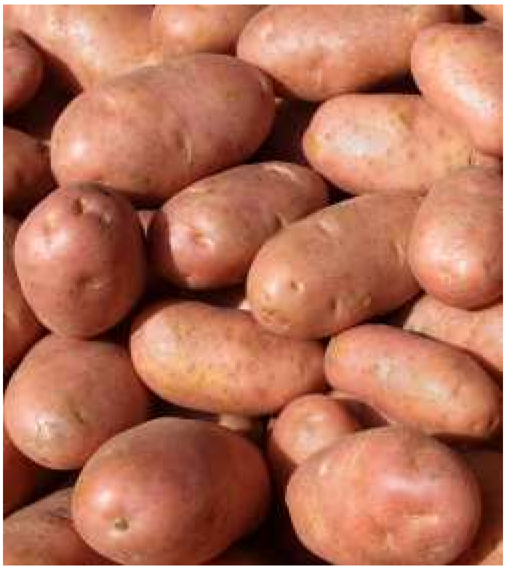
Functional validation of late blight resistance genes and their complementarity in stacks



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Course code: PBR-80436 MSc Thesis Plant Breeding

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Abstract

Phythophthora infestans (*Pi*) is the causal agent of potato late blight disease. Functional stacking of multiple resistance (*R*) genes could offer an effective approach for more durable resistance to this disease. This project aimed at generating optimized *R* gene stacks by characterizing newly identified *R* genes, *Rpi-chc1* homologs, to find their level of complementarity. *Rpi-tar1* was found to display a complementary spectrum, thus could be stacked with *Rpi-chc1*. Seven combinations of two well characterized *R* genes, *Rpi-edn2: Rpi-sto1*, *Rpi-blb3: Rpi-edn2*, *Rpi-chc1: Rpi-sto1*, *Rpi-blb3: Rpi-chc1*, *Rpi-chc1: Rpi-edn2*, *Rpi-vnt1: Rpi-blb3* and *Rpi-vnt1-Rpi-edn2* were successfully cloned into single binary vector pBINPLUS-PASSA and transformed into *Agrobacterium*. Triple *R* genes cassette, pBINPLUS-PASSA: *Rpi-vnt1: Rpi-blb3: Rpi-chc1* was transformed into susceptible cultivar Desiree. The functionality of stacked *R* genes, *Rpi-sto1: Rpi-blb3* in 24 cisgenic Desiree plants and *Rpi-vnt1: Rpi-chc1* in 19 transgenic Desiree plants was validated. Detached leaf assay and agroinfiltration of avirulence (*Avr)* genes showed that 10 of the double *R* gene cisgenic plants have a functional *Rpi-sto1* while none of them displayed proper expression of *Rpi-blb3*. In transgenic plants harboring *Rpi-vnt1: Rpi-chc1*, only three showed that both *R* genes were active whereas most of the other transformants have only functional *Rpi-vnt1*.

Keywords: Potato, *Phythophthora infestans*, durable resistance, *R* gene, functionality

Table of contents

List of tables

List of figures

General introduction

Potato is one of the important non-cereal crop plants in the world food production (Foster et al., 2009; Kim et al., 2011; Rauscher et al., 2006). Currently, it is the third crop for human consumption worldwide after wheat and rice. However, until now potato growers are continually suffering from the devastating late blight disease caused by the fungus-like oomycete *Phytophthora infestans* (*Pi)*. The first reports of this disease date from more than a century ago, when it spread from the west of Ireland to the rest of Europe and caused the Irish potato famine. Currently, late blight is estimated to cost $6.7 billion losses annually worldwide with $3.5 billion annually in developing countries alone (Haverkort et al., 2008; Shaw, 2010).

Resistant varieties were successfully made through introgression of a single resistance genes (*R* gene) from wild species (Li et al., 2011). However, the ability of *Pi* to rapidly evolve by mutation of effectors caused the resistances provided by the *R* genes to be broken. The application of fungicides is the only relevant way to combat late blight in most of the developed countries although the cost is high and unfavorable to environment. The capacity of *Pi* to overcome single *R* genes (Black et al., 1953) and the appearance of new fungicide resistant isolates (Grünwald et al., 2001) have increased the interest of potato breeders to stack multiple *R* genes into cultivars. This could offer prospects for a more durable resistance and provide a useful alternative to environmentally damaging chemical fungicides.

In order to achieve *R* gene mediated broad spectrum recognition of *Pi*, it is more desirable to combine genes from different *R* gene clusters which will result in different types and timing of HR reactions (Douglas and Halpin, 2010; Halpin, 2005). In the last few decades, major quantitative trait loci (QTL) for resistance against potato late blight disease have been successfully mapped on potato chromosomes (Gebhardt and Valkonen, 2001). Based on genetic maps, so far, several *R* genes have been identified and cloned from related wild *Solanum* species. For instance, *R1* (Ballvora et al., 2002), *R2* (Lokossou et al., 2009) , *R3a* (Huang et al., 2005) and *R3b* (Li et al., 2011) were cloned from the species *Solanum demissum*. There are also *R* genes originating from other wild species such as *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* from *Solanum bulbocastanum* (Lokossou et al., 2009; Song et al., 2003; van der Vossen et al., 2003; van der Vossen et al., 2005), *Rpi-ber* (Rauscher et al., 2006) *, Rpi-ber1* and *Rpi-ber2* (Park et al., 2009) from *Solanum berthaultii, Rpi-sto1* from *S. stoloniferum* (Vleeshouwers et al., 2008) and *Rpi-vnt1.1* from *Solanum venturii* (Foster et al., 2009; Pel et al., 2009). Using a selected set of late blight isolates it has been observed that some of the *R* genes displayed a broader resistance spectrum than others. This differential ability in conferring resistance to *Pi* could be utilized by breeders through stacking of multiple *R* genes from wild species into a potato cultivar. Hence, potato crops with a continuous resistance to *Pi* could be produced and maintained in the field.

Furthermore, many *R* genes in potato are located in clusters and genetically co-localize with *R* gene homologs. These homologs may possess different functionality or could also act together in conferring resistance against wide ranges of *Pi* strains (Vleeshouwers et al., 2011). Many *Rpi-chc1* homologs, derived from *S. chacoense*, *S. tarijense* and *S. berthaultii* have been identified using *Rpi-chc1* paralog/allele mining studies (Fig. 1). The different *Rpi-chc1* homologs were expressed in *N. benthamiana* and examined for their ability to recognize different effectors from *Pi*. The results showed that those different *Rpi-chc1* homologs have different recognition specificities towards different *Pi* effectors (Table 1; Lokossou 2010, unpublished data). Transgenic plants harboring those homologs were developed by transformation of the susceptible potato cultivar Desiree. Among the different transgenic events, in previous studies the most resistant events were selected (Sidrat Abdullah, 2012).

Plants react to various types of pathogen invasion through defense mechanism mediated by resistance proteins encoded by *R* genes which are members of the NBS-LRR (Nucleotide-binding site-Leucine-rich repeats) protein family (DeYoung and Innes, 2006). Inside the plant, *Pi* feeds on potato cells through specialized structures (haustoria) in the host cell plasma membrane and brings effector molecules inside the plant cell. Then, the *R* proteins of the plant recognize the pathogen derived effectors and activate the innate immunity followed by induction of programmed cell death responses to prevent pathogen growth (McHale et al., 2006). The hypersensitive response (HR) reactions by the plant cells are induced when the *R* gene and their cognate *Avr* effectors are interacting. The resistance that is brought about by this specific recognition is also referred to in literature as gene for gene interaction or race specific resistance (Flor, 1971). In managing late blight this type of resistance is known as vertical (monogenic) resistance. It has been proven previously that *Pi* can easily break the resistance and lower the durability of the single *R* gene (Malcolmson and Black, 1966). Another type of resistance is known as horizontal (polygenic) resistance. In contrast to vertical resistance, this type of resistance is controlled by multiple genes and is a non-race specific and therefore expected to provide more durability (Gebhardt, 1994; Tan et al., 2008). However, the level of horizontal resistance known so far is lower than the resistance provided by vertical resistance. Multiple broad spectrum (vertical) *R* genes can be combined into single genotype by stacking and produce high levels of horizontal resistance that is potentially very durable.

Cultivars with stacked *R* genes can be developed by traditional breeding or genetic modification (GM). The introduction of different combinations of *R* genes into cultivars is observed to be more efficient by using genetic transformation than using conventional breeding which is highly time consuming. Besides, linkage drag of undesirable genes from wild potato genome, as often occurred in conventional breeding, can also be prevented. However, strict regulations and public unawareness limit the application of the GM method in Europe. Scientists have proposed cisgenic modification methods where *A. tumefaciens* is used to insert only natural genes from crossable wild species. This approach could provide an alternative way for breeding of durable potato cultivars against *Pi*.

In functional stacking of late blight *R* genes in potato cultivars, the concept of gene for gene interaction can be applied to examine the biological activity of stacked *R* genes in order to determine the efficiency of the stacking process. The functionality of each *R* gene can be examined by investigating the relationship between the disease resistance and HR reaction to their corresponding *Avr* effectors in agro-infiltration test (Vleeshouwers et al., 2008). So far, several pairs of potato *R* genes and cognate *Pi Avr* effectors have been identified. For example, *Avr2 (Avr-blb3)/Rpi-blb3* (Lokossou et al., 2009; van Poppel et al., 2008), *Avrvnt1/Rpi-vnt1 (Pel et al., 2009)* and *Avr-sto1(=IPI-O1)/Rpi-sto1*(Vleeshouwers et al., 2008).

This research aimed firstly to characterize new late blight *R* genes in order to find out if it is worthwhile to stack these genes. The level of complementarity of the *R* genes was assessed by using transgenic plants and by comparing their effector and *Pi* isolate recognition spectra. Then, several combinations of well characterized *R* genes were made in one binary vector. These new constructs can be used to introduce multiple combinations of two *R* genes into susceptible potato cultivar Desiree by *A. tumefaciens*-mediated transformation. The functionality of stacked *R* genes was validated by disease tests and agro-infiltration using corresponding avirulence (*Avr)* effectors.

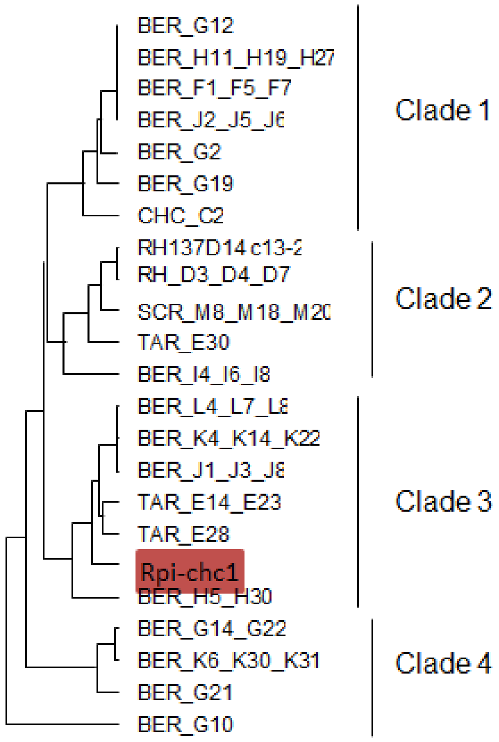
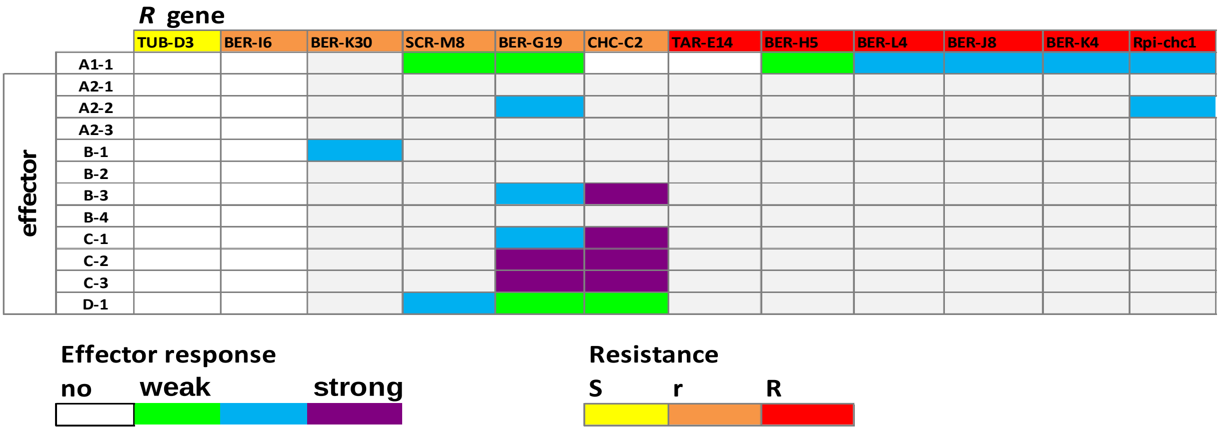


