Improving late blight (*Phytophthora infestans*) resistance in potato using resistance genes from wild potato relatives and from the Sarpo Mira potato variety.

ANNEX 1

This annex contains details of the experiments that have been (or will be) done to characterise the genetically modified plants referred to in the associated release application by The Sainsbury Laboratory.

Included here are details of a number of independently generated transgenic lines carrying *Rpi-vnt1.1* or *Rpi-amr3*.

SECTION 1: RESULTS FROM CHARACTERISATION OF TRANSGENIC PLANTS

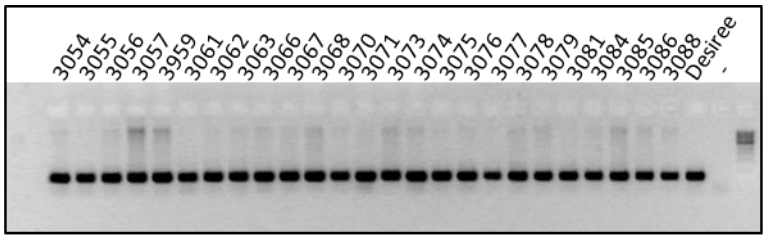
(A) Molecular characterization of plants carrying *Rpi-vnt1.1*

Included here are details of a number of independently generated *Rpi-vnt1.1* transgenic lines. However, only line PL3056 (*Rpi-vnt1.1*) is nominated for release.

Genomic DNA quality determination

All genomic DNAs analysed for the presence of insert and vector backbone sequences were tested for their quality. We used primers designed to amplify the potato plasma membrane ATPase (primers #3 and #4, Annex 1, Section 2; designed on HPA1 from *Solanum tuberosum*). The desired product was amplified from all genomic DNAs indicating that they are suitable templates of sufficient quality for use in characterisation of the transgenic plants by PCR (Figure 1).

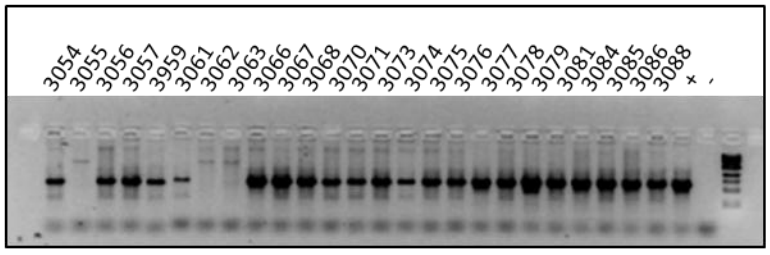
Figure 1*: Amplification of HPA1 from genomic DNA extracted from Desiree and Rpi-vnt1.1 transgenic plants.*



Analysis of the presence of transgene in potato lines (Desiree) transformed with pSLJ21152 (Rpi-vnt1.1)

The presence of insert in *Rpi-vnt1.1* transgenic plants was confirmed using primers against *Rpi-vnt1* (primers #14 and #15, Annex 1, Section 2). According to this analysis 22 plants transformed with pSLJ21152 were shown to contain the inserted sequence (Figure 2).

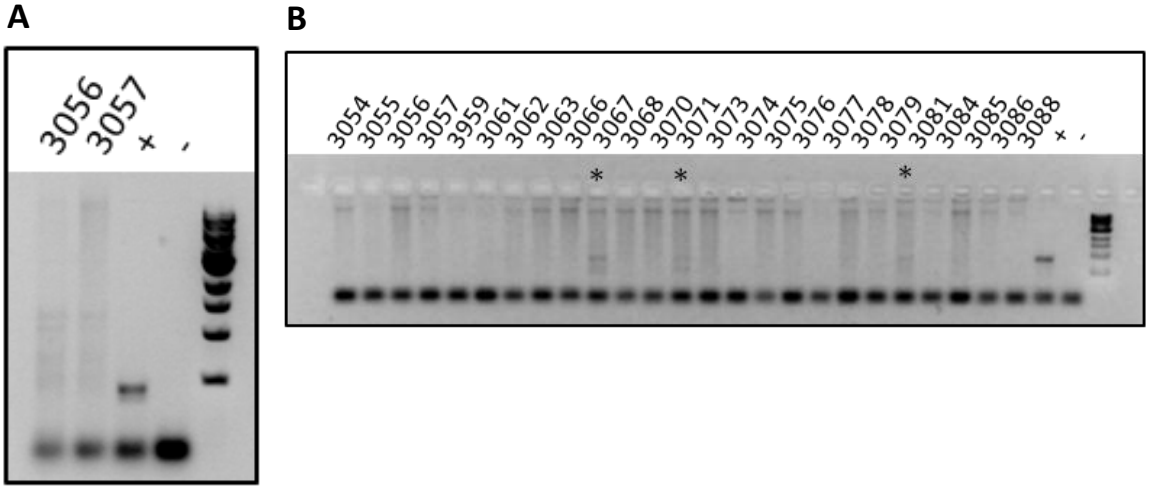
Figure 2: *Presence of insert in Desiree and Rpi-vnt1.1 transgenic plants: Amplification of Rpi-vnt1 gene (positive control is pSLJ21152).*



