located next to the vector's left border.

Plasmids SLJ24897, SLJ24909 (S/L), SLJ24918 (S/L) and SLJ24933 (S/L) carry an extra T-DNA left-border sequence in between the *ALS/CSR* gene and the vector's left border. This sequence derives from an *Agrobacterium tumefaciens* octopine-type Ti plasmid. It was added with the aim of decreasing the chances of backbone integration due to left-border 'read-through' during T-DNA transfer.

Plasmids SLJ24895 and SLJ24896 (S/L) contain *Rpi-amr3i* and *Rpi-amr1e* respectively. Plasmid SLJ24896 has two versions, 'S' (for 'short') and 'L' (for 'long'). The only difference between them is the length of the *Rpi-amr1e* gene cloned. Gene-expression data suggest that the transcripts of this gene undergo alternative splicing and consequently, both a short and a long variant of the protein are generated. These variants only differ in 11 amino acids located at the C-terminus: 2 of those residues are polymorphic between the short and the long variants while the other 9 constitute an extension only present in the longer version of the Rpi-amr1e protein. Only the short variant will be produced from the gene cloned in SLJ24896 (S) but both the long and the short versions will be produced from the gene cloned in SLJ24896 (L). The '(S/L)' tag added to the name of a plasmid will hereinafter always refer to the short and long versions of the *Rpi-amr1e* gene included in the

corresponding plasmids. Both *Rpi-amr3i* and *Rpi-amr1e* were isolated from the wild potato relative *Solanum americanum* (Witek et al, 2016).

Plasmid SLJ24897 carries *Rpi-vnt1.1* which originates from the wild South American potato relative *Solanum venturii* (Foster et al, 2009). They are plant resistance (*R*) genes of the CC-NB-LRR class which confer resistance to some isolates of the late blight pathogen *Phytophthora infestans*. The expression of these genes is under the control of endogenous regulatory sequences.

Plasmid SLJ24904 encodes Oc-I∆D86, a variant of the rice cysteine proteinase inhibitor Oc-I (Urwin et al, 1995) and a repellent of synthetic origin (Winter et al, 2002). These proteins confer resistance to potato cyst-nematodes (Urwin et al, 2003; Lilley et al, 2004; Liu et al, 2005; Lilley et al, 2011; Green, 2012). Both *Oc-I∆D86* and the repellent gene are under the control of promoters that target gene expression to roots. The root-specific promoters used are from a serine-threonine kinase gene (*ARSK1*) and from the *MDK4-20* gene (Lilley et al, 2004; Lilley et al, 2011). They provide expression in roots and at root tips respectively. In addition, a signal sequence from the calreticulin gene of *Nicotiana plumbaginifolia* is linked to the repellent sequence to favour its release from roots (Liu et al, 2005; Green, 2012). Finally, regulatory sequences from the *ARSK1* and *MDK4-20* genes are also included as transcriptional terminators. These sequences (as well as any other terminator sequence included in this application) are **not related** to any technology that prevents seed propagation of plants.

Plasmid SLJ24909 (S/L) carries a stack of the three late blight resistance genes *Rpi-vnt1.1*, *Rpi-amr3i* and *Rpi-amr1e* (short or long version respectively).

Plasmid SLJ24933 (S/L) carries a stack of the three late blight resistance genes (*Rpi-vnt1.1*, *Rpi-amr3i* and *Rpi-amr1e* in its short or long version respectively) plus the two genes conferring resistance to potato cyst-nematodes (*Oc-I∆D86* and the gene coding for the repellent peptide).

Plasmid SLJ24918 (S/L) carries a stack of the three late blight resistance genes (*Rpi-vnt1.1*, *Rpi-amr3i* and *Rpi-amr1e* in its short or long version respectively), the two genes conferring resistance to potato cyst-nematodes (*Oc-I∆D86* and the gene coding for the repellent peptide) plus two gene-silencing modules. These gene-silencing modules are designed to silence the polyphenol oxidase gene *Ppo* (Rommens et al, 2006), the asparagine synthetase-1 gene *Ast1* (Chawla et al, 2012) and the vacuolar acid invertase gene *Vlnv* (Ye et al, 2010; Bhaskar et al, 2010) in a tuber-specific manner. Silencing of these genes in potato tubers aims to prevent browning upon bruising, accumulation of asparagine and cold-induced potato sweetening, lowering the potential for blackening and acrylamide formation upon cooking.

Both gene-silencing modules contain two tuber-specific promoters in convergent orientation: one from the ADP-glucose pyrophosphorylase (*Agp*) gene (Muller-Rober et al, 1994) and the other from the granule-bound starch synthase (*Gbss*) gene (Visser et al, 1991). The first gene-silencing module in the stack contains sense and antisense sequences from the *Vlnv* gene in between the convergent promoters. The second gene-silencing module contains sense and antisense sequences from the *Ppo* and the *Ast1* genes in between the convergent promoters. In this case, sense and antisense sequences are separated by a 'spacer' sequence. The structure of these modules is such that they direct the transcription of RNA molecules with complementary sequences. The consequent formation of double-stranded RNA triggers the post-transcriptional silencing of the above-mentioned genes via the endogenous silencing machinery of plants.

All the plasmids described above contain an 'end-linker' sequence in their T-DNAs. This element is part of the Golden Gate cloning toolbox (Engler et al 2014) and its sole function is to link the genes of interest to the vector backbone. Also, as indicated in the table below, some of the plasmids contain 'dummy-linkers' in their T-DNAs. These linkers are sometimes required to connect genetic elements when assembling the T-DNAs by the Golden Gate cloning technique (Engler et al 2014). Some of the 'dummy-linkers' used are part of the Golden Gate cloning toolbox (Engler et al 2014) and these are labelled as 'Dummy-Linker A' in the table below. On the other hand, linkers labelled as 'Dummy-Linker B' were specially design for this project, and contain stop codons in the six possible reading frames. None of the linker sequences used encode proteins.

Finally, 'silent' nucleotide changes (i.e., changes that do not affect the protein sequence) have been introduced into some of the genes mentioned above, to make them amenable to cloning.

Table of genetic elements in T-DNA:

Abbreviation	Name & Function	Size (bp)	Origin
SLJ24895			
T-DNA		9019	
p-*CSR*	Promoter of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1207	*Solanum lycopersicum*
CSR	Coding region of acetolactate synthase / chlorsulfuron	1980	*Solanum*

	resistance gene (*ALS/CSR*)		*lycopersicum*
t-*CSR*	Terminator of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	564	*Solanum lycopersicum*
Dummy-Linker A	Linker sequence required for Golden Gate cloning	23	Synthetic
p-*Rpi-amr3i*	Promoter region of *Rpi-amr3i* gene	1901	*Solanum americanum*
Rpi-amr3i	Coding region of *Rpi-amr3i* gene	2664	*Solanum americanum*
t-*Rpi-amr3i*	Terminator region of *Rpi-amr3i* gene	644	*Solanum americanum*
End-Linker	Linker sequence required for Golden Gate cloning	36	Synthetic

SLJ24896 (S)			
T-DNA		9498	
p-*CSR*	Promoter of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1207	*Solanum lycopersicum*
CSR	Coding region of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1980	*Solanum lycopersicum*
t-*CSR*	Terminator of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	564	*Solanum lycopersicum*
Dummy-Linker A	Linker sequence required for Golden Gate cloning	23	Synthetic
Dummy-Linker B	Linker sequence required for Golden Gate cloning	28	Synthetic
p-*Rpi-amr1e*	Promoter region of *Rpi-amr1e* gene	1632	*Solanum americanum*
Rpi-amr1e	Coding region of *Rpi-amr1e*	3698	*Solanum*

	gene		*americanum*
t-*Rpi-amr1e*	Terminator region of *Rpi-amr1e* gene	330	*Solanum americanum*
End-Linker	Linker sequence required for Golden Gate cloning	36	Synthetic

SLJ24896 (L)			
T-DNA		9498	
p-*CSR*	Promoter of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1207	*Solanum lycopersicum*
CSR	Coding region of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1980	*Solanum lycopersicum*
t-*CSR*	Terminator of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	564	*Solanum lycopersicum*
Dummy-Linker A	Linker sequence required for Golden Gate cloning	23	Synthetic
Dummy-Linker B	Linker sequence required for Golden Gate cloning	28	Synthetic
p-*Rpi-amr1e*	Promoter region of *Rpi-amr1e* gene	1632	*Solanum americanum*
Rpi-amr1e	Coding region of *Rpi-amr1e* gene	4810	*Solanum americanum*
t-*Rpi-amr1e*	Terminator region of *Rpi-amr1e* gene	907	*Solanum americanum*
End-Linker	Linker sequence required for Golden Gate cloning	36	Synthetic

SLJ24897			
T-DNA		7811	
Extra LB	Extra left border sequence derived from an octopine-type Ti	25	*Agrobacterium*

	plasmid		*tumefaciens*
p-*CSR*	Promoter of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1207	*Solanum lycopersicum*
CSR	Coding region of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1980	*Solanum lycopersicum*
t-*CSR*	Terminator of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	564	*Solanum lycopersicum*
p-*Rpi-vnt1.1*	Promoter region of *Rpi-vnt1.1* gene	709	*Solanum venturii*
Rpi-vnt1.1	Coding region of *Rpi-vnt1.1* gene	2676	*Solanum venturii*
t-*Rpi-vnt1.1*	Terminator region of *Rpi-vnt1.1* gene	614	*Solanum venturii*
End-Linker	Linker sequence required for Golden Gate cloning	36	Synthetic

