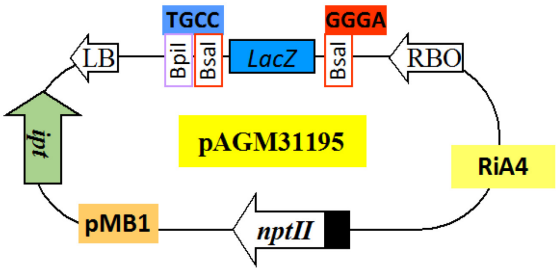
Improving late blight (*Phytophthora infestans*) resistance in potato using resistance genes from wild potato relatives.

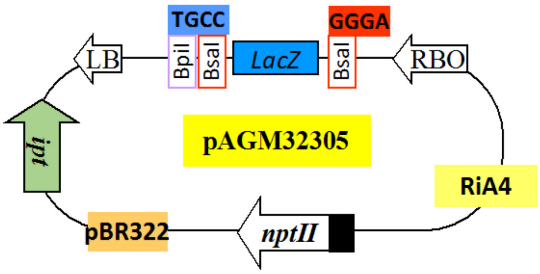
Annex 1

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. It also provides vector maps and details of the structure of the T-DNAs in each plasmid used for plant transformation.

Section 1: Maps of vectors pAGM31195 and pAGM32305.
Section 2: Structure of T-DNAs in plasmids SLJ24895, SLJ24896, SLJ24897, SLJ24904, SLJ24909, SLJ24933 and SLJ24918.
Section 3: Results from the characterisation of transgenic plants carrying *Rpi-amr3i*.
Section 4: Methods used for the characterisation of transgenic plants.

Section 1: Maps of vectors pAGM31195 and pAGM32305



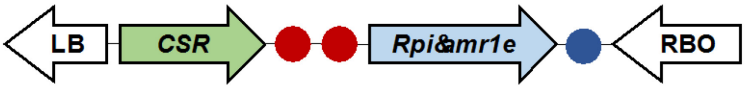


A detailed description of vector elements has been presented in Section 11 of Part A1. Note that the LacZ gene in the vector's Golden Gate cassette is removed upon cloning.

Section 2: Structure of T-DNAs in plasmids SLJ24895, SLJ24896, SLJ24897, SLJ24904, SLJ24909, SLJ24933 AND SLJ24918

