Durable late blight resistance in potato (*Solanum tuberosum*):

R gene mapping and stacking

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1. Abstract

Potato late blight, caused by the oomycete *Phytophthora infestans*, is the most devastating disease of potato resulting in billion-dollar losses annually. For decades, disease management of late blight has mainly been based on the application of fungicides. Although this applications have desirable results in the field, there are some major drawbacks, like high costs, non-durable and harmful effects on the environment. As an alternative, breeding for new potato cultivars possessing genetic resistance, which is more sustainable and durable, has been suggested. Over the last few decades, resistance breeding, deploying qualitative resistance (*R*) genes from wild *Solanum* species have been the primary aim to address late blight. In this study an effort was made to fine map an *R* gene of *Solanum pinnatisectum*, a wild relative of the cultivated potato originating from Mexico, using two Bacterial Artificial Chromosome (BAC) libraries. Several BAC pools were found to potentially contain the *R* gene allele and can be used to identify candidate genes. Furthermore, the deployability of *R* genes was determined by phenotyping late blight resistance in transgenic potato populations. Results of *R9a* transgenic potatoes, derived from six common cultivars, indicated that genetic background influences the expression of *R9a* and thus late blight resistance. The influence of transgene regulatory elements, focusing on the size of the promoter and terminator, was determined using two *Rpi-chc1* transgenic Desiree populations. Unexpectedly, extending the size of either the promoter or terminator decreased *R* gene expression in both transgenic populations.

2. Introduction

Potato (*Solanum tuberosum*), along with wheat, maize and rice, is one of the most cultivated crops worldwide and, therefore, a very important source of nutrition (FAO, 2013). The origins of the potato can be traced to the Andean region in South America where they were domesticated 8000 years ago (Bakker et al., 2010). In the 16th century ancestors of *S. tuberosum* were introduced and cultivated in Europe. As a consequence of four centuries of cultivation, especially in Northern Europe, the crop became adapted to the long summer days and evolved to such an extent that it was classified as subspecies *tuberosum* (Bradshaw and Ramsay, 2005). Since the 17th century potatoes have been spread and cultivated globally and have become a major crop. Most of potatoes were produced in developed countries, like Europe and North America, but since 2005 the total potato production of developing countries exceeded that of the developed countries. Nowadays more than one third of the global potato production takes place in China and India (Haverkort et al., 2009). The global potato production increased with 100 Megatons the last 50 years reaching a total of 370 Megatons in 2012 (FAO, 2013). Although this increase seems promising for the future, where the world will face a growing food demand, the global potato production is continuously at risk.

The major biotic threat for potato cultivation is the oomycete *Phytophthora infestans* which causes late blight, resulting in global yield losses up to 16% (Haverkort et al., 2009; Haas et al., 2009). The Great Irish Famine in the 19th century is probably the most devastating and known example of what *P. infestans* is capable of. The blight symptoms are brown and black lesions occurring on the entire plant, including stems, leaves and tubers (Fry, 2008). Once infected, the size of the edible tuber is reduced dramatically, up to 80%, depending on the potato variety (Lung'aho et al., 2008). Due to the relatively short life cycle of *P. infestans*, a potato field can be completely blighted within one or two weeks after infection when not treated. *P. infestans*, in addition to other pathogens and pests, was accidently introduced at the same time the potato was introduced in Europe. Since the majority of the cultivated potatoes nowadays originated from only a small set of Andean ancestors, the genetic diversity among potato varieties is low, making the potato production sector very susceptible to disease outbreaks.

For decades, disease management of *P. infestans* has mainly been based on the application of fungicides. Although this applications have desirable results in the field, there are some major drawbacks, like high costs and harmful effects on the environment. Besides, the frequent application of fungicides, e.g. metalaxyl, had led to the evolution of pathogenic strains possessing resistance to these compounds (Davidse et al., 1981). As an alternative, breeding for new potato cultivars possessing genetic resistance has been suggested. Late blight resistance occurs in many wild tuber-bearing *Solanum* species, since they coevolved with the pathogen. These species, originated in Central- and South America, possess a broad spectrum of resistance specificities which can be utilized in breeding programs to develop new resistant potato cultivars.

Two types of host plant resistance to *P. infestans* can be distinguished, qualitative and quantitative resistance. Qualitative- or race-specific resistance is derived from so called resistance (*R*) genes. *R* genes function in a gene-for-gene manner which indicates that each *R* gene forms a couple with a corresponding avirulence (*Avr*) effector gene which is present in the pathogen (Flor, 1971). The *R* genes encode receptors ensuring defence by recognizing the *Avr* transcripts of the pathogen once infected (McDowell and Woffenden, 2003). A pathogen containing the *Avr* effector cannot infect a host plant which contains the corresponding *R* gene. In such conditions the resistant potato cultivar induce a cell death-associated defence reaction, also known as hypersensitive response (HR), at an early stage of *P. infestans* infection (Kamoun et al., 1999). In this way the pathogen cannot spread to other parts of the plants and will eventually die along with the infected cell. *S. demissum* was the first wild potato species that was used to characterize and employ *R* genes to *P. infestans* (*Rpi*). This species was chosen because of its high level of resistance (>11 *Rpi*'s discovered) and ease of introgression. While breeding for this kind of resistance is relatively easy and fast, such resistant is not durable. In the field compatible *P. infestans* races are rapidly selected and after several cycles the majority of the pathogen population will no longer be hampered by the resistance. Besides, *P. infestans* possess a remarkable capacity to rapidly adapt to resistant plants (Vleeshouwers et al., 2011).

The other type of resistance is quantitative- or race non-specific resistance, which is based on multiple genes, residing in multiple genomic loci, each having a small effect rendering the plant partially resistant to a broad spectrum of different pathogen races. The occurrence of quantitative resistance has been observed in several wild *Solanum* species, including *Solanum demissum* (Kuhl et al., 2001). This type of resistance is considered more durable since the pathogen can still infect the host plant, although on a much lower rate, the *P. infestans* population in the field will not be subjected, in theory, to negative selection. However, quantitative resistance is difficult to characterize, is largely influenced by the environment and appears to be associated with late foliage maturity, which makes this approach unfavourable in practice (Visker, 2005).

At the moment breeding for resistance in potato is mainly focussing on qualitative resistance since durable resistance through quantitative resistance loci is not yet available and difficult to accomplish. Several strategies to make the application of *R* genes more durable are suggested, in particular stacking of *R* genes. By stacking multiple broad spectrum *R* genes, the cultivar will have a much broader resistance spectrum than when only a single *R* gene is present. In addition, the pathogen must overcome more than one *R* gene to become infectious, thereby the durability of the resistance is highly increased. Recently, three cisgenic *R* genes were stacked in a susceptible potato cultivar (Zhu et al., 2012). A cisgene, which is a gene derived from the gene pool of sexually compatible species, in this case an *R* gene of a wild *Solanum* species, is introduced in a host plant by genetic transformation. After successful transformation the resistance spectrum of the transformed host was found to be the sum of the spectra of the three individual *R* genes. *R* gene stacking is not feasible with classical breeding, since it takes around 12 years on average to introgress only a single *R* gene (Jacobsen and Schouten, 2007). Cisgenic breeding enables fast transfer of multiple R genes and prevents linkage drag, which is a real issue when using classical breeding (Zhu et al., 2012). The success of this approach in the future is heavily dependent on the availability of R genes. For this reason the urge to find novel *Rpi* genes is high and many wild resistant *Solanum* species are being characterized for this purpose.

Map-based cloning, also referred to as positional cloning, is a technique for localising and isolation a gene of interest with the help of genetics and molecular markers. The development of molecular biology and the construction of genetic maps in the early 1990s made it applicable in genetic studies (Young, 1990). This approach has been proven to be very suitable for cloning *R* genes from wild *Solanum* species since the majority of all novel *R* genes were cloned through this approach (Lokossou, 2010). The first step in map-based cloning is finding closely linked markers to the *R* gene with the help of a segregating population obtained from a cross between a resistant and susceptible genotype. Subsequently a Bacterial Artificial Chromosome (BAC) library is needed for physical mapping. A BAC library consist of the entire genome of the resistant genotype. The genome is cleaved into relatively small DNA segments (~100kb) and inserted into plasmids of *Escherichia coli* to enable rapid cloning. The BAC library must cover the whole genome of the resistant genotype and a coverage of at least 10

times of each part of the genome has to be reached for accurate mapping (Verzaux, 2010). The BAC library is screened for closely linked markers to identify individual positive BAC clones which are subsequently sequenced to determine the exact physical location of the *R* gene. Then a genetic transformation technique is used for transferring the *R* gene into a host plant.

Plant transformation mediated by *Agrobacterium tumefaciens* is the most widely used method to introduce foreign genes into plants (Fig. 1). This bacteria is capable of transferring a DNA segment (T-DNA), located on a tumour-inducing (Ti) plasmid, into the genome of its host plant cell. This plasmid contains virulence genes (*vir*-genes) encoding a set of proteins required for the excision, transfer and integration of the T-DNA during infection. Isolated *R* genes are integrated in a functional DNA vector including a promoter and terminator. Recent research revealed that vector properties, like the length of the promoter and terminator, influence the expression of the *R* gene and therefore late blight resistance (Vossen, 2013). The vector is introduced into the T-DNA of the Ti-plasmid and subsequently cloned in the *A. tumefaciens*. *In vitro*, plant cells derived from a potato cultivar are incubated with transformed Agrobacteria to allow infection and transfer of the T-DNA into the plant genome. Transformed plant cells are selected and chronologically regenerated into callus, plantlets and eventually into genetically modified (GM) plants with the use of plant tissue culture techniques (Stanton, 2003; Hoekema et al., 1984).

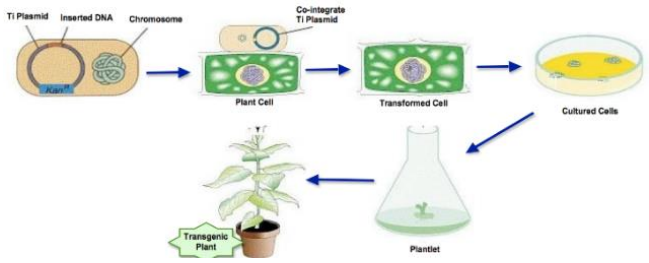


Figure 1. Overview of Agrobacterium-mediated Plant Transformation (University of Toronto, 2000)

Subsequently, mature greenhouse-grown GM plants are screened for resistance to *P. infestans*, using a detached leaf assay (DLA). Although only the resistance of the leaves is tested instead of the entire plant, the DLA turns out to be a good alternative for field testing and speeds up the resistance screening. In this test, leaves from the plants are detached and incubated with spores of the pathogen. After about one week the total percentage of leaf area covered with lesions can be measured and rated on a linear infection scale, ranging from fully resistant to extremely susceptible (Vleeshouwers et al., 1999). At the final stage field trials can be performed prior to cultivation of the novel durable resistance potato.

Research objectives

This thesis covers three subtopics in the cisgenic breeding pipeline aiming at late blight resistance in potato. The objectives and background of the subtopics are listed below.

Physical mapping of a late blight resistance gene, *Rpi-pnt1*, in two BAC libraries of *Solanum pinnatisectum*, a diploid species originating from Mexico. Late blight resistance was mapped to chromosome 7 using a segregation population (Kuhl et al. 2001). Subsequently, a relatively small segregation population (n=76) was made to map more closely linked markers to *Rpi-pnt1* located between TG20a and CP56 (Fig. 2). The DNA segment covered by markers cos 54310 and cos 17200 was sequenced to develop 8 novel markers (A-H). Recombination events of a large segregation population (n=1400), derived from *S. pinnatisectum* accession 204-1, showed that markers E, F, G and H are closely linked to *Rpi-pnt1* (Oortwijn and Arens). Markers E, F and G are linked in coupling and H in repulsion with respect to *Rpi-pnt1*. The goal is to identify a BAC clone which covers the entire DNA fragment including *Rpi-pnt1*. Such a BAC clone can then be sequenced to identify candidate *R* genes on chromosome 7, and ultimately the *Rpi-pnt1* gene can be identified.

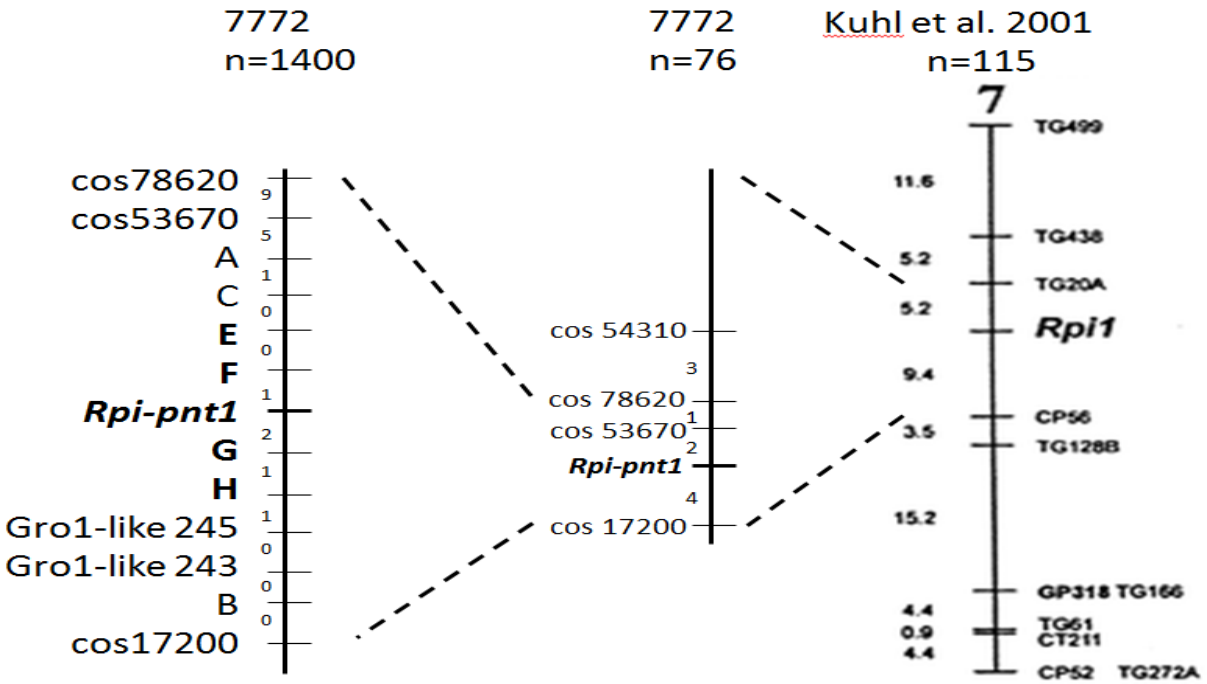


Figure 2. Genetic map of late blight resistance gene, *Rpi-pnt1*, from *S. pinnatisectum*. *Rpi-pnt1* was mapped to chromosome 7. Based on data of DNA sequence between marker cos 78620 and cos17200, 8 novel markers were developed (A-H). Recombination events showed that markers E, F, G and H are closely linked to *Rpi-pnt1* (Kuhl et al., 2001).