Fig 1: Phylogenetic tree of *Rpi-chc1* homologs (Vossen, unpublished)

Table 1: Effectors response and resistance to *Pi* of different *Rpi-chc1* homologs after transient expression in *N. benthamiana*



***R* gene**

effector	TUB-D3 🟨	BER-I6 🟧	BER-K30 🟧	SCR-M8 🟧	BER-G19 🟧	CHC-C2 🟧	TAR-E14 🟥	BER-H5 🟥	BER-L4 🟥	BER-J8 🟥	BER-K4 🟥	Rpi-chc1 🟥
A1-1	⬜	⬜		🟩	🟩	⬜	⬜	🟩	🟦	🟦	🟦	🟦
A2-1	⬜	⬜										
A2-2	⬜	⬜			🟦							🟦
A2-3	⬜	⬜										
B-1	⬜	⬜	🟦									
B-2	⬜	⬜										
B-3	⬜	⬜			🟦	🟪						
B-4	⬜	⬜										
C-1	⬜	⬜			🟦	🟪						
C-2	⬜	⬜			🟪	🟪						
C-3	⬜	⬜			🟪	🟪						
D-1	⬜	⬜		🟦	🟩	🟩						

Effector response

no ⬜ weak 🟩 🟦 🟪 strong

Resistance

S 🟨 r 🟧 R 🟥

(Lokossou, unpublished)

Materials and methods

Plant material

Thirty-five transgenic plants of potato Desiree harboring different *Rpi-chc1* homologs that were developed earlier in house were used for validation and characterization of newly cloned *R* genes (Table 2). Several events from each clone were selected based on previous work. Clone A17-27 and susceptible potato Desiree were indicated as positive and negative control respectively. The donor plants of each clone were also included in the experiment to compare the resistance spectrum. Nine isolates (H30PO4, IPO-C, USA 618, NL 7245, Pic 99183, Dinteloord, Katshaar, NL 7379 and NL 7135) were used in detached leaf assay and seven effectors were infiltrated in agro-infiltration (Table 3).

Cisgenic Desiree plants (A26; *Rpi-sto1* and *Rpi-blb3*) and transgenic Desiree plants (A19; *Rpi-vnt1* and *Rpi-chc1*) were used to functionally validate *R* gene stacking. Two different effectors and two different isolates were selected based on their compatibility to each single *R* gene in the respective constructs. For A26 (*Rpi-sto1* and *Rpi-blb3)* plants, IPO-O and IPO-C were used in DLA and *Avr-sto1* and *Avr2* effectors in agro-infiltration. For A19 (*Rpi-vnt1* and *Rpi-chc1*) plants, EC1 and Dinteloord were used in DLA and *A2-2* and *Avr-vnt1* in agro-infiltration.

Table 2: Coding of transgenic plants used in this study

Transgenic Plants	Event numbers	Promotor : Genotype/Clone
A32	-126, -56, -66, -78	chc-short:324-2/J8
A33	-112, -31, -32, -33, -34, -51, -52, -53, -72	chc-short:852-5/E14
A34	-11, -13, -41, -62, 79	chc-short:487-1/I6
A35	-02, -14, -86, -88, -90	chc-short :493-7/G19
A36	-13, -35, -74, -91	chc-short:493-9/H5
A37	-23, 44	chc-short :543-5/C2
A40	-04, -28, -31, -44, -51, -53	chc-short:852-5/E28
A17 (resistant control)	-27	chc-long:chc1
Desiree (susceptible control)		

Methods

Multiplication of plants

MS (Murashige skoog) 20 media which consists of MS medium, vitamins and sucrose were used for multiplication of transgenic and donor plants. After being prepared, the media were autoclaved at 121°C for 15 minutes and then poured into plastic pots (diameter 15cm). Fresh shoots or single nodes were cut and transplanted on media under sterile air flow in a laminar flow cabinet to avoid any contamination. After two weeks of rooting in climate chamber at 25°C, the *in vitro* plantlets were transferred into the greenhouse and grown in the trays. After one week, they were transferred into big pots and grown for two to four weeks.

Detached Leaf Assay

Detached Leaf Assay (DLA) was used to determine the response of the plants to different *Pi* isolates. Two weeks before inoculation, a fresh patch of mycelium was placed on rye medium supplemented with 20g/l sucrose. After two weeks, the mycelium had grown on the plate and sterile water was added to the plate to wash off the sporangiospores. The sporangiospores suspension was transferred into new tube and incubated at 4°C for at least two hours. After two hours, the concentration of the suspension was adjusted to $5*10^4$ zoospores ml $^{-1}$ for inoculation.

Fully expanded primary leaves with petiole length 2cm were excised from the greenhouse grown plants. After the petioles were cut to make it sharp, then they were inserted into 4cm*4cm green foam which was soaked in water for 20 min. Two leaves from the same transgenic event were put on each foam with adaxial face down and placed on water-soaked filter paper. Label for each event was put and fixed on the foam with a wooden toothpick. Each leaf was inoculated with six 10µl droplets of a zoospore suspension (50,000 spores/ml) of a specific *Pi* isolate (Vleeshouwers et al., 1999) onto the abaxial side of the leaf. After seven days of inoculation, the leaves were evaluated and scored in terms of necrosis. Plants were scored 1-2.5: Resistance (R), 2.6- 3.5-Moderate Resistance (MR), and grouped as susceptible, in between scores 3.6 -7.

Agro-infiltration

The assay for hypersensitive response (HR) to the cognate *Avr* effectors was performed by using Agro-infiltration (Bendahmane et al., 2000; Vleeshouwers et al., 2008). Two media were used to grow the effectors, LB and YEB media. The LB media was prepared with 1% (w/v) bactopeptone, 1% (w/v) NaCl and 0.5% (w/v) yeast extract. The YEB media was prepared with 0.5% (w/v) beef extract, 0.5% (w/v) bactopeptone, 0.5% (w/v) sucrose, 0.1% (w/v) yeast extract and 0.2% (v/v) 1M $MgSO_4$. Two days before infiltration, *A.tumefaciens* strain *AGL1* containing *R* genes and *Avr* genes were inoculated from glycerol stock in 3ml LB medium with appropriate antibiotics and grown overnight at 30°C. Then, optical density at wavelength 600nm (OD_{600}) was measured and 10ml of YEB medium was inoculated with x µl of LB culture (according to the following calculation) and grown overnight at 30°C in order to get $OD_{600} = 1$ on the next day.

X=V*Z/OD of the preculture

where $Z=800/2^{(\Delta t/td)}$,
X=volume in microliter to be inoculated
V=culture volume in milliliter
Δt= desired culture time, td-doubling time

On the following day, MMA media was prepared with 2% (w/v) sucrose, 0.5% (w/v) MS salts (no vitamins), 1% (v/v) 1M Morpholino-ethane-Sulfonic acid (MES) pH 5.5 and 0.1% (v/v) acetosyringone. The YEB culture was centrifuged for 8 mins at 4000rpm. The pellet was re-suspended with 5 ml of MMA and OD_{600} of 2.0 was reached by adjusting with MMA. During infiltration, three leaves on each plant were infiltrated with specific effectors at different OD_{600}, obtained by dilution with MMA, using 1ml syringe. After 4 days, the leaves were scored in terms of percentage of cell death or hypersensitive response (HR).

Table 3 List with available RD12 homologs in pK7WG variants

Effector	Tree code	Vector	A.tum strain	Antibiotics
PITG_16245	*A1-1*	pK7WG**F**2	*GV3101*	*Rif+Spec*
PITG_20336	*A2-1*	pK7WG2	Agl1	C.amph.+Spec
PITG_20934	*A2-2*	pK7WG2	Agl1	C.amph.+Spec
PITG_23230	*A2-3*	pK7WG2	Agl1	C.amph.+Spec
PITG_16243	*B1*	pK7WG2	Agl1	C.amph.+Spec
PITG_16248	*B2*	pK7WG2	Agl1	C.amph.+Spec
PITG_16248	*B2*	pK7WG**F**2	*GV3101*	*Rif+Spec*
PITG_23069	*B3*	pK7WG2	Agl1	C.amph.+Spec
PITG_23069	*B3*	pK7WG**F**2	*GV3101*	*Rif+Spec*
PITG_23074	*B4*	pK7WG2	Agl1	C.amph.+Spec
PITG_16242	*C1*	pK7WG2	Agl1	C.amph.+Spec
PITG_16242	*C1*	pK7WG**F**2	*GV3101*	*Rif+Spec*
PITG_16235	*C2*	pK7WG2	Agl1	C.amph.+Spec
PITG_16424	*C3*	pK7WG2	Agl1	C.amph.+Spec
PITG_16428	*D1*	pK7WG2	Agl1	C.amph.+Spec
PITG_09577	*D2*	pK7WG**F**2	*GV3101*	*Rif+Spec*

Starter construct and isolation of Plasmid-DNA

In order to design double *R* gene constructs, six plasmids; pBINPLUS: *Rpi-sto1*, pBINPLUS: *Rpi-chc1*, pBINPLUS: *Rpi-vnt1*, pBINPLUS: *Rpi-blb3*, pRIAB1.2-PASSA and pBINPLUS-PASSA were used as the basis material. Three donor vectors; pUC-PASSA, pUC-ASAS, and pUC-PSAA were utilized to shuttle several *R* genes to acceptor vectors. *N. benthamiana* cultivars were used for functional analysis of newly-developed *R* gene cassettes against *Phytophthora infestans.*

The QIAGEN® Plasmid Midi Kit was used to isolate plasmid DNA of construct containing *R* gene(s). A 1µl aliquot of culture from glycerol stocks was picked and inoculated as a starter culture of 3ml LB medium containing the appropriate antibiotic. The cultures were grown at 37°C for overnight with vigorous shaking. The cells then were harvested by centrifugation at 4500 x g for 15min. Each bacterial pellet was suspended in 10ml Buffer P1+RNase. Next, 10ml of Buffer P2 was added to each tube and mix thoroughly by inverting 4-6 times. Then, the tubes were incubated at room temperature for 5min. 10ml of Buffer P3 was added to each tube and immediately mix by gently inverting 4-6 times and incubate on ice for 15min. The tubes were then centrifuged at 20,000 x g for 30min at 4°C. The supernatant containing plasmid DNA was removed and filtered using Mira cloth. Next, QIAGEN-tips 100 were equilibrated by applying 4ml Buffer QBT and the columns were allowed to empty by gravity flow. Then, the filtered supernatants were allowed to enter the resin by gravity flow. The QIAGEN-tips were washed with 2 x 10ml Buffer QC. The DNA was eluted with 5 x 1ml Buffer QF and precipitated by 3.5 ml isopropanol. Then, the DNA was mixed and centrifuged at 15,000 x g for 30min at 4°C. The supernatant were carefully decanted. The DNA pellet was washed with 2ml of 70% ethanol and centrifuged at 15,000 x g for 10min. The supernatants were decanted without disturbing the pellet. Finally, the pellets were air-dried until all liquid disappeared and re-dissolved in a 100µl of Mili-Q water.

Digestion of Plasmid-DNA

Before digestion of Plasmid-DNA was performed, the concentration of isolated plasmid-DNA was measured by using Nanodrop. Then, 1µg of DNA was added in 30µl of digestion reaction, including 3µl of suitable buffer (10x), 1µl of enzyme A and 1µl of enzyme B. The reaction mixture was put at 37°C for at least 2 hours to allow for a complete digestion of DNA. For donor vector, 1µl of Thermosensitive Alkaline Phosphatase (TSAP) was added after digestion and put in the incubator for another 15 minutes at 37°C in order to avoid self-ligation in the vector itself. The digestion was then inactivated at 60°C for 20min. After that, the digestion products were run on the gel to check digestion efficiency and quantity of DNA.

Ligation of digested DNA

For ligation of digested DNA, T4 ligase was used to join the 5'phosphate and the 3'-hydroxyl groups of double stranded DNA molecules. Firstly, the concentrations of vector and insert were estimated based on the gel electrophoresis results of digestion step. In order to increase the chance of insertion into a vector, the insert was added 3-fold molar excess more than the vector. When estimating the molar ratio of 1:3, the size of the insert was considered in order to get molar equivalents of insert and vector. Then, 8µl of reaction was made including 1µl of ligase buffer and 1µl of ligase. The mixture were centrifuged at 4600 rpm and incubated at 16°C for overnight.