Analysis of potato lines (Desiree) transformed with pSLJ21152 (Rpi-vnt1.1) for vector backbone sequences

PCR experiments were done to show that sequences outside of the T-DNA borders are absent from the plant line to be released that contains pSLJ21152 (*Rpi-vnt1.1*). To determine this, PCR primer pairs designed to amplify regions from the vector backbone close to the left and right borders were used (primers #114/#115 and #116/#117, Annex 1, Section 2). PCR products were not obtained from either of the plant lines tested (PL3056 and PL3057) using primers #114/#115 close to the right border (Figure 3A). PCR using primers #116/#117 designed against sequence close to the left border did not yield a PCR product even in the positive control. However, we also tested this plant line using primers #22/23 (Annex 1, Section 2) designed to amplify the *nptIII* bacterial selection marker present in the vector backbone. No PCR products were obtained from the majority of plants (the only exception being lines PL3067, PL3071 and PL3081) demonstrating the absence of this antibiotic marker (Figure 3B). Plant line PL3056 is the line containing *Rpi-vnt1.1* chosen for release.

Figure 3: *Testing for absence of vector backbone in transgenic plants containing Rpi-vnt1.1* ***(A)*** *Amplification of sequences close to right borders from lines containing Rpi-vnt1.1 (Positive control is pSLJ21152)* ***(B)*** *Amplification of nptIII gene in Rpi-vnt1.1 transgenic lines (Positive control is pSLJ21152).*

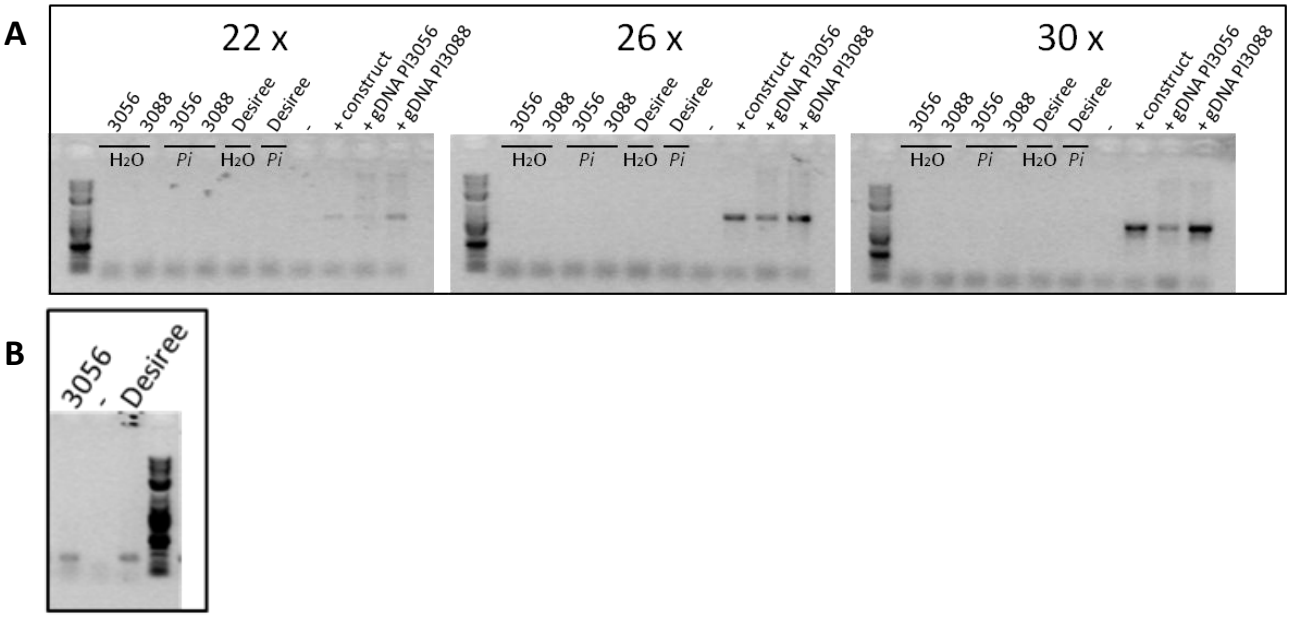


Expression analysis of Rpi-vnt1.1

To analyse expression levels of the transgenic lines, primers against *Rpi-vnt1* (primer #14 and # 15, Annex 1, Section 2) were used in semi-quantitative RT-PCR. Plants of the transgenic lines PL3056 and PL3058 carrying *Rpi-vnt1.1* were inoculated with either water (as a negative control) or spores of *Phytophthora infestans*. 18 hours after inoculation, RNA was extracted and RT-PCRs done for 22, 26 and 30 cycles. As shown in Figure 4A, expression of *Rpi-vnt1.1* was undetectable even after 30 cycles. In contrast, expression of the constitutively expressed reference gene *PHA1* was

detectable from 22 cycles (Figure 4B). Plant line PL3056 is the line carrying *Rpi-vnt1.1* chosen for release.