Deployability of R genes by transformation to potato cultivars

Requirement of regulatory elements in *Rpi-chc1*, a late blight resistance gene isolated from *S. chacoense*. A previous study showed that only 50% of the transgenic events of a *Rpi-chc1* transgenic Desiree populations showed late blight resistance, while in transgenic Premiere plants less than 5% disease resistant events were found. Transgenic events with the highest transgene copy number and highest expression levels were most resistant. These results indicate that expression of late blight resistance through the *R* gene is a limiting factor. Since the expression of a gene is largely dependent on its corresponding promoter and terminator, properties of these regulatory elements on late blight resistance were studied. The vector used in the previous study had a 3kb promoter and a 700bp terminator. In this study we tested constructs with an extended (4kb) upstream and an extended downstream region (1.2kb). These two constructs were transformed to Desiree previously and transgenic plants were tested for late blight resistance using a detached leaf assay (DLA). The aim was to determine the influence of a larger promoter or terminator size on late blight resistance through the expression of *Rpi-chc1*.

The influence of the genetic background of a potato cultivar on the expression of the integrated late blight *R* gene is determined. The *R9a* gene, also referred to as *Rpi-edn2*, isolated from *S. demissum* (Kwang Ryong Jo, 2013) and from *S. edinense* (Verzaux, 2010), was inserted in six common potato cultivars through Agrobacterium-meditated transformation. These plants were tested for late blight resistance using a DLA. The presence of other *R* genes previously introgressed in some of the used cultivars have been taken into account for choosing the appropriate *P. infestans* isolates. Also a potential correlations between DLA resistance, vector backbone integration and T-DNA copy number was investigated. Ultimately, transgenic plants without vector backbone integrations and proper DLA resistance were selected.

3. Materials and Methods

3.1 BAC library screening

Three BAC libraries, two of *S. pinnatisectum* (4628-D and 4804-pnt*)* constructed from pnt204-1 and one of *S. agrimonifolium* were characterized. The *S. pinnatisectum* genome was cleaved with restriction endonucleases *HindIII* and *BamHI* and had average insert sizes of approximately 100 and 110 kb for 4628-D and 4804-pnt respectively. The *S. agrimonofolium* BAC library was cleaved with *HindIII* and had an average insert size of 130kb. The DNA segments were integrated into *E. coli* pBeloBAC11 vectors, containing a gene encoding chloramphenicol resistance. BAC libraries were made by Bio S&T Inc®, Canada. Both *S. pinnatisectum* libraries consisted of four 96 well plates containing 384 BAC pools each containing 500 different *E. coli* clones. The *S. agrimonifolium* BAC library consisted of six 96 wells plated containing a total of 576 BAC pools each containing 500 different *E. coli* clones. The BAC pools were stored in 15% glycerol at -80 ºC. DNA of the BAC clones was isolated with a QIAprep Spin Miniprep Kit; Qiagen Cat. No. 27104. The coverage of the *Solanum* genome by the libraries was tested by using markers TG63 and TG20, located on chromosome 10 and 7 respectively.

The *S. pinnatisectum* BAC libraries where then screened for 4 CAPS markers flanking *Rpi-pnt1*. On each side of the gene two flanking CAPS markers were used for screening the BAC pools, marker G and H on the right and marker E and F on the left (Table 1). The genome coverage and *Rpi-pnt* screening was carried out with the following thermal cycling conditions: an initial denaturation step at 94 ºC for 5min; then 30 amplifying cycles of 94 ºC for 30s, Ta for 30s, 72 ºC for 1min; and a final extension step at 72 ºC for 5min (Table 1). PCR products were run on a 1% w/v agarose gel at 100 V for 25 minutes.

Table 1. Markers used for screening and validating the BAC libraries.

Marker		Nucleotide sequence (5' – 3')	T_a (ºC)	Size (bp)	Enzyme
TG63	F	CCC AGA GTC CCC CTT CCT ATT	55	~1000	n/a
	R	CGA GAT GTT GAA TTT GCG TAA GA			
TG20	F	GTG CAA TCC TGC TGT TGG AAC C	56	500	n/a
	R	CCA GCA TTA CAT AGG GTT ACC AT			
CAPS E	F	ACC TCC TGG CAA TGT AAA C	60	~1400	*Hpy*FigVI
	R	GAT CGG TTT TCC CCA TTT CT			
CAPS F	F	AAT GAC ATG GAC TCC CCT TG	60	~1200	*Hin*F1
	R	CAA TGT TCC ACG TTG GTG TC			
CAPS G	F	TTT TTG CTT GCC GTT TAT GA	60	~1200	*Hpy*CH4IV
	R	TAG TGA GCG TGA GGC TTG TG			
CAPS H	F	TAA ATC CAG GCT CCC ACA CT	62	~1000	*Hin*F1
	R	CAG GGG GAA GGG GAA ATT AT			

BAC pools containing one of the 4 CAPS markers were cleaved with the corresponding restriction enzymes to determine if the resistant (*Rpi-pnt1*) or susceptible allele was present (Table 2). 5 µl CAPS marker PCR product was cleaved with 1 µl restriction enzymes and incubated for 2 hours at 37 ºC. The restriction digests were visualized on a 1% w/v agarose gel at 100 V.

Subpools of the positive BAC pools were made to find the single BAC clone containing *Rpi-pnt1*. Glycerol stocks of the corresponding E. coli BAC pool were diluted in Lysogene borth (LB) medium (10 g/L Tryptone, 5 g/L yeast extract, 5 g/L NaCl). Several dilutions (10x, 100x, 1000x) were made to measure cell density of the glycerol stock at 600 nm using a spectrophotometer. Based on the cell density dilutions were made of the glycerol stock of positive BAC pools in LB medium. A certain amount of the dilution, corresponding to approximately 250 *E. coli* clones, was plated out on a series of petri dishes filled with 20 ml lysogene borth agarose (LBA) medium (10 g/L Tryptone, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L Agar, Bacteriological, 0,01 mM chloramphenicol) and cultivated overnight at 37 °C. The following day 1 ml LB was added to each petri dish and a blue spreader was used to loosen the E. coli colonies to obtain a suspension. 750 µl of the LB suspension of each petri dish was transferred to a 1,5 ml eppendorf tube. A PCR with the corresponding CAPS primers (Table 2) was performed on the subpools and visualized on a 1% w/v agarose gel using GelRed™ staining. The subpooling step was repeated until the BAC pool was reduced to less than 100 *E.coli* clones. At that stage PCR reactions were performed on single BAC clones by picking single colonies of the petri dish.

3.2 Deployability of *R* genes by transformation to potato cultivars

Plant material

Plant populations derived from 6 common potato cultivars, Desiree, Premiere, Aveka, Atlantic, JV18 and JV19, transformed with *R9a* were evaluated for late blight resistance (Table 2). Furthermore, there were two Desiree populations, A58-x and A59-x, both transformed with *Rpi-chc1*. A total of 240 plants, each population consisted of 30 transformed individuals, obtained through Agrobacterium-mediated transformation, were present in the greenhouse. The transformed callus were grown *in vitro* in a climate room at 24 ºC with 16-h light and 8-h darkness for two weeks and thereafter moved to the greenhouse. Each individual plant originated from different callus tissue (event number) to acquire independent transformation.

Transgenic populations	Cultivar	Endogenous R genes
A73.4	Desiree	n/a
B73.4	Premiere	R10
C73.4	Aveka	R10
H73.4	Atlantic	n/a
P73.4	JV18	R2
V73.4	JV19	n/a

Table 2. The 6 potato cultivars with information about pedigree and R gene content. (Wur potato pedigree database).

Detached leaf assay

Prior to the DLA plants have grown for five weeks in the greenhouse (seven weeks for the second DLA). From each individual, two petioles with fully expanded leaves were collected and put on filter paper, saturated with tap water, in a plastic tray (50 cm x 30 cm). Every two petioles of an individual received a single label, indicating potato cultivar, event number and the *P. infestans* isolates they received. In the laboratory the petioles were translocated to new plastic trays, containing water-saturated filter paper and blocks of florist foam (Oasis®). The petioles were diagonally cut in such way that on each contrary side of the block a petiole could be fitted (Fig. 3). The leaves are placed with the abaxial side up to enhance infection. The label was pinned on the block of florist foam with a skewer.

Figure 3. A plastic tray covered with saturated filter paper, containing blocks of florist foam and potato petioles with corresponding labels.

The *P. infestans* isolates, IPO-C, NL08532 and 90128, were multiplied and maintained on Rye Sucrose Agar (RSA) medium at 15 ºC in the dark (table 3). Two weeks prior to the detached leaf assay (DLA) inoculum was transferred to a new petri dish plate (90mm x 15 mm) containing RSA medium and incubated. To prepare the inoculum, 10 ml of cold tap water was added and a glass spatula was subsequently used to loosen the pathogenic spores from the plate. The spore suspension was transferred to a 50 ml blue cap tube and incubated for 2 hours at 4 ºC to induce germination of the zoospores. After incubation the zoospore concentration was determined, using a light microscope, and further diluted with cold tap water to obtain a final concentration of 50.000 spores per ml.

Table 3. List of isolates used in the detached leaf assay.

Isolate	Country of Origin	Virulence*	Reference
IPO-C	Belgium	1,2?,3,4,5,6,7,10,11	Champouret et al. 2009
NL08532	The Netherlands	1,2?,3,4,5,6?,7?,8,10,11	Vossen J., Kessel G., 2008
90128	The Netherlands	1,2,3,4,7,8,10,11	Champouret et al. 2009

* Virulence spectrum is based on ability to infect the differential set of potato carrying R1 to R11 (Black et al. 1953).

The abaxial side of the leaves was inoculated with several 10 µl droplets, containing approximately 500 spores, of the selected *P. infestans* isolates. The leaflets or *R9a* transformed plants were inoculated with isolates IPO-C and NL08532. The leaves on the left side of both petioles in addition to the left side of both upper leaves were inoculated with two droplet IPO-C isolate per leaf. The same applies to the leaves on the right side of both petiole and the right side of the upper leaf only these were

inoculated with isolate NL08532. The *Rpi-chc1* transformed leaves of Desiree, two petioles, were inoculated with isolates IPO-C and NL08532. The second DLA was performed 2 weeks later with isolates 90128 and IPO-C. The trays were enclosed with a transparent plastic bag to acquire a 100% humidity favoring conditions for infection. Subsequently the tray was placed in a climate cell at 15 ºC with 16-h light and 8-h darkness.

After six days of incubation in the climate cell the leaves were screened for infection, using a disease severity scale, ranging from the most vulnerable V8, through V7, V6, V5, R6, R7, R8 to R9, being the most resistant response (Table 4). Weighting factor were subsequently used for analyzing the late blight resistance score of each isolate-genotype combination.

Table 4. *P. infestans* disease severity scale used for the detached leaf assay.

Score	Observation	Diameter of (HR) / Lesion (mm)	Weighting factor	Group*
R9	Non visible Hypersensitive response (HR)	0	0	R
R8	Small HR	~ 1.0 - 2.5	1	R
R7	Medium HR	~ 2.6 - 10	2	R
R6	Large HR	> 10	3	MR
V5	Large dry lesion, no sporulation	< 20	4	MR
V6	Large water soaked lesion, no sporulation	> 20	6	S
V7	Large lesion, sporulation on adaxial side	> 20	8	S
V8	Large lesion, sporulation on both sides	> 20	9	S

*Individuals were grouped in Resistant (R), Moderate resistance (MR) or Susceptible (S).

DNA isolation

Two grinding balls (3175-mm) were added to each collection microtube (Collection Microtubes, racked, 96-wells; Qiagen cat no. 19560). Unexpanded young leaves were collected from 261 plants, which have grown for 8 weeks in the greenhouse, and were put into tubes and then capped (Collection Microtube Caps, 960 in strips of 8; Qiagen cat. no. 19566). The tubes were stored at minus 81 °C. After 5 days the tubes were taken out and cooled with liquid nitrogen prior to grinding of the leaves. The leaves were grinded for 2 min at 20Hz (Tissuelyser II; Qiagen cat. no. 85300). Then 400 µl isolation buffer (per 100 samples, ~ 40 ml: 16.8 ml lyses buffer [pH 7.5], 16.8 ml extraction buffer [pH 7.5], 6.7 ml 5% sarkosyl, 100 mg sodiumbisulfite (table 5 and 6)) was added to each sample. The samples were mixed by gently inverting the racks a couple of times and incubated at 65 ºC for 1 hours while mixing occasionally. Then 400 µl chloroform/isoamylalcohol (24/1) was added to each sample and mixed well. The samples were centrifuged (Heraeus™ Multifuge 3SR) at 4600 rpm for 10 min. 350 µl supernatant of each sample was transferred to a new microtube. Then 200 µl isopropanol was added to each tube and closed with new strips of 8 caps. The tubes were mixed by gently inverting the racks a couple of times and centrifuged at 4600 rpm for 5 min. The supernatant was then poured off. 300 µl

70% ethanol was added to the pellet and centrifuged at 4600 rpm for 5 min. The supernatant was poured off and the pellet was air dried at room temperature overnight. The following day each pellet was dissolved in 100 µl TE-RNase (10 mM Tris [pH 8], 1 mM EDTA [pH 8], RNase 10 µg/ml). The DNA samples were then diluted 10 times with MilliQ and stored at -20 ºC.

Table 5: Lyses buffer preparation.

Compound	Final concentration
Tris-HCl (pH 7.5)	0.2 M
EDTA (pH 8.0)	0.05 M
NaCl	2 M
CTAB	2%

Table 6: Extraction buffer preparation.

Compound	Final concentration
Tris-HCl (pH 7.5)	100 mM
EDTA (pH 8.0)	5 mM
Sorbitol	0.35 M

Vector backbone screening

Transgenic Desiree populations A58 and A59 were screened for *Rpi-chc1* and transgenic populations A73.4, B73.4, C73.4, P73.4, and V73.4 were screened for *R9a* (Table 7). Besides, all transgenic plants were also screened for the presence of *npt*III (Table 7). Genotypes not containing this selection marker, kanamycin resistance, were selected and screened for backbone markers present in the vector (Fig. 4; Table 7). The following thermal cycling conditions were used for the PCR: an initial denaturation step at 94 ºC for 5min; then 30 amplifying cycles of 94 ºC for 30s, Ta for 30s, 72 ºC for 30s; and a final extension step at 72 ºC for 5min (Table 8 and 9). PCR products were run on a 1% w/v agarose gel at 120 V for 25 minutes.