Transformation of E.coli

Competent MegaX DH10B cells (Invitrogen, Netherlands) were used for *E.coli* transformation. Firstly, the inactivated ligation mixture was diluted with Mili-Q water to reduce the amount of salt which was introduced during previous steps. High amount of salt in the DNA might cause sample to arce when electroporating which eventually causes cells death and significantly reduces the transformation efficiency. 12 µl of the cells was pipetted into the pre-cooled tube containing 1 µl of desalted ligation mixture. The mixture were then electroporated at 1.40 V by using Gene PulserII (Bio-Rad) which was set at 25 µ F capitance and 200 MOhm. The electroporation was successful with a time constant between 4 to 5 ms. After adding 2 X of 500µl of LB media, the cell mixtures were incubated in 37°C shaker for at least 1 hour for recovery.

Blue/white selection

Agar plates containing LB broth and supplemented with appropriate antibiotics, X-gal (5-bromo-4chloro-3-indolyl-β-D-galactopyranoside) and IPTG (Isopropyl-beta-thio galactopyranoside) were prepared. 100 µl of transformation products was spread onto LB-agar plate. The remaining 900 µl of culture was spread on another plate to make a comparison with the 100 µl spread after overnight culture in 37°C incubator. On the next day, white colonies were picked up by sterile tip and transferred into 96-well plate containing LB medium supplemented with appropriate antibiotics. The plate was put into incubator overnight at 37°C to allow for the potential positive colonies to grow.

Colony PCR

Polymerase Chain Reaction (PCR) was used to screen for the presence or absence of specific genes in the plasmid transformants. Specific primers were used for each *R* gene. PCR was performed in a 25µl reaction, including 1µl of forward primer (10µM), 1µl of reverse primer (10µM), 1µl of dNTPs (5mM), 2.5µl of Dream Taq buffer (5mM) and 0.1µl of Dream Taq polymerase (Fermentas). The detailed information of the specific primers can be found in table 4. 1 µl of potential positive colonies grown in 96-well plate were added into PCR reaction mixture and colony PCR was carried out with the following program: 94°C for 5min, 94°C for 30s, Tm for 30s, 72°C for 1min, 72°C for 5min during 30 cycles. Then, the PCR products were run on gel electrophoresis to check for positive colonies.

Table 4: Overview of the specific primers for colony PCR

Name	Gene (*Rpi*)	Sequence (5' - 3')	Tm (°C)	Product
MA524	blb3	F AGGTTGCCAATGGAAGACGGAATA	57	400bp
MA525		R GAAAGAATGGAGGATGTGGCTGAC		
MA508	sto1	F AGAGGATGTGGGCGATGAAGTATG	57	500bp
MA509		R GCTTCTTTTGCGTCCTTATCATTC		
MA537	vnt1	F GCCCCCTCCAATCGCACAT	57	500bp
MA538		R AAATTCAAGATGGATGTGGTAAGG		
MA446	edn2	F GCATCATGTCTGCACCTATG	54	380bp
MA447		R CTTTGATGTGGATGGATGGTG		
MN586	chc1	F ACAGATAATAATTTTCAACTGC	57	320bp
MN587		R ATTTGGGACATTCTGATATAAG		

F – Forward primer
R – Reverse primer

Single colony PCR for confirming the construct

Two colonies that were positive in the colony PCR were re-streaked and re-grown again in agar plates containing LB medium supplemented with appropriate antibiotics to get a single colony. The single colony was tested again with PCR and checked on the gel to confirm the positive colonies.

Transformation of A. tumefaciens and colony PCR

The successfully built constructs were transformed to *A.tumefaciens AGL1+pVirG* by electroporation. Before that, the plasmid DNA from construct in *E.coli* was isolated with QIAGEN® Plasmid Midi Kit. Then, 20 µl of the cells was pipetted into the pre-cooled tube containing considerable amount of DNA which was calculated to be equal to approximately 15ng. The mixture were then electroporated at 1.40 V by using Gene PulserII (Bio-Rad) which was set at 25 µ F capitance and 200 MOhm. The electroporation was successful with a time constant between 4 to 5 ms. After adding 2 X of 500µl of LB media, the cell mixtures were incubated in 37°C shaker for at least 3 hour for recovery.

Stability test of constructs

After the single and double *R* gene cassettes were made, the stability of these cassettes in *A. tumefaciens* strains was tested. Firstly, the *A.tumefaciens* transformants (*AGL1+pVirG)* were cultured in selective medium containing Kanamycin, Chloramphenicol and Carbenicilin. After two or three days, four colonies obtained from each construct were grown in 3ml LB+antibiotic culture. From each culture, glycerol stock was made and the remaining culture (1µl) was grown in new 3ml LB+antibiotic culture. When the OD of the culture reaches 1.0, it was spread into agar selective medium to obtain 20 colonies. 20 colonies from each 4 cultures were then tested with colony PCR to see whether both *R* genes were stably harbored.

Validation of new R gene stacks using agroinfiltration in N. benthamiana

The co-infiltration of newly developed construct in *A.tumefaciens* harboring candidate *R* gene(s) with their corresponding *Avr* was performed to screen for the functionality of the *R* genes in the construct. The procedure for *A.tumefaciens* strain preparation is same as described in previous experiments.

Plant transformation

An *R* gene cassette (*Rpi-vnt1: Rpi-blb3: Rpi-chc1*) that has been developed in previous work was transformed into susceptible potato cultivar Desiree by using *Agrobacterium*-mediated transformation. Two days before transformation, *A. tumefaciens* containing the stably inserted triple *R* genes construct was grown in 50 ml liquid LB medium with selection in order to prepare for transformation. The explants of susceptible potato cultivar Desiree were cut where the internodia with a size of 2-5 mm were used. The explants were transferred to R3B medium containing 4.4g of MS, 30g of saccharose, 8g of agar, 2ml of 1-1 naphthaleneacetic acid (NAA) and 1ml of BAP in 1L volume. On each of the petridish with R3B medium, 2 sterile filter papers were put with 1.5ml of liquid PACM medium (4.4g MS, 2g caseine hydrolysate, 30g saccharose, 1ml 2,4 D and 0.5ml kinetine in 1l volume). On the next day, the grown culture was centrifuged for 10 min at 2500 rpm. After that, 1.5 of the original volume of LB without addition of antibiotics was poured into the pellet. The explants from R3B medium were transferred into bacteria solution and left for 5-10 min. After that, the explants were dried and transferred back on the R3B plate. After 2 days, the explants were transferred to Zcvk medium which contained 4.4g MS, 20g saccharose, 8g agar, 1ml zeatine, 2ml claforan, 2ml vancomycine and 2ml of kanamycine in 1L volume. Three controls were added which consists of plate with i) no bacteria, no selection; ii) with bacteria, no selection; iii) no bacteria, with selection. After 2 weeks, the explants were transferred to fresh Zcv-selection medium. This step was repeated until sufficient transformants were harvested.

Results

Functional validation of newly cloned late blight resistance genes

Seven groups of transgenic plant harboring *Rpi-chc1* homologs were screened to validate the different *Rpi-chc1* homologs and to find their recognition specificities with various isolates and effectors of *Phytophthora infestans*. The screenings of the plants were done by using detached leaf assay and agro-infiltration.

DLA screening of transgenic events containing Rpi-chc1 homologs

In total, 35 transgenic events of different *Rpi-chc1* homologs were examined for their level of resistance towards nine isolates. Most of transgenic events tested for A32 (324-2/J8) and A33 (852-5/E14) were found to be resistant to H30PO4 while only one or two were resistant to IPO-C and Pic 99183 (Table 5). There was no resistance displayed by most of the plants tested with other isolates except for A40 (852-5/E28). The frequency of finding resistant events was very low in A34 (487-1/I6), A35 (493-7/G19), A36 (493-9/H5) and A37 (543-5/C2). In contrast to other groups, A40 (852-5/E28) showed at least two events to be resistant to all isolates. Among the A40 transgenic events tested, all gave full resistance to H30PO4 and Pic 99183, four were resistant to IPO-C and three or two were resistant to other isolates.

A17-27 plants gave considerable resistance levels to most of the isolates except for NL 7245 and Dinteloord. An expected result was displayed by Desiree plants when they were found to be susceptible to all isolates (Figure 3). No spectrum similarity was observed between any of the donor plants.

Table 5: Isolate recognition specificities among different *Rpi-chc1* homologs

TE	Genotype /Gene	Events tested(#)	H30PO4	IPOC	USA 618	NL 7245	Pic 99183	Dinteloord	Katshaar	NL 7379	NL 7135
A32	324-2/J8	4	4	1	-	-	1	-	-	-	-
A33	852-5/E14	9	8	1	-	-	2	-	-	-	-
A34	487-1/I6	5	1	-	-	-	1	-	-	-	-
A35	493-7/G19	5	1	-	-	-	-	-	-	-	-
A36	493-9/H5	4	-	-	-	-	-	-	-	-	-
A37	543-5/C2	2	-	-	-	-	1	-	-	-	-
A40	852-5/E28	6	6	4	2	3	6	3	2	3	2
A17-27	*Rpi-chc1* long		R	R	R	MR	R	S	R	R	R

Desiree	S	S	S	S	MS	S	S	S	S
94-2031	HR	R	R	R	R	S	R	HR	R
852-5	S	S	S	MR	MR	MR	MR	MR	MR
493-7	HR	R	S	S	S	MR	R	R	S
493-9	HR	R	S	R	R	R	S	MR	S
543-5	MR	S	MR	R	R	MR	R	R	R
852-5	S	S	S	MR	MR	MR	MR	MR	MR

Plants scored 1-2.5: Resistance (R) and 2.6- 3.5-Moderate Resistance (MR), and grouped as susceptible, in between scores 3.6 -7.

94-2031 Donor plant for A32 and A17-27

852-5 Donor plant for A33

493-7 Donor plant for A35

493-9 Donor plant for A36

543-5 Donor plant for A37 and A17-27

852-5 Donor plant for A40

Screening of transgenic events containing Rpi-chc1 homologs by using agro-infiltration

All Avr-*chc1 homologs* available in house (Table 3) need to be tested first for their ability to give any necrosis in the absence of the *R* gene. Sixteen effectors were examined through agro-infiltration on susceptible potato cultivar Desiree at three different ODs. *Avr3b* and *Avr3b+R3b* were included as negative control and positive control respectively (Fig. 2). *D2* and *C1* (*GV3101*) were observed not to cause necrosis at any OD used in this experiment. In contrast, *B4*, *C2* and *D1* gave a relatively higher necrosis than other effectors. *C2* and *D1* showed auto-necrosis at all ODs tested. Lower ODs reduced the extent of autonecrosis. Remarkably, three of the effectors that are available in two different *A. tumefaciens* strains; *B2*, *B3* and *C1* displayed more necrosis in *Agl1* than *GV3101* strains. Other effectors such as *A1-1*, *A2-1* and *B1* were observed to give minimum a-specific cell death ranging from 0.1 to 0.2 in different OD. Seven effectors were selected and the OD was determined based on the one that causes minimum or no auto-necrosis.