SLJ24904			
T-DNA		6784	
p-*CSR*	Promoter of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1207	*Solanum lycopersicum*
CSR	Coding region of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1980	*Solanum lycopersicum*
t-*CSR*	Terminator of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	564	*Solanum lycopersicum*
Dummy-Linker A	Linker sequence required for Golden Gate cloning	23	Synthetic
Dummy-Linker B	Linker sequence required for Golden Gate cloning	28	Synthetic

p-*ARSK1*	Promoter of the serine-threonine kinase gene *ARSK1*	811	*Arabidopsis thaliana*
Oc-I∆D86	Coding sequence of an allele of the *Oc-I* gene	306	*Oryza sativa*
t-*ARSK1*	Terminator of the serine-threonine kinase gene *ARSK1*	334	*Arabidopsis thaliana*
p-*MDK4-20*	Promoter of the *MDK4-20* gene	924	*Arabidopsis thaliana*
Signal sequence	Signal sequence from the calreticulin gene	81	*Nicotiana plumbaginifolia*
Repellent	Sequence coding for a repellent peptide	30	Synthetic
t-*MDK4-20*	Terminator of the *MDK4-20* gene	460	*Arabidopsis thaliana*
End-Linker	Linker sequence required for Golden Gate cloning	36	Synthetic

SLJ24909 (S)			
T-DNA		18680	
Extra LB	Extra left border sequence derived from an octopine-type Ti plasmid	25	*Agrobacterium tumefaciens*
p-*CSR*	Promoter of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1207	*Solanum lycopersicum*
CSR	Coding region of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1980	*Solanum lycopersicum*
t-*CSR*	Terminator of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	564	*Solanum lycopersicum*
p-*Rpi-vnt1.1*	Promoter region of *Rpi-vnt1.1* gene	709	*Solanum venturii*
Rpi-vnt1.1	Coding region of *Rpi-vnt1.1*	2676	*Solanum venturii*

	gene		
t-*Rpi-vnt1.1*	Terminator region of *Rpi-vnt1.1* gene	614	*Solanum venturii*
p-*Rpi-amr3i*	Promoter region of *Rpi-amr3i* gene	1901	*Solanum americanum*
Rpi-amr3i	Coding region of *Rpi-amr3i* gene	2664	*Solanum americanum*
t-*Rpi-amr3i*	Terminator region of *Rpi-amr3i* gene	644	*Solanum americanum*
p-*Rpi-amr1e*	Promoter region of *Rpi-amr1e* gene	1632	*Solanum americanum*
Rpi-amr1e	Coding region of *Rpi-amr1e* gene	3698	*Solanum americanum*
t-*Rpi-amr1e*	Terminator region of *Rpi-amr1e* gene	330	*Solanum americanum*
End-Linker	Linker sequence required for Golden Gate cloning	36	Synthetic

SLJ24909 (L)			
T-DNA		18680	
Extra LB	Extra left border sequence derived from an octopine-type Ti plasmid	25	*Agrobacterium tumefaciens*
p-*CSR*	Promoter of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1207	*Solanum lycopersicum*
CSR	Coding region of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1980	*Solanum lycopersicum*
t-*CSR*	Terminator of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	564	*Solanum lycopersicum*
p-*Rpi-vnt1.1*	Promoter region of *Rpi-vnt1.1* gene	709	*Solanum venturii*

Rpi-vnt1.1	Coding region of *Rpi-vnt1.1* gene	2676	*Solanum venturii*
t-*Rpi-vnt1.1*	Terminator region of *Rpi-vnt1.1* gene	614	*Solanum venturii*
p-*Rpi-amr3i*	Promoter region of *Rpi-amr3i* gene	1901	*Solanum americanum*
Rpi-amr3i	Coding region of *Rpi-amr3i* gene	2664	*Solanum americanum*
t-*Rpi-amr3i*	Terminator region of *Rpi-amr3i* gene	644	*Solanum americanum*
p-*Rpi-amr1e*	Promoter region of *Rpi-amr1e* gene	1632	*Solanum americanum*
Rpi-amr1e	Coding region of *Rpi-amr1e* gene	4810	*Solanum americanum*
t-*Rpi-amr1e*	Terminator region of *Rpi-amr1e* gene	907	*Solanum americanum*
End-Linker	Linker sequence required for Golden Gate cloning	36	Synthetic

SLJ24933 (S)			
T-DNA		21626	
Extra LB	Extra left border sequence derived from an octopine-type Ti plasmid	25	*Agrobacterium tumefaciens*
p-*CSR*	Promoter of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1207	*Solanum lycopersicum*
CSR	Coding region of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1980	*Solanum lycopersicum*
t-*CSR*	Terminator of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	564	*Solanum lycopersicum*
p-*Rpi-vnt1.1*	Promoter region of *Rpi-vnt1.1*	709	*Solanum venturii*

	gene		
Rpi-vnt1.1	Coding region of *Rpi-vnt1.1* gene	2676	*Solanum venturii*
t-*Rpi-vnt1.1*	Terminator region of *Rpi-vnt1.1* gene	614	*Solanum venturii*
p-*Rpi-amr3i*	Promoter region of *Rpi-amr3i* gene	1901	*Solanum americanum*
Rpi-amr3i	Coding region of *Rpi-amr3i* gene	2664	*Solanum americanum*
t-*Rpi-amr3i*	Terminator region of *Rpi-amr3i* gene	644	*Solanum americanum*
p-*Rpi-amr1e*	Promoter region of *Rpi-amr1e* gene	1632	*Solanum americanum*
Rpi-amr1e	Coding region of *Rpi-amr1e* gene	3698	*Solanum americanum*
t-*Rpi-amr1e*	Terminator region of *Rpi-amr1e* gene	330	*Solanum americanum*
p-*ARSK1*	Promoter of the serine-threonine kinase gene *ARSK1*	811	*Arabidopsis thaliana*
Oc-I∆D86	Coding sequence of an allele of the *Oc-I* gene	306	*Oryza sativa*
t-*ARSK1*	Terminator of the serine-threonine kinase gene *ARSK1*	334	*Arabidopsis thaliana*
p-*MDK4-20*	Promoter of the *MDK4-20* gene	924	*Arabidopsis thaliana*
Signal sequence	Signal sequence from the calreticulin gene	81	*Nicotiana plumbaginifolia*
Repellent	Sequence coding for a repellent peptide	30	Synthetic
t-*MDK4-20*	Terminator of the *MDK4-20* gene	460	*Arabidopsis thaliana*
End-Linker	Linker sequence required for Golden Gate cloning	36	Synthetic

SLJ24933 (L)			
T-DNA		21626	
Extra LB	Extra left border sequence derived from an octopine-type Ti plasmid	25	*Agrobacterium tumefaciens*
p-*CSR*	Promoter of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1207	*Solanum lycopersicum*
CSR	Coding region of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1980	*Solanum lycopersicum*
t-*CSR*	Terminator of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	564	*Solanum lycopersicum*
p-*Rpi-vnt1.1*	Promoter region of *Rpi-vnt1.1* gene	709	*Solanum venturii*
Rpi-vnt1.1	Coding region of *Rpi-vnt1.1* gene	2676	*Solanum venturii*
t-*Rpi-vnt1.1*	Terminator region of *Rpi-vnt1.1* gene	614	*Solanum venturii*
p-*Rpi-amr3i*	Promoter region of *Rpi-amr3i* gene	1901	*Solanum americanum*
Rpi-amr3i	Coding region of *Rpi-amr3i* gene	2664	*Solanum americanum*
t-*Rpi-amr3i*	Terminator region of *Rpi-amr3i* gene	644	*Solanum americanum*
p-*Rpi-amr1e*	Promoter region of *Rpi-amr1e* gene	1632	*Solanum americanum*
Rpi-amr1e	Coding region of *Rpi-amr1e* gene	4810	*Solanum americanum*
t-*Rpi-amr1e*	Terminator region of *Rpi-amr1e* gene	907	*Solanum americanum*

p-*ARSK1*	Promoter of the serine-threonine kinase gene *ARSK1*	811	*Arabidopsis thaliana*
Oc-I∆D86	Coding sequence of an allele of the *Oc-I* gene	306	*Oryza sativa*
t-*ARSK1*	Terminator of the serine-threonine kinase gene *ARSK1*	334	*Arabidopsis thaliana*
p-*MDK4-20*	Promoter of the *MDK4-20* gene	924	*Arabidopsis thaliana*
Signal sequence	Signal sequence from the calreticulin gene	81	*Nicotiana plumbaginifolia*
Repellent	Sequence coding for a repellent peptide	30	Synthetic
t-*MDK4-20*	Terminator of the *MDK4-20* gene	460	*Arabidopsis thaliana*
End-Linker	Linker sequence required for Golden Gate cloning	36	Synthetic