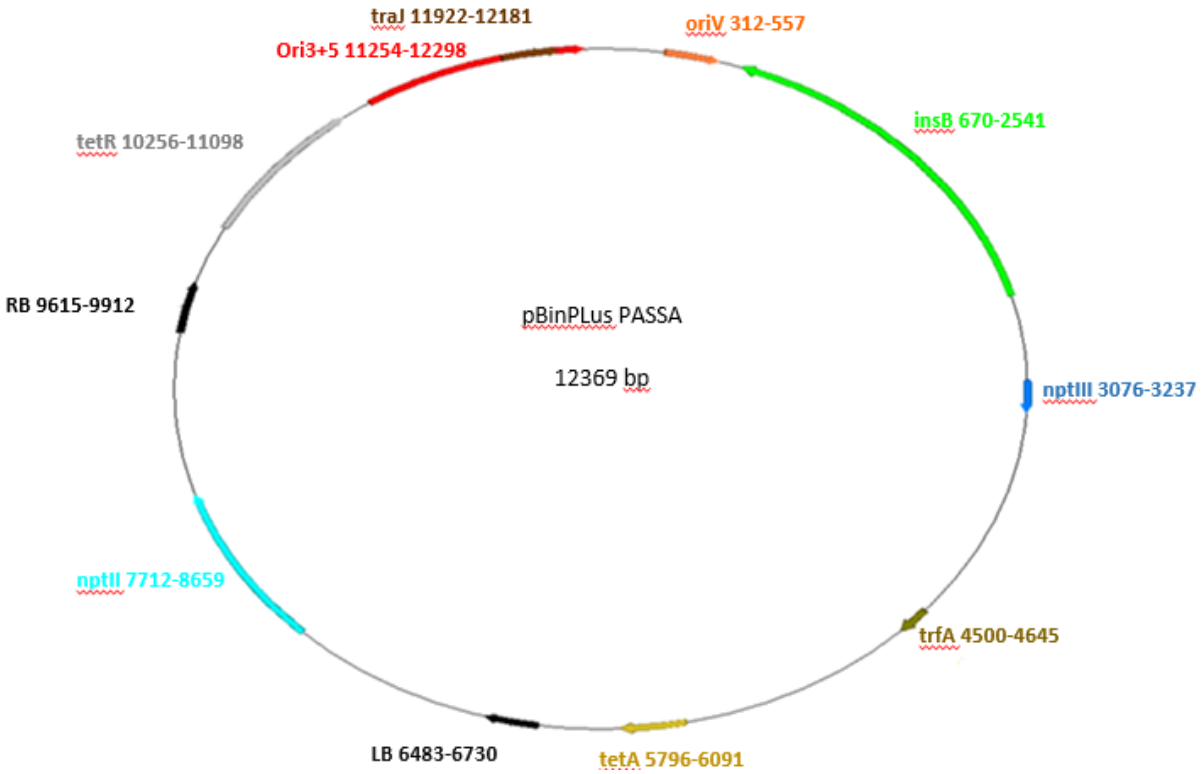


Figure 4. Location of the markers on the pBinPlus PASSA vector. The image was created using plasmid editor software ApE®, version 2.0.47 (Davis MW, 2013).

Table 7. *Rpi* gene- and vector backbone markers.

Marker		Nucleotide sequence (5' – 3')	T_a (°C)	Size (bp)
R9a	F	GCA TCA TGT CTG CAC CTA TG	54	450
	R	CTT TGA TGT GGA TGG ATG GTG		
Rpi-chc1	F	ACA GAT AAT AAT TTT CAA CTG C	57	400
	R	ATT TGG GAC ATT CTG ATA TAA G		
nptIII	F	GAA AGC TGC CTG TTC CAA AG	61	162
	R	GAA AGA GCC TGA TGC ACT CC		
RB	F	CCT GGC GTT ACC CAA CTT AAT	60	298
	R	ACC CGC CAA TAT ATC CTG TCA		
traJ	F	ACG AAG AGC GAT TGA GGA AA	63	260
	R	CAA GCT CGT CCT GCT TCT CT		
oriV	F	ATA AGT GCC CTG CGG TAT TG	61	246
	R	GCA GCC CTG GTT AAA AAC AA		
insB	F	GCG CTA TCT CTG CTC TCA CT	63	1872
	R	AAC GGC CTC ACC CCA AAA A		
trfA	F	CGT CAA CAA GGA CGT GAA GA	62	146
	R	CCT GGC AAA GCT CGT AGA AC		
tetA	F	CTG CTA GGT AGC CCG ATA CG	61	296
	R	CCG AGA ACA TTG GTT CCT GT		
tetR	F	GGG GGA GGG GAT GTT GTC TA	60	843
	R	AGG GGT ATG TTG GGT TTC AC		
oriv3+5	F	TGC GGC GAG CGG TAT CAG	63	1045
	R	CTT CTT GAT GGA GCG CAT GGG		
LB	F	CAA CGC TCT GTC ATC GTT A	64	248
	R	TTA CAC CAC AAT ATA TCC TGC C		
virG1s	F	CAA TAG TAG CTG TAA CCT CG	55	692
	R	ACC TGC CGT AAG TTT CAC C		
nptII	F	CTC CTG TCA TCT CAC CTT GC	58	948
	R	TAA TCAT CGC AAG ACC GGC		

Real-time quantitative PCR

To quantify the transgene copy number of *R9a* in the transformed plants a real-time quantitative PCR was performed. *NptII* transgenic-specific primers were used to estimate *R9a* transgene copy number (Table 8). The assay was performed with the IQ SYBR green biorad supermix (Bio-Rad, Hercules, CA, U.S.A) in a 22.5 ul total volume, following manufacturer's recommendations, containing 4.5 ul (10-100 ng/ul) DNA template. *R9a* copy number were normalized relative to housekeeping gene *EF1*-alpha (Table 10). Each reaction was performed in duplo (2x 10ul PCR mix) and assayed in a Biorad CFX-96 qPCR (Bio-Rad, Hercules, CA, U.S.A). The following thermal cycling conditions were used

for the qPCR: an initial denaturation step at 95 ºC for 3min; then 40 amplifying cycles of 95 ºC for 30s and 60 ºC for 1min (+ plate read); and a final melting curve evaluation. Results were visualized with Bio-Rad CFX manager software v1.6.541.1028 (Bio-Rad Laboratories, CA, U.S.A.). The cycle threshold (C_t) value was determined by the Biorad CFX-96 qPCR machine by taking the amplification curves of each reaction into account. Three transgenic Desiree controls with known *nptII* copy numbers (#), A03-10(1), A03-12(1) and A03-01(2), were included in the analysis. These controls function as calibrators and are used to convert the obtained ΔC_t values into transgene copy numbers.

Table 8. Overview of the primers used for the real-time quantitative PCR.

Marker	Nucleotide sequence (5' – 3')	T_a (ºC)	Size (bp)
NptII	F GTC CCG CTC AGA AGA ACT CGT C	57	~125
	R CGT TGG CTA CCC GTG ATA TTG C		
EF1-α	F ATT GGA AAC GGA TAT GCT CCA	60	~125
	R TCC TTA CCT GAA CGC CTG TCA		

4. Results

4.1 Validation of BAC libraries

Prior to the screening for *Rpi-pnt*, the *S. pinnatisectum* genome coverage of both BAC libraries were determined. Marker TG63, located on chromosome 10, was used for this purpose. When taking into account a diploid genome with a size of 800 mb, a BAC library consisting of four 96 wells each well (pool) containing 500 *E. coli* BAC clones with an average insertion size of 100 kb, a 12 times coverage is expected. In total, TG63 was present in 92 and 115 pools which indicates a genome coverage of 11.5 and 14.4 in BAC library I and BAC library II respectively. Both BAC libraries meet the requisite of having a coverage of at least 10 times the *S. pinnatisectum* genome.

Furthermore, the genome coverage of a *S. agrimonifolium* BAC library was determined. Previous research have shown that this species also harbors late blight resistance due to the presence of an *R* gene, *Rpi-agf1*. Screening the BAC library for this *R* gene was not performed in this study. Since this *Solanum* species is a tetraploid, a larger BAC library was made to achieve a sufficient coverage of the entire genome. Marker TG20, located on chromosome 7, was used for screening the genome coverage. When taking into account a tetraploid genome with a size of 800 mb, a BAC library consisting of six 96 wells each well (pool) containing 500 *E. coli* BAC clones with an average insertion size of 130 kb, a 11.7 times coverage is expected. Markers TG20 was present in 142 BAC pools, which indicates a 5.9x coverage of the *S. agrimonifolium* genome. This coverage is rather low compared to the other two *S. pinnatisectum* BAC libraries.

4.2 Screening of *S. pinnatisectum* BAC libraries with flanking markers

In a previous study a *S. pinnatisectum* BAC clone, 2A4, flanking *Rpi-pnt1* was isolated from a BAC library. By analyzing the sequence of the insert of this BAC clone, it was concluded that it contained markers E and F and thus the left flanking region of *Rpi-pnt1* (Fig. 5). In this study an attempt was made to identify a BAC clone which covers the entire DNA fragment including the *R* gene allele. Several BAC pools were found to contain one or both flanking regions (Fig. 5).

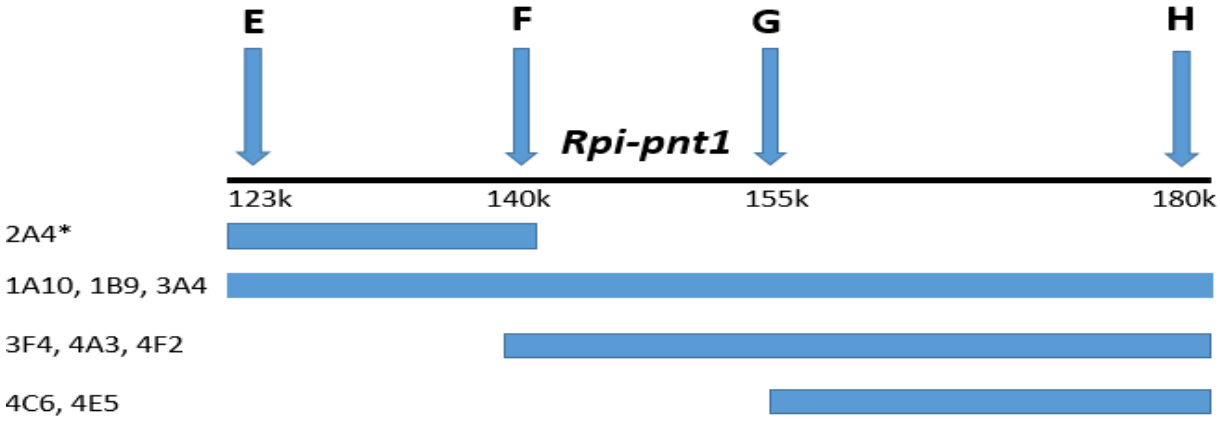


Figure 5. A physical map of *Rpi-pnt1* with locations of flanking markers E, F, G, and H on chromosome 7 of *S. pinnatisectum*. BAC clone 2A4 (*) containing the region on the right side of the R gene was isolated and sequenced in a previous study. Multiple BAC pools containing one or both flanking regions in coupling phase with *Rpi-pnt1* have been found. BAC pools 1A10, 1B9 and 3A4 contain all four markers and thus both flanking regions. Other BAC pools contain a part of the flanking regions; 3F3, 4A3, 4F2 contain 3 flanking markers and 4C6 and 4E5 contain the right flanking region.

The first BAC library of *S. pinnatisectum* was restricted with *HindIII* and had an average insert size of 100kb. The screening for *Rpi-pnt1* was performed on 48 columns pools, each containing 8 BAC pools belonging to a single column of one of the four 96 well plates. The column pools were screened for the Rpi-pnt1 flanking markers E, F, G, and H (Appendix I). Although the coverage was sufficient in unlinked genomic regions, only 2 positive BAC pools were found in the *Rpi-pnt1* region, (pool D4 and B11 for marker H and E respectively; Fig. 6). Genomic DNA of a resistant parent and susceptible parent were included as well as a positive control as to determine if the resistant allele was present in one of the column pools. The latter was determined by analysing digestion patterns after restriction by CAPS enzymes.

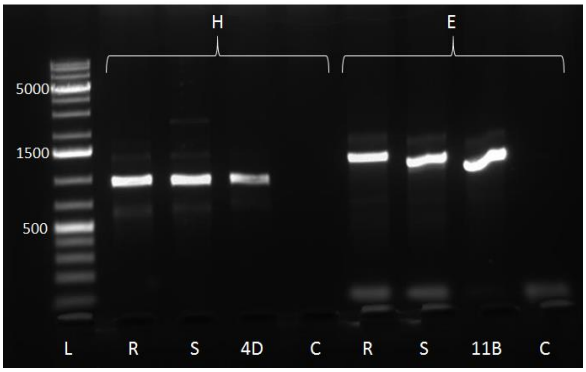


Figure 6. Digital image of *Rpi-pnt1* flanking CAPS marker (H and E) run on a 1% agarose gel, stained with GelRed™. From left to right: 1kbp Ladder, Resistant parent (R), Susceptible parent (S), positive column pool and negative control (C).

PCR products H and E were cleaved with restriction enzymes *Hin*fI and *HpyFig*VI, respectively. The results indicate that the susceptible allele is present in column pool D4 since marker H is linked in repulsion to *Rpi-pnt1* (Fig. 7A). The restriction digests of marker E indicates that column pool B11 also contains the susceptible allele (Fig 7B).

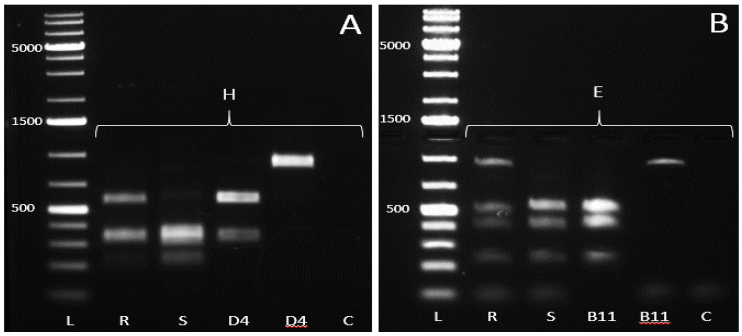


Figure 7. Digital image of restriction digests of *Rpi-pnt1* flanking CAPS marker (H and E) run on a 1% w/v agarose gel, stained with GelRed™. From left to right: 1kbp Ladder, Resistant parent, Susceptible parent, BAC pool, non-cleaved BAC pool and negative control. (A) CAPS Marker H, BAC pool 4D digests matches with resistant parent. (B) CAPS Marker E, BAC pool 11B digests matches with susceptible parent.

The second BAC library of *S. pinnatisectum* was cleaved with *BamHI* and had an average insert size of 110kb. Unlike the first BAC library the screening was performed on each individual BAC pool instead of column pools. The number of BAC pools containing E, F, G and H was respectively 35, 24, 37 and 60. This numbers indicate that the markers are not equally represented in the BAC library. The scoring results of each individual BAC pool can be found in the Appendix II. 18 BAC pools, selected on the basis of the presence of multiple CAPS markers, were restricted by CAPS enzyme to determine if the resistant allele was present (Table 9, Appendix III). Three BAC pools, 1A10, 1B9 and 3E4, cover both flanking regions including *Rpi-pnt1* since there restriction patterns correspond with the restriction pattern of the resistant parent, taking into account the phase of the markers. BAC pools, 2F9 and 4B3, also contain both flaking regions although in this case the susceptible allele is present (Table 9).

Table 9. Allelic status of 18 BAC pools containing one or multiple *Rpi-pnt1* flanking CAPS markers.