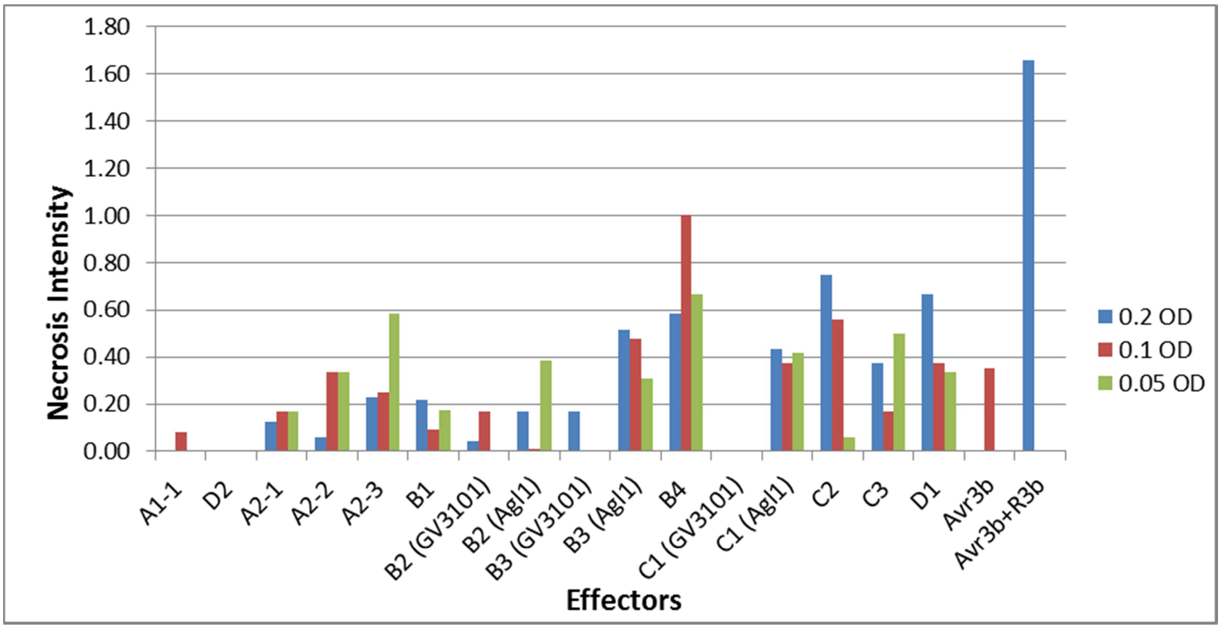


Fig 2: Effector responses on susceptible potato cultivar Desiree at different OD after 4 dpi. Sixteen available *Avr* effectors were tested for auto-necrosis response. *Avr3b* and *Avr3b+R3b* act as negative control and positive control respectively.

A2-2 gave HR in the majority of A40 (852-5/E28) events while *C2* gave a very minimum HR in one plant from A34 (487-1/I6) and A37 (543-5/C2) which was 0.1 and 0.2 respectively. Control plants, A17-27, also showed significant HR to *A2-2*. *Avr3b* and *Avr3b+R3b* were used as negative and positive control respectively in this experiment (Table 6).

Table 6: *Avr* effectors recognition specificities among different *Rpi-chc1* homologs

TE	Genotype /Gene	Events tested(#)	Effectors responsiveness				
			A2-2 (0.2 OD)	C2 (0.05 OD)	C3 (0.1 OD)	Avr3b (0.1 OD)	Avr3b+R3b (0.2 OD)
A32	324-2, 94-2031/J8	4	-	-	-	-	1
A33	852-5/E14	9	-	-	-	-	3
A34	487-1/I6	5	-	1	-	-	1
A35	493-7/G19	5	-	-	-	1	1
A36	493-9/H5	4	-	-	-	-	2
A37	543-5/C2	2	-	1	1	-	-
A40	852-5/E28	6	4	-	-	-	2
A17-27	*Rpi-chc1* long		HR	-	-	-	HR
Desiree			-	-	-	-	HR
94-2031			HR	0.2	-	0	0.6
493-7			nd	nd	nd	nd	nd
493-9			0.2	0.2	-	0.1	0.2
543-5			HR	HR	HR	0.4	0.9
852-5			HR	0.6	-	0.4	0.6

Plants scored 1-2 for indicating 50-100% cell death as strong HR are considered as incompatible effectors of the specific transgenic events. 94-2031 Donor plant for A32 and A17-27; 852-5 Donor plant for A33; 493-7 Donor plant for A35; 493-9 donor plant for A36; 543-5 Donor plant for A37 and A17-27; 852-5 Donor plant for A40

None of the events from A32 (324-2, 94-2031/J8), A33 (852-5/E14), A34 (487-1/I6), A35 (493-7/G19) and A36 (493-9/H5) transgenics showed significant HR to any effectors (Table 5, Appendix 3 and 4). Thus, no correlation can be made to isolate resistance. In contrast, one of the A37 (543-5/C2) plants which was A37-23 displayed a significant HR to effector *C3* in the first agro-infiltration test (Table 6). This finding was expected for transgenic plants containing *C2* gene as they are anticipated to respond to C class effectors. However, an inconsistent result was obtained when in the repeat experiment; only a minimum HR response can be seen to the same effector. Besides A37 (543-5/C2) plants, A35 (493-7/G19) plants were also expected to respond to C class effectors. Nonetheless, the result showed that nearly none of the transgenic events from A35 (493-7/G19) recognised any of the C class effectors (Appendix 3 and 4). For *A2-2* effector, a very low HR response can be observed by

A37 (543-5/C2) plants. In disease resistance test, A37-23 transgenic event showed disease response to only one of the isolates which was to Pic 99183 with a moderate resistance (Table 5). Based on this result, it can be observed that low HR response in *A2-2* effector would considerably cause the plants to be resistant to less number of isolates. In case of A36 (493-9/H5) plants, it is expected that these plants would give response to *A1-1* effector. But, it was found that none of the transgenic events from this plant group showed HR response to *A1-1* (Appendix 3 and 4).

For A40 (852-5/E28), six transgenic events were used in detached leaf assay and agro-infiltration test (Table 7). Four of the transgenic events gave significant HR to *A2-2* effector in agro-infiltration. In contrast to previous groups, a high HR in *A2-2* had led to more responses in disease resistance assay. Among all transgenic events, most of them showed resistance to all nine isolates especially A40-31(Fig 3 and 4), A40-44 and A40-53. Whereas, A40-4 and A40-28 gave resistance to less number of isolates as their effector recognition to *A2-2* was relatively less. From another point of view, among nine isolates, all events appeared to cause resistance response to H30PO4 and Pic 99183 while only two events conferred resistance to USA618 and Katshaar.

Table 7: Correlation between disease resistance and *Avr* response in A40 (852-5/E28)

TE	*Pi* inoculation									Effector recognition
	H30 PO 4	Pic 99183	IPO C	NL 7245	Dinteloord	NL 7379	USA 618	Katshaar	NL 7135	A2-2 (0.2)
A40-4	R	MR	S	S	S	S	S	S	S	0
A40-28	R	R	MR	S	S	S	S	S	S	0.5
A40-53	R	R	R	MR	R	MR	S	R	R	1.0
A40-44	HR	R	HR	R	R	R	MR	S	S	1.9
A40-31	R	R	R	MR	MR	R	R	R	R	2.0
A40-51	R	R	S	S	S	S	S	S	S	1.2
A17-27	R	R	R	MR	S	R	R	R	R	1.6
Desiree	S	MS	S	S	S	S	S	S	S	0

Plants scored 1-2.5: Resistance(R) and 2.6- 3.5-Moderate Resistance (MR), and grouped as susceptible, in between scores 3.6 -7. In agro-infiltration plants scored ‘’1-2’’ indicating strong HR (Hypersensitive Response).

The donor plant for each group of transgenic events harboring *Rpi-chc1* homologs was included in this study (Table 5 and 6). However, the result for donor plant of A34 (487-1/I6) need to be ignored because it showed responses to all isolates and effectors including the negative control (Appendix 1, 3 and 4). This plant was most likely in stressed condition during the experiment. Generally, based on the results of disease resistance assay, none of the set of the transgenic plants demonstrated the same isolates recognition pattern with their respective donor plants. As for agro-infiltration test, A40 (852-5/E28) transgenic events showed about the same pattern for *A2-2* effector recognition with their donor plant (852-5) where almost all of them gave relatively high HR. Whereas, other *Rpi-chc1* homologs were observed to give no HR response to *A2-2* which was not in the case of their donor plants where they showed clear HR response.

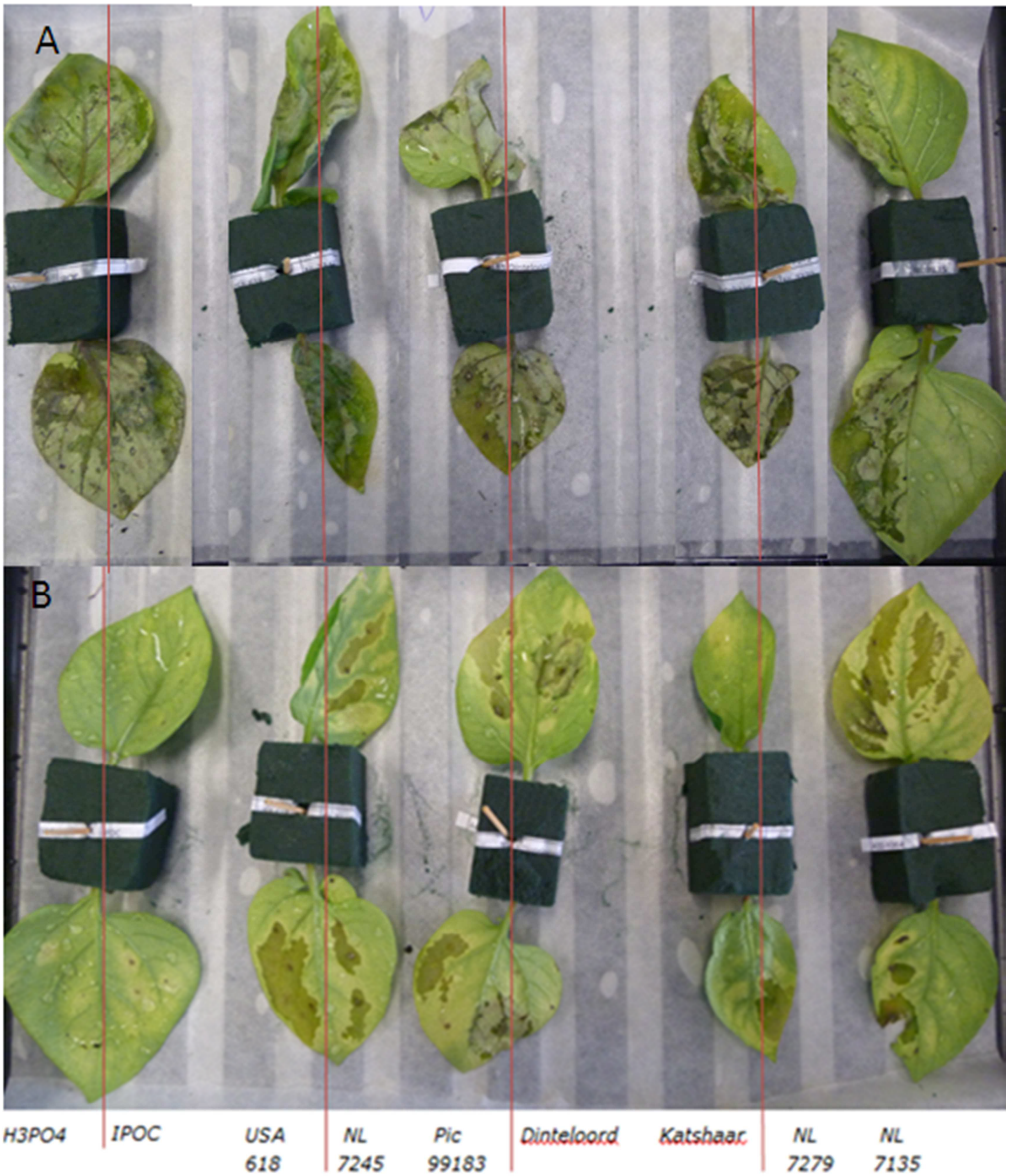


Fig 3: Detached leaf assay in susceptible Desiree plant (A) and A40-31 (B) by using *Pi* isolates in which six 10µl droplets of a zoospore suspension (50,000 spores/ml).

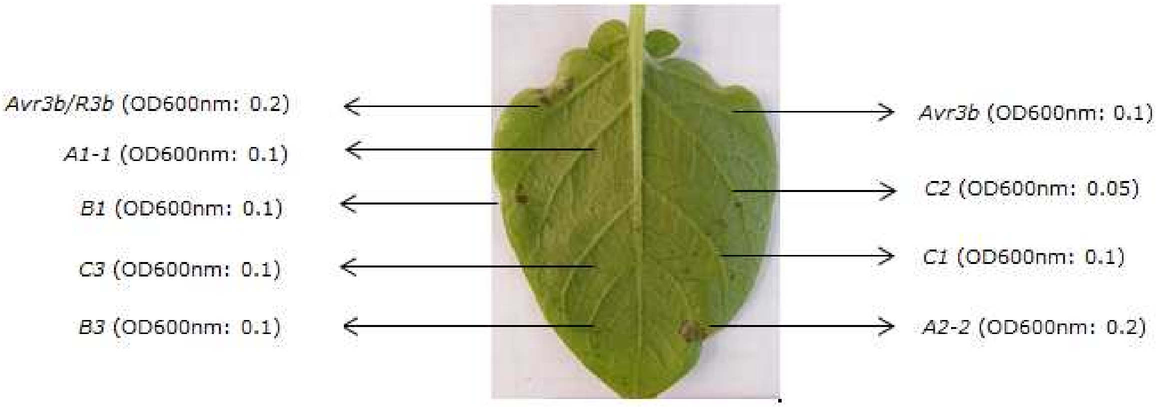


Fig 4: Agro-infiltration of *Avr* effectors in A40-31. Co-infiltration of *Avr3b/R3b* served as a positive control and *Avr3b* alone as a negative control.