Figure 4: *Expression analysis of the Rpi-vnt1.1 transgene.* ***(A)*** *RT-PCR of Rpi-vnt1.1 from plants of lines PL3056, PL3058 and non-transformed Desiree inoculated with either water (H_20) or P. infestans (Pi). Positive controls include pSLJ21152 plasmid DNA and genomic DNA from the transgenic plants PL3056 and PL3058.* ***(B)*** *RT-PCR of the reference gene PHA1 from PL3056 and non-transformed Desiree (results from 22 cycles shown).*



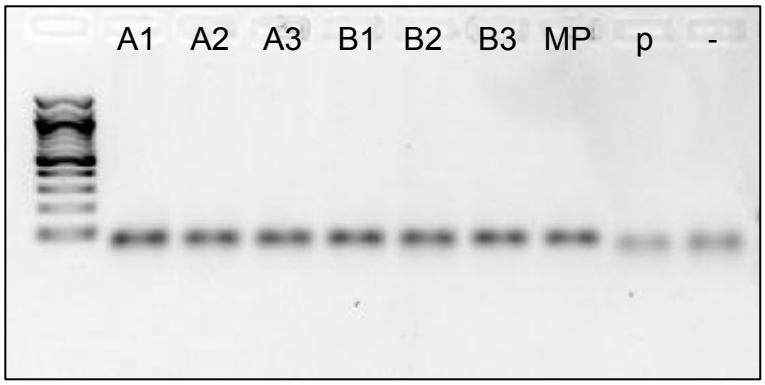
(B) Molecular characterization of plants carrying *Rpi-amr3*

Included here are details of a number of independently generated *Rpi-amr3* transgenic lines.

<u>*Genomic DNA quality determination*</u>

All genomic DNAs analysed for the presence of insert and vector backbone sequences were tested for their quality. We used primers designed to amplify the potato elongation factor 1 (*EF1*, primers EF1_F/EF1_R, Annex 1, Section 2; designed on *EF1* from *Solanum tuberosum*). The desired product was amplified from all genomic DNAs indicating that they are suitable templates of sufficient quality for use in characterisation of the transgenic plants by PCR (Figure 5). Faint bands on ‘p’ and ‘-‘ correspond to primer dimers.

***Figure 5**: Amplification of EF1 from genomic DNA extracted from Maris Piper (MP) and Rpi-amr3 transgenic plants (A1, A2, A3, B1, B2, B3). ' p ' refers to plasmid pSLJ24119 (Rpi-amr3) and ' – ' refers to a PCR reaction where no template was included (negative control).*



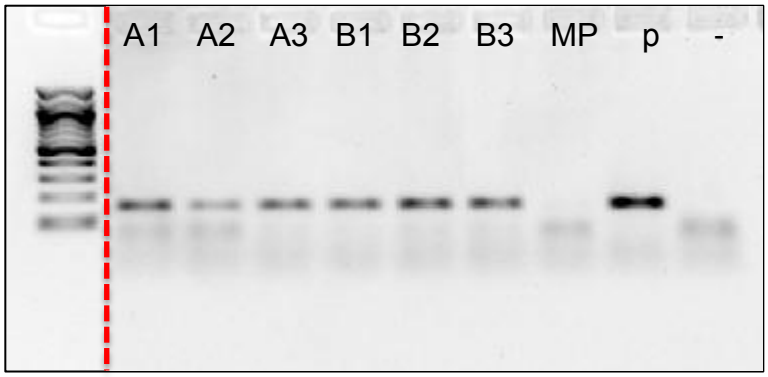
Elongation Factor 1 (EF1) - Control

Analysis of the presence of the T-DNA in potato lines (Maris Piper) transformed with pSLJ24119 (Rpi-amr3)

The presence of the T-DNA insert in *Rpi-amr3* transgenic plants was confirmed using primers against *Rpi-arm3* (primers MP015/MP016, Annex 1, Section 2) and against the *nos* promoter and coding sequence of the *bar* selectable marker present in pSLJ24119 (primers MP017/MP018, Annex 1, Section 2). According to this test, all the 6 lines analysed contain the T-DNA corresponding to pSLJ24119 (*Rpi-amr3*) (Figure 6). No product was obtained with Maris Piper WT plants or in the negative control while the positive control yielded the expected amplification product.