Marker / BAC pool	CAPS E	CAPS F	↓ *Rpi-pnt1*	CAPS G	CAPS H
1A10*	C	C		C	R
1B9*	C	C		C	R
2A5				R	C
2F9	R	R		R	C
2G1				C	
3E4	C	C		C	R
3F4		C		C	C
4A3		C		C	C
4B1	C?	C		C	R
4B3	R	R		R	C
4B12				C	
4C1		R		R	C
4C6				C	R
4D9		R		R	C
4E5				C	R
4E12				C	
4F2*		C		C	R
4F9	R			R	C
Resistant parent	C	C		C	R
Susceptible parent	R	R		R	C

CAPS markers E, F and G are linked in coupling (C) and H in repulsion (R) with respect to *Rpi-pnt1*. Pools colored dark grey potentially contain a BAC clone in which the resistant *Rpi-pnt1* allele is present. * Selected pools for isolation of single coupling phase BAC clones.

4.3 Isolation of single BAC clones

Effort was made to isolate the BAC clone, containing marker H, out of column pool 4D in the first *S. pinnatisectum* BAC library. Although marker H was present in coupling phase in this pool and thus does not contain the *Rpi-pnt1*, it is of scientific interest to know the difference between the susceptible and resistant allele of *Rpi-pnt1* and single BAC clone isolation was pursued. The pool was reduced from 500 to approximately 150 BAC clones. However, insufficient time was left to reduce the pool size further and isolate the single BAC clone.

Isolation of coupling phase single BAC clones (1A10, 1B9 and 4F2) from the second BAC library was outsourced to Bio S&T Inc®, Canada, who made the BAC libraries (Table 9).

4.4 Employment of R genes in potato cultivars: influence of regulatory elements

A previous study showed that 50% of a *Rpi-chc1* transgenic Desiree population, containing a 3kb promoter and 700bp terminator, was late blight resistant to *P. infestans* isolate 90128. In order to determine the influence of regulatory elements two *Rpi-chc1* transgenic Desiree populations (A58: 4kb promoter and a 700bp terminator, A59: 3kb promoter and a 1.2kb terminator) varying in promoter and terminator size were evaluated for late blight resistance. A similar DLA, with the same *P. infestans* isolates, was performed on the two transgenic Desiree populations transformed with late blight resistance gene *Rpi-chc1*. The results of the DLA are shown in table 10 and Appendix V.

Table 10. Number of resistant (R), moderate resistant (MR) and susceptible (S) individuals of both transgenic *Rpi-chc1* Desiree populations, varying in promoter and terminator size, for three different *P. infestans* isolate.

Isolate / Genotype	IPO-C		NL8532		90128	
	R MR S*	%R+MR**	R MR S	%R+MR	R MR S	%R+MR
A58 (4kb pro. / 700bp ter.)	4 : 4 :16	33.3 (b)	5 : 3 : 16	33.3 (b)	6 : 9 : 8	65.2 (b)
A59 (3kb pro. / 1.2kb ter.)	1 : 1 : 28	6.7 (a)	1 : 2 : 27	10.0 (a)	0 : 3 : 26	10.3 (a)
Desiree	*All S*		*All S*		*All S*	

* Plants were scored based on the average DLA score: R<2, MR; 2-3.9, S; >4. The presence of certain *R* and *Avr* genes in respectively genotype and isolate is given. **An ANOVA test has been performed on these percentages of resistant (R and MR) transgenic events to see if there are significantly differences, indicated by a, b, between the genotypes for each of the isolates.

A high proportion of susceptible transgenic events were observed especially in the A59 population containing an extended terminator (Table 10). Only a few transgenic events of this population showed late blight resistance. The results suggest that enlarging the size of the promoter or terminator do not contribute to increased late blight resistance, since less than 50% resistant transgenic events were observed among both transgenic lines. An exception to this are the results of A58 populations tested for isolate 90128, where a high level of late blight resistance was observed. A larger terminator appears to have a more detrimental effect on late blight resistance than a larger promoter.

4.5 Deployability of R genes in potato cultivars: influence of genetic background

The influence of the genetic background of a potato cultivar on the expression of the integrated *R* gene was determined. For this purpose, a detached leaf assay was performed to phenotype late blight resistance of *R9a* transgenic potato populations, derived from six common potato cultivars. Three *P. infestans* isolates, IPO-C, NL08532 and 90128, were used for inoculating the leaflets (Table 4). Since some of the cultivars already contain *R* genes (*R10* in Aveka, Premiere; *R2* in JV18), the isolates were selected on their virulence spectrum. In this way only the *R9a* provides late blight resistance towards the selected isolates in the DLA on the transgenic plants. IPO-C does not contain *Avr2* and *Avr10* and is thus virulent on all non-transgenic cultivars. However, this was not the case for the other two isolates. Isolates, 90128 and NL08532, both contain *Avr2* and are avirulent on JV18. The results of the DLA are shown in Table 11 and Appendix IV. A distinction was made between complete and moderate late blight resistance by grouping R9, R8, R7, and R6, V5 plants respectively (Table 4). The percentage of transgenic events per cultivar possessing late blight resistance, combining complete and moderate, to a certain isolate are also given. Controls, comprising the results of two non-transformed plants, are included in the table to verify if late blight resistance to a certain isolate was already present in the genotypic background of the cultivar. An unbalanced ANOVA test indicated that there was significant differences between late blight resistance among the genotypic backgrounds (Table 11; Appendix V).

Table 11. Number of complete resistant (R), moderate resistant (MR) and susceptible (S) *R9a* transgenic events per genotype - *P.infestans* isolate treatment.

Isolate / Genotype	NL08532 (*Avr2*) R MR S*	%R+MR**	IPO-C R MR S	%R+MR	90128 (*Avr2*) R MR S	%R+MR
A73.1 *Desiree*	14 : 3 : 12 *All S*	56.6 (c)	14 : 7 : 9 *All S*	70.0 (bc)	3 : 11 : 16 *All S*	46.7 (c)
B73.1 Premiere (R10)	16 : 5 : 9 *All S*	70.0 (b)	8 : 10 : 12 *All S*	60.0 (c)	3 : 3 : 23 *All S*	20.7 (d)
C73.1 Aveka *(R10)*	18 : 8 : 1 *All S*	96.3 (a)	11 : 13 : 3 *All S*	88.5 (a)	17 : 2 : 7 *All S*	73.1 (b)
H73.1 Atlantic	13 : 10 : 6 *All S*	79.3 (b)	2 : 19 : 8 *All S*	67.3 (bc)	0 : 0 : 29 *All S*	0.00 (d)
P73.1 JV18 *(R2)*	**28 : 0 : 0 *All R***	**100 (a)**	22 : 5 : 1 *All S*	91.1 (a)	**25 : 1 : 1 *All R***	**96.3 (a)**
V73.1 JV19	9 : 7 : 6 *All S*	72.7 (b)	6 : 11 : 5 *All S*	74.1 (bc)	0 : 3 : 18 *All S*	14.3 (d)

(*) Plants were scored based on the average DLA score: R<2, MR; 2-3.9, S; >4. The presence of certain R and Avr genes in respectively genotype and isolate is given. (**) An ANOVA test has been performed on these percentages of resistant (R and MR) transgenic events to see if there are significantly differences, indicated by a, b, c, d, between the genotypes for each of the isolates. Bold font type indicates that the observed resistance is not caused by the transgene but is due to resistance in the background.

Most of the transgenic events derived from cultivars Aveka and JV18 show either complete or moderate resistance to all three *P. infestans* isolates (Table 11). The majority of the transgenic events of the other four cultivars, Desiree, Premiere, Atlantic and JV19, show resistance to isolate NL08632 and IPO-C. This indicates that the inserted late blight resistance gene *R9a* is actively expressed, since the non-transformed controls of these cultivars are all susceptible to both isolates. Although the percentages

of transgenic events derived of these four cultivars showing resistance to isolate IPO-C, 70.0, 60.0, 67.3, 74.1 for Desiree, Premiere, Atlantic, JV19 respectively, are not significantly different (at P=0.05), the ratio between complete and moderate resistant transgenic events differ considerably among the cultivars. Atlantic and Aveka have relatively many transgenic events with moderate resistance to IPO-C in comparison with Desiree and Premiere. Many of the transgenic events of the four last mentioned cultivars show susceptibility to isolate 90128. This is especially true for Atlantic and JV19 where only 0% and 14.7% of the transgenic events respectively show moderate resistance to 90128. Overall, 90128, showed very limited avirulance towards *R9a*.

4.6 Effects of transgene copy number and vector backbone integration

Since there was significant difference between late blight resistances among, but also within the *R9a* transgenic populations, molecular analyses were performed to find out the underlying cause (table 10). Therefore, *R* gene copy number of each transgenic event was determined by quantitative real-time PCR to see if this number correlates with late blight resistance. Mean transgene copy number among the 6 genotypic backgrounds was significantly different (F_{prop}: 0.035; Appendix V). On average, the lowest transgene copy numbers were found in populations A73.4 (Desiree) and V73.4 (JV19), while the highest copy numbers were found in populations B73.4 (Premiere) and H73.4 (Atlantic). Although the large variety in copy number, ranging from 1 to 50, an unbalanced ANOVA test (treatment: copy number, blocking: genotype) did not show any significant correlation between transgene copy number and late blight resistance for any of the isolates. However, the data suggest a trend between transgene copy number and late blight resistance for IPO-C, since most of the resistant individuals for this isolate had a relatively high copy number (Appendix V).

Subsequently, the potential correlation between late blight resistance and the presence of vector backbone of the plasmid was examined. Also cultivar correlation with vector backbone integration was studied. Another purpose of this analysis was to select transgenic events which does not contain any vector backbone for a follow-up study. A figure of the plasmid used for Agrobacterium transformation can be found in the material and method section (Fig. 4). Each individual was screened for the presence of the kanamycin resistance gene *nptIII*, which supply *A. tumefaciens* resistance to the antibiotic. The *Rpi* is located between the left and right border of the T-DNA and *nptIII* on the vector backbone. Transgenic events derived from a successful transformation do not contain any vector backbone and therefore also no *nptIII*. With an ANOVA test no significant correlation was observed between the presence of vector backbone and late blight resistance among and within the six *R9a* transgenic populations. There was also no significant cultivar correlation with respect to vector backbone integration. However, despite of this result the analysis was useful for making a selection of backbone free transgenic events for a follow-up study where field resistance will be tested, see Paragraph 4.5.

4.7 Selection of transgenic events for testing field resistance

Transgenic events free of vector backbone were selected for follow-up studies. The aim of this study was not to obtain marker-free transgenic plants since *nptII*, located on the T-DNA besides the *R* gene, is present in each plant. However, a requirement for performing a field experiment to test late blight resistance is that the plants do not contain any vector backbone. In table 12, the backbone-free *R9a* transgenic plants are listed with corresponding results of late blight resistance and transgene copy number. As aforementioned in the previous paragraph, table 12 shows that transgene copy number is higher among transgenic Atlantic individuals. Although a correlation between transgene copy number and late blight resistance was not significantly different the results in table 12 do suggest a trend. Among the backbone free transgenic events of Premiere only the event with the highest copy number was resistant to IPO-C. Furthermore, when examining the backbone free Atlantic events, complete resistance (R) was only observed among the two events with the highest copy numbers.

Table 12. Late blight resistance and transgene copy number in events without vector backbone.

Transgenic event	Genotype	NL08532	IPO-C	90128	*R9a* Copy number
A73.4-01	Desiree	R	R	S	1
A73.4-18	Desiree	S	S	S	3
A73.4-19	Desiree	R	R	R	4
A73.4-44	Desiree	R	R	MR	6
A73.4-45	Desiree	MR	MR	MR	1
A73.4-50	Desiree	MR	MR	S	11
A73.4-51	Desiree	S	MR	S	2
B73.4-04	Premiere	MR	S	S	1
B73.4-32	Premiere	R	R	S	11
B73.4-51	Premiere	S	S	S	9
B73.4-58	Premiere	R	S	S	3
C73.4-02	Aveka	MR	MR	S	6
C73.4-20	Aveka	R	R	R	9
C73.4-21	Aveka	R	R	R	7
C73.4-23	Aveka	R	R	R	1
C73.4-24	Aveka	MR	S	S	1
C73.4-34	Aveka	R	MR	R	4
C73.4-39	Aveka	MR	MR	R	3
H73.4-05	Atlantic	MR	MR	S	6
H73.4-17	Atlantic	MR	S	S	2
H73.4-21	Atlantic	R	MR	S	30
H73.4-23	Atlantic	MR	MR	S	6
H73.4-29	Atlantic	R	R	S	13
H73.4-30	Atlantic	MR	MR	S	16
H73.4-34	Atlantic	MR	MR	S	4
P73.4-02	JV18	R	R	R	3
P73.4-03	JV18	R	R	R	5
P73.4-04	JV18	R	R	R	15
P73.4-23	JV18	R	R	R	4
P73.4-49	JV18	R	MR	R	7
V73.4-02	JV19	R	MR	S	9
V73.4-05	JV19	R	R	S	17
V73.4-22	JV19	R	S	S	7
V73.4-32	JV19	MR	R	MR	3
V73.4-38	JV19	S	S	S	4

5. Discussion and Conclusions

5.1 Screening of a late blight resistance gene in BAC libraries of *S. pinnatisectum*

The genome coverage of both *S. pinnatisectum* Bacterial Artificial Chromosome (BAC) libraries was adequate for screening *Rpi-pnt1*. Several BAC pools have been found to potentially contain both or a part of the flanking regions of *Rpi-pnt1* on chromosome 7 in *S. pinnatisectum* (Table 9). However, a BAC pool consists of approximately 500 clones thus it may be that the observed flanking region, derived from the presence of the closely linked markers, originates from two different BAC clones. Therefore, it is necessary to isolate the single BAC clone out of the pool to verify if it contains the entire genomic region. Subsequently the insert of the BAC clone can be sequenced to identify candidate genes.

Remarkably, all coupling phase pools were found in the second BAC library. This may be due to the fact that different enzymes were used for cleaving the *S. pinnatisectum* genome prior to developing both libraries. When a lot of restriction sites of a certain enzyme are located in a genomic region, this region will be cleaved in relatively small fragments (>100kb). Since only inserts of around 100kb were selected, these genomic regions are barely or not represented by the BAC library.

5.2 Deployability of *R* genes in potato cultivars: influence of regulatory elements

A high level of late blight susceptibility was observed among a *Rpi-chc1* transgenic Desiree population, transformed with a construct with an extended promoter (4kb). Two-thirds of the transgenic events was susceptible to both IPO-C and NL08532. A much higher proportion (65.5%) of the populations showed resistance to isolate 90128. However, the latter results did not significantly differ from the results of a *Rpi-chc1* transgenic Desiree population, transformed with a normal sized upstream (3kb) and downstream (1.2kb) region. A previous study showed that 50% of this transgenic Desiree populations was resistant to 90128. Therefore, it appears that extending the promoter does not alters the expression of *Rpi-chc1* in a transgenic plant.