SLJ24895)T-DNA

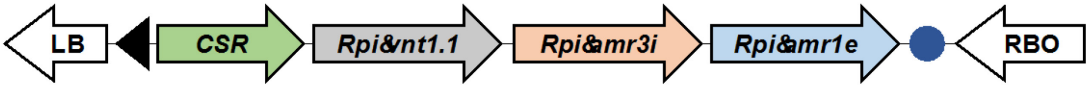
SLJ24896)T-DNA



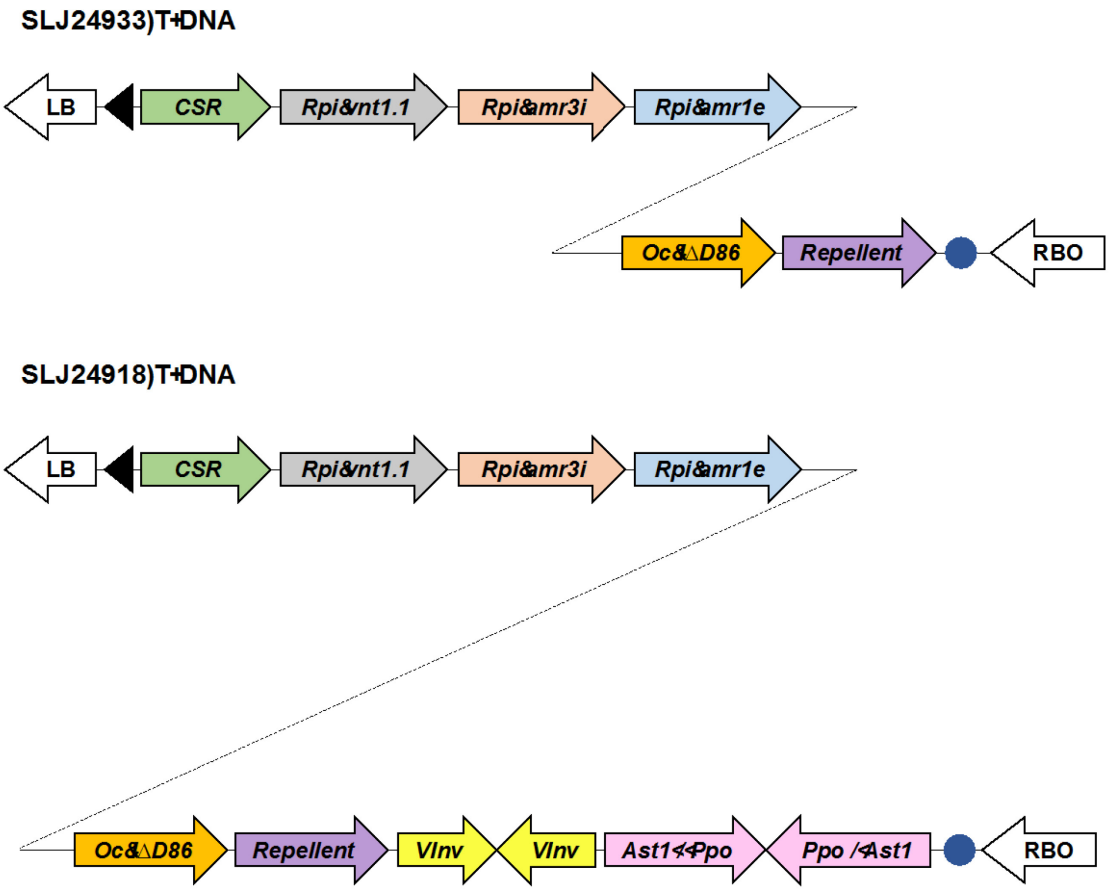
SLJ24897)T-DNA

SLJ24904)T-DNA

SLJ24909)T-DNA



A detailed description of T-DNA elements has been presented in Section 12 of Part A1, including information on regulatory sequences linked to the cloned genes.
Black triangles represent extra left border sequences. Red circles indicate dummy-linkers and blue circles represent end-linkers. Rpi-amr1e will be cloned both as a long and a short version.



A detailed description of T-DNA elements has been presented in Section 12 of Part A1, including information on regulatory sequences linked to the cloned genes and gene-silencing modules. Black triangles represent extra left border sequences and blue circles indicate end-linkers. Rpi-amr1e will be cloned both as a long and a short version.

Section 3: Results from the characterisation of transgenic plants carrying *Rpi-amr3i*

This section includes details of selected *Rpi-amr3i* transgenic lines generated with construct **SLJ24895**. Similar analyses will be carried out with the other transgenic plants included in this application.

Test for presence of T-DNA and assessment of vector backbone insertion

All genomic DNAs analysed for the presence of T-DNA and vector backbone sequences were first tested for their quality. We used primers designed to amplify the potato elongation factor 1 alpha (*EF1*α, primers MP075/MP076, Annex 1, Section 4; designed on *EF1*α from *Solanum tuberosum*). The desired product was amplified from all genomic DNAs (lines 5c, 9a and 14, in addition to Maris Piper wild type plants). This indicates they are suitable templates of sufficient quality for use in characterisation of the transgenic plants by PCR (Figure 1). As expected, the PCR negative control and empty vector pAGM31195 did not yield any amplification product.

The presence of the T-DNA insert in *Rpi-amr3i* transgenic plants was confirmed using a forward primer binding to the selectable marker gene (*CSR*) terminator and a reverse primer binding to the *Rpi-amr3i* promoter (primers MP037/amr3_prom_R, Annex 1, Section 4). According to this test, all the 3 lines analysed contain the T-DNA corresponding to **SLJ24895** (Figure 1). As expected, no product was obtained with Maris Piper wild type samples, the PCR negative control or the empty vector pAGM31195.

PCR experiments were also done to test whether sequences outside of the T-DNA borders are present in the *Rpi-amr3i* transgenic plants. To determine this, PCR primer pairs were designed to amplify regions from the vector backbone close to the left border (*ipt* gene, primers MP115/MP116) and the right border (RiA4 replicon, primers MP110/MP111). In addition, primers that amplify the *nptII* gene used for bacterial selection were also included in the analysis (primers MP112/MP113). The sequences of all these primer pairs are listed in Annex 1, Section 4.

When using the primer pairs encompassing backbone sequences, only the positive control (empty vector pAGM31195) yielded a product of the expected size (Figure 1). No product was obtained using genomic DNA from the transgenic lines, which indicates they do not carry the backbone sequences described above. As expected, no product was obtained with Maris Piper wild type samples or with the PCR negative control.