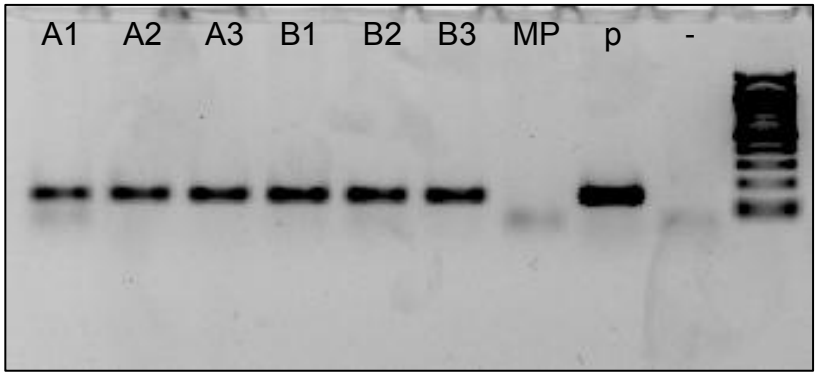
***Figure 6:** Presence of T-DNA insert in Maris Piper (MP) and Rpi-amr3 transgenic plants (A1, A2, A3, B1, B2, B3): PCR amplification of a fragment of the Rpi-amr3 gene **(A)** and the pnos::bar selectable marker **(B)**. ' p ' refers to plasmid pSLJ24119 (Rpi-amr3) (positive control) and ' – ' refers to a PCR reaction where no template was included (negative control).*

A



T-DNA (Rpi-amr3)

B



T-DNA (Selectable marker)

Analysis of potato lines (Maris Piper) transformed with pSLJ24119 (Rpi-amr3) for vector backbone sequences

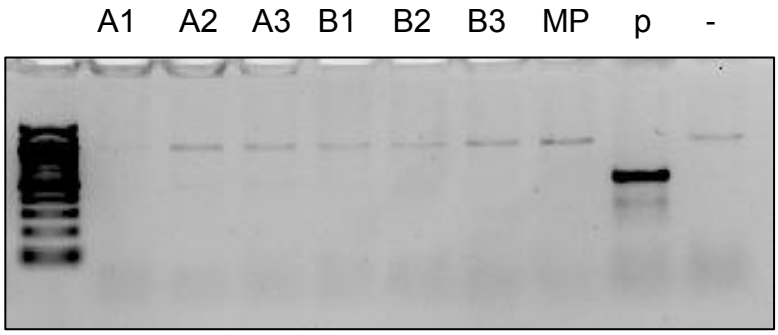
PCR experiments were done to test whether sequences outside of the T-DNA borders are present in the *Rpi-amr3* transgenic plants. To determine this, PCR primer pairs designed to amplify regions from the vector backbone close to the left and right borders were used (primers MP007/MP008 for right border region and MP011/MP012 for left border region, Annex 1, Section 2).

When using the primers binding close to the right border, only the positive control (‘ p ‘, pSLJ24119) yielded a product of the expected size (Figure 7A). When using the primers binding close to the left border, the positive control and three of the transgenic lines yielded a product of the expected size (Figure 7B). In addition, two lines (as well as the Maris Piper WT control) show extremely faint bands (which might be a consequence of cross-contamination) an a sixth line shows no band.

To further investigate this, additional tests are in progress. We will extract new genomic DNA samples from these lines and we will test them for the presence of the *nptIII* selectable marker gene located in the pICSLUS0001 backbone. We will use primers that amplify the *nptIII* full-length sequence (primers US_nptIII_F/US_nptIII_R, Annex 1, Section 2). Lines giving a positive result in that test will be excluded from the proposed trial.

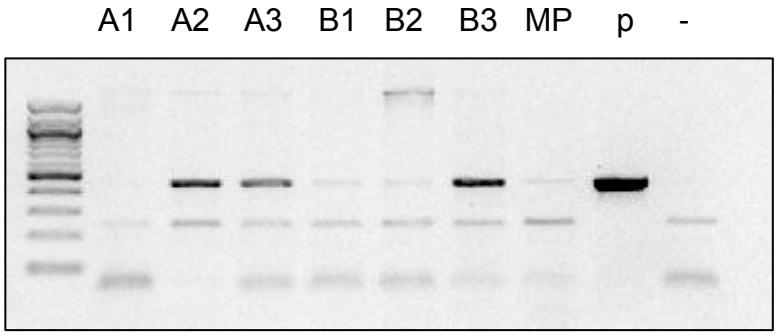
Figure 7: *Testing for absence of vector backbone in transgenic plants containing Rpi-amr3: PCR amplification of sequences close to right borders* ***(A)*** *and left borders* ***(B)*** *of the pICSLUS0001 vector backbone in Maris Piper (MP) and Rpi-amr3 transgenic plants (A1, A2, A3, B1, B2, B3). ' p ' refers to plasmid pSLJ24119 (Rpi-amr3) (positive control) and ' – ' refers to a PCR reaction where no template was included (negative control).*

A



Backbone - Right Border Region

B



Backbone - Left Border Region

Expression analysis of Rpi-amr3

Expression of the *Rpi-amr3* transgene was assessed by quantitative PCR (qPCR) using primers MP015/MP016 (Annex 1, Section 2). *Elongation factor 1* gene (*EF1*) was used as control for the normalization of the qPCR data. The expression of *EF1* was assessed

with primers EF1_F/EF1_R (Annex 1, Section 2) and the result is expressed as [Number of mRNA molecules of *Rpi-amr3* per 1 Million mRNA molecules of *EF1* control].