Unlike the extension of the promoter region, it appears that enlarging the terminator region alters the expression of *Rpi-chc1* dramatically. A *Rpi-chc1* transgenic Desiree populations, transformed with a construct with an extended terminator (1.2kb), showed high levels of late blight susceptibility. A vast majority of the transgenic events showed susceptibility to each of the *P. infestans* isolates. This indicates that extending the downstream region decreases the functionality of *Rpi-chc1* in transgenic Desiree plants resulting in reduced late blight resistance.

Overall, the altered properties of the regulatory elements studied here, appears to have no effect or even decrease the functionality of *Rpi-chc1* in transgenic Desiree plants. Taking into account the high susceptibility levels, it is doubtful if the construct with and extended terminator was functional. To verify

this, agrobacteria with stable *Rpi-chc1* plasmid insertions can be used to check the functionality of the construct by co-infiltration with the corresponding *Avr* effector, *Avr-chc1 (*Zhu et al., 2012). After successful Agrobacterium-mediated transformation, the stability and functionality of the *R* gene in the transgenic potato plant must be determined. This can be done by measuring mRNA levels and thus the actual degree of expression of the *R* gene. This is of importance to verify if *Rpi-chc1* is actually transcribed in the transgenic plants. Besides there are many other factors which might influence the functionality of a transgene. These and the influence of genetic background on the deployability of *R* genes in potato cultivars are discussed in the next paragraph.

5.3 Deployability of *R* genes in potato cultivars: influence of genetic background

Evaluation of six R9a *Solanum* transgenic populations, derived from six common potato cultivars, showed that the genetic background has influence on the degree of late blight resistance. The amount of transgenic events, derived from each cultivar, having late blight resistance were significantly different among the cultivars. Most of the transgenic events derived from cultivars Aveka and JV18 show either complete or moderate resistance to all three *P. infestans* isolates (Table 10). The proportion of resistant transgenic events derived from these cultivars was significantly higher than the other four cultivars. However, the results for JV18 were somewhat skewed since the cultivar already contain *R2* providing resistance to 90128, used in the detached leaf assay. This seems also be the case for isolate NL08532, since the JV18 control plants were resistant to this isolate. Although in the beginning it was assumed that this isolate did not contain *Avr2*. In addition, Champouret (2010) stated that isolate IPO-C also contains *Avr2*. It is remarkable that both untransformed JV18 controls plants were susceptible to IPO-C. Nonetheless, it is assumed that the observed late blight resistance in JV18 for the three isolates was not caused by the transgene but is due to the presence of *R2* in its genetic background.

The genetic background of Aveka might also contain resistance to NL08532, since only a single transgenic event derived from this cultivar was susceptible, although the controls suggest otherwise by being susceptible to this isolate. Unlike JV18 this cultivar does not contain *R2.* Furthermore, in contrast to the results of JV18 a relatively high proportion of the Aveka events showed moderate resistance instead of complete resistance to NL08532, suggesting that a different mechanism plays a role in this background.

Among the transgenic events derived from the other four genetic backgrounds, Desiree, Premiere, Atlantic and JV19, lower late blight resistance levels were observed for each of the *P. infestans* isolates. This was especially the case for isolate 90128, where a majority of the transgenic events of these four cultivars showed susceptibility. Verzaux (2010) indicated that late blight resistance obtained through *R9a* can be broken by isolate 90128. In this study, two F1 individuals, derived from a cross between *S. edinense* 150-4 and cv. Concurrent, were tested for late blight resistance to 90128. Results

of a detached leaf assay showed that both F1 individuals, containing *R9a*, showed susceptibility to 90128 (Verzaux, 2010). However, this loss of resistance was not observed in the Aveka background where a majority of the transgenic events showed resistance, although the proportion of 90128 resistant events was slightly lower than that of the other two isolates, NL08532 and IPO-C (Table 10). Overall, the background of Aveka, excluding the biased results of JV18, appears to be most beneficial for the functionality of *R9a* in a transgenic potato.

So far, studies focused on expression of three *R* genes, *R3a*, *RB* and *Rpi-phu1,* indicated that genetic factors influence the expression level (Sliwka et al., 2012). This studies determined the expression of the *R* gene by measuring the amount of mRNA with a real-time PCR. However, it must be noted that the genetic background of a cultivar does not only influence transcription of the *R* gene and thereby the quantity of mRNA, but also post-transcriptional regulation of the mRNA. For instance, posttranscriptional processes like splicing, polyadenylation, nuclear transport or stability of the mRNA influence the final quantity of the resulting protein (Koziel et al., 1996). Additionally, post-translational events, like protein folding and translocation, are necessary to obtain a mature active protein. It is, therefore, important to take all these factors into account when studying *R* gene transformation.

Besides the influence of the genetic background, there are other factors associated with plant transformation which may influence the degree of expression of a transgene. A previous study showed that presence of vector backbone of the plasmid in the transgenic line can decrease the expression of the transgene or might induce transgene rearrangements, resulting in an inactive or unstable mature protein (Fu et al., 2000). Also transgene copy number can either be positively or negatively associated with transgene expression (Xu-Gang et al., 2003; Bradeen et al., 2009). It has been observed that when multiple copies of a transgene are integrated into a plant genome, some or all of the copies can be inactivated or silenced (Zhong, 2000). Thus a higher copy number does not necessarily lead to a higher expression level. No significant correlation between transgene copy number and/or the presence of vector backbone with late blight resistance was found. However, since the size of the populations was rather small, significance was hard to detect. The data of backbone free *R9a* transgenic plants, listed in table 12, do suggest a correlation between transgene copy number and late blight resistance. This corresponds to a previous study in which a similar correlation was observed in a different *R9a* transgenic Desiree population (unpublished data; Kodde, L). Remarkably, a significant correlation was found between cultivar and transgene copy number, suggesting that the genetic background effects the amount of successful transgene insertions through Agrobacterium-mediated transformation.

6. Recommendations

6.1 Identifying candidate genes for *Rpi-pnt1*

The BAC pools that have been identified to contain the right and left flanking region with respect to *Rpi-pnt1* have to be reduced to a level of single BAC clones. By sequencing the insert of these BAC clones candidate genes for *Rpi-pnt1* can be identified to determine the physical location of the *R* gene on chromosome 7 of *S. pinnatsectum*. In a later stage the *R* gene can be isolated and integrated into a vector in order to perform transformation studies.

6.2 Further characterization of *R* gene transgenic potato lines

A field test has to be performed on the selected vector backbone free transgenic events to verify if the DLA results correlates with late blight resistance in the field. To measure the actual transcript level of each transgenic event, RNA should be isolated to perform a real-time quantitative PCR. This is in particular relevant for the *Rpi-chc1* transgenic Desiree lines in which most of the transgenic events were susceptible to late blight. In this way it can be determined if the *R* gene is expressed and in what degree under the control of the extended promoter and terminator. Furthermore, it is of importance to verify if the vector with an extended terminator which was used for transformation is functional.

7. References

Bakker E., Borm, T., Prins P., van der Vossen E., Uenk G., Arens M., de Boer J, van Eck H, Muskens M, Vossen J, van der Linden G, van Ham r, Klein-Lankhorst R, Visser R, Smant G, Bakker J, Goverse A (2011). A genome-wide genetic map of NB-LRR disease resistance loci in potato. *Theor Appl Genet* 123: 493-58.

Black W, Mastenbroek C, Mills WR, Peterson LC (1953). A proposal for an international nomenclature of races of Phytophthora infestans and of genes controlling ummunity in Solanum demissum derivates. Euphytica 2:173-240.

Bradshaw JE and Ramsay G (2005). Utilisation of the commonwealth potato collection in potato breeding. *Euphytica* 146:9-19.

Bradeen JM, Lorizzo M, Mollow DS, Raasch K, Kramer LC, Millet BP, Austin-Phillips S, Jiang J, Carputo D (2009). Higher copy numbers of the potato RB transgeven correspond to enhanced transcript and late blight resistance levels. Mol Plant Microbe Interact, 2009 Apr; 22(4): 437-446.

Champouret N (2010). Functional genomics of phytophthora infestans effectors and Solanum resistance genes. pHD thesis Wageningen University, Wageningen.

Davidse LC, Looijen D, Turkensteen LJ, van der Wal D (1981). Occurence of metalaxyl-resistant strains of Phytophthora infestand in dutch potato fields. Netherlands journal of plant pathology vol. 87: 65-68.

Flor HH (1971). Current status of gene-for-gene concept. *Annu Rev Phytopatholy* 9: 275-296.

Fry W (2008). *Phytophthora infestans*: the plant (and *R* gene) destroyer. *Mol. Plant Pathol.* 9:385–42.

Fu X, Duc LT, Fontana S, Bong BB, Tinjuangjun P, Sudhakar D, Twyman RM, Christou P, Kohli A (2000). Linear transgene constructs lacking vector backbone sequencing generate low-copy-number transgenic plants with simple integration patterns. Transgenic Res. 2000 Feb; 9(10): 11-9

Haas BJ et al. (2009) Genome sequence and analysis of the Irish potato famine pathogen Phytophthora infestans. *Nature* 461: 393-398.

Haverkort A, Struik P, Visser R, Jacobsen E. (2009). Applied biotechnology to combat late blight in potato caused by *Phytophthora infestans*. *Potato Res.* 52:249–264.

Hoekema A, Hooykaas PJJ, Schilperoort RA (1984). Transfer or the octopine T-DNA segment to plant cells mediated by different types of *Agrobacterium* tumour- or root-inducing plasmids: Generality of virulence systems. *J. Bacteriol.* 158: 383-385.

Jacobsen E and Schouten HJ (2007). Cisgenesis strongly improves introgression breeding and induced translocation breeding of plants.

Kamoun S, Huitema E, Vleeshouwers VGAA (1999). Resistance to oomycetes: A general role for the hypersensitive response? *Trends in Plant Science* 4: 196-21.

Koziel MG, Carozzi NB, Desai N (1996). Optimizing expression of transgenes with an emphasis on post-transcriptional events. Plant molecular Biology 32: 393-405.

Kuhl JC, Hanneman Jr. Havey MJ (2001). Characterization and mapping of *Rpi1*, a late blight resistance locus from diploid (1EBN) Mexican *Solanum pinnatisectum*. Mol. Genetics Genomics (2001) 265: 977-985

Lokossou A (2010). Dissection of the major late blight resistance cluster on potato linkage group IV. PhD thesis Wageningen University, Wageningen.

Lung'aho C, Nyongesa M, Lemaga B (2008). Yield and economic losses caused by potato late blight in central kenya. International Potato Centre-SSA, Kampala, Uganada.

McDowell JM and Woffenden BJ (2003). Plant disease resistance genes: recent insights and potential applications. *Trends Biotechnol* 21: 178-183.

Sliwka, J, Swiatek M, Tomczynska I, Stefanczyk E., Chmielarz M, Zimnoch-Guzowska E (2013). Influence of genetic background and plant age on expression of the potato late blight resistance gene *Rpi-plu1* during incompatible interactions with *Phytophthora infestans.* Plant Pathology (2013) 62: 1072-1080

Stanton BG (2003). Agrobacterium-mediated plant transformation: the biology behind the "Gene-jockeying" tool. *Microbiol. Mol. Biol. Rev*. 67: 16-37.

Vleeshouwers GAA, van Dooijeweert W, Keizer PLC, Sijpkes L, Govers F, Colon LT (1999). A laboratory assay for Phytophthora infestans resistance in various Solanum species reflects the field situation. *European Journal of Plant Pathology* 15: 241-25.

Vleeshouwers VGAA, Raffalea S, Vossen JH, Champouret N, Oliva R, Segretin ME, Rietman H, Cano LM, Lokossou A, Kessel G, Pel MA, Kamoun S (2011). Understanding and exploiting late blight resistance in the age of effectors. *Annu Rev Phytopathology* 49: 57-531.

Verzaux, E (2010). Resistance and susceptibility to late blight in Solanum: gene mapping, cloning and stacking. PhD Thesis, Wageningen University, Wageningen.

Visker MHPW (2005). Association between late blight resistance and foliage maturity type in potato – Physiological and genetic studies. PhD Thesis, Wageningen University, Wageningen.

Xu-Gang LI, Song-Biao C, Zi-Xian L, Tuan-Jie C, Qian-Chun Z, Zhen ZHU (2002). Impact of copy number on transgene expression in tobacco. Acta botanica Sinica 2002, 44(10): 120-123.

Young ND (1990). Potential applications of map-based cloning to plant pathology. *Physiological and Molecular Plant Pathology* 37: 81-94.

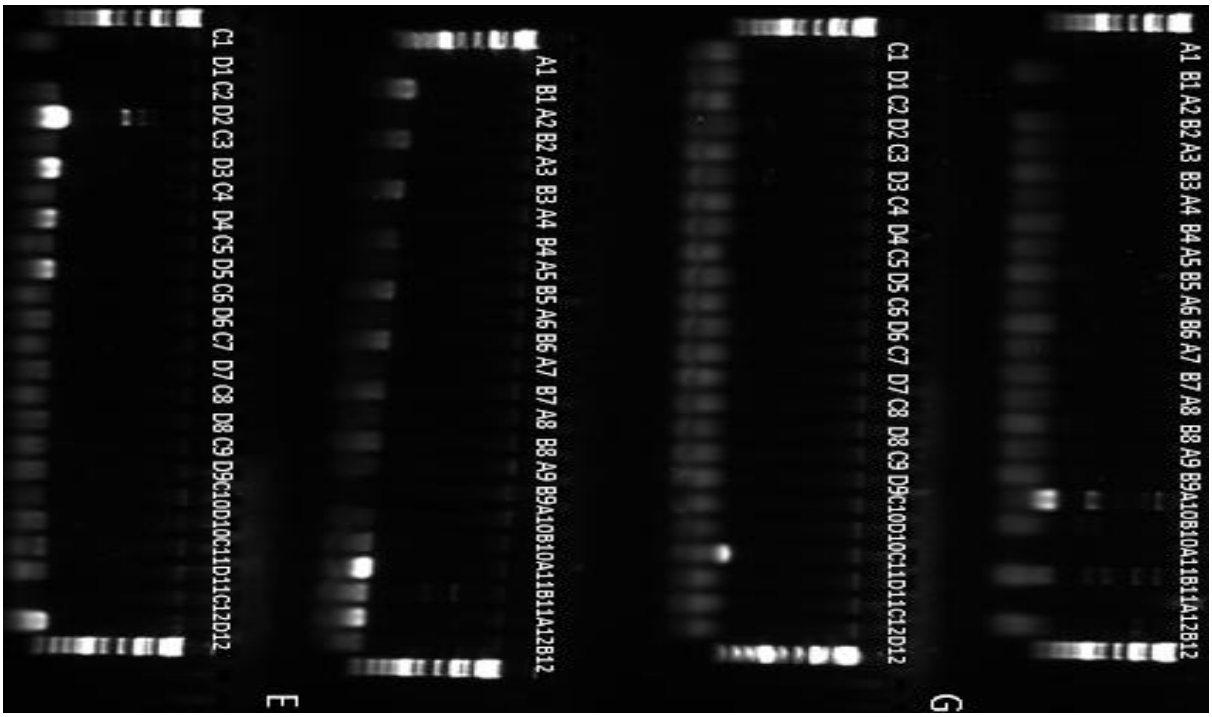
Zhong GY (2000). Genetic issues and pitfalls in transgenic plant breeding. *Euphytica* 118: 137-144.