SLJ24918 (S)			
T-DNA		30198	
Extra LB	Extra left border sequence derived from an octopine-type Ti plasmid	25	*Agrobacterium tumefaciens*
p-*CSR*	Promoter of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1207	*Solanum lycopersicum*
CSR	Coding region of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1980	*Solanum lycopersicum*
t-*CSR*	Terminator of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	564	*Solanum lycopersicum*
p-*Rpi-vnt1.1*	Promoter region of *Rpi-vnt1.1* gene	709	*Solanum venturii*
Rpi-vnt1.1	Coding region of *Rpi-vnt1.1*	2676	*Solanum venturii*

	gene		
t-*Rpi-vnt1.1*	Terminator region of *Rpi-vnt1.1* gene	614	*Solanum venturii*
p-*Rpi-amr3i*	Promoter region of *Rpi-amr3i* gene	1901	*Solanum americanum*
Rpi-amr3i	Coding region of *Rpi-amr3i* gene	2664	*Solanum americanum*
t-*Rpi-amr3i*	Terminator region of *Rpi-amr3i* gene	644	*Solanum americanum*
p-*Rpi-amr1e*	Promoter region of *Rpi-amr1e* gene	1632	*Solanum americanum*
Rpi-amr1e	Coding region of *Rpi-amr1e* gene	3698	*Solanum americanum*
t-*Rpi-amr1e*	Terminator region of *Rpi-amr1e* gene	330	*Solanum americanum*
p-*ARSK1*	Promoter of the serine-threonine kinase gene *ARSK1*	811	*Arabidopsis thaliana*
Oc-I∆D86	Coding sequence of an allele of the *Oc-I* gene	306	*Oryza sativa*
t-*ARSK1*	Terminator of the serine-threonine kinase gene *ARSK1*	334	*Arabidopsis thaliana*
p-*MDK4-20*	Promoter of the *MDK4-20* gene	924	*Arabidopsis thaliana*
Signal sequence	Signal sequence from the calreticulin gene	81	*Nicotiana plumbaginifolia*
Repellent	Sequence coding for a repellent peptide	30	Synthetic
t-*MDK4-20*	Terminator of the *MDK4-20* gene	460	*Arabidopsis thaliana*
p-*Agp*	Promoter of the ADP-glucose pyrophosphorylase gene (*Agp*)	2259	*Solanum tuberosum*
Sense-*Vlnv*	Fragment of the vacuolar acid invertase gene (*Vlnv*) in sense orientation	680	*Solanum tuberosum*

Antisense-*Vlnv*	Fragment of the vacuolar acid invertase gene (*Vlnv*) in antisense orientation	504	*Solanum tuberosum*
p-*Gbss*	Promoter of the granule-bound starch synthase gene (*Gbss*)	923	*Solanum tuberosum*
p-*Agp*	Promoter of the ADP-glucose pyrophosphorylase gene (*Agp*)	2259	*Solanum tuberosum*
Antisense-*Ast1*	Fragment of the asparagine synthetase-1 gene (*Ast1*) in antisense orientation	405	*Solanum tuberosum*
Antisense-*Ppo*	Fragment of the polyphenol oxidase gene (*Ppo*) gene in antisense orientation	144	*Solanum tuberosum*
Spacer	Spacer sequence that creates a loop in between the sense and antisense fragments of the hairpin structure	162	*Solanum tuberosum*
Sense-*Ppo*	Fragment of the polyphenol oxidase gene (*Ppo*) gene in sense orientation	144	*Solanum tuberosum*
Sense-*Ast1*	Fragment of the asparagine synthetase-1 gene (*Ast1*) in sense orientation	406	*Solanum tuberosum*
p-*Gbss*	Promoter of the granule-bound starch synthase gene (*Gbss*)	686	*Solanum tuberosum*
End-Linker	Linker sequence required for Golden Gate cloning	36	Synthetic

SLJ24918 (L)			
T-DNA		30198	
Extra LB	Extra left border sequence derived from an octopine-type Ti plasmid	25	*Agrobacterium tumefaciens*
p-*CSR*	Promoter of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1207	*Solanum lycopersicum*

CSR	Coding region of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	1980	*Solanum lycopersicum*
t-*CSR*	Terminator of acetolactate synthase / chlorsulfuron resistance gene (*ALS/CSR*)	564	*Solanum lycopersicum*
p-*Rpi-vnt1.1*	Promoter region of *Rpi-vnt1.1* gene	709	*Solanum venturii*
Rpi-vnt1.1	Coding region of *Rpi-vnt1.1* gene	2676	*Solanum venturii*
t-*Rpi-vnt1.1*	Terminator region of *Rpi-vnt1.1* gene	614	*Solanum venturii*
p-*Rpi-amr3i*	Promoter region of *Rpi-amr3i* gene	1901	*Solanum americanum*
Rpi-amr3i	Coding region of *Rpi-amr3i* gene	2664	*Solanum americanum*
t-*Rpi-amr3i*	Terminator region of *Rpi-amr3i* gene	644	*Solanum americanum*
p-*Rpi-amr1e*	Promoter region of *Rpi-amr1e* gene	1632	*Solanum americanum*
Rpi-amr1e	Coding region of *Rpi-amr1e* gene	4810	*Solanum americanum*
t-*Rpi-amr1e*	Terminator region of *Rpi-amr1e* gene	907	*Solanum americanum*
p-*ARSK1*	Promoter of the serine-threonine kinase gene *ARSK1*	811	*Arabidopsis thaliana*
Oc-I∆D86	Coding sequence of an allele of the *Oc-I* gene	306	*Oryza sativa*
t-*ARSK1*	Terminator of the serine-threonine kinase gene *ARSK1*	334	*Arabidopsis thaliana*
p-*MDK4-20*	Promoter of the *MDK4-20* gene	924	*Arabidopsis thaliana*
Signal sequence	Signal sequence from the calreticulin gene	81	*Nicotiana plumbaginifolia*

Repellent	Sequence coding for a repellent peptide	30	Synthetic
t-*MDK4-20*	Terminator of the *MDK4-20* gene	460	*Arabidopsis thaliana*
p-*Agp*	Promoter of the ADP-glucose pyrophosphorylase gene (*Agp*)	2259	*Solanum tuberosum*
Sense-*Vlnv*	Fragment of the vacuolar acid invertase gene (*Vlnv*) in sense orientation	680	*Solanum tuberosum*
Antisense-*Vlnv*	Fragment of the vacuolar acid invertase gene (*Vlnv*) in antisense orientation	504	*Solanum tuberosum*
p-*Gbss*	Promoter of the granule-bound starch synthase gene (*Gbss*)	923	*Solanum tuberosum*
p-*Agp*	Promoter of the ADP-glucose pyrophosphorylase gene (*Agp*)	2259	*Solanum tuberosum*
Antisense-*Ast1*	Fragment of the asparagine synthetase-1 gene (*Ast1*) in antisense orientation	405	*Solanum tuberosum*
Antisense-*Ppo*	Fragment of the polyphenol oxidase gene (*Ppo*) gene in antisense orientation	144	*Solanum tuberosum*
Spacer	Spacer sequence that creates a loop in between the sense and antisense fragments of the hairpin structure	162	*Solanum tuberosum*
Sense-*Ppo*	Fragment of the polyphenol oxidase gene (*Ppo*) gene in sense orientation	144	*Solanum tuberosum*
Sense-*Ast1*	Fragment of the asparagine synthetase-1 gene (*Ast1*) in sense orientation	406	*Solanum tuberosum*
p-*Gbss*	Promoter of the granule-bound starch synthase gene (*Gbss*)	686	*Solanum tuberosum*
End-Linker	Linker sequence required for Golden Gate cloning	36	Synthetic

Part IV

Information relating the genetically modified plant

13. A description of the trait or traits and characteristics of the genetically modified plant which have been introduced or modified.

Plants transformed with plasmids SLJ24895, SLJ24896 (S/L) and SLJ24897 contain introduced plant *R* genes (*Rpi-amr3i, Rpi-amr1e* and *Rpi-vnt1.1* respectively*)*, which were isolated from the wild potato relatives *Solanum americanum* and *Solanum venturii*. The introduced genes confer useful resistance against different isolates of the late blight pathogen. *R* genes enable plants to recognise certain isolates of the pathogen, which possess a specific corresponding avirulent effector gene. The recognition event triggers a signalling cascade culminating in expression of the plant defense response, which acts to prevent further pathogen growth within the host plant.

Plants transformed with plasmid SLJ24909 (S/L) contain a stack of the three plant *R* genes *Rpi-amr3i, Rpi-amr1e* and *Rpi-vnt1.1*. Deployment of *R*-gene stacks has the potential to confer a more efficient and durable resistance by combining different recognition specificities (Haverkort et al, 2016; Jo et al, 2016).

Plants transformed with plasmid SLJ24904 contain a stack of two genes conferring resistance to potato cyst nematodes (PCN). The expression of these genes is targeted to the plant root system, which is the organ invaded and affected by PCN. The first gene (*Oc-I∆D86*) encodes a variant of the rice cysteine proteinase inhibitor (‘cystatin’) Oc-I, from which one amino acid has been deleted (Urwin et al, 1995). The cystatin *Oc-I* gene is normally expressed in rice seeds. When *Oc-I∆D86* is expressed in potato roots it confers resistance against PCN thanks to its antifeedant activity (Lilley et al, 2004; Green et al, 2012). The second gene codes for a repellent peptide that is not derived from the gene of an organism (Winter et al, 2002). It has no known lethal effects as used. It merely prevents PCN from invading roots from soil (Liu et al, 2005; Lilley et al, 2011; Green et al, 2012). As consequence the nematodes deplete their lipid reserves and die. This is also the normal fate of PCN that fail to locate and invade wild type roots after hatching from dormant eggs. Deployment of a gene-stack conferring resistance by two different mechanisms has the potential to be a more efficient and durable strategy against PCN, compared to deployment of the individual genes (Fuller et al, 2008).

Plants transformed with plasmid SLJ24933 (S/L) contain the stack of three plant *R* genes (*Rpi-amr3i, Rpi-amr1e* and *Rpi-vnt1.1*) plus the stack of the two genes conferring resistance to PCN (*Oc-I∆D86* and repellent).

Plants transformed with plasmid SLJ24918 (S/L) contain the stack of three plant *R* genes (*Rpi-amr3i, Rpi-amr1e* and *Rpi-vnt1.1*), the stack of two genes conferring resistance to PCN (*Oc-I∆D86* and repellent) and two gene-silencing modules. These modules are designed to silence the polyphenol oxidase gene *Ppo* (Rommens et al, 2006), the asparagine synthetase-1 gene *Ast1* (Chawla et al, 2012) and the vacuolar acid invertase gene *Vlnv* (Ye et al, 2010; Bhaskar et al, 2010) in a tuber-specific manner.