RNA of suitable quality for cDNA synthesis and qPCR was only obtained from leaves of lines A1, A2 and A3. Plants corresponding to lines B1, B2 and B3 were senescent at the moment of collecting the samples, so the expression of the *Rpi-amr3* transgene could not be determined with confidence. New RNA samples will be extracted once plants are regenerated from tubers and the analysis will be repeated. In addition to RNA from the *Rpi-amr3* lines, an RNA sample from the *Solanum americanum* accession from which *Rpi-amr3* was isolated was included as control (SP1102). Accession SP1102 is diploid and contains 2 copies of *Rpi-amr3* per diploid genome.

The normalized expression values for the different *Rpi-amr3* transgenic lines and the SP1102 control are indicated in the table below:

Line	Number of *Rpi-amr3* mRNAs / 1M *EF1* mRNAs
A1	488
A2	367
A3	164
B1	83
B2	2
B3	48
SP1102	526

As shown in the table, the level of expression of the *Rpi-amr3* transgene in lines A1, A2 and A3 is not higher than the level of expression of *Rpi-amr3* in the resistant accession from which this gene has been isolated. In addition, and in agreement with the results previously obtained with *Rpi-vnt1.1* plants, more than 30 qPCR cycles were required to detect the expression of this transgene.

(C) Molecular characterization of plants carrying *Rpi-amr1e, Rpi-amr1k, Rpi-Smira1 and Rpi-Smira3*

Constructs carrying *Rpi-amr1e, Rpi-amr1k, Rpi-Smira1* and *Rpi-Smira3* are in the pipeline for plant transformation at the moment. The plants obtained after the transformation will be characterized in the same way as the plants carrying *Rpi-amr3* (described above).

Genomic DNA samples will be extracted from several independent lines of each genotype. The quality of the genomic DNA will be assessed by PCR with primers designed to amplify the potato elongation factor 1 (*EF1*, primers EF1_F / EF1_R, Annex 1, Section 2; designed on *EF1* from *Solanum tuberosum*).

The presence of the transgenes will be assessed by PCR with primers specific to the resistance gene sequences. To further confirm the insertion of the T-DNA, primers

MP017/MP018 (Annex 1, Section 2) will be used to assess the presence of the T-DNA selectable marker in *Rpi-amr1e* and *Rpi-amr1k* lines and primers GG_nos_F/GG_nptII_R (Annex 1, Section 2) will be used for the same purpose in *Rpi-Smira1 and Rpi-Smira3* lines.

To determine whether vector backbone sequences have also been integrated, primers MP007/MP008, MP011/MP012 and US_nptIII_F/US_nptIII_R (Annex 1, Section 2) will be used to test *Rpi-amr1e* and *Rpi-amr1k* lines and primers GG_RB_F/GG_RB_R, GG_LB_F/GG_LB_R and GG_nptIII_F/GG_nptIII_R (Annex 1, Section 2) will be used to assess *Rpi-Smira1 and Rpi-Smira3* lines. Any line giving a positive result in the PCR test for the presence of the *nptIII* backbone selectable marker will be excluded from the trial.

Finally, to assess the expression and insert copy number of *Rpi-amr1e, Rpi-amr1k, Rpi-Smira1* and *Rpi-Smira3* in the different transgenic lines we will perform qPCR and ddPCR assays as described above for the *Rpi-amr3* lines, using primers specific to the resistance gene sequences.

SECTION 2: METHODS USED FOR CHARACTERISATION OF TRANSGENIC PLANTS

(A) Molecular characterization of plants carrying *Rpi-vnt1.1*

Genomic DNA isolation

Genomic DNA was isolated by using the Retsch DNA isolation protocol detailed in Park et al (2005). DNA was eluted in 75 µl DNAse free water.

PCR amplification

All PCR reactions were performed in 30 ul with (3µl 10x PCR buffer, 3 µl 0.8 mM dNTPs, 0.25 µl Taq polymerase (homemade) and 2 µl of genomic DNA or 5 µl cDNA per PCR reaction). The following PCR program was used for all PCR reactions: 94 ^{o}C, 30'' - 55 ^{o}C, 30'' – 72 ^{o}C, 2'30'' – 26 - 33x. 7,5 µl of this reaction was loaded and run on a 1.2 % TAE agarose gel.

RNA isolation

RNA was isolated from young fresh leaves using the Tri reagent (Sigma, T9424). After DNAse treatment, the pellet was disolved in 100 µl RNA free water. Subsequently, 2.5 µg of total RNA was used to make first strand cDNA using Superscript III Reverse Transcriptase (Invitrogen, 18080-444). 100 µl water was added after cDNA synthesis. 5 µl was used as template per RT-PCR reaction.