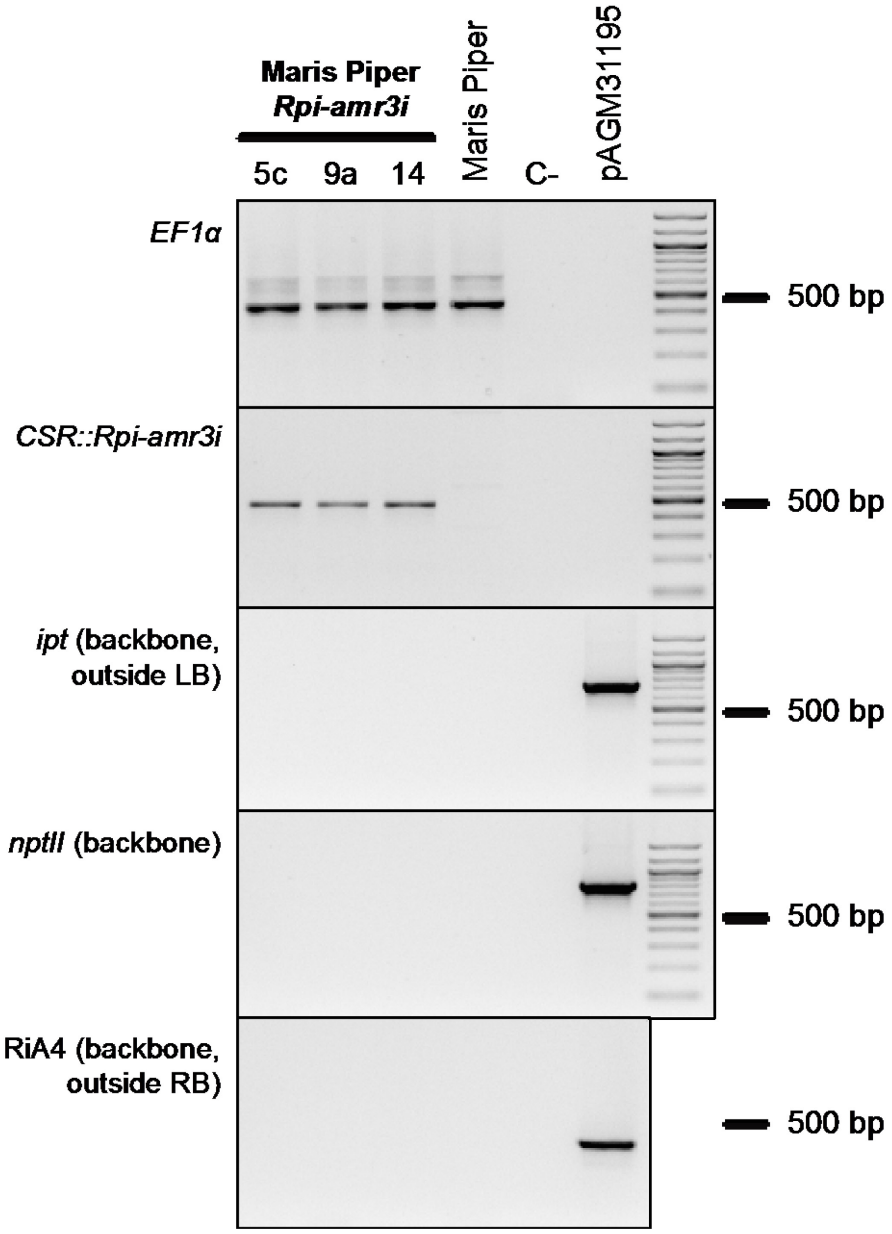


***Figure 1**: Test for presence of T-DNA and assessment of vector backbone insertion by PCR. Genomic DNA was extracted from Maris Piper / Rpi-amr3i lines 5c, 9a and 14, as well as from Maris Piper wild type plants. Empty vector pAGM31195 was included as control. 'C-' refers to a PCR reaction where no template was included (negative control). PCR reactions were carried out with primer pairs that amplify sequences from potato EF1α (genomic DNA quality control), sequences spanning the junction between the selectable marker gene CSR and Rpi-amr3i in the*

T-DNA of SLJ24895 (CSR::Rpi-amr3i) and different regions of the vector backbone (ipt gene, nptII gene, RiA4 replicon).

Assessment of Rpi-amr3i expression

Expression of the *Rpi-amr3i* transgene was assessed by quantitative PCR (qPCR) using specific primers MP015/MP016 (Annex 1, Section 4). The *Elongation factor 1 alpha* gene (*EF1*α) was used as control for the normalization of the qPCR data. The expression of *EF1*α was assessed with primers EF1_F/EF1_R (Annex 1, Section 4) and the result is 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.