The enzyme Ppo plays a major role in tuber discolouration after impact-induced bruising. Upon mechanical damage of the tuber, Ppo-mediated oxidation of polyphenols leads to the precipitation of black or brown pigment deposits. This phenomenon has a negative impact on tuber quality. One of the gene-silencing modules in plasmid SLJ24918 (S/L) contains sense and antisense sequences derived from the 3'UTR of the *Ppo* allele *POT32*. POT32 is the predominant Ppo variant in tubers (Thygessen et al, 1995) and its silencing significantly decreases enzymatic browning upon bruising (Rommens et al, 2006).

Cold storage of tubers triggers the accumulation of reducing sugars (i.e., glucose and fructose). This process, known as cold-induced potato sweetening, is responsible for the potato blackening upon cooking at temperatures above 120 ºC in low-moisture environments. Blackening is the result of the accumulation of dark (and bitter-tasting) compounds, which are products of the non-enzymatic Maillard reaction between reducing sugars and amino acids. The enzyme VINV hydrolyses sucrose to glucose and fructose in the vacuole and its activity correlates with potato sweetening during cold storage. Silencing of the *Vlnv* gene decreases potato blackening upon cooking and may be correlated with increased sucrose levels (Ye et al, 2010; Bhaskar et al, 2010). To silence this gene in tubers, a second gene-silencing module in plasmid SLJ24918 (S/L) contains sense and antisense sequences derived from the potato *Vlnv* gene flanked by convergent tuber-specific promoters.

The Maillard reaction also leads to the formation of acrylamide from reducing sugars and asparagine, which is the predominant free amino acid in potato tubers. Acrylamide is a neurotoxic compound and potential carcinogen (Friedman, 2003). The Food Standards Agency (FSA) has recently released a report on foods with high potential for acrylamide formation, advising on actions to reduce dietary intake of this compound (FSA, Chief Scientific Advisor Science Reports, 2015). Silencing of the *Vlnv* gene in tubers contributes to a reduction in the acrylamide-forming potential, since it decreases the availability of reducing sugars (Ye et al, 2010).

On the other hand, the enzyme Ast1 is the main responsible for asparagine formation in tubers. Therefore, silencing of the *Ast1* gene can further diminish the production of acrylamide by reducing the levels of asparagine in tubers (Chawla et al, 2012). This is coupled to an increase in the levels of glutamine, which doesn't affect the quality of potato as food. To silence *Ast1*, the *Ppo*-gene-silencing module

in plasmid SLJ24918 (S/L) also contains sense and antisense sequences from the potato *Ast1* gene, all flanked by convergent tuber-specific promoters.

In addition to the previous traits, all the transgenic plants proposed for release in this application will also contain the *CSR* gene which confers resistance to some herbicides (sulfonylureas and imidazolinones). This trait will be used **only** for the ***in vitro*** selection of transgenic lines during tissue culture and these plants remain sensitive to other herbicides.

Even though the plasmids described above are designed to modify diverse traits, the goal of the proposed trial is to evaluate resistance to circulating *P. infestans* isolates in field conditions. Plants transformed with plasmid SLJ24904 will be used as negative controls. Resistance to PCN and tuber quality traits in the above described transgenic plants will be evaluated independently by our collaborators.

14. The following information on the sequences actually inserted or deleted:

(a) the size and structure of the insert and methods used for its characterisation, including information on any parts of the vector introduced into the genetically modified plant or any carrier or foreign DNA remaining in the genetically modified plant,

The sizes of the T-DNA sequences to be inserted are approximately as follows:

SLJ24895: 9 kb

SLJ24896 (S/L): 9.5 kb (S) / 11.1 kb (L)

SLJ24897: 7.8 kb

SLJ24904: 6.8 kb

SLJ24909 (S/L): 18.7 kb (S) / 20.3 kb (L)

SLJ24933 (S/L): 21.6 kb (S) / 23.2 kb (L)

SLJ24918 (S/L): 30.2 kb (S) / 31.8 kb (L)

Plasmids SLJ24895, SLJ24896 (S/L) and SLJ24897 were assembled in vector pAGM31195. Plasmids SLJ24904, SLJ24909 (S/L), SLJ24933 (S/L) and SLJ24918 (S/L) were assembled in vector pAGM32305. Vector maps corresponding to pAGM31195 and pAGM32305 can be found in Annex 1 to this application (Section 1) and a detailed description of vector elements has been presented in Section 11 of this document.

In all cases, the selectable marker gene *CSR* is located next to the LB of the T-DNA, while the genes of interest are located next to the RBO. A schematic representation of T-DNA structures of plasmids SLJ24895, SLJ24896 (S/L), SLJ24897, SLJ24904, SLJ24909 (S/L), SLJ24933 (S/L) and SLJ24918 (S/L) is presented in Annex 1 to this application (Section 2) and a detailed description of T-DNA elements has been presented for each plasmid in Section 12 of this document.

The initial molecular characterization of plants carrying the above-mentioned plasmids is done by polymerase chain reactions (PCR). Genomic DNA samples are extracted from several independent lines of each genotype. The quality of the genomic DNA is assessed by PCR with primers designed to bind to the potato elongation factor 1 alpha (*EF1*α) gene. The presence of the transgenes is assessed by PCR with primers specific to the sequences of the selectable marker gene and genes of interest. Amplicons have been designed within the transgenes and spanning the junctions between the different genes in the T-DNAs.

Sequences outside the T-DNA are not expected to integrate, however this may still happen in some instances. For that reason, we have optimised the design of the transformation vectors to reduce the chances of unintended backbone integration and also to be able to counter-select plants in which this has happened.

Transference of the T-DNA to the plant normally starts in the RB region and finishes in the LB region. We have included a full overdrive sequence in the RB region of vectors pAGM31195 and pAGM32305 (RBO) to ensure an efficient transfer of T-DNAs in the correct orientation (Peralta et al, 1986; Shurvinton and Ream, 1991). In addition, plasmids SLJ24897, SLJ24909 (S/L), SLJ24933 (S/L) and SLJ24918 (S/L) carry an extra T-DNA LB sequence in between the selectable marker gene and the vector's LB. This sequence was added with the aim of decreasing the chances of backbone integration due to LB 'read-through' during T-DNA transfer. Finally, both pAGM31195 and pAGM32305 carry the *Agrobacterium tumefaciens isopentenyl transferase* (*ipt*) gene in the backbone region just outside the LB. This gene codes for an enzyme that drives the synthesis of the natural cytokinin isopentenyl adenosine. Production of this cytokinin during tissue culture induces a characteristic shooting phenotype that allows to easily counter-select plants where the backbone has been integrated (Richael et al, 2008).

In addition to the *ipt* gene, the backbone also contains the *neomycin phosphotransferase II (nptII)* gene for bacterial selection only. This gene is

expressed as an enzyme that inactivates the antibiotics neomycin, kanamycin, geneticin (G418), and paromomycin by phosphorylation. The expression of this gene is driven by a bacterial promoter and the protein encoded has been shown to be bio-safe, non-toxic and poses no risk to human or animal health (The EFSA Journal, 2009, 1034: 66-82).

The rest of genetic elements in the vectors' backbone are regulatory sequences that do not encode proteins. Border sequences (LB and RBO) derive from *Agrobacterium tumefaciens*. Replicons pMB1 and pBR322 derive from *Escherichia coli* while replicon RiA4 derives from *Agrobacterium rhizogenes*. The function of these replicons is to serve as origins of replication in bacterial cells. Finally, the promoter region of the *beta-lactamase* (*bla*) gene derives from *Escherichia coli* and drives the expression of *nptII* in bacterial cells. All these elements have been detailed in Section 11 of this document.

In summary, in the unlikely event of backbone integration, none of the elements in the vectors' backbone poses a significant risk. Both the *ipt* and the *nptII* genes are already widely present in the environment and the use of the *nptII* gene has been deemed safe by EFSA (The EFSA Journal, 2004, 48: 1-18; The EFSA Journal, 2009, 1034: 66-82). The rest of the elements are also present in the environment and do not code for proteins.

After analysing the presence of the T-DNAs, transgenic plants are also assessed for the presence of backbone sequences by PCR. Amplicons span the *ipt* coding sequence next to the LB region, the *nptII* coding sequence and the RiA4 replicon next to the RBO region.

Most of the transgenic plants described in this application are currently in the transformation pipeline. A few lines transformed with plasmid SLJ24895 (carrying *Rpi-amr3i)* have been already characterised at the genotypic and phenotypic levels. Details of the molecular characterization of these plants are presented in Annex 1 to this application (Section 3). Briefly, genomic DNA was extracted from three selected lines transformed with SLJ24895 and the quality of the genomic DNA was assessed by PCR, using control primers. The presence of the T-DNA was confirmed in all the lines tested, using primers encompassing the junction between the selectable marker gene and *Rpi-amr3i*. In addition, PCR experiments were done to test whether sequences outside of the T-DNA borders are present in those transgenic lines (Annex 1, Section 3). Primer pairs binding to different regions of the vector backbone were used, encompassing the *ipt* gene (outside the left border), the *nptII* gene, and the RiA4 replicon (outside the right border). The three selected lines were negative for the presence of any of the backbone regions tested.

The rest of the transgenic lines generated will be characterized in the same way as the plants carrying *Rpi-amr3i*, analysing the presence of the T-DNA insert and the integration of backbone sequences as described above. Further details on methods and primers used in PCR tests can be found in Annex 1 to this application (Section 4).

(b) the size and function of the deleted region or regions,
Not applicable.

(c) the copy number of the insert, and

Copy number (CN) of the insert will be determined by Droplet Digital PCR (ddPCR). Details of the protocol followed and the primers used can be found in Annex 1 to this application (Section 4).