List of primers used to characterise *Rpi-vnt1.1* transgenic plants

Construct	Gene/Region	Primer number	Sequence 5'-3'
Desiree	*PHA1*	3	TGCTGCAATCGAAGGAATTGGC
Desiree	*PHA1*	4	CTTCACCACTGATTCCACGTGAC
pSLJ21152	*nptIII*	22	CGTCGATACTATGTTATACGCC
pSLJ21152	*nptIII*	23	ATATCCTCCCTGATCGACCGG
pSLJ21152	*Rpi-vnt1*	14	TTCAACGTTTGTTATTCATGC
pSLJ21152	*Rpi-vnt1*	15	ATACTCTCAAGTACTCTGTTC
pSLJ21152	Outside LB	116	CGCTGTGCAGCCGCTGATGGTC
pSLJ21152	Outside LB	117	TAAGCTGCCGGGTTTGAAACAC
pSLJ21152	Outside RB	114	ACGATCCGACAGCGCGCCCAGC
pSLJ21152	Outside RB	115	CCTGAAGTGCCAGTAAAGCGC

(B) Molecular characterization of plants carrying *Rpi-amr3, Rpi-amr1e, Rpi-amr1k, Rpi-Smira1 and Rpi-Smira3*

Genomic DNA isolation

Plant genomic DNA extraction is performed according to the Doyle and Doyle CTAB-based method (Doyle and Doyle, 1987).

PCR amplification from genomic DNA

All PCR reactions are performed in a final volume of 25µl with 2.5µl of 10x PCR buffer, 0.5µl of 10mM dNTPs, 1µl of 10µM forward and reverse primers, 0.3µl of Taq polymerase (homemade) and 100-150ng of genomic DNA per PCR reaction. The following PCR program is used: 95°C 5min, [95°C 30'' - 60°C or 61°C 30'' - 72°C 30'' or 45''] 32x - 72°C 5min. After the amplification, 10µl of this reaction is loaded and run on a 1.5 % TBE agarose gel.

Droplet Digital PCR (ddPCR) amplification for copy number determination

All ddPCR reactions are performed with 15ng of genomic DNA as template. The reaction mix is set up using the BIO-RAD QX200 ddPCR 2X EvaGreen Supermix following the manufacturer's recommended protocol. The final concentration of primers is 100nM and 2U of EcoRI enzyme are added per reaction to separate tandem gene copies and improve template accessibility. Droplets are generated with the BIO-RAD QX200 Droplet Generator and PCR amplification is performed in a BIO-RAD C1000 Touch Thermal Cycler. The following PCR program is used: 95°C 5min, [95°C 30'' – 60.5°C 1min - 72°C 1min] 40x - 4°C 5min - 90°C 5min. All the steps are performed with a temperature ramp of 2°C/sec and a lid temperature of 105°C. After amplification, droplets are analysed in the BIO-RAD QX200 Droplet Reader device and the number of positive droplets obtained for the gene of interest is compared to the number of positive droplets obtained for the control gene (*Vacuolar Invertase*), which corresponds to 4 copies / potato tetraploid genome.