Development of *R* genes constructs

In order to develop double *R* genes constructs, two vectors were used; pUC vector with ampicillin selection and pBINPLUS vector with kanamycin selection. In pUC vector, three sets of specific restriction enzyme recognition sites were used which were *PacI-AbsI-SrfI-SbfI-AscI* (PASSA), *AbsI-SbfI-AscI-SrfI* (ASAS) and *PacI-SbfI-AscI-AbsI* (PSAA) to provide unique orders of cutting sites. Furthermore, two *R* genes that are cloned using the same restriction sites can be combined in one vector. For instance, *Rpi-edn2* and *Rpi-sto1* are cloned using the same restriction sites which are *SbfI* and *AscI*. These two *R* genes can be harbored in one vector by using pUC-ASAS to shuttle *Rpi-edn2* in between *AbsI* and *SrfI* and sub cloning it into pBINPLUS-PASSA which was already inserted with *Rpi-sto1* in *SbfI-AscI* position (Table 9). There were 11 single *R* gene constructs that had been made and eight of them were used to develop seven double *R* genes constructs (Table 8 and 9). Both single and double *R* gene vectors that have been made after enzyme digestion and ligation except for pUC-PSAA vector were transformed into *E.coli* (Competent MegaX DH10B cells) by electroporation. Then, all successfully built pBINPLUS-PASSA construct containing single and double *R* genes were transformed into *A. tumefaciens* strain *AGL1+pVirG* (Table 8 and 9).

Table 8: Overview of construction of single *R* gene vector

Single *R* gene vector	Cloning steps	Location of the gene	Size
pUC-PASSA:edn2	pRIAB-PASSA:edn2 is digested with *SbfI-AscI* and cloned into pUC-PASSA	*PacI-AbsI-SrfI-SbfI-edn2-AscI*	±10.7kb
pUC-PASSA:chc1	pBINPLUS:chc1 is digested with *SbfI-AscI* and cloned into pUC-PASSA	*PacI-AbsI-SrfI-SbfI-chc1-AscI*	±10.7kb
pUC-ASAS:edn2	pRIAB-PASSA:edn2 is digested with *SbfI-AscI* and cloned into pUC-ASAS	*AbsI-SbfI-edn2-AscI-SrfI*	±10.7kb
pUC-ASAS:chc1	pBINPLUS:chc1 is digested with *SbfI-AscI* and cloned into pUC-ASAS	*AbsI-SbfI-chcI-AscI-SrfI*	±11kb
pUC-ASAS:sto1	pBINPLUS:sto1 is digested with *SbfI-AscI* and cloned into pUC-ASAS	*AbsI-SbfI-stoI-AscI-SrfI*	±9.7kb
pUC-PSAA:edn2	pRIAB-PASSA:edn2 is digested with *SbfI-AscI* and cloned into pUC-PSAA	*PacI-SbfI-edn2-AscI-AbsI*	±10.7kb
pUC-PSAA:chc1	pBINPLUS:chc1 is digested with *SbfI-AscI* and cloned into pUC-PSAA	*PacI-SbfI-chc1-AscI-AbsI*	±10.7kb
pUC-PSAA:sto1	pBINPLUS:sto1 is digested with SbfI-AscI and cloned into pUC-PSAA	*PacI-SbfI-sto1-AscI-AbsI*	±9.7kb
pBINPLUS-PASSA:edn2	pUC-PASSA:edn2 is digested with *SbfI-AscI* and cloned into pBINPLUS-PASSA	*PacI-AbsI-SrfI-SbfI-edn2-AscI*	±20.1kb
pBINPLUS-PASSA:sto1	pUC-PASSA:sto1 is digested with *SbfI-AscI* and cloned into pBINPLUS-PASSA	*PacI-AbsI-SrfI-SbfI-sto1-AscI*	±19.1kb
pBINPLUS-PASSA:blb3	pUC-PASSA:blb3 is digested with *SrfI-SbfI* and cloned into pBINPLUS-PASSA	*PacI-AbsI-SrfI-blb3*-Sbf*I-AscI*	±20.9kb

Table 9: Overview of construction of double *R* genes vector

R genes combination (pBINPLUS-PASSA)	Cloning steps	Location of the gene	Size
edn2-sto1	pUC-ASAS:edn2 is digested with *AbsI-SrfI* and cloned into pBINPLUS-PASSA:sto1	*PacI-AbsI-SbfI-*edn2*-AscI-SrfI-SbfI-*sto1*-AscI*	±26.4kb
blb3-edn2	pUC-PASSA:edn2 is digested with Sbf*I-AscI* and cloned into pBINPLUS-PASSA:blb3	*PacI-AbsI-SrfI*-blb3-Sbf*I*-edn2-Asc*I*	±28.2kb
chc1-sto1	pUC-ASAS:chc1 is digested with *XhoI(*inside *AbsI)-XmaI(*inside *SrfI)* and cloned into pBINPLUS-PASSA:sto1	*PacI-AbsI-SbfI-*chc1*-AscI-SrfI-SbfI-*sto1*-AscI*	±26.7kb
blb3-chc1	pUC-PASSA:chc1 is digested with Sbf*I-AscI* and cloned into pBINPLUS-PASSA:blb3	*PacI-AbsI-SrfI*-blb3-Sbf*I*-chc1-Asc*I*	±28.5kb
chc1-edn2	pUC-ASAS:chc1 is digested with *XhoI(*inside *AbsI)-XmaI(*inside *SrfI)* and cloned into pBINPLUS-PASSA:edn2	*PacI-AbsI-SbfI-*chc1*-AscI-SrfI-SbfI-*edn2-*AscI*	±27.7kb
vnt1-blb3	pUC-PASSA:blb3 is digested with *XmaI(*inside Srf*I)-SbfI* and cloned into pBINPLUS-PASSA:vnt1	*PacI-AbsI-*vnt1*-SrfI-*blb3-Sbf*I*-Asc*I*	±25.18kb
vnt1-edn2	pUC-PASSA:edn2 is digested with *SbfI-AscI* and cloned into pBINPLUS-PASSA:vnt1	*PacI-AbsI-*vnt1*-SrfI-* Sbf*I*- edn2-Asc*I*	±24.38kb

R gene cloning into donor and acceptor vectors

After isolation of plasmid DNA from donor vector (pUC vector) and acceptor vector (pBINPLUS PASSA vector), digestion step was carried out in order to prepare for ligation reaction. For example, to construct a double *R* gene vector of pBINPLUS-PASSA: vnt1:edn2, pUC-PASSA: edn2 as a donor vector was digested with *SbfI-AscI*. When compared with the marker ladder in lane M, a brightest band in lane C and D clearly shows the digested vector fragment is at the right position of 7.7 kb (Figure 5). An extra bright band appeared above the position of the right size band which indicates partial digestion of vector. In addition, a weak band with a size of 3 kb is also seen which represents pUC vector fragment. Besides that, pBINPLUS-PASSA: vnt1 as an acceptor vector was also digested with *SbfI-AscI*. A bright band is seen from the gel in lane A and B with the right size between 10 kb and 20 kb while the small fragments with several nucleotides length that was cut out are not visible (Figure 5). From the gel image, it is concluded that the quality and amount of digested products were sufficient for ligation in next step. Next, the digestion products from both vectors were ligated by T4 ligase enzyme.

After ligation step, the product was transformed into *E.coli* and grown in selective medium. 96 single white colonies were picked up from the plate and re-grown in 96-well plate containing selective medium overnight. Then, colony PCR was carried out to screen for positive recombinants in ligation as well as positive transformants in *E.coli*. From 22 colonies, only one showed no band in colony PCR when specific forward and reverse primer for *Rpi-vnt1* were used. The positive transformants gave products with size of 500 bp (Figure 6a). The presence of *Rpi-edn2* gene was also examined in other PCR test by using specific primer for *Rpi-edn2*. Nineteen colonies from 22 colonies showed positive bands in colony PCR with the right size of 380 bp (Figure 6b). Two positive colonies from initial PCR screen were selected and restreaked in plate in order to purify them. Then, PCR test was done again to confirm the successful transformants (Figure 7).

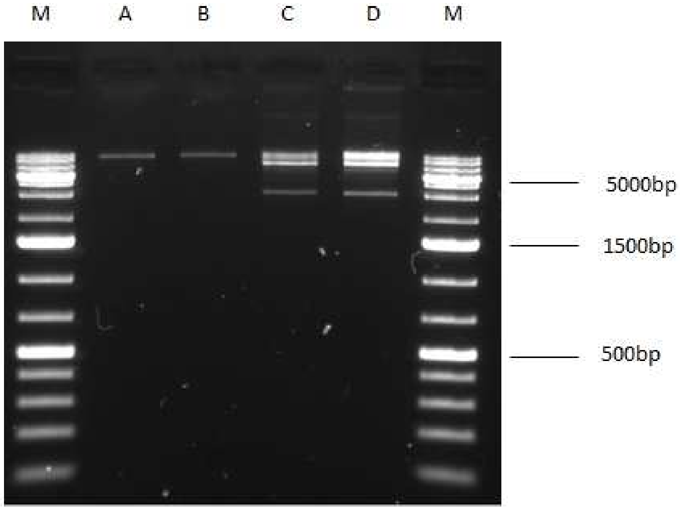
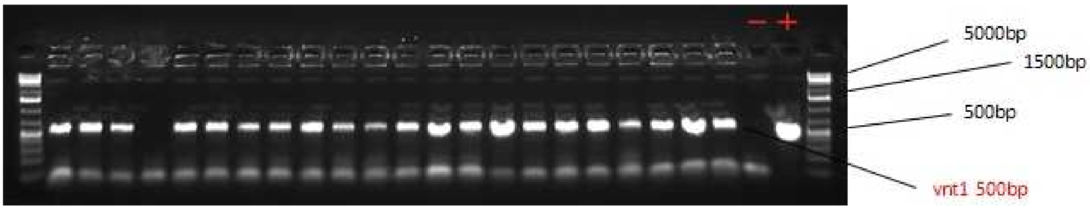


Fig 5: Example of preparation of donor and acceptor plasmids for shuttle reaction.
Digestion of acceptor vector pBINPLUS-PASSA: vnt1 with *Sbf*I and *Asc*I (lane A, B). Digestion of donor vector pUC-PASSA: *edn2* with *Sbf*I and *Asc*I (lane C, D). Lane M O'GeneRuler 1kb plus DNA Ladder.

a)



b)

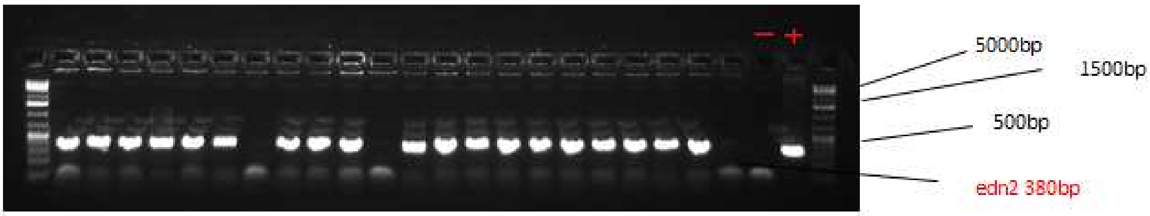


Fig 6: Example of subcloning efficiency.
A: Colony PCR check for *Rpi-vnt1* (500bp) in construct PBINPLUS-PASSA: vnt1:edn2. B: Colony PCR checks for *Rpi-edn2* (380bp) in construct PBINPLUS-PASSA: vnt1-edn2. – indicates negative control for PCR (no template added). + indicates positive control (template from isolated DNA of pUC vector)

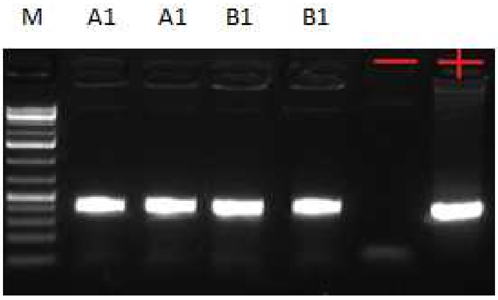


Fig 7: Example of colony purification.
Colonies that were positive after initial PCR screen were purified by restreaking and testing single colonies again using PCR. Positive clones of pBINPLUS-PASSA:vnt1:edn2, lane A1 from single colony A1 and lane B1 from single colony B1, lane M O'GeneRuler 1kb Plus DNA Ladder.