As described below, expression of the *Rpi-amr3i* transgene was assessed by quantitative PCR (qPCR) in selected lines transformed with plasmid SLJ24895. Expression of the transgene was very low, with more than 30 qPCR cycles required to detect it. Therefore, we expect the CN of the insert to be low (i.e., no more than 3 copies).

Since all the plasmids carry the same *Agrobacterium* origin of replication (the low-copy-number RiA4 replicon), we expect the CNs in all the transgenic lines to be similar to those of SLJ24895 (if not lower). Lines carrying *Rpi-amr3i* were generated with the AGL1 *A. tumefaciens* strain, and the other strain used in this work (GALLS) is reported to mediate a lower number of insertions in comparison to AGL1 (Walt Ream, personal communication). In addition, a lower CN is expected for the larger T-DNAs included in this application.

(d) the location or locations of the insert or inserts in the plant cells (whether it is integrated in the chromosome, chloroplasts, mitochondria, or maintained in a non-integrated form) and the methods for its determination.

As plants were transformed using *A. tumefaciens,* all transformation events will result in a nuclear location for the transgenes. The lines already available have shown stability of the inserted sequence during propagation, which is associated with nuclear integration.

15. The following information on the expression of the insert -

(a) information on the developmental expression of the insert during the lifecycle of the plant and methods used for its characterisation,

The selectable marker gene *CSR* is an allele of the tomato acetolactate synthase (*ALS*) gene that has been cloned under the control of its native regulatory elements. Studies performed in the related solanaceous species *Nicotiana tabacum* revealed that *ALS* transcripts have higher levels in seedlings, younger tissues and developing organs compared to mature tissues and older organs (Keeler et al, 1993).

The expression of the late blight resistance genes *Rpi-vnt1.1*, *Rpi-amr3i* and *Rpi-amr1e* in the transgenic plants proposed for release is governed by their respective native promoters and terminators. Resistance genes of the same class (NB-LRR; nucleotide binding site-leucine rich repeat) have previously been shown to exhibit very low expression levels in vegetative parts of the plant (Tan et al, 2007).

The *Oc-I∆D86* cystatin gene and the repellent gene are under the control of the root-specific promoters from the *Arabidopsis thaliana* serine-threonine kinase (*ARSK1*) and *MDK4-20* genes, respectively (Lilley et al, 2004; Lilley et al, 2011).

The *ARSK1* promoter activity was studied in transgenic potato plants using a promoter/beta-glucoronidase (GUS) reporter construct. This showed that the *ARSK1* promoter is active in root vascular tissue and at the base of lateral roots. No expression was detected in aerial parts but some was observed within the vascular tissue of developing tubers (Lilley et al, 2004). Expression of *Oc-I∆D86* under the *ARSK1* promoter in transgenic potato lines showed that the level of cystatin in protein extracts from whole roots was barely detectable (Lilley et al, 2004).

The *MDK4-20* promoter was also tested in transgenic potato plants using a promoter/GUS reporter construct (Lilley et al, 2011). Expression was detected in the outer cell layers of the root tip and extended from the root cap towards the zone of elongation.

The gene-silencing modules in plants transformed with plasmid SLJ24918 (S/L) are controlled by two convergent potato promoters: a short (2.2-kb) version of the ADP-glucose pyrophosphorylase (*Agp*) promoter and the promoter of the granule-bound starch synthase (*Gbss*) gene. Both have at least a 100-fold higher activity in tubers than in leaves (Rommens et al, 2008; Visser et al, 1991; Muller-Rober et al, 1994).

A 3.2-kb version of the *Agp* promoter is active in many but not all starch-containing cells. Studies with a promoter/GUS reporter construct showed that expression from this promoter is restricted to cell types that are net importers of photoassimilates, while no activity was observed in the main exporters of

photoassimilates (Muller-Rober et al, 1994). A similar study carried out with the *Gbss* promoter shows high expression in stolons and tubers with low activity in other plant organs (Visser et al, 1991).

The characterisation of the transgenic plants proposed for release includes the assessment of expression of the genes of interest by quantitative PCR (qPCR). As an example of this analysis, details of the expression levels of *Rpi-amr3i* in some of the lines transformed with SLJ24895 are included in Annex 1 to this application (Section 3). Information on the protocol and primers used are also included in Section 4 of Annex 1.

Briefly, expression of the *Rpi-amr3i* transgene was assessed by using specific primers. In addition, primers binding to the elongation factor 1 alpha gene (*EF1*α) were used as control for the normalization of the qPCR data. The result was expressed as [Number of mRNA molecules of *Rpi-amr3i* per 1 Million mRNA molecules of *EF1*α control]. In addition to RNA from the *Rpi-amr3i* lines, an RNA sample from lines carrying the *Rpi-amr1e* transgene was included as negative control. As shown in Annex 1, Section 3, expression of the transgene was very low, with more than 30 qPCR cycles required to detect it. As expected, no *Rpi-amr3i* expression was detected in the lines carrying *Rpi-amr1e.*

The rest of the transgenic lines generated will be characterized in the same way as the plants carrying *Rpi-amr3i*, analysing the expression of the genes of interest by qPCR.

(b) the parts of the plant where the insert is expressed, such as roots, stem or pollen.

As mentioned above, the selectable marker gene used in all the plasmids included in this application is controlled by native tomato regulatory elements. Analysis of *ALS* transcripts in the related solanaceous species *Nicotiana tabacum* showed that expression occurs in all tissues tested (Keeler et al, 1993), with higher levels in seedlings and expanding leaves, intermediate levels in stems and flowers and low levels in mature leaf samples. Root tips show higher expression than older root tissues and *ALS* transcripts have also been detected in tobacco seeds.

Resistance genes are known to usually be expressed, albeit at very low levels, in all vegetative parts of plants (Tan et al, 2007).

As mentioned above, both the *ARSK1* and the *MDK4-20* promoters are active in potato roots. No expression from a *ARSK1* promoter/GUS reporter construct was detected in aerial parts of transgenic potato plants, but some

was observed within the vascular tissue of developing tubers (Lilley et al, 2004). In addition, no activity from the *MDK4-20* promoter was detected in transgenic potato samples corresponding to leaves, stem, petiole, floral organs, stolon or tubers (Lilley et al, 2011).

Previous studies with a 3.2-kb version of the *Agp* promoter showed it is active in tubers, starch sheath cells of stem and petioles, mature pollen grains, ovaries and guard cells but not in the mesophyll cells of leaves (Muller-Rober et al, 1994). The same report identified regions of the promoter required for the expression in petioles/stems, tubers and guard cells. Removal of the distal 1.2-kb fragment abolished expression in the starch sheath of the vascular tissue while the internal and proximal fragments (which are present in the 2.2-kb *Agp* promoter in SLJ24918 (S/L)) drive the expression in tubers and guard cells.

Finally, promoter/GUS reporter assays with the *Gbss* promoter show high expression in stolons and tubers with relatively low activity in leaves, stems or roots from greenhouse-grown plants (Visser et al, 1991).

16. Information on how the genetically modified plant differs from the parental or recipient plant in the following respects -

(a) mode or modes and/or the rate of reproduction,

The inserted sequences are not expected to alter either the mode or rate of reproduction of the genetically modified plants.

(b) dissemination,

The dissemination capacity of the genetically modified plants is not expected to differ from the parental lines.

(c) survivability.

The genetically modified plants transformed with plasmids SLJ24895, SLJ24896 (S/L), SLJ24897, SLJ24909 (S/L), SLJ24933 (S/L) and SLJ24918 (S/L) will have enhanced survivability in the field due to an increase in the range of *P. infestans* isolates to which they are resistant. This increased survivability will only be apparent in the event that the local *P. infestans* population is comprised of isolates against which the plants are resistant. Should the local population comprise of genotypes which are not recognised either by the introduced *R* genes, or by *R* genes already present in the genome of the potato plant, no increase in survivability will be apparent.

In a similar way, the genetically modified plants transformed with plasmids SLJ24904, SLJ24933 (S/L) and SLJ24918 (S/L) would have enhanced survivability in the field if cysts of the PCN *Globodera pallida* are present in the soil. No difference would be observed if cysts from the PCN *Globodera rostochiensis* are present, since the Maris Piper parental line is already resistant to that species. In any case, there's no evidence of PCN contamination in the soil of our experimental field and the absence of PCN will be tested before planting.

The gene-silencing modules in plants transformed with plasmid SLJ24918 (S/L) are designed to modify tuber quality traits that are important in post-harvest management and processing of the potato tubers. They are not expected to affect the survivability of the transgenic plants.

The transgenic plants proposed for release will also contain the *CSR* gene which confers resistance to some herbicides (sulfonylureas and imidazolinones). This trait will be used **only** for the ***in vitro*** selection of transgenic lines during tissue culture. The herbicide used for tissue culture selection is the sulfonylurea chlorsulfuron. These plants remain sensitive to other herbicides such as glyphosate or glufosinate, which could readily be used to eliminate them in the field.

Finally, in the unlikely event of backbone integration, no effect in survivability is expected from any of the elements in the vector backbone. The *nptII* gene is driven by a bacterial promoter and no antibiotic will be used in the field. The *ipt* gene will be used to counter-select plants where the backbone has been integrated and this will be performed *in vitro*, at the tissue culture stage. If the *ipt* gene is fully integrated and expressed, the enzyme IPT stimulates the production of natural cytokinins during tissue culture of transformed plants. This induces a characteristic shooting phenotype that allows the easy identification of such plants (Richael et al, 2008).