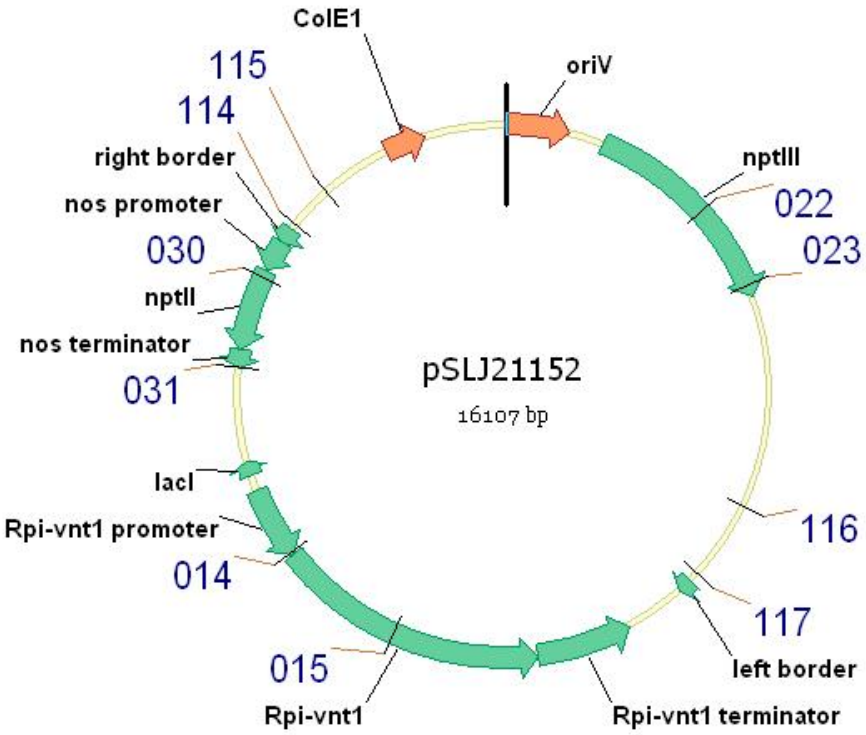
RNA extraction and qPCR amplification

RNA is isolated using the RNeasy Mini Kit (Qiagen, 74104) and treated with DNAse. All qPCR reactions are performed in a final volume of 20µl with 10µl of Sigma SYBR Ready Mix and 1µl of 10µM forward and reverse primers. cDNA synthesis is performed with 2.5µg total RNA using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen, 18080-400) in a final volume of 20µl. The cDNA is diluted 1/5 and 1µL of the dilution is used as template for qPCR amplification. The following qPCR program is used: 95°C 2min, [95°C 20'' - 58°C 20'' - 72°C 30''] 40x - 72°C 5min. The result is expressed as [Number of mRNA molecules of *Rpi-amr3* per 1 Million mRNA molecules of *EF1* control].

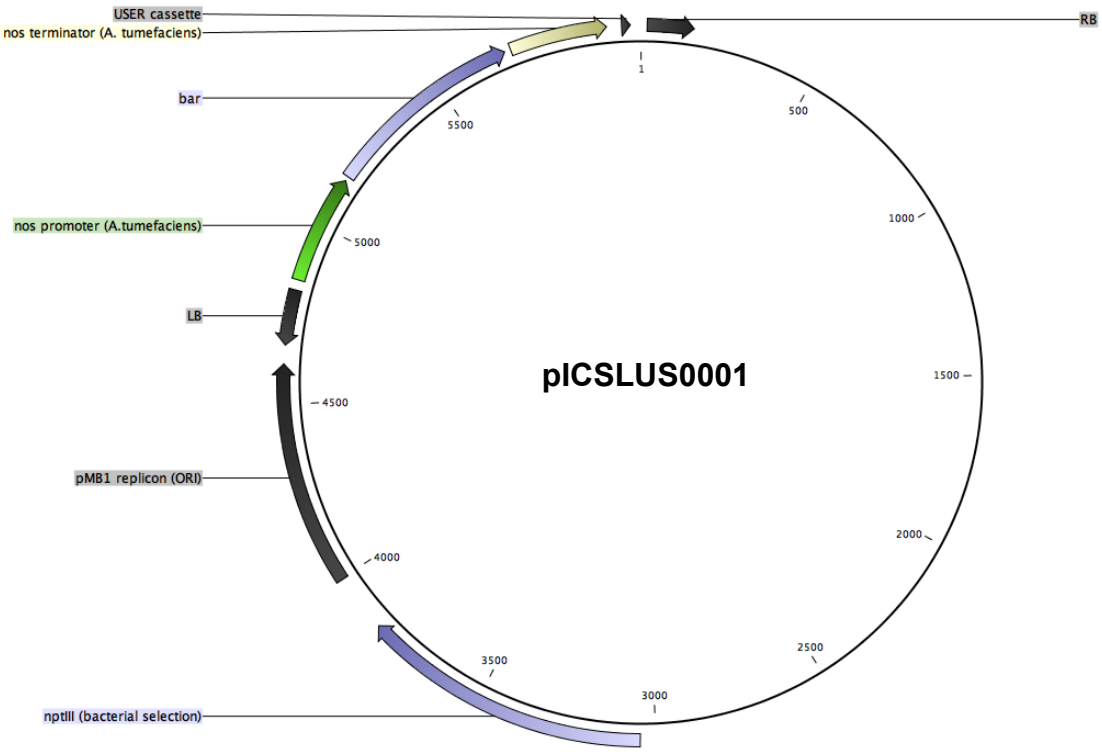
List of primers used to characterise *Rpi-amr3, Rpi-amr1e, Rpi-amr1k, Rpi-Smira1 and Rpi-Smira3* transgenic plants