Transformation of R gene constructs into A. tumefaciens

All pBINPLUS-PASSA constructs harboring single and double *R* genes were transformed into *A.tumefaciens*. After transformation, it was examined whether the constructs could replicate stably in *A. tumefaciens* transformant clones. From selective solid LB medium, four colonies were picked for each construct and grown in selective liquid LB medium. Then, from each colony, a glycerol stock was made and the remaining was used to inoculate a new culture tube with selective liquid LB medium. After three days, the cultures were streaked on selective solid LB medium in order to get 20 single colonies. Those 20 colonies were picked and tested with PCR to check presence of the *R* gene (s). The colonies of all single *R* gene vectors tested gave stable result where all 20 colonies from each culture showed the target band in PCR reaction with the specific primers. However, for double *R* genes constructs, four out of seven constructs transformed without deletions (Table 10). One construct (pBINPLUS-PASSA: vnt1:blb3) showed that some colonies lacked either of both genes. Two constructs always showed deletion of the second gene.

Table 10: Overview of stability test of single and double *R* genes vector in *A. tumefaciens*

	Gene 1				Gene 2			
Vector (in pBINPLUS PASSA)	Colony 1	Colony 2	Colony 3	Colony 4	Colony 1	Colony 2	Colony 3	Colony 4
Rpi-edn2	S	S	S	S				
Rpi -sto1	S	S	S	S				
Rpi -blb3	S	S	S	S				
Rpi vnt1-blb3	S	S	S	N	S	S	S	N
Rpi -edn2-sto1	S	S	S	S	S	S	S	S
Rpi -chc1-sto1	S	S	S	S	S	S	S	S
Rpi -chc1-edn2	S	S	S	S	S	S	S	S
Rpi -vnt1-edn2	S	S	S	S	S	S	S	S
Rpi -blb3-chc1	S	S	S	S	N	N	N	N
Rpi -blb3-edn2	S	S	S	S	N	N	N	N

20 colonies were tested with PCR, S indicating stable; 18-20 colonies show presence of the gene, N: not stable; 0-17 colonies show presence of the gene.

Co-infiltration of new double and triple R gene constructs with their corresponding Avr effectors

The new constructs in *A. tumefaciens* with stable plasmid insertions were tested for their functionality of the genes by co-infiltration with their corresponding *Avr* effector in *N. benthamiana* (Appendix 19). However, from the results obtained, no conclusion can be drawn since the positive and negative control did not show the expected result. A repeat experiment need to be done in susceptible Desiree plants because the constructs containing *Rpi-vnt1* show autonecrosis in *N. benthamiana*.

Transformation of stably inserted triple R genes construct into susceptible potato cultivar Desiree

Stable insertion of the triple *R* genes construct, pBINPLUS-PASSA: vnt1: blb3: chc1 that was developed in previous work by Marjon Arens was transformed into susceptible potato cultivar Desiree by using *Agrobacterium*-mediated transformation method (Figure 8).

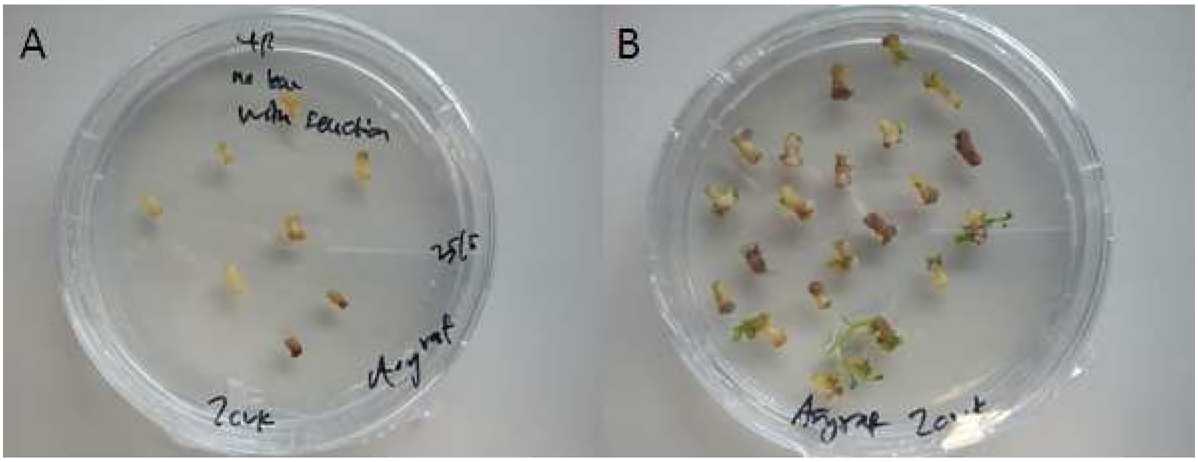


Fig 8: Transformation of susceptible potato cultivar Desiree after 15 weeks.
A: Explants grown on kanamycin containing medium without co-cultivated with *Agrobacterium* as a control. B: Explants with regenerating shoots after co-cultivation with *Agrobacterium* containing pBINPLUS-PASSA:vnt1:blb3:chc1 vector and grown on kanamycin containing medium.

Functional characterization of double *R* genes stacked in transgenesis and cisgenesis Desiree plants

Firstly, functionality of 24 of A26 plants containing *Rpi-blb3* and *Rpi-sto1* were validated by isolates test and agro-infiltration of corresponding *Avr* effectors (Table 11). In DLA test, two isolates were used, IPO-O which was reported to break *Rpi-sto1* and to be recognized by *Rpi-blb3* and IPO-C which was vice versa. IPO-O was recognized *Rpi-blb3* in all A26-plants except for A26-1386 while IPO-C was recognized only half of the plants. Eight out of 24 plants gave cell-death response to both isolates. Susceptible Desiree plants showed a clear susceptibility to both isolates. Besides, several control plants were also included in this test such as A10-43 (*Rpi-blb3* and *Rpi-sto1)* and A14-16 (*Rpi-vnt1*, *Rpi-blb3* and *Rpi-sto1)*, A03-142 (*Rpi-blb3)* only and three A09 (*Rpi-sto1)* events. All control plants displayed the expected results except for A09 plants which showed resistance to both isolates. In agro-infiltration test, two *Avrs* were infiltrated into the leaves which were *Avr-sto1* that corresponding to *Rpi-sto1* and *Avr*2 that corresponding to *Rpi-blb3*. In addition, co-infiltration of *Avr3b* and *R3b* was also included to serve as a positive control while only *Avr*3*b* as a negative control. Remarkably, only one of the A26 plants tested responded well to *Avr*2 and half to *Avr-sto1*. For control plants, all of them showed clear response to their corresponding *Avr*s.

Table 11: Activity of *R* genes in cisgenic Desiree plants (A26 plants harboring *Rpi blb3:Rpi sto1*) in relation to resistance and effector recognition.

Transgenic event	*Pi* inoculation		Effector recognition by measuring HR			
	IPO-C	IPO-O	Avr-sto1 (0.1 OD)	Avr2 (0.2 OD)	Avr3b (0.1 OD)	Avr3b+R3b (0.2 OD)
A26-1023	MR	HR	0.8	0.0	0.0	0.9
A26-1065	S	HR	1.2	0.0	0.0	0.3
A26-1172	S	HR	0.0	0.4	0.0	0.8
A26-1206	R	R	1.5	0.0	0.0	0.2
A26-1263	S	MR	0.0	0.0	0.0	0.1
A26-1369	S	R	0.0	0.0	0.0	0.3
A26-1389	S	R	0.9	0.1	0.0	0.2
A26-1554	R	R	1.0	0.1	0.0	0.4
A26-1679	R	HR	1.5	0.0	0.0	0.3
A26-1735	S	HR	1.1	0.1	0.0	0.4
A26-2351	S	R	0.0	0.0	0.0	0.2
A26-528	R	HR	1.7	0.0	0.0	0.4
A26-767	R	R	1.3	0.0	0.0	0.6
A26-768	S	R	0.0	0.0	0.0	0.2
A26-871	R	R	1.5	0.0	0.0	0.4
A26-882	MR	R	1.0	0.0	0.0	0.7
A26-915	R	HR	1.2	0.2	0.0	0.4
A26-941	S	R	0.0	0.0	0.0	0.8
A26-946	S	R	0.0	0.0	0.0	0.4

A26-947	S	R	0.0	0.0	0.0	0.8
A26-948	S	R	0.0	0.0	0.0	0.5
A26-97	R	HR	1.8	1.8	0.0	0.4
A26-1386	S	S	nd	nd	nd	nd
A26-1018	S	R	nd	nd	nd	nd
A03-142	S	HR	0.0	1.6	0.0	1.0
A09-277	R	HR	0.9	0.1	0.0	0.1
A10-43	R	HR	1.5	1.1	0.0	0.7
A14-16	HR	HR	1.8	1.9	0.0	0.4
Desiree	S	S	0.0	0.0	0.0	1.2
A09-268	R	R	1.6	0.1	0.0	0.7
A09-283	R	HR	1.2	0.0	0.0	0.7

In *Pi* inoculation the plants scored 0-0.9: Highly Resistance (HR), 1-2.5: Resistance(R) and 2.6- 3.5-Moderate Resistance (MR), and grouped as susceptible, in between scored 3.6 -7.0 In agro-infiltration plants scored 1.0-2.0: HR (Hypersensitive Response),'nd' means not determined.

The second set of transgenic Desiree plants that have been tested for their functionality was A19 plants harboring *Rpi-chc1* and *Rpi-vnt1* gene (Table 12). Isolate EC1 and Dinteloord were selected to be used in DLA due to the fact that isolate EC1 is compatible on *Rpi-vnt1* but incompatible on *Rpi-chc1* and isolate Dinteloord is compatible on *Rpi-chc1* but incompatible on *Rpi-vnt1*. Out of 19 A19 plants, only three plants displayed resistance to both isolates while two plants gave susceptible responses to both isolates. A17-27 plant that acts as a positive control for *Rpi-chc1* showed a clear resistance to isolate EC1. In plant A13-13 the *Rpi-vnt1* detected, as expected, the Dinteloord isolate. In agro-infiltration test, two *Avrs*; *A2-2* and *Avr-vnt1* that corresponding to *Rpi-chc1* and *Rpi-vnt1* respectively were used. The positive and negative controls were also included in this experiment similar as was done in the DLA experiment. Almost all A19 plants tested responded to *Avr-vnt1* but only one plant clearly responded to *A2-2* even after the OD had been increased from 0.1 to 0.2 in the repeat experiment.

Table 12: Activity of *R* genes in transgenic Desiree plants (A19 plants harboring *Rpi chc1-Rpi vnt1*) in relation to resistance and effector recognition.

Transgenic event	*Pi* inoculation		Effector recognition by measuring HR			
	EC1	Dinteloord	A2-2 (0.2 OD)	Avr-vnt1 (0.1 OD)	Avr3b (0.1 OD)	Avr3b+R3b (0.2 OD)
A19-2	R	R	0.0	1.9	0.0	0.5
A19-4	S	S	0.0	0.5	0.1	1.2
A19-5	S	R	0.0	2.0	0.1	0.5
A19-23	R	R	1.7	2.0	0.1	0.9
A19-35	S	R	0.0	1.8	0.0	0.4
A19-46	R	R	0.4	1.9	0.0	1.1
A19-63	S	R	0.1	2.0	0.0	0.6
A19-66	S	R	0.0	1.9	0.1	1.2
A19-67	S	R	0.2	2.0	0.0	0.7
A19-73	S	S	0.0	1.6	1.2	1.3
A19-77	S	R	0.0	1.6	0.0	0.0

A19-94	S	R	0.0	1.9	0.1	0.5
A19-95	S	R	0.0	2.0	0.0	0.1
A19-96	S	R	0.2	2.0	0.1	0.8
A19-99	S	R	0.0	2.0	0.0	0.6
A19-105	S	R	0.0	2.0	0.0	0.7
A19-115	S	R	0.4	1.9	0.0	0.9
A19-118	S	R	0.0	1.0	0.0	0.0
A19-120	S	R	0.0	2.0	0.0	0.1
A17-27	R	S	1.6	0.0	0.0	0.4
A13-13	S	R	0.0	1.9	0.0	0.8
Desiree	S	S	0.0	0.0	0.1	0.5

In *Pi* inoculation the plants scored 0-0.9:Highly Resistance (HR),1-2.5:Resistance(R) and 2.6-3.5-Moderate Resistance (MR) and grouped as susceptible in between scored 3.6 -7.0 In agro-infiltration plants scored 1.0-2.0: HR(Hypersensitive Response),'nd' means not determined.