17. The genetic stability of the insert and phenotypic stability of the genetically modified plant.

Agrobacterium-mediated transformation generates stable insertions. No genetic or phenotypic instability has been observed in the lines that are already growing in the glasshouse. The plants that will be taken to the field if the permit is granted, will be either the original transformed lines grown in the glasshouse for around two months (after at least 3 rounds of *in vitro* propagation) or regenerated from tubers obtained from the original transformed lines. Any plant showing genetic or phenotypic instability when grown in the glasshouse will be excluded from the trial. Only lines for which no changes in phenotype are evident will be selected for field testing.

18. Any change to the ability of the genetically modified plant to transfer genetic material to other organisms.

The only mechanism by which potatoes could conceivably transfer genetic material to other organisms would be via uptake of potato DNA from dead plant material by soil living bacteria, by transfer of DNA to bacteria in the stomachs of animals that consume potatoes or by cross-pollination of compatible wild species. The transfer of genetic material from the potato plants to soil microorganisms, and their successful expression and long-term establishment is very improbable under field conditions (Schlüter et al, 1995). The transfer and subsequent establishment and expression of genetic material in bacteria or in cells of the gastrointestinal tract in man or animals after unintended consumption of plant parts derived from the potato plants to be released is very improbable under natural conditions (van den Eede, 2004). In any case, due to the toxicity of the above ground plant parts, animals do not feed on this material. The tubers produced by the transgenic plants released will not be used for animal feed and will be destroyed following harvest. There are no wild Solanaceous species in the UK with which the potatoes could outcross. The modifications made to the transgenic plants are not predicted to alter the ability to transfer genetic material by any of these routes.

19. Information on any toxic, allergenic or other harmful effects on human health arising from the genetic modification.

Potato plants transformed with plasmids SLJ24895, SLJ24896 (S/L), SLJ24897, SLJ24909 (S/L), SLJ24933 (S/L) and SLJ24918 (S/L) contain genes conferring increased resistance to potato late blight (*Rpi-vnt1.1*, *Rpi-amr3i* and/or *Rpi-amr1e*). The proteins encoded by these genes are not expected to exert any toxic, allergenic or other harmful effects on human health.

Rpi-vnt1.1, *Rpi-amr3i* and *Rpi-amr1e* are members of a class of resistance (*R*) genes (NB-LRR; nucleotide binding site-leucine rich repeat) that are already known to be abundant within potato and other plant genomes. This particular class of *R* genes contains the majority of plant *R* genes identified thus far and they all possess highly similar protein structures. Many of the European cultivated potato varieties already contain additional *R* genes of the NB-LRR class that were derived from the wild potato species *Solanum demissum* (Wastie, 1991). Thus far, no member of the NB-LRR class of *R* genes has been shown to confer toxic or allergenic properties. The abundance of this class of genes in plants, many of which are food crops, suggests that there is no particular hazard associated with their presence in the genome. Considering plants for which a complete genome sequence is available, *Arabidopsis thaliana* is known to possess approximately 200 *R* genes and *R* gene homologues (Meyers et al, 2003), while rice possesses around 500 (Zhou et al, 2004). Within the potato genome, a set of 438 NB-LRR-type genes has been

predicted (Jupe et al, 2012), and further analysis showed that the doubled monohaploid reference potato genome encodes ~ 750 NB-LRR proteins (Jupe et al, 2013). The expression of the *R* genes in the transgenic potatoes to which this application for release applies is under the control of native promoters. This correlates with very low levels of expression, comparable with what is known for other native resistance genes.

Potato plants transformed with plasmids SLJ24904, SLJ24933 (S/L) and SLJ24918 (S/L) contain two genes conferring resistance to potato cyst nematodes (PCN): *Oc-I∆D86*, which encodes a variant of the rice cystatin Oc-I from which one amino acid has been deleted (Urwin et al, 1995) and a gene coding for a repellent peptide (Winter et al, 2002). The expression of these genes is targeted to the plant root system.

Cystatins are present in many plants (Benchabane et al, 2010). They are, for example, expressed in rice seeds and maize kernels. The lack of toxicity of the cystatin Oc-I∆D86 to mammals has already been established (Atkinson et al, 2004). It is readily degraded by boiling and upon exposure to simulated gastric fluid. Similarly, it is not an allergen (Meredith and Atkinson, 2000). The repellent to be used is not lethal to nematodes (Winter et al, 2002) and it merely prevents plant parasitic species from invading roots. It is easily destroyed by heat and upon exposure to simulated intestinal fluid or nonsterile soil (Roderick et al, 2012). In addition, the peptide sequence is not flagged as a potential allergen (Roderick et al, 2012).

Potato plants transformed with plasmid SLJ24918 (S/L) also contain two gene-silencing modules. As described previously, these modules only include potato sequences and their structure is such that they do not code for proteins. Instead, they direct the transcription of RNA molecules with complementary sequences and their mode of action is based on using the endogenous post-transcriptional silencing machinery of plants to reduce the expression of the *Ppo*, *Ast1* and *VInv* genes in tubers. No toxic or allergenic potential is therefore expected and nucleic acids (such as the endogenous RNA and DNA molecules of plants) are readily degraded by human digestive fluids (Liu et al, 2015). It is also worth noting that transgenic potatoes developed with an equivalent technology have been approved for commercialization in the US.

All the plasmids used to generate the plants included in this application carry the selectable marker gene *CSR. CSR* is an allele of the tomato acetolactate synthase (*ALS*) gene that has been cloned under the control of its native regulatory elements. It codes for a variant of the ALS enzyme that is resistant to inhibition by some herbicides (sulfonylureas and imidazolinones). Resistance to ALS-inhibiting herbicides is present in several commercially-available crops, including wheat,

soybean, rice, canola and sunflower (Green and Owen, 2011; Hanson et al, 2014). In all of them, resistance is due to mutations in the *ALS* gene. This is also the case for the tomato *ALS* allele introduced in the plants proposed for release. Resistance to these herbicides has been typically achieved by traditional breeding methods but at least one transgenic event that includes a resistant *ALS* allele has been deregulated in the US (Green and Owen, 2011). Therefore, no harmful effects are predicted to arise from the use of this marker gene.

Furthermore, linker sequences used to assemble the plasmids included in this application do not code for proteins so no toxic or allergenic potential is predicted.

Finally, as mentioned above, several measures have been taken to avoid backbone integration in the transgenic plants to which this application refers. In the unlikely event of backbone sequences being inserted, the only two protein-coding genes present in the vectors' backbones are the marker gene *nptII* and the *ipt* gene.

The marker gene *nptII* (or *aph*(3')-IIa) is under the control of a bacterial promoter and is used for bacterial selection **only**. It is expressed as an enzyme (aminoglycoside 3-phosphotransferase II or neomycin phosphotransferase II) that inactivates the antibiotics neomycin, kanamycin, geneticin (G418), and paromomycin by phosphorylation. The protein encoded by the gene has been shown to be bio-safe, non-toxic and poses no risk to human or animal health. The following passage is taken from Appendix A of the Statement of EFSA on the "Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants" (The EFSA Journal, 2009, 1034: 66-82):

"The safety of the *aph*(3')-IIa gene and its protein product APH(3')-IIa has been verified by a number of studies. The exposure of humans and animals to the gene and protein via food and feed is very low due to the initially low levels in plants and further losses during processing. The protein is readily digested in the gastrointestinal tract. Bioinformatic analyses indicate no concerns as regards toxicity or allergenicity. Lack of toxicity has been verified by acute oral toxicity in mice. The *aph*(3')-IIa gene has been used in human gene therapy studies with no clinical signs of toxicity. Subchronic toxicity study on rats and nutritional studies on broilers and heifers with plant material containing APH(3')-IIa provide further assurance of safety."

In summary no toxicity of the NPTII protein has been observed and in simulated digestive fluids this protein is rapidly degraded. The characteristics of the transgenic protein NPTII involve no outstanding safety issues and derived products are no more likely to cause adverse effects on human and animal health than conventional potato (The EFSA Journal, 2006, 323: 1-20).

The *isopentenyl transferase (ipt*) gene derives from the soil bacteria *Agrobacterium tumefaciens.* This gene codes for an enzyme that catalyses the synthesis of the cytokinin isopentenyl adenosine, which naturally occurs in plants (Sakakibara et al, 2005). Plants have their own *isopentenyl transferase* genes for cytokinin production, some of which are expressed in edible parts of crops like maize kernels (Brugiere et al, 2008).

In this case, the presence of the *ipt* gene in the vector backbone of pAGM31195 and pAGM32305 allows the counter-selection of plants where the backbone has been integrated. The enzyme IPT encoded by the backbone *ipt* gene stimulates the production of natural cytokinins during tissue culture of transformed plants. This induces a characteristic shooting phenotype that allows the easy identification of such plants (Richael et al, 2008).

If the backbone *ipt* gene is not significantly expressed due to positional effects or has been only partially inserted, it is possible that plants where parts of the backbone have been integrated 'scape' the counter-selection step. Therefore, plants selected for release will be screened for the presence of backbone sequences in any case. However, in line with the above discussion on the *nptII* backbone gene, no harmful effects are expected in relation to this gene either. If the gene is normally expressed and the IPT enzyme produced, plants will display an abnormal development and will be discarded. In addition, the IPT enzyme sequence is not flagged as a potential allergen by Allergenonline (www.allergenonline.com). An '80mer Sliding Window Search' was carried out and it yielded no matches of significant identity. Such search is described as 'a precautionary search using a sliding window of 80 amino acid segments of each protein to find identities greater than 35% (according to CODEX Alimentarius guidelines, 2003)'.

Furthermore, integration coding sequences from *Agrobacterium* spp. into plant genomes is a phenomena that occurs in nature. For example, it has been recently described that the cultivated sweet potato's genome contains *Agrobacterium* T-DNA sequences with expressed genes (Kyndt et al, 2015).