Construct	Gene/Region	Primer ID	Sequence 5'-3'
Maris Piper	*EF1*	EF1_F	GGAAGCTGCTGAGATGAACAAGA
Maris Piper	*EF1*	EF1_R	CTCACGTTCAGCCTTAAGTTTGT
Maris Piper	*Vacuolar Invertase*	MP013	CTGGGTCAAGTACAAAGGCAAC
Maris Piper	*Vacuolar Invertase*	MP014	CATTTTGGGGTCCGGTCCAA
pSLJ24119 (*Rpi-amr3*)	*Rpi-amr3*	MP015	CTGAGGATTCTGCACGAGAGATTG
pSLJ24119 (*Rpi-amr3*)	*Rpi-amr3*	MP016	TCATCATAACTTCAAGGAGGTAAG
pICSLUS0001 T-DNA	*nos promoter*	MP017	TGACGTTCCATAAATTCCCCTC
pICSLUS0001 T-DNA	*bar*	MP018	ATATCAGCCTCAGTAGCCCTTC
pICSLUS0001 backbone	*Outside RB*	MP007	TGGCCGGTTTGTCTGATG
pICSLUS0001 backbone	*Outside RB*	MP008	TGTCGTAAGGGCTTGGCTG
pICSLUS0001 backbone	*Outside LB*	MP011	TAGCACCGCCTACATACCTCG
pICSLUS0001 backbone	*Outside LB*	MP012	GTCCAACAGATCCGAGCAGG
pICSLUS0001 backbone	*nptIII*	US_nptIII_F	CCCTGATAAATGCTTCAATAATATTG
pICSLUS0001 backbone	*nptIII*	US_nptIII_R	TATATATGAGTAAACTTGGTCTGAC
pICSL86900 T-DNA	*nos promoter*	GG_nos_F	TCTGGAGTTTAATGAGCTAAGCAC
pICSL86900 T-DNA	*nptII*	GG_nptII_R	CGATTGTCTGTTGTGCCCAGTC
pICSL86900 backbone	*Outside RB*	GG_RB_F	CCTGGCCGGCCAGCTTGGCCGCTG
pICSL86900 backbone	*Outside RB*	GG_RB_R	TAAGGGCTTGGCTGCACCGGAATC
pICSL86900 backbone	*Outside LB*	GG_LB_F	TGCTGCCAGTGGCGATAAGTCGTG
pICSL86900 backbone	*Outside LB*	GG_LB_R	TTGCTGGCGTTTTTCCATAGGCTC
pICSL86900 backbone	*nptIII*	GG_nptIII_F	GACAATAACCCTGATAAATGCTTC
pICSL86900 backbone	*nptIII*	GG_nptIII_R	ATATATGAGTAAACTTGGTCTGAC

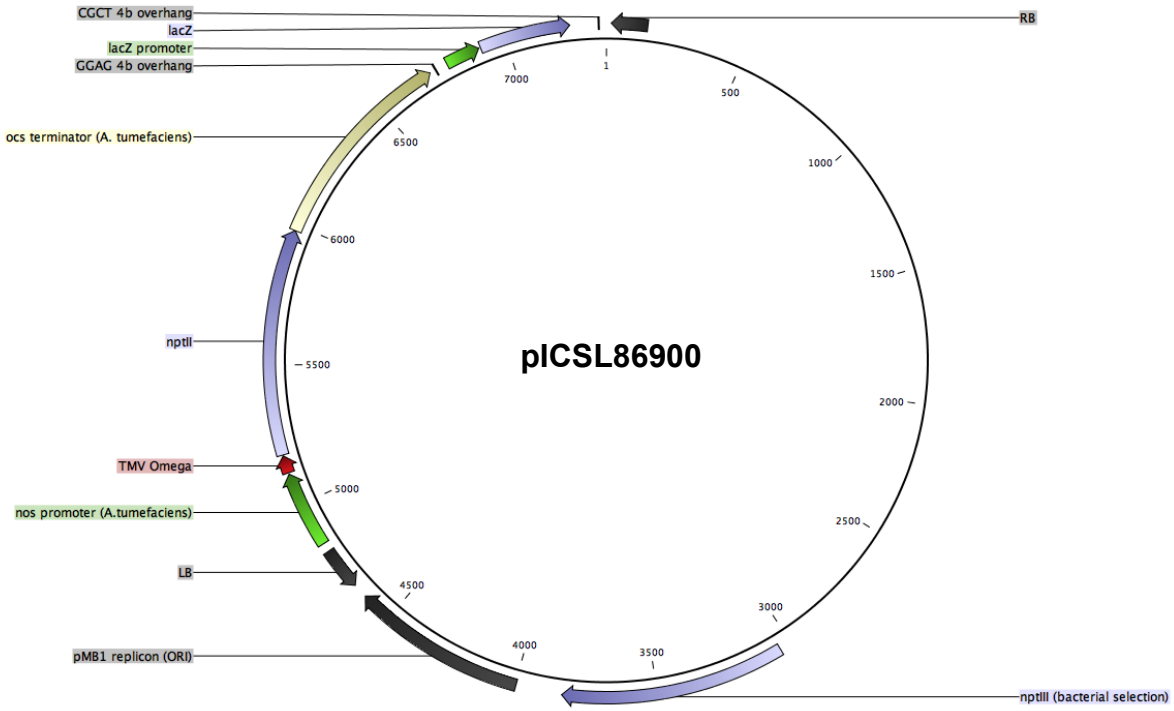
SECTION 3: VECTOR MAP OF pSLJ21152 (*Rpi-vnt1.1*)



SECTION 4: VECTOR MAP OF pICSLUS0001 (VECTOR USED TO GENERATE PLASMIDS pSLJ24119, pSLJ24156 AND pSLJ24478)



SECTION 5: VECTOR MAP OF pICSL86900 (VECTOR USED TO GENERATE PLASMIDS pSJL24466 AND pSJL24468)



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Park TH, Vleeshouwers VG, Huigen DJ, van der Vossen EA, van Eck HJ, Visser RG (2005) Characterization and high-resolution mapping of a late blight resistance locus similar to *R2* in potato. *Theor. Appl. Genet.* 111: 591-597.