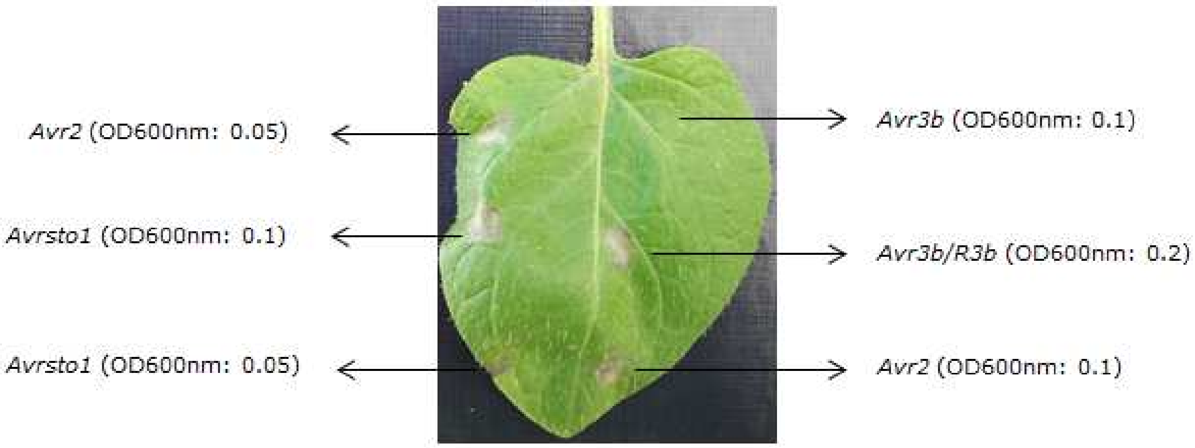


Fig 9: Agro-infiltration of *Avr* effectors in double *R* gene regenerant A26-97 using different densities of inoculum of *Avr2* and *Avrsto1*. Co-infiltration of *Avr3b/R3b* served as a positive control and *Avr3b* alone as a negative control.

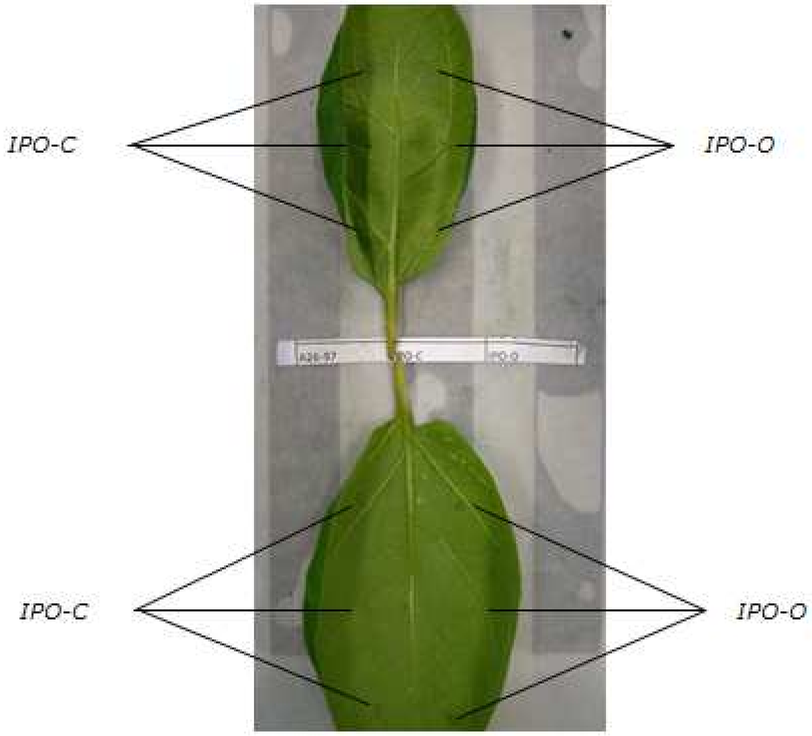


Fig 10: Detached leaf assay of double *R* gene regenerant A26-97 by using *Pi* isolates *IPO-C* and *IPO-O* in which six 10µl droplets of a zoospore suspension (50,000 spores/ml).

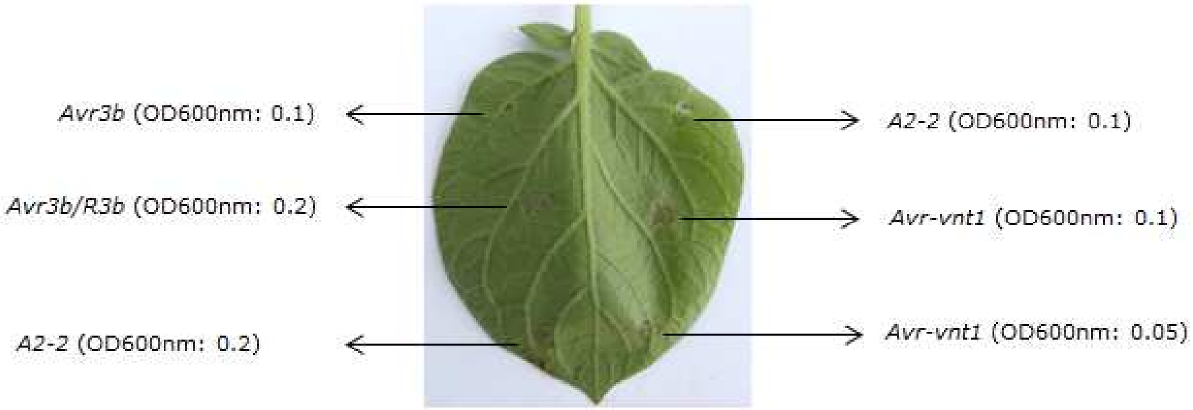


Fig 11: Agro-infiltration of *Avr* effectors in double *R* gene regenerant A19-23 using different densities of inoculum of *A2-2* and *Avr-vnt1*. Co-infiltration of *Avr3b/R3b* served as a positive control and *Avr3b* alone as a negative control.

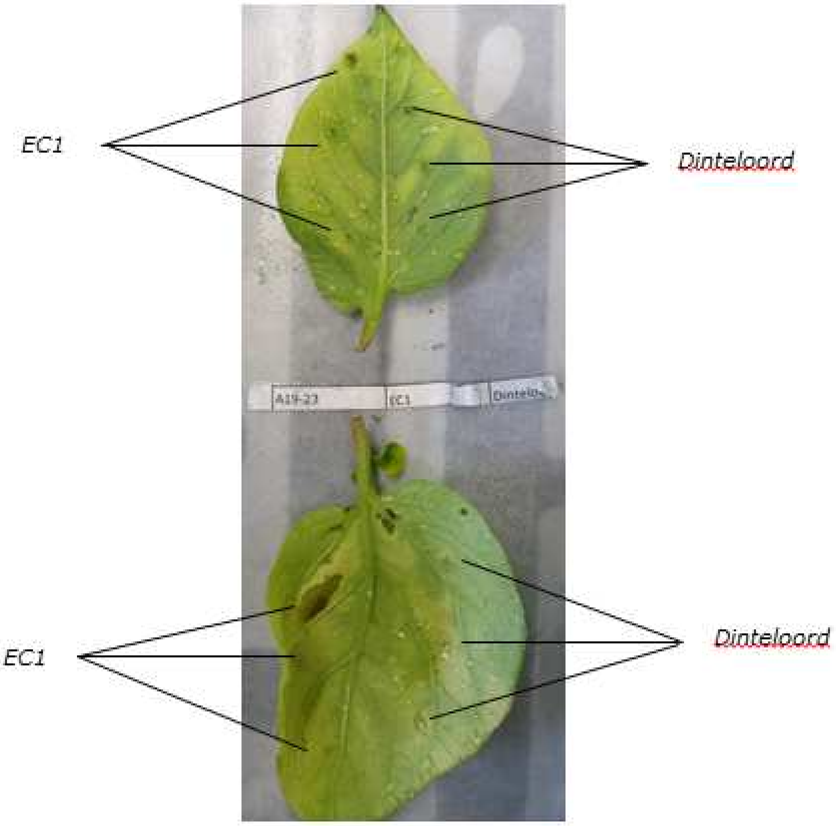


Fig 12: Detached leaf assay of double *R* gene regenerant A19-23 by using *Pi* isolates *EC1* and *Dinteloord* in which six 10µl droplets of a zoospore suspension (50,000 spores/ml).

Discussion

Complementarity in activity spectra of *Rpi-chc1* homologs

Transgenic plants harboring *Rpi-chc1* homologs were developed by transformation of the susceptible potato cultivar Desiree. The most resistant events were selected among the different transgenic events in previous studies (Sidrat Abdullah, 2012). In this research, the functionality of the resistance of these events was continued and compared to wild type species which were the donor plants of the *Rpi-chc1* homologs by using various isolates and effectors of *Pi*. The *Rpi-chc1* homologs that showed different and complementary resistance spectrum against *Pi* will be selected for stacking into potato cultivar. A17-27 (*Rpi-chc1*) plant was used as a positive control to compare with different homologs of *Rpi-chc1*. From detached leaf assay, it was found that A17-27 plants demonstrated a significant resistance against all isolates used in this study except for isolate NL7245 and Dinteloord. (Appendix 1 and 2). The observation of high resistance to most of the isolates was expected as it has been described in previous studies that A17 plants were efficiently expressed the *Rpi-chc1* gene due to long promoter used in the plasmid (Tiwari Abdullah, 2011). However, the moderate resistance response against NL7245 was contrasting with previous study as this clone was found to be more susceptible (Sidrat Abdullah, 2012). In agro-infiltration test, it was also examined that the A17-27 plants harboring *Rpi-chc1* recognized the effector *A2-2* with a strong HR. This result is well-supported by previous studies done in *N. benthamiana* where *Rpi-chc1* recognizes its corresponding *Avr* effector, *A2-2* (Lokossou, Abd-el Haliem, unpublished). Moreover, donor plants for A17-27 (543-5) which were also included in this studies did also give cell-death responses to all isolates except for IPO-C and Dinteloord in 543-5 and 94-2031 respectively (Table 2). These two isolates somehow broke the resistance of the plants, probably by changing of *Avr* effectors in order to evade recognition by the plants as described in the literature (Jones and Dangl, 2006). This was in agreement with previous studies (Tiwari Abdullah, 2011). It was however unexpected that NL7245 showed an incompatible interaction with *Rpi-chc1*.

From *Pi* inoculation test of *Rpi-chc1* homologs, it can be seen that most of the isolates were virulent to the transgenic plants except for H30PO4 and Pic 99183 (Table 5, Appendix 1 and 2). The most likely reason to explain this phenomenon is that the expression level of *Rpi-chc1* homologs in those plants was not sufficient to cause resistance responses. This finding was in contrast with previous study where it was found that the A37 (543-5/C2) plants were resistant to NL7245 (Sidrat Abdullah, 2012). However, in case of H30PO4, it can be observed that this isolates was incompatible to all transgenic events in A32 (324-2/J8) and A40 (852-5/E28) as well as most of the events in A33 (852-5/E14). This may suggest that these plants show reproducible resistance to this particular isolate and J8 and E14 have similar activities. On the other hand, almost none of the transgenic events in A34 (487-1/I6), A35 (493-7/G19), A36 (493-9/H5) and A37 (543-5/C2) showed incompatible responses to H30PO4. It seems that those plant sets contain a broken *R* gene to H30PO4. Some regenerants showed resistance to Pic 99183, although this isolates, did not cause too much virulence on Desiree control plants (Appendix 1 and 2) either and therefore the results for this isolate were ignored.

For A32 (324-2/J8) plants, the results demonstrated that most of the transgenic events did not respond well in detached leaf assay. As for their donor plant (94-2031) which contained *Rpi-ber*, it gave a good response to all isolates except for Dinteloord while in agro-infiltration, only *A2-2* effector has been well recognized (Appendix 1, 2, 3 and 4). As explained before, A17-27 gave also a strong HR to *A2-2* effector and this clearly indicates that *Rpi-ber* is same as *Rpi-chc1*. The insufficient responses in A32 (324-2/J8) regenerants towards *Pi* isolates might be explained by low expression of the gene in transgenic events. Similar to A32 (324-2/J8), it was found that A34 (487-1/I6) and A36 (493-9/H5) plants which derived from *S. berthaultii* did not give high resistance to isolates used in this experiment. A34 (487-1/I6) transgenic events which harbor an *Rpi-chc1* homolog from donor plant 487-1showed only moderate resistance response to H30PO4 and Pic 99183. It has been described in the literature that *Rpi-ber1* and *Rpi-ber2* are in the location of chromosome *10* of *S. berthaultii* and very close to *Rpi-ber* (Park et al., 2009). These two *R* genes are derived from the same accession as *Rpi-ber* (Tan, 2010). However, based on disease resistance test of A32 (324-2/J8) and A34 (487-1/I6) plants, no strong conclusion can be drawn since the isolate spectrum is not informative when comparing with their donor plants.