In addition to the absence of known harmful properties of any of the genetic elements present in the modified potatoes, no harmful properties are expected to emerge when the above-mentioned genes and traits are combined. Finally, tubers will be destroyed at harvest and thus there will be no risk of the genetically modified material entering the food chain.

20. Information on the safety of the genetically modified plant to animal health, particularly regarding any toxic, allergenic or other harmful effects arising from the genetic modification, where the genetically modified plant is intended to be used in animal feeding stuffs.

The modified plants are not intended to be used as animal feed and tubers will be destroyed at harvest. Thus there will be no risk of the genetically modified material entering the food chain.

21. The mechanism of interaction between the genetically modified plant and target organisms, if applicable.

The target organism of the plants carrying *Rpi-vnt1.1*, *Rpi-amr3i* and/or *Rpi-amr1e* is *Phytophthora infestans*, the cause of potato late blight. The goal of the proposed trial is to assess the level of resistance of such plants to circulating strains of the late blight pathogen. It is expected that this interaction will be manifested by a reduction in the ability of *Phytophthora infestans* to infect the genetically modified potatoes. Resistance levels throughout the season are predicted to be higher in plants carrying the three-*R*-gene stack, if the *R* genes have different specificities and work cooperatively. This is in fact one of the variables that will be analysed during the proposed trial. The benefits of this approach have been extensively reported in the scientific literature, including examples of potato plants carrying *R*-gene stacks that have been field-trialled within the European Union (Haverkort et al, 2016; Jo et al, 2016).

R genes encode molecules with both recognition and signal transduction properties (Takken and Goverse, 2012). Current models indicate that the LRR (leucine-rich repeat) region recognises specific molecules secreted by the pathogen (effectors or avirulence factors) which are intended to help the pathogen cause disease on the host. Recognition by the LRR region results in a signal transduction event that culminates in the triggering of plant defence responses. These result in localised host plant cell death and prevent spread of the pathogen through host tissues (Jones and Dangl, 2006). The recognition and triggering of defence responses may also induce expression of defence-related genes in distant parts of the plant to the original infection site (Heil and Bostock, 2002).

Even though the assessment of resistance against potato cyst nematodes (PCN) is not a goal of the proposed trial, some of the plants included in this application carry two genes that target PCN. These genes have been deployed as stacks with the late blight resistance genes mentioned above, or they have been introduced into plants that will be used as negative controls. One of the genes codes for a cystatin and the other for a repellent peptide. Cystatins inhibit cysteine proteinases, which have an important role as digestive enzymes in many invertebrates but not in mammals. As a consequence, they limit growth and hence fecundity of the root feeding parasitic females of PCN, which are sedentary in roots (Urwin et al, 1995; Lilley et al, 2004). On the other hand, the repellent merely prevents the invading stage of PCN from entering roots (Winter et al, 2002; Lilley et al, 2011). These effects will be manifested

if cysts of the PCN *Globodera pallida* are present in the soil of the experimental field. However, there's no evidence of PCN contamination in the soil of our field and the absence of PCN will be tested before planting. Finally, the use of the repellent peptide in combination with a maize kernel cystatin to confer resistance against pathogenic nematodes in plantain (*Musa* spp.) has been previously reported and tested in the field (Roderick et al, 2012; Tripathi et al, 2015).

The gene-silencing modules incorporated in some of the plants proposed for release do not target organisms and are expected to improve tuber quality only. It is worth noting that the enzyme PPO has been linked to plant defence responses, however, silencing of *Ppo* in tubers does not enhance susceptibility to the late blight pathogen (Rommens et al, 2006). Also, commercial potatoes were the *Ppo* gene has been silenced by the same mechanism don't show increased disease susceptibility. This is probably due to an incomplete suppression of the browning process and to other plant defence mechanisms.

22. The potential changes in the interactions of the genetically modified plant with non-target organisms resulting from the genetic modification.

Resistance genes of the NB-LRR class initiate a resistance response upon recognition of the target organism (Jones and Dangl, 2006). For recognition to take place, a specific avirulence factor has to be injected into the plant by the pathogen. Based on current knowledge about the resistance genes that target *P. infestans* and on the information available on *P. infestans* avirulence factors (Vleeshouwers et al, 2011; Rodewald and Trognitz, 2013), it is not expected that any of the introduced late blight *R* genes (alone or in combination) will affect other non-target organisms that could be in contact with these plants during the trial.

The potential non-target effects of expressing the cystatin Oc-I∆D86 in potato to provide protection against cyst nematodes have been extensively studied as part of previous work (Cowgill et al, 2002a, 2002b, 2004; Cowgill and Atkinson, 2003; Celis et al, 2004; Kiezebrink and Atkinson, 2004; Green et al, 2012). These studies were performed with plants that expressed the cystatin from the constitutive 35S promoter or from the root-specific *ARSK1* promoter (which is the promoter used in the plants proposed for release). Potential non-target effects were assessed in relation to non-target insects and their predators and parasitoids, soil micro-organisms and micro-arthropods, earthworms and non-target nematodes. These works established considerable advantages to soil organisms relative to nematicide use and did not detect any issues of environmental concern.

The repellent peptide used in this work is not toxic to target nematodes but merely prevents root invasion, therefore reducing the damage associated with this process and subsequent development of the potato cysts (Winter et al, 2002; Lilley et al,

2011). The root-specific promoter driving the expression of the repellent-coding gene ensures only limited expression in root tips, and previous studies have shown that this strategy confers resistance against potato cyst nematodes without impact on non-target nematodes (Green et al, 2012).

Further, as mentioned above, the gene-silencing modules incorporated in some of the plants proposed for release do not target organisms and are expected to improve tuber quality only. In a similar way, the selectable marker gene is not expected to affect the interaction of the plants with target or non-target organisms.

Overall, no effects on non-target organisms are expected other than those that also apply to the interaction of non-genetically modified potatoes with non-target organisms under conventional agricultural practice. Due to a reduced use of anti-fungal chemicals in the experimental field, an increase in the populations of non-target organisms that are normally affected by anti-fungal treatments might be expected. No other changes in interactions are anticipated. Further, the trial will provide an opportunity to investigate any potential changes in the interactions with non-target organisms via observations on disease and pest susceptibility.

23. The potential interactions with the abiotic environment.

None of the introduced genes is predicted to have any effect on frost, drought or salt tolerance and therefore we do not expect the genetically modified potatoes to differ in any of these respects to other potato varieties or crops. With the exception of a reduced fungicide input, the field trial will be treated no differently to a standard potato crop. We do not expect the modified plants to respond any differently to any standard agricultural practices such as fertiliser application.

24. A description of detection and identification techniques for the genetically modified plant.

PCR primers for specific detection of the introduced T-DNAs are available and details are given in Annex 1 (Section 4).

25. Information about previous releases of the genetically modified plant, if applicable.

None of the plants included in this application have been previously released. However, similar potato lines carrying some of the genes and/or gene-silencing modules used in this work have been previously tested in the field in the UK or in the US by us or others. Further, some of those lines have been approved for commercialization in the US.

Désirée potato plants carrying *Rpi-vnt1.1* were tested in field trials in the UK and the results of those experiments are in the public domain (Jones et al, 2014). They showed that *Rpi-vnt1.1* could confer resistance to the races of the late blight pathogen that circulated in the UK at the time of the trial.

Désirée plants carrying the *Oc-I∆D86* cystatin gene or the repellent-coding gene under the control of the root-specific promoters *ARSK1* and *MDK4-20* respectively, have also been previously tested in field trials in the UK. Results of those trials have been reported in Lilley et al (2004), Kiezebrink and Atkinson (2004) and Green et al (2012) and showed that those genes conferred resistance against potato cyst nematodes without detrimental effects on non-target organisms.

Finally, potato plants carrying gene-silencing constructs targeting *Ppo, Ast1* or *VInv* have been described in Rommens et al (2006), Ye et al (2010) and Chawla et al (2012) and have been the subject of field trials in the US. Plants containing these gene-silencing constructs in different combinations have been approved for commercialization or are in the last steps of the deregulation process in that country. In particular, one of those lines carries modules to silence the three genes mentioned above in combination with the late blight resistance gene *Rpi-vnt1* included in this application.

Part V

Information relating to the site of release

(Applications for consent to release only)

26. The location and size of the release site or sites.

The plants will be released on an area of arable land no larger than 1000 metres squared located at the John Innes Centre (JIC, Ordnance Survey map grid reference TG 1707). Each year the area planted with the genetically modified plants will be approximately 100 metres squared. In accordance with potato planting practice, the plot will rotate within the release site each year of the trial.

27. A description of the release site ecosystem, including climate, flora and fauna.

The release site (Ordnance Survey map grid reference TG 1707) is arable land located at the John Innes Centre (JIC); some areas are bordered by deciduous hedges or trees. Flora in the immediate vicinity will be unknown until decisions on other local (non-GM) field trials are made each year but will likely be limited to cereals (wheat/barley) and peas. With the exception of a surrounding guard crop of

Désirée no potatoes will be grown within the accepted distance of 20 metres from the release site. The guard crop is in place to protect the release plants against edge effects such as wind and rain. As the Désirée tubers are red skinned (Maris Piper is white skinned) these guard potatoes will also serve a useful visual marker during harvesting of the trial. With respect to planting and waste management these potatoes will be treated as part of the trial and will be disposed of as described for the transgenic potatoes.

28. Details of any sexually compatible wild relatives or cultivated plant species present at the release sites.

There are no sexually compatible wild Solanaceous relatives present on the release site. If present at all nearby the trial site, other related Solanaceous wild species will be limited to the boundary hedge/field margins of the trial site and thus will be separated by a distance of more than 20 metres from the genetically modified crop.