The normalized expression values for three selected *Rpi-amr3i* transgenic lines and the *Rpi-amr1e* negative control are indicated in the table below:

Line	Number of *Rpi-amr3i* mRNAs / 1M *EF1*α mRNAs
Rpi-amr3i – 5c	8
Rpi-amr3i – 9A	21
Rpi-amr3i – 14	3
Rpi-amr1e	0

As shown in the table, expression of the *Rpi-amr3i* transgene was very low compared to the *EF1*α control. In addition, more than 30 qPCR cycles were required to detect *Rpi-amr3i* expression. As expected, no *Rpi-amr3i* expression was detected in the lines carrying *Rpi-amr1e*.

Section 4: Methods used for characterisation of transgenic plants

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 25 µl with 2.5 µl of 10x Standard *Taq* PCR buffer (New England BioLabs), 0.5 µl of 10 mM dNTPs, 1 µl of 10 µM forward and reverse primers, 0.125 µl of *Taq* polymerase (New England BioLabs) and 100-150 ng of genomic DNA per PCR reaction. The following PCR program is used: 95°C 3 min, 30x [95°C 30'' > $T^{o}_{annealing}$ 30'' > 68°C for corresponding elongation time], 68°C 5 min. $T^{o}_{annealing}$ is within 55°C to 60°C depending on the melting temperature of the primer pairs. Elongation time is within 30'' to 50'' depending on the length of the amplicons. After the amplification, 10 µl of the PCR reactions (including DNA loading buffer) are loaded and run on a 1.5 % TAE agarose gel next to a DNA molecular-weight ladder (100 bp, New England BioLabs).

Droplet Digital PCR (ddPCR) amplification for copy number determination
All ddPCR reactions are performed with 15 to 30 ng 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 2-5 U 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 5 min, 40x [95°C 30'' – 60.5°C 1 min - 72°C 1 min] - 4°C 5 min - 90°C 5 min. 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) and treated with DNAse (Qiagen). All qPCR reactions are performed in a final volume of 20 µl with 10 µl of KAPA SYBR® FAST qPCR Mix and 1 µl of 10 µM forward and reverse primers. cDNA synthesis is performed with 2-2.5 µg total RNA using the SuperScript II First-Strand Synthesis SuperMix (Invitrogen) and oligo-dT primer in a final volume of 20 µl. The cDNA is diluted 1/5 and 1 µL 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-amr3i* per 1 Million mRNA molecules of *EF1* control].

List of primers used in PCR, ddPCR and qPCR tests

Construct	Gene/Region	Test	Primer ID	Sequence 5'-3'
Maris Piper (Control)	*EF1α*	PCR	MP075	GGAAGCTGCTGAGATGAACAAGA
Maris Piper (Control)	*EF1α*	PCR	MP076	CCTTCACAATTTCATCATACCTAGCC
SLJ24895	*CSR terminator*	PCR	MP037	TACAGATGGACAAGATCATTTACC
SLJ24895	*Rpi-amr3i promoter*	PCR	amr3_prom_R	GGCATGTTCAAGGCTACAAGATTA
pAGM31195 and pAGM32305 Backbone	*ipt* (outside LB)	PCR	MP115	AAAACTTATGGATCTGCGTC
pAGM31195 and pAGM32305 Backbone	*ipt* (outside LB)	PCR	MP116	GGAGCTGGTGCAAACTAATAC
pAGM31195 and pAGM32305 Backbone	*nptII*	PCR	MP112	GAAGAGTATGATTGAACAAGATGG
pAGM31195 and pAGM32305 Backbone	*nptII*	PCR	MP113	ATATATGAGTAAACTTGGTCTGAC
pAGM31195 and pAGM32305 Backbone	RiA4 (outside RBO)	PCR	MP110	CAAATAACAGTTGGGTGGAG
pAGM31195 and pAGM32305 Backbone	RiA4 (outside RBO)	PCR	MP111	CATGCTAACATTCAACTCTGGC
Maris Piper (Control)	*EF1α*	qPCR	EF1_F	GGAAGCTGCTGAGATGAACAAGA
Maris Piper (Control)	*EF1α*	qPCR	EF1_R	CTCACGTTCAGCCTTAAGTTTGT
SLJ24895	*Rpi-amr3i*	qPCR	MP015	CTGAGGATTCTGCACGAGAGATTG
SLJ24895	*Rpi-amr3i*	qPCR	MP016	TCATCATAACTTCAAGGAGGTAAG
Maris Piper (Control)	*Vacuolar Invertase*	ddPCR	MP013	CTGGGTCAAGTACAAAGGCAAC
Maris Piper (Control)	*Vacuolar Invertase*	ddPCR	MP014	CATTTTGGGGTCCGGTCCAA
All constructs	*CSR*	ddPCR	MP035	GAACTGTTATCAAAATCGCTAAAGC
All constructs	*CSR*	ddPCR	MP036	AGGAAAAGACTTATTTACCCTACATC

References

Doyle JJ and Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11-15.