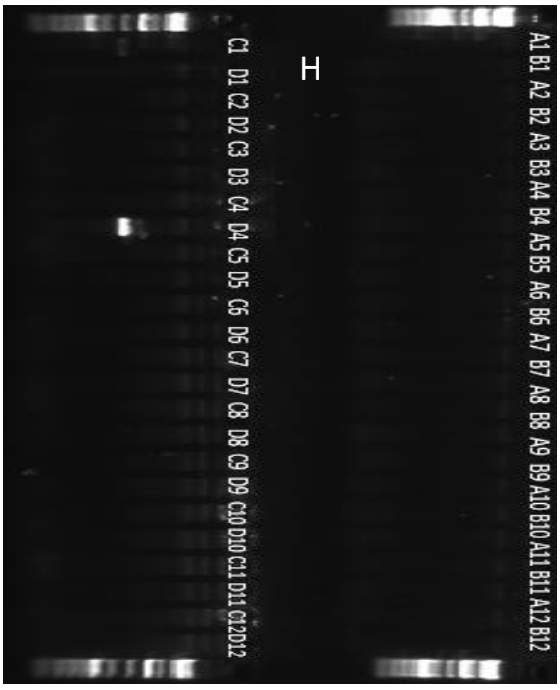
Zhu S, Li Y, Vossen JH, Visser RGF, Jacobsen E (2012). Functional stacking of three resistance genes against *Phytophthora infestans* in potato. *Transgenic Res.* 21: 89-99.

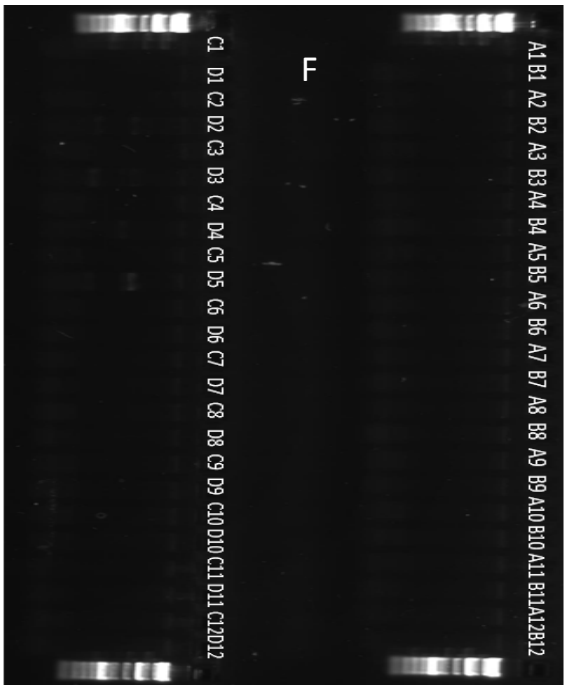
Appendix

I. Screening *S. pinnatisectum* BAC library I (4628-D) for *Rpi-pnt1* with 4 flanking markers

The BAC library consisted of 48 column BAC pools. Each pool was screened for the 4 CAPS markers (E, F, G, H) flanking *Rpi-pnt1*.CAPS markers used are given in each gel picture. Column pools were named after their specific row (A-H) / column (1-12) location in the plate.

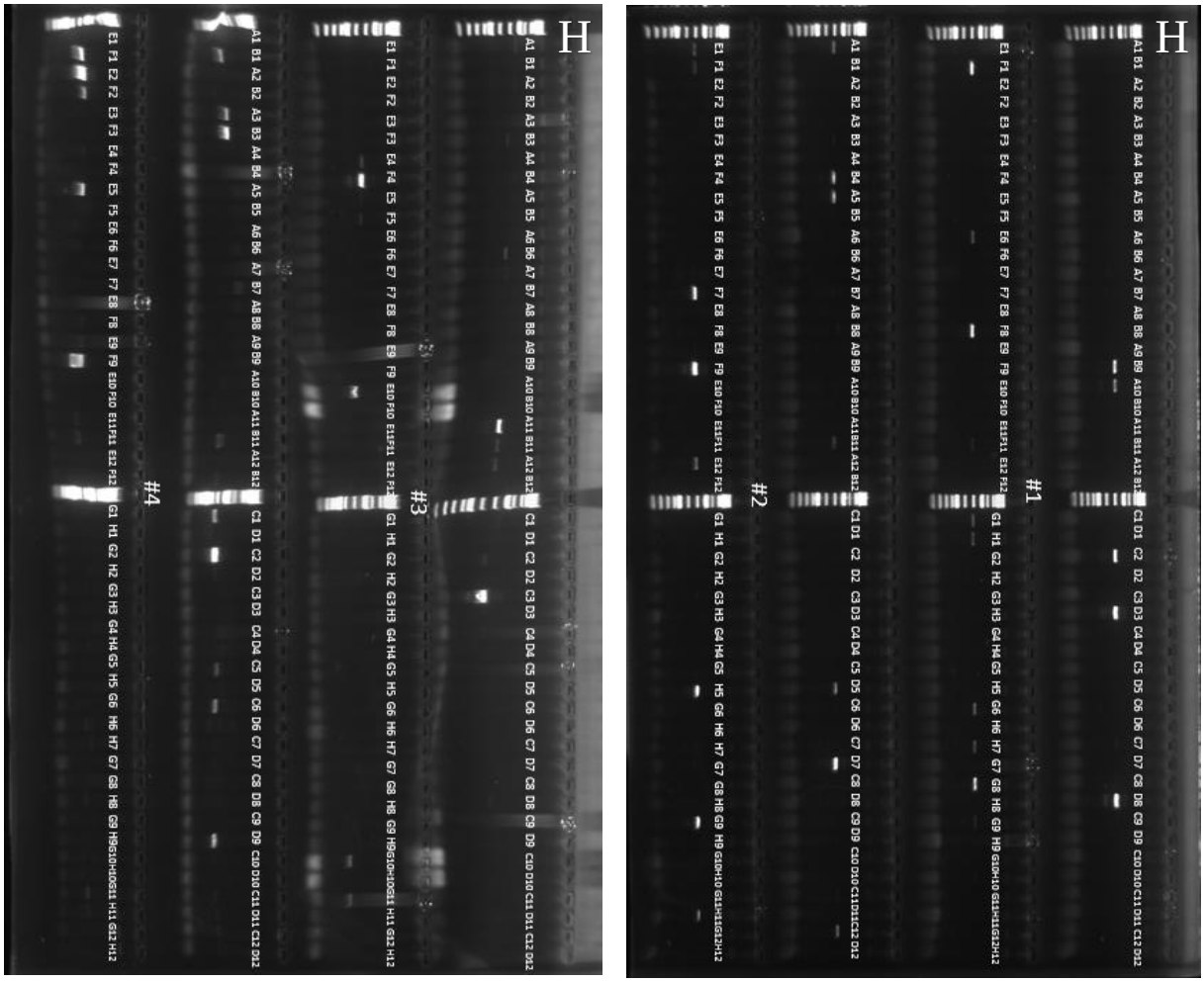


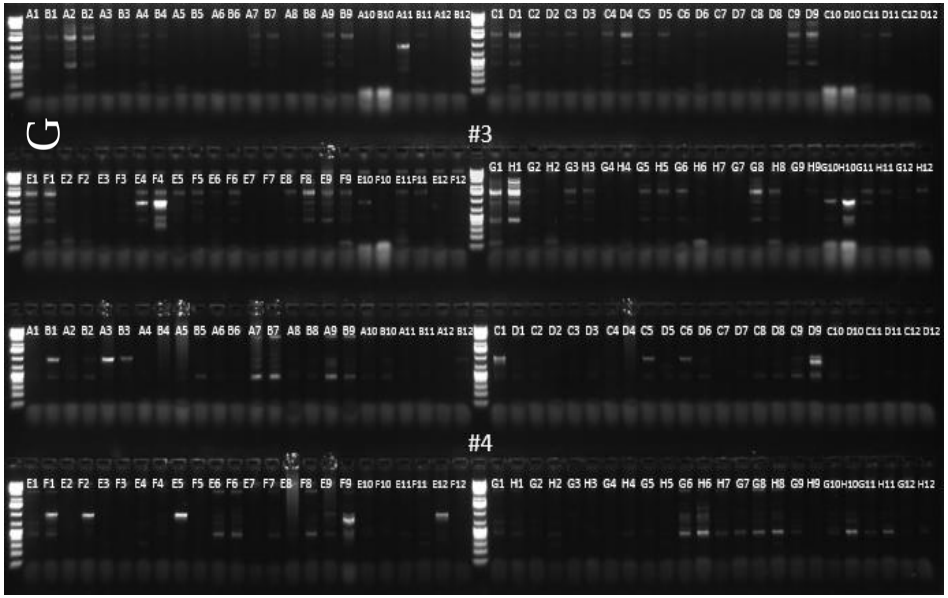


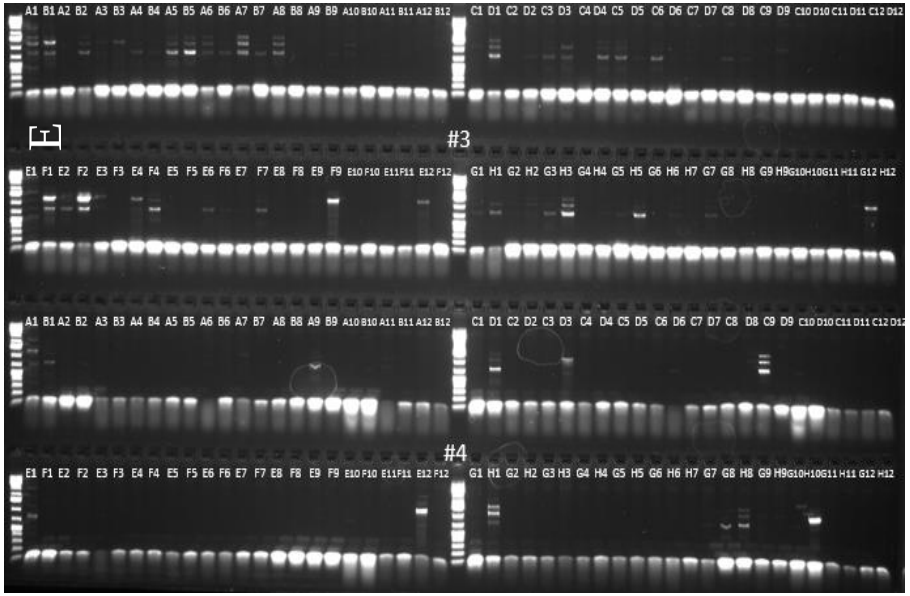


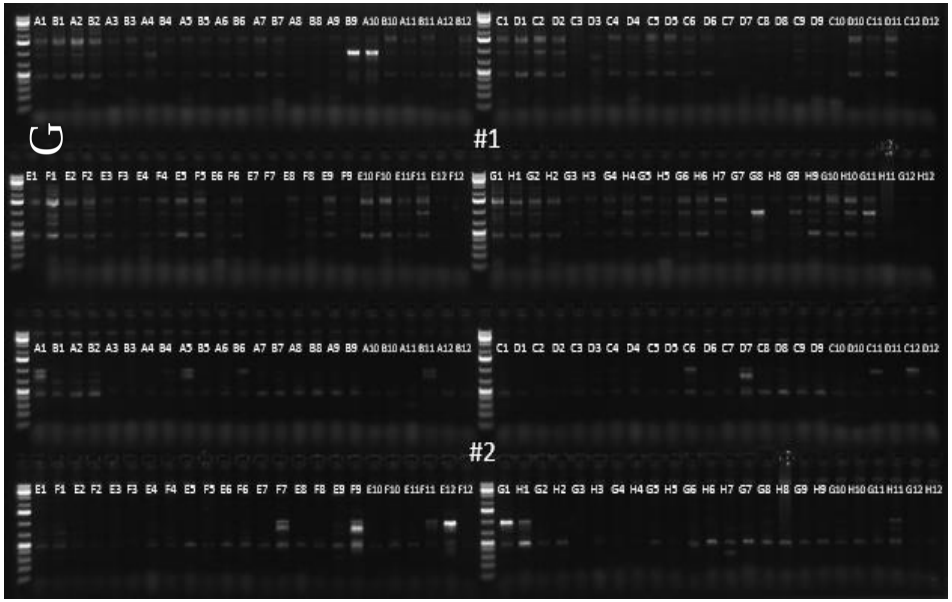
II. Screening *S. pinnatisectum* BAC library II (4804-pnt) for Rpi-pnt1 with 4 flanking markers

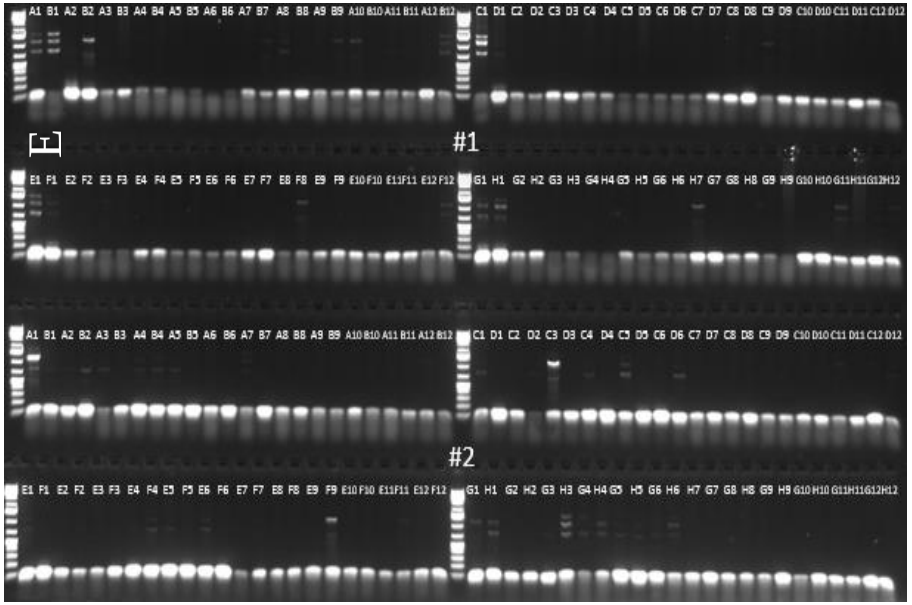
The BAC library consisted of 384 BAC pools distributed over four 96 wells plates. Each pool was screened for the 4 CAPS markers (E, F, G, H) flanking *Rpi-pnt1*.Plate number # (1, 2, 3, 4) and CAPS markers used are given in each gel picture. Pools were named after their specific row (A-H) / column (1-12) location in the plate.