29. The proximity of the release sites to officially recognised biotopes or protected areas which may be affected.

There are no officially recognised biotopes, protected areas or Sites of Special Scientific Interest (SSSIs) within 4 km of the release site. Given that potato pollen is not normally disseminated more than 10 metres from the parent plants, this distance equates to 400 times the normal dissemination distance. The closest SSSI to the release site is Sweet Briar Road Meadows which is ~4 km away and is a series of unimproved wet meadows with permanent water-logging and thus very unlikely to host any potato plants. Potato does not hybridise with any British native plants. This combined with the fact that potato is not a wind-pollinated plant and is not visited frequently by pollinators such as honeybees (due to lack of nectar production) mean that there is no risk to any officially recognised biotopes or protected areas listed by Natural England.

Part VI

Information relating to the release

30. The purpose of the release of the genetically modified plant, including its initial use and any intention to use it as or in a product in the future.

Since 2001, we have been working towards identifying, mapping and isolating resistance (*R*) genes from potato that confer resistance against potato late blight (*Phytophthora infestans*). This research has been publicly funded.

The genes identified are potentially valuable weapons in the fight against potato late blight as they confer resistance against many different isolates of this pathogen, including the strains which are currently responsible for major potato losses in the UK and Europe. Thus there is a need to test these genes in a 'real' environment.

Previously, the gene *Rpi-vnt1.1* was isolated from the wild potato relative *Solanum venturii* (Foster et al, 2009). This gene was transformed into the potato cultivar Désirée and successfully tested in the field (Jones et al, 2014; Consent 10/R29/01). Recently, two other genes were isolated from the wild potato relative *Solanum americanum: Rpi-amr3i* (Witek et al, 2016) and *Rpi-amr1e* (manuscript in preparation).

These three genes have now been transformed into Maris Piper potato, both as single genes or as a three-gene stack. Some of the plants proposed for release have the three-*R*-gene stack combined with genes conferring resistance against potato cyst nematodes (PCN) with or without gene-silencing modules conferring increased tuber quality. Plants proposed as negative controls in the field trial will only carry the PCN resistance trait.

The aims of the trial are:

1) to demonstrate that the transferred late blight resistance genes offer a valuable method for controlling late blight of potatoes which does not rely on agricultural inputs (pesticides);
2) to confirm that the transferred resistance genes still function in a 'real life' situation (i.e. in a field as opposed to a lab/greenhouse);
3) to evaluate the performance of the three-*R*-gene stack in comparison to the *R* genes deployed individually;
4) to expose plants containing the newly identified genes to the local populations of late blight to confirm that they are indeed useful;
5) if infection does result in disease, to isolate the corresponding pathogen race.

Even though some of the plants will also carry genes related to nematode resistance and improvement of tuber quality, none of these traits are within the scope of the proposed trial. Those characteristics will be evaluated independently by our collaborators in the project.

Recently, the British Biotechnology and Biological Sciences Research Council (BBSRC) put in place the Horticulture and Potato Initiative (HAPI). This program is part of a BBSRC's strategy to support innovative developments in bioscience. The goal of the HAPI is to address challenges faced by the horticulture and potato industries in the UK, and funding has been granted for collaborative works between research institutions and industrial partners. One of those BBSRC's research grants supports this work. This means that if the project yields good results, the industrial

collaborators within the partnership will support the steps towards commercialization, to make the benefits of the programme available to farmers, processors and consumers. Please see http://www.bbsrc.ac.uk/innovation/collaboration/collaborative-programmes/hapi/ for more details on the HAPI.

31. The foreseen date or dates and duration of the release.

If consent is granted, this year's field trial will start in late May 2017 and will continue until 30th November 2017. The trial will then proceed for 3 more years (2018-2020), from 1st May until 30th November in each year. The exact timing of sowing of the trial will depend upon weather conditions at the time. Harvesting of tubers will take place during September or October of each year of the field trials.

32. The method by which the genetically modified plants will be released.

Tubers or small glasshouse-grown plants will be planted in the field by hand following a randomised block trial design.

33. The method for preparing and managing the release site, prior to, during and after the release, including cultivation practices and harvesting methods.

The ground will be prepared by staff from The Operations Centre (John Innes Centre, JIC) who look after field trials on the Norwich Research Park (NRP) site according to normal agricultural practices for potato. Ground preparations will consist of existing grass being sprayed with herbicide to clear the ground. Manure will be applied if necessary and the ground will be prepared for planting using a power harrow. Harvest will occur late September/October depending on weather conditions at the time (if the plants senesce prior to this then harvesting will be brought forward). Plants will be lifted by haulms and harvesting of tubers will be by fork and hand to ensure removal of all GM material. The plot will be monitored for groundkeepers during the remainder of the year and will be shallow tilled to remove weeds and encourage germination of any shed true potato seed. Any groundkeepers identified will be removed by hand and destroyed by autoclaving within the Sainsbury Laboratory. The monitoring of the plot for groundkeepers will be continued for a period of 2 years following the 4-year experiment in accordance with DEFRA guidance. During this time the plot will be left fallow to enable easy identification and removal of groundkeepers.

34. The approximate number of genetically modified plants (or plants per square metre) to be released.

For each year of the field trial we estimate that the release will not exceed 250 transgenic plants. These will include different lines of the different genotypes described in this application.

Part VII

Information on control, monitoring, post-release and waste treatment plans

35. A description of any precautions to -

(a) maintain the genetically modified plant at a distance from sexually compatible plant species, both wild relatives and crops.

Although there are no sexually compatible wild relatives capable of hybridising with potato present in the UK, transgenic plants will be isolated from any other Solanaceous relatives, including other potato crops, by a distance of at least 20 metres. The release site will be routinely monitored for volunteers and any discovered will be destroyed. Post-harvest, the plot will be left fallow to allow identification of volunteers. For a two-year period following the trial the only crops grown on the release site will be those that allow easy identification and destruction of volunteers.

(b) any measures to minimise or prevent dispersal of any reproductive organ of the genetically modified plant (such as pollen, seeds, tuber).

Pollen will be allowed to be produced and to disperse but it's low viability and the distance of the transgenic plants from other potato crops (at least 20 metres) will ensure that the only recipients will be local potatoes within the trial. Prior to planting, plant and/or tubers will be transported to the release site in a vehicle not used for general transport purposes and the plants will not be mixed with either other plants or with equipment used for working on other agricultural land. Any equipment used for the planting (and harvesting) of transgenic material will be thoroughly cleaned after use. Harvesting of tubers will be by fork and hand which will minimise dispersal. The use of a guard crop with a different tuber skin colouration will help identify that all transgenic tubers have been harvested.

36. A description of the methods for post-release treatment of the site or sites.

Harvest will occur late September/October depending on weather conditions at the time (if the plants senesce prior to this then harvesting will be brought forward). Harvesting will be by fork and hand to ensure removal of all GM material. The plot

will be then left fallow, monitored for groundkeepers during the remainder of the year and sprayed with a systemic broadleaf herbicide. Any groundkeepers identified will be destroyed by herbicide treatment (e.g. glyphosate) or removed by hand and destroyed by autoclaving as described below. The monitoring of the plot for groundkeepers will be continued at monthly intervals by walking the trial site for a period of 2 years following the 4-year experiment in accordance with DEFRA guidance. During this time the plot will be left fallow to enable easy identification and removal of groundkeepers.

37. A description of the post-release treatment methods for the genetically modified plant material including wastes.

All harvested material (plant tops and tubers) will be placed in sealed bags or containers and removed from site to an authorised waste disposal facility. Disposal will be carried out by incineration through our contractor SRCL.

38. A description of monitoring plans and techniques.

The purpose of the monitoring plan is to enable early detection of any unintended effects related to the release of the transgenic potato plants.

The release site will be visited by trained laboratory personnel who are working on the project at no less than weekly intervals. Visits will usually occur more frequently. Any unexpected occurrences that could potentially result in adverse environmental effects or the possibility of adverse effects on human health will be notified to the national inspectorate immediately. Should the need arise to terminate the release at any point the emergency plans detailed below will be followed.

Post-trial the release site will remain fallow to enable easy identification of volunteers. The site will be inspected monthly between April and November (the growing season of potato) and any volunteers identified will be immediately destroyed either by application of a systemic broadleaf herbicide or by hand pulling plants and digging out tubers/root systems. These will then be autoclaved within the Sainsbury Laboratory. If volunteers are found at the end of the 2-year period, DEFRA recommendations will be followed for the management of the release site.

39. A description of any emergency plans.

At any time point post planting, should the release need to be terminated, any plant material will be sprayed with an appropriate systemic broadleaf herbicide and tubers dug up by fork and hand and transferred to an authorised waste facility for disposal by deep burying or incineration. Should the release site be subject to vandalism, care will be taken to ensure that all uprooted plant material within and outside of the trial site is identified and destroyed accordingly as described above.

40. Methods and procedures to protect the site.
Potatoes are not grazed on by animals due to the toxic nature of the above ground plant parts. The release site will be fenced to protect against animal damage and entry by unauthorised persons. The site will also be monitored by remote security cameras visible from the John Innes Centre (JIC) reception which is manned throughout the day by JIC reception staff and by security guards out of normal working hours.

Part VIII

Information on methodology

41. A description of the methods used or a reference to standardised or internationally recognised methods used to compile the information required by this Schedule, and the name of the body or bodies responsible for carrying out the studies.

Methods are detailed in appropriate references listed at the end of this application or are included in Annex 1 (Sections 3 and 4) where results are also detailed. Results included in Annex 1 where obtained at The Sainsbury Laboratory (Norwich, UK).

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