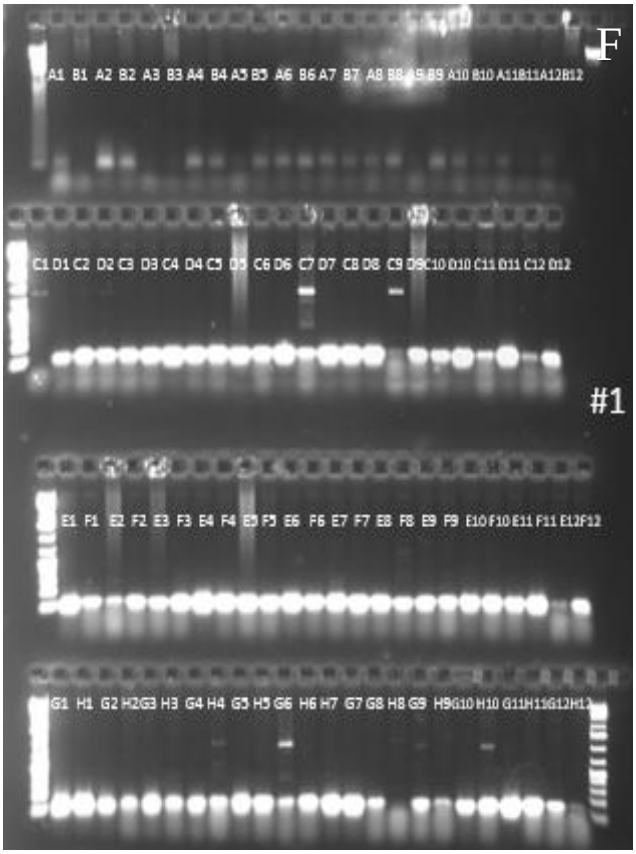


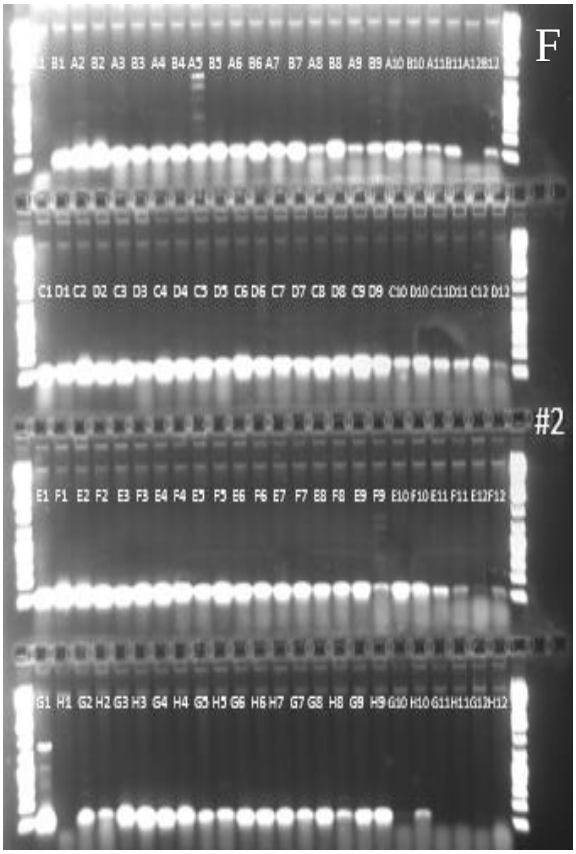

G
#3
#4

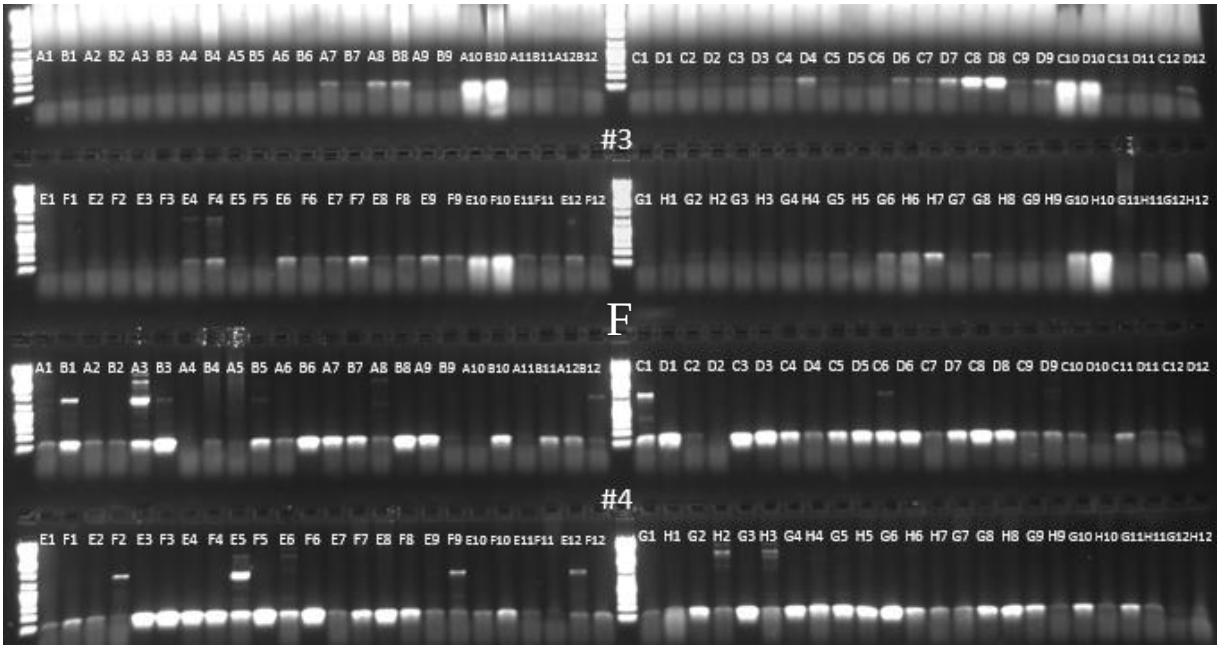

H
#3
#4


G
#1
#2


H
#1
#2

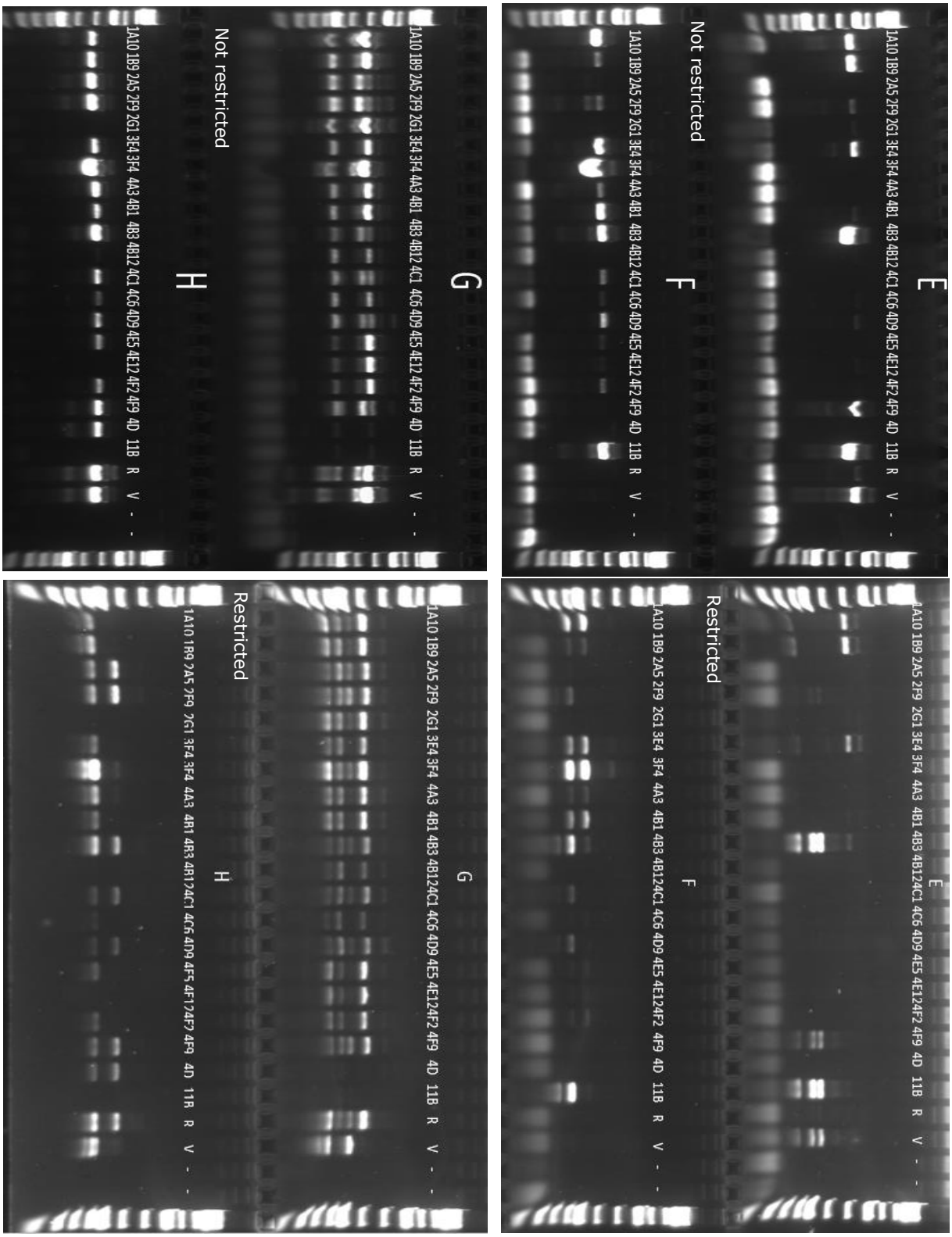

F
A1 B1 A2 B2 A3 B3 A4 B4 A5 B5 A6 B6 A7 B7 A8 B8 A9 B9 A10 B10 A11B11A12B12
C1 D1 C2 D2 C3 D3 C4 D4 C5 D5 C6 D6 C7 D7 C8 D8 C9 D9C10 D10 C11 D11 C12 D12
#1
E1 F1 E2 F2 E3 F3 E4 F4 E5 F5 E6 F6 E7 F7 E8 F8 E9 F9 E10 F10 E11 F11 E12F12
G1 H1 G2 H2G3 H3 G4 H4 G5 H5 G6 H6 H7 G7 G8 H8 G9 H9G10 H10 G11H11G12H12


F
C1 D1 C2 D2 C3 D3 C4 D4 C5 D5 C6 D6 C7 D7 C8 D8 C9 D9 C10 D10 C11D11 C12 D12
#2
E1 F1 E2 F2 E3 F3 E4 F4 E5 F5 E6 F6 E7 F7 E8 F8 E9 F9 E10 F10 E11 F11 E12F12
G1 H1 G2 H2 G3 H3 G4 H4 G5 H5 G6 H6 H7 G7 G8 H8 G9 H9 G10 H10 G11H11G12H12


A1 B1 A2 B2 A3 B3 A4 B4 A5 B5 A6 B6 A7 B7 A8 B8 A9 B9 A10 B10 A11B11A12B12
C1 D1 C2 D2 C3 D3 C4 D4 C5 D5 C6 D6 C7 D7 C8 D8 C9 D9 C10 D10 C11 D11 C12 D12
#3
E1 F1 E2 F2 E3 F3 E4 F4 E5 F5 E6 F6 E7 F7 E8 F8 E9 F9 E10 F10 E11F11 E12 F12
G1 H1 G2 H2 G3 H3 G4 H4 G5 H5 G6 H6 H7 G7 G8 H8 G9 H9 G10 H10 G11H11G12H12
F
A1 B1 A2 B2 A3 B3 A4 B4 A5 B5 A6 B6 A7 B7 A8 B8 A9 B9 A10 B10 A11B11A12B12
C1 D1 C2 D2 C3 D3 C4 D4 C5 D5 C6 D6 C7 D7 C8 D8 C9 D9 C10 D10 C11 D11 C12 D12
#4
E1 F1 E2 F2 E3 F3 E4 F4 E5 F5 E6 F6 E7 F7 E8 F8 E9 F9 E10 F10 E11F11 E12 F12
G1 H1 G2 H2 G3 H3 G4 H4 G5 H5 G6 H6 H7 G7 G8 H8 G9 H9 G10 H10 G11H11G12H12

III. Restriction analysis on 18 selected pools of *S. pinnatisectum* BAC library 2

18 BAC pools from *S. pinnatisectum* BAC library 2 (4804-pnt) were selected for a restriction analysis, since they contain one or more flanking CAPS markers (Table 9, Appendix 2.1). The last 6 wells contain in the following order: column pool 4D (BAC library 1), column pool 11B (BAC library 2), Resistant parent (R), Susceptible parent (S), MQ (-) and MQ (-).



IV. Experimental results of *R9a* transgenic potato populations

In this table the experimental results obtained from each transgenic events derived from each genotype are listed. Transgenic events refer to their corresponding genotypic background, A: Desiree, B: Premiere, C: Aveka, H: Atlantic, P: JV18, V: JV19. Detached leaf results for each transgenic event, using the scoring assay of table 4, are given. In the lab, isolated genomic DNA of each transgenic event was used to determine transgenic copy number and the presence of R9a, nptIII and vector backbone.

Genotype	IPO-C (1)	IPO-C (2)	NL08532	90128	R9a	nptIII	Copy number	Backbone free
A73.4-01	1,5	2	1,5	6	1	0	1	yes
A73.4-02	1,5	3	3,5	6	1	1	4	no
A73.4-03	1	1	2	2,5	1	1	12	no
A73.4-05	2,5	8	5	8	1	1	45	no
A73.4-15	2	1	3,5	3	1	1	1	no
A73.4-16	2,1	0	1	1	1	0	23	no
A73.4-17	2	1	1	2	1	1	6	no
A73.4-18	7	2	5	6	1	0	3	yes
A73.4-19	1	1	0	2	1	0	4	yes
A73.4-20	3,5	1,5	2	3	1	1	4	no
A73.4-25	1	2	1	3	0	0	13	no
A73.4-26	2	2	1,5	3	1	1	4	no
A73.4-27	2	3	1,5	6	1	1	23	no
A73.4-31	4	2,5	4	6	1	1	6	no
A73.4-32	8	6	8	8	1	1	4	no
A73.4-33	7	3	6	3	1	1	9	no
A73.4-34	8	6	8	6	1	1	19	no
A73.4-35	6	7	4	7	1	1	2	no
A73.4-40	5	2,5	4	6	1	1	23	no
A73.4-41	6	2	6	3	1	1	17	no
A73.4-42	6,22	8,5	6,5	6	1	1	17	no
A73.4-43	2,5	1	2	5	1	0	5	no
A73.4-44	2	1	1	3	1	0	6	yes
A73.4-45		3		2,5	1	0	1	yes
A73.4-46	2	1	1	2,5	1	1	10	no
A73.4-50	4	2,5	3	6	1	0	17	yes
A73.4-51	5	2,5	6	6	1	0	2	yes
A73.4-52	2,5	1,5	1	6	1	1	5	no
A73.4-53	8	5	8	6	1	1	2	no
A73.4-54	3	1,5	2	3,5	1	0	10	no
B73.4-01	1,66	3	1,2	6	1	1	23	no
B73.4-02	2,88	8	6,8	6	1	1	43	no
B73.4-03	2,27	1,5	0,1	5	1	1	17	no
B73.4-04	2	7	2,5	8	1	0	1	yes
B73.4-05	2	1,5	1,75	6	1	1	10	no
B73.4-08	2,5	7	2,4	7	0	1	5	no
B73.4-09	1,85	1	1,1	1	1	1	4	no
B73.4-10	4,5	X	1,4	X	1	0	8	no
B73.4-11	3	9	3	7	1	1		no
B73.4-12	1	2	0	2,5	1	1	19	no
B73.4-13	1	3	0	6	1	1	27	no

Genotype	IPO-C (1)	IPO-C (2)	NL08532	90128	R9a	nptIII	Copy number	Backbone free
B73.4-14	3	4	3	4	0	0	5	no
B73.4-28	2,5	2,5	2	3	1	1	18	no
B73.4-29	2	2	1	6	1	1	47	no
B73.4-30	3	2	2	3	1	1	17	no
B73.4-31	5	1	3	2	1	1	50	no
B73.4-32	1	3	1	6	1	0	11	yes
B73.4-33	8	2,5	6	6	1	1	8	no
B73.4-38	7	7	6	8	1	0	1	no
B73.4-39	8	7	6	6	1	1	4	no
B73.4-40	3	1	1,5	1	1	1	13	no
B73.4-41	8	9	7	8	1	0	0	no
B73.4-42	2,5	2	1	6	1	1	9	no
B73.4-48	3	1	1	4	1	1	6	no
B73.4-49	8	6	8	5	1	1	44	no
B73.4-50	8	6	6	6	1	0	5	no
B73.4-51	6	6	4	7	1	0	9	yes
B73.4-58	4	6	2	7	1	0	3	yes
B73.4-60	4	2,5	1	6	1	1	2	no
B73.4-61	8	1	6	6	1	1	7	no
C73.4-01	3	2,5	2,8	5	1	1	49	no
C73.4-02	2,8	3	2,5	6	1	0	6	yes
C73.4-03	1,9	1	0,4	2	1	1	9	no
C73.4-04	3	X	2,5	X	1	0	7	no
C73.4-05	2,5	1	0,5	1,5	1	1	18	no
C73.4-06	3	7	2,5	6	0	0	3	no
C73.4-07	2,5	1	1	1,2	1	1	16	no
C73.4-12	2,8	1	1,2	1	1	1	26	no
C73.4-13	2	0	1,4	2	1	1	40	no
C73.4-14	2	0,5	1	2	1	1	15	no
C73.4-15	2	1	1	2	1	1	13	no
C73.4-19	2	1,5	1	2	1	1	35	no
C73.4-20	1	1	0	1	1	0	9	yes
C73.4-21	1,5	2	0,75	1	1	0	7	yes
C73.4-22	2,25	3	1,8	5	1	1	19	no
C73.4-23	2,62	1	1,6	1,5	1	0	1	yes
C73.4-24	5,43	8	2,75	8	1	0	1	yes
C73.4-29	2,6	0	1,25	0,5	1	1	23	no
C73.4-30	2,5	1	2,6	1	1	1	6	no
C73.4-31	1,9	2	1	2	1	0	4	no
C73.4-32	1,6	8	0,75	4,5	1	1	26	no
C73.4-33	1,75	1	7	1	1	0	12	no
C73.4-34	4,5	1	1,75	2	1	0	4	yes
C73.4-35	5,25	1,5	3,1	6	1	0	5	no
C73.4-36	2,75	3	1	3	1	1	35	no
C73.4-39	3,4	2	2,25	2	1	0	3	yes
C73.4-40	2	3	1,8	2,5	1	0	1	no
H73.4-01	8,5	7	6,25	4	1	1	41	no
H73.4-02	6	6	5,4	8	1	1	0	no
H73.4-03	7,2	7	7,8	7	1	1	50	no
H73.4-05	2,7	3,5	2,7	6	1	0	6	yes
H73.4-06	2,7	1,5	1,5	6	1	1	19	no
H73.4-07	3,375	6	2,25	6	1	1	46	no
H73.4-08	3,5	4	0,8	6,2	1	1	15	no

Genotype	IPO-C (1)	IPO-C (2)	NL08532	90128	R9a	nptIII	Copy number	Backbone free
H73.4-09	3	1	1,75	6	1	1	12	no
H73.4-11	4,5	2	2,5	6	1	1	48	no
H73.4-12	3	1	1,5	5	1	1	41	no
H73.4-13	3,5	2	0	6	1	1	13	no
H73.4-14	8	6	7	7	0	0	5	no
H73.4-15	2,5	2,5	2	6	1	1	39	no
H73.4-16	2,5	2	2	6	0	0	10	no
H73.4-17	3	6	2,5	6	1	0	2	yes
H73.4-18	2,5	2,5	2	5	1	1	18	no
H73.4-19	1,5	2	2	5	1	1	24	no
H73.4-21	3	1,5	1,5	6	1	0	30	yes
H73.4-22	7,2	5	3,5	5	1	1	39	no
H73.4-23	3	2,5	3	5	1	0	6	yes
H73.4-24	3	1,5	2,5	5	1	0	13	yes
H73.4-26	1,5	4	2,5	6	1	1	23	no
H73.4-29	2,5	1,5	1,5	5	1	0	13	yes
H73.4-30	2,5	2,5	2,5	6	1	0	16	yes
H73.4-31	4	8	4	7	1	1	37	no
H73.4-32	3	2	1,5	6	1	1	19	no
H73.4-33	2,25	3	2	7	1	1	6	no
H73.4-34	2,5	2,5	3	6	1	0	4	yes
H73.4-51	5,6	6	5,6	8	1	0	3	no
P73.4-01	1	2	1	1	1	1	7	no
P73.4-02	0,3	2	0,2	0	1	0	3	yes
P73.4-03	0,3	2,5	0,2	1	1	0	5	yes
P73.4-04	0,3	1	0	0,5	1	0	15	yes
P73.4-05	0,3	3	0	1,5	1	1	28	no
P73.4-06	4,5	6	0	0,5	1	1	3	no
P73.4-07	3	2,5	0	1	1	1	12	no
P73.4-08	1	1	1	1	1	1	14	no
P73.4-09	2,75	1,5	0,4	0	1	1	2	no
P73.4-10	2,9	1,5	1	0	1	1	27	no
P73.4-11	1	3	1	0,5	1	1	41	no
P73.4-12	1	2	1	0,5	1	1	12	no
P73.4-13	1	6	1	1	1	1	5	no
P73.4-18	1,5	1,5	0,5	1	1	1	16	no
P73.4-20	1,5	1,5	0,5	1	1	0	12	no
P73.4-21	2	3,5	0	2,5	1	1	1	no
P73.4-22	1,8	2	1	1	1	1	39	no
P73.4-23	1,9	1	0,25	1	1	0	4	yes
P73.4-24	1	2,5	0,25	1	1	1	18	no
P73.4-25	0,6	0	0,2	1	1	1	27	no
P73.4-26	0,2	2	0	0,5	1	1	13	no
P73.4-27	0,6	3	1	1	1	1	26	no
P73.4-28	1	3	0,4	0	1	1	4	no
P73.4-29	2,75	3	1,25	X	1	1	6	no
P73.4-37	1,2	3	0,25	1	1	1	7	no
P73.4-47	0,75	4,5	0	1	1	1	45	no
P73.4-49	2,25	2	0	1	1	0	7	yes
P73.4-57	0,5	7	0	4	1	1	24	no
V73.4-01	2,8	3	1,75	7	1	1	9	no
V73.4-02	2,75	2,5	2	7	1	0	9	yes
V73.4-03	2	0,5	0,5	6	1	1	16	no

Genotype	IPO-C (1)	IPO-C (2)	NL08532	90128	R9a	nptIII	Copy number	Backbone free
V73.4-04	6	2,5	3	6	1	1	8	no
V73.4-05	2,5	1,5	1	5	1	0	17	yes
V73.4-11	7,75	6	7,75	8	1	1	3	no
V73.4-12	2	1,5	3	5	1	1	5	no
V73.4-15	5	8	X		1	1	7	no
V73.4-16	1,5	1,5	2,5	6	1	1	9	no
V73.4-18	2,8	6	2,5	7	1	1	28	no
V73.4-19	2,5	2	0,4	7	1	1	20	no
V73.4-20	2,75	1,5	0,8	4,5	1	0	4	no
V73.4-22	2,5	6	2	8	1	0	7	yes
V73.4-23	4	X	2,5	7	1	0	6	no
V73.4-26	3	X	4	X	1	1	3	no
V73.4-28	1	2,5	1	6	1	1	18	no
V73.4-29	8	8	8	8	1	1	5	no
V73.4-30	3	2,5	4	3,5	1	1	5	no
V73.4-31	2,5	1,5	1	6	1	1	5	no
V73.4-32	2	2	3,25	3	1	0	3	yes
V73.4-33	5,8	3	8,2	3	1	1	1	no
V73.4-37	2	3	2,8	7	1	1	23	no
V73.4-38	3,2	6	5,6	7	1	0	4	yes

V. Experimental results of *Rpi-chc1* transgenic potato lines

In this table the experimental results obtained from transgenic events derived from each *Rpi-chc1* transgenic Desiree line are listed. Detached leaf results for each transgenic event, using the scoring assay of table 4, are given. In the lab, isolated genomic DNA of each transgenic event was used to determine the presence of *Rpi-chc1*, *nptIII* and vector backbone.

Genotype	IPO-C (1)	IPO-C (2)	NL08532	90128	Chc1	nptIII	Backbone free
A58-01	7	0,5	1,5	0	1	0	no
A58-02	7	X	2,5	5	1	0	yes
A58-03	9	2	8	2,5	1	0	no
A58-04	8	9	8	8	0	0	no
A58-05	9	8,5	9	7	1	0	no
A58-06	3	3	3	2,5	1	0	yes
A58-08	8	8	8	3	0	0	no
A58-11	7	2	7	3	1	1	no
A58-12	7	6	7	3	1	0	no
A58-15	2	1,5	1	2,5	1	1	no
A58-16	8	5	8	2,5	1	0	no
A58-17	7,1	2	1	1	1	0	yes
A58-18	6	X	2,5	X	0	0	no
A58-22	8	2	6	3	1	0	yes
A58-23	2,5	1	2	1,5	1	1	no
A58-24	8,5	6	7,3	6	1	1	no
A58-25	1	1,5	1	2	1	1	no

Genotype	IPO-C (1)	IPO-C (2)	NL08532	90128	Chc1	nptIII	Backbone free
A58-26	8	8,5	8	6	0	0	no
A58-27	8	8	8	6	0	0	no
A58-28	8	8	8	7	0	1	no
A58-32	8	3	6	2	1	0	yes
A58-33	8	7	8	6	1	0	no
A58-34	7	4	6	1	0	0	no
A58-35	8	2	6	3	0	0	no
A59-01	7,7	8	8,4	4	1	1	no
A59-02	9	8	9	X	1	0	yes
A59-03	2,6	6	8	6	0	0	no
A59-04	3,5	7	3	6	0	0	no
A59-05	5,5	7	8	6	1	0	yes
A59-06	8	7	8	6	1	1	no
A59-07	8	8	8	6	1	1	no
A59-08	8	8	8	6	1	0	yes
A59-11	8	9	8	9	1	0	yes
A59-12	7,6	9	7,4	8	1	0	yes
A59-13	7,4	8,5	8,3	7	1	0	no
A59-14	8,4	9	8,2	8	1	1	no
A59-15	9	9	9	7	1	0	yes
A59-16	8	8	6	7	0	0	no
A59-17	8	9	8	8	1	1	no
A59-18	8	9	8	8,5	1	0	yes
A59-21	9	9	9	6	1	1	no
A59-22	8	9	6	8	1	0	no
A59-23	8	9	8	8	1	0	yes
A59-24	8	8	8	6	1	0	yes
A59-27	8	8	8	6	1	1	no
A59-28	9	8,5	8	7	1	0	yes
A59-31	8	7	3	3	1	1	no
A59-32	8	7	8	5	1	1	no
A59-33	8	3	7	2,5	1	1	no
A59-34	6	6	2	6	1	0	yes
A59-35	8	3	8	3	1	1	no
A59-41	8	9	6	8	1	1	no
A59-44	8	8	6	6	0	0	no
A59-47	8	7	6	7	1	0	no

V. Experimental results of control potato populations

In this table the experimental results obtained from control lines used for the experiment are listed. Detached leaf results for each transgenic event, using the scoring assay of table 4, are given. In the lab, isolated genomic DNA of each control line was used to determine the presence of *Rpi-chc1/R9a* and *nptIII*.

Genotype	IPO-C (1)	IPO-C (2)	NL08532	90128	Chc1/R9a	nptIII
A17-27	1,9	2,5	1	2,5	1	0
A17-27	2	2	1	2,5	1	0
Atlantic	9	8,5	9	8	0	0
Atlantic	9	9	8	8,5	0	0
Aveka	7	5	7	1,5	0	0
Aveka	8	7	8	2,5	0	0
Desiree	7	7	X	8	0	0
Desiree	6,5	7	X	7	0	0
Premiere	8,5	8	8,5	8	0	0
Premiere	8,5	7	8,5	9	0	0
JV18	5,8	6	0,75	1	0	0
JV18	7	5	1.25	1,5	0	0
JV19	8,25	7	6	7	0	0
JV19	8,4	6	7,2	6	0	0
B17-64	4,75	5	1	3	1	0
B17-64	6	5	1	6	1	0
C17-2	3,75	1	2,4	1,5	1	0
C17-2	5,7	1,5	2	2	1	0
P17-11	0,75	1	0,25	1	1	0
P17-11	0,6	1	0,2	1,5	1	0
V17-9	4,5	1	1,25	1	1	1
V17-9	1	0	0	1,5	1	1
A73.1-11	2,5	2	1,5	2	0	1
A73.1-11	3	2,5	1	4	1	1

VI. Statistic results of experimental data

Outcome of ANOVA testing correlation between late blight resistance for each *P. infestans* isolate and genotypic background. Significant differences was found for each *P. infestans* isolate (F<0.001).

Variate: 90128

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	5	476.948	95.390	38.94	<.001
Residual	137	335.615	2.450		
Total	142	812.563			

Variate: IPO-C

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	5	124.574	24.915	7.01	<.001
Residual	141	501.021	3.553		
Total	146	625.595			

Variate: NL08532

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	5	157.784	31.557	8.04	<.001
Residual	141	553.314	3.924		
Total	146	711.097			

Outcome of ANOVA testing correlation between transgene (*R9a*) copy number and genotypic background. Mean transgene copy number was significantly different between the 6 genotypic backgrounds.

Variate: Copy_nptII

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	5	3793.7	758.7	2.47	0.035
Residual	141	43360.0	307.5		
Total	146	47153.7			

Transgenic cultivar	Mean	
V73.4	8.49	a
A73.4	9.68	a
P73.4	15.00	ab
C73.4	17.39	ab
B73.4	20.14	b
H73.4	22.45	b

Outcome of ANOVA testing correlation between late blight resistance for isolate 90128 and transgene copy number (Blocking: Genotype). No significant correlation was found.

Variate: IPO-C

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	5	124.574	24.915	12.46	0.212
Copy number	140	499.021	3.564	1.78	0.544
Residual	1	2.000	2.000		
Total	146	625.595	4.285		