From all groups of *Rpi-chc1* homologs tested, only A40 (852-5/E28) displayed good resistance responses to a range of isolates especially to H30PO4 and Pic 99183 where all transgenic events gave cell-death responses (Table 5, Appendix 1 and 2). Transgenic events, A40-31(Fig 3 and 4), A40-44 and A40-53 demonstrated almost a full resistance spectrum to all isolates. Whereas, A40-4, A40- 28 and A40-51 showed the least resistance responses to all isolates. This observation could be explained by the individual recognition to effector *A2-2* in agro-infiltration test. A40-4 and A40-28 had less recognition of effector *A2-2* than other transgenic events. In case of A40-51, although it showed a strong HR response to *A2-2*, a considerably high response indicated by negative control should also be taken into account. However, it was unexpected that race specificity was not found for this resistance gene.

In effector recognition tests, several available effectors were tested first for their ability to cause autonecrosis in absence of *R* gene. It was found that *A1-1* effector in the pK7WGF2 vector gave minimum a-specific cell death. This result was contrasting with previous work where *A1-1* in pGR106 (PVX virus) caused high autonecrosis (Sidrat Abdullah, 2012). The different vector of *A1-1* effector used in this study could be the reason to explain this difference in effector responses. After being tested with selected *Avr* effectors, very few transgenic plants did recognize the effectors except for A40 (852-5/E28) (Table 6, Appendix 3 and 4). In previous studies done in *N. benthamiana*, A35 (493-7/G19) plants were reported to give strong HR to *C2* and *C3* effectors (Lokossou, unpublished).

However, there was no recognition specificities found to *C2* and *C3* for A35 (493-7/G19) transgenic potato plants in this experiment. This finding was also in contrast with previous work where it was found that A35 (493-7/G19) plants did respond to *C2* and *C3* (Sidrat Abdullah, 2012). The most likely reason to explain this observation is that the gene was not strongly expressed or the OD used in this study was too low to cause HR.

Cloning of single and double *R* genes constructs

Several combinations of well characterized *R* genes against *Pi* were stacked in one binary vector. These new constructs can be used to introduce multiple combinations of *R* genes into susceptible potato cultivar Desiree by *A. tumefaciens*-mediated transformation. The addition of specific enzyme sites PASSA into pBINPLUS vector offered possibility to stack two *R* genes that have same restriction sites. Besides, the unique cutting sites of PASSA also provide the potential to stack more *R* genes in future work. After transformation into *E*.coli, each gene in all double *R* genes vectors was present as shown in PCR test. However, after transformation of the new constructs into *A.tumefaciens* and tested for stability, two of them appeared to be losing one of the genes (Table 10). Second gene from pBINPLUS-PASSA: blb3:chc1 and pBINPLUS-PASSA: blb3:edn2 were absent in all colonies even after being tested twice. Also another construct which was pBINPLUS-PASSA:vnt1:blb3 showed unstable gene integration in colony 4 for both genes. Apparently, the constructs that contained *Rpi-blb3* replicate less stably in *A. tumefaciens*. Apart from that, the size of the vector could also influence the stability after being transformed into *A.tumefaciens*. The two unstable constructs have sizes of 28.2kb and 28.5kb which are the largest among all constructs that were made.

Functional characterization of double *R* genes stacked in transgenesis and cisgenesis Desiree plants

The introduction of new combinations of double *R* genes into susceptible potato cultivar Desiree by *A. tumefaciens*-mediated transformation normally would take long time. In this study, the functionality of stacked double *R* genes was carried out in two available sets of cisgenic and transgenic Desiree plants made in previous work. Both agro-infiltration and DLA were used to assess the level of functional expression of the *R* genes in these stacks. Twenty-four transformants bearing *Rpi-blb3* and *Rpi-sto1* (A26 plants) were tested in this study. Most of them showed resistance in detached leaf assay for isolate that recognizes *Rpi-blb3* but none gave HR after agro-infiltration using corresponding *Avr* effector except for A26-97 (Fig. 9 and Fig. 10). However, based on previous data, it was found that in A26-97 plant a wrong gene construct was inserted (*Rpi-sto1: Rpi-vnt1*: *Rpi-blb3: NPTII*). The fact that none of the plants with the *Rpi-blb3: Rpi-sto1* insert showed *Avr2* recognition could indicate that a mutant form of *Rpi-blb3* was used for this construct. The resistance displayed in DLA test for isolate IPO-O was not as expected since the A09 control plants that contain only *Rpi-sto1* should not give resistance to this isolate (Zhu et al., 2012). IPO-O was reported to break *Rpi-sto1* and was recognized by *Rpi-blb3*. So, from DLA result by using the IPO-O isolates, it cannot be concluded that A26 showed proper expression of *Rpi-blb3*. The most likely reason for that is the *R* gene used in developing the construct was mutated and cause gene expression to be uncertain. On the other hand, the biological activity (IPO-C resistance) of *Rpi-sto1* in this plant set showed 80% match with the agro-infiltration induced hypersensitivity reaction (Table 11). Transformants that displayed cell-death response in DLA would also give HR response to corresponding *Avr* effector in agro-infiltration. This observation indicates that almost half of the A26 plants have an active *Rpi-sto1* since the positive control plant for this gene (A03-142) showed the expected susceptibility to IPO-C.

Nineteen transformants harboring *Rpi-chc1* and *Rpi-vnt1* gene were tested for their functionality. From 19 plants, only one showed that both integrated *R* genes were functioned properly based on resistance in DLA and agro-infiltration of matching *Avr* effectors (Fig. 11 and Fig. 12). Meanwhile, two transformants appeared to have both genes active according to *Pi* inoculation test, but not supported in effector recognition test when only one *Avr* produced HR. This shows that specific complementary detection from isolates in DLA is more effective than from effector in agro-infiltration. Most of other transformants seems to have only one *R* gene active which was *Rpi-vnt1* characterized by isolate Dinteloord and *Avr-vnt1*.

Conclusions and future perspectives

R **gene activity spectra**

A17-27 (*Rpi-chc1* long promoter) plants had a full resistance spectrum to almost all isolates and a high response to *A2-2* effector. Transgenic clones containing *Rpi-chc1* homologs showed a group specific response to isolate H30PO4 where all A32 (324-2/J8) and almost all A33 (852-5/E14) plants displayed resistance to H30PO4. None of the transgenic events from A34 (487-1/I6), A35 (493-7/G19), A36 (493-9/H5) and A37 (543-5/C2) showed resistance to H30PO4. It is therefore concluded that J8, E14 and E28 are genuine resistance genes and that J8 and E14 have similar activities. The genes 852-5/E28 was the most effective homologs of *Rpi-chc1*. The A40 (852-5/E28) transgenic events showed resistance to a wide range of isolates in disease resistance test. Since *Rpi-tar1* was found to display complementary spectra with *Rpi-chc1,* therefore these two *R* genes could be stacked to give a broad resistance spectrum. In comparison with the respective donor plants, isolates spectrum for all transgenic events is not informative due to low expression levels from the *Rpi-chc1* short promotor that drives expression in the transgenic plants. For future work, all *Rpi-chc1* homologs clones need to be repeated in agro-infiltration with higher OD of effectors in order to get more clear HR responses.

R **gene cassettes**

In general, all single and double *R* gene cassettes in pBINPLUS-PASSA vector were successfully made and could be stably used in transformation except for pBINPLUS-PASSA: blb3:chc1 and pBINPLUS-PASSA:blb3:edn2. For these two constructs, other strategies need to be done in the future in order to achieve more stably inserted genes. For instance, pUC-ASAS could be used in an intermediate step to shuttle *Rpi-chc1* and *Rpi-edn2* instead of pUC-PASSA. In this way, the order of the genes in the new vector would be changed and may lower the chance of problematic sequence occur in T-DNA of plasmid. Besides, during transformation of construct into *A.tumefaciens* agrobacterium, other strains could also be used such as *GV3101* instead of *AGL1+pVirG*.

R **gene activity in cassettes**

Half of the cisgenic Desiree plants harboring *Rpi-blb3: Rpi-sto1* (A26) have biologically active *Rpi-sto1* gene. The other genes, *Rpi-blb3* need to be validated in the future work with a more reliable result of positive control in order to draw a strong conclusion regarding the functionality of the gene. For A26-97 clone, it needs to be co-infiltrated with *Avr-vnt1* to characterize *Rpi-vnt1* in order to confirm that a wrong gene construct (*Rpi-sto1: Rpi-vnt1*: *Rpi-blb3:* NPTII) was inserted. Only one of the transformants in transgenic Desiree plants bearing *Rpi-chc1: Rpi-vnt1* has both biologically active genes while the others have only an active *Rpi-vnt1*. In general, *R* gene cassettes must be validated extensively before transformation and in order to select sufficient clones expressing *Rpi-chc1* in a proper way, many more cis/transgenic events must be made and tested.

Acknowledgment

First and foremost, I would like to express my greatest gratitude and thanks to my main supervisor, Dr. Jack Vossen for his supervision, endless guidance, motivation, encouragements and suggestion throughout the whole research period. Also, I would like to say thousand thanks to my supervisor during development of constructs, Marjon Arens and potato transformation, Marjan Bergervoet. Not to forget, to all technicians and colleagues in the lab for made the whole experiment being easier. In addition, to the whole Plant Breeding Department and those that helping me directly or indirectly which I always remember but could not mention, thank you very much for your endless support to me, before, during and after the whole process of the project. My special thanks and appreciation also goes to my beloved family for their truthful support, continuous pray and encouragement that motivate and inspire me during my whole degree time. My acknowledgement is also due to my examiner, Prof. Evert Jacobsen for spending his valuable time to check and examine my thesis.

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Appendix

1. DLA 1 of different *Rpi-chc1* homologs

TE	H3PO4	IPOC	USA 618	NL 7245	Pic 99183	Dinteloord	Katshaar	NL 7379	NL 7135
A32-126	1.2	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
A32-56	1.0	4.3	7.0	7.0	1.0	1.7	1.0	6.7	6.5
A32-78	2.0	1.2	6.8	6.0	6.2	6.5	6.5	6.5	7.0
A33-112	4.7	7.0	7.0	7.0	7.0	7.0	7.0	7.0	6.8
A33-31	2.0	6.7	7.0	7.0	7.0	7.0	7.0	7.0	7.0
A33-32	4.7	6.8	7.0	7.0	7.0	7.0	7.0	7.0	7.0
A33-33	1.7	6.5	7.0	7.0	5.3	7.0	6.0	7.0	7.0
A33-34	1.3	6.3	7.0	4.7	7.0	6.5	6.5	6.0	6.0
A33-51	1.2	3.5	6.0	6.3	1.8	6.8	5.7	7.0	6.5
A33-52	2.7	7.0	7.0	7.0	7.0	7.0	5.0	6.7	7.0
A33-53	1.2	4.5	7.0	7.0	0.7	6.2	5.7	6.0	6.0
A34-11	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
A34-13	2.7	3.8	7.0	7.0	7.0	7.0	7.0	7.0	7.0
A35-02	4.3	7.0	7.0	7.0	7.0	7.0	6.0	6.0	6.0
A35-14	4.0	7.0	7.0	7.0	5.0	7.0	5.7	6.0	6.2
A35-86	5.3	7.0	7.0	7.0	7.0	7.0	7.0	6.0	6.0
A35-88	1.3	7.0	7.0	7.0	6.0	6.8	6.8	7.0	6.7
A35-90	5.5	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
A36-13	4.0	7.0	7.0	7.0	5.0	7.0	6.2	6.0	7.0
A36-35	4.7	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
A36-74	6.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
A36-91	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	6.7
A37-23	7.0	7.0	7.0	7.0	2.0	7.0	7.0	7.0	7.0
A37-44	3.5	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
A40-4	1.3	4.2	7.0	7.0	4.7	4.7	3.7	6.7	5.3

A40-28	1.7	4.0	7.0	7.0	3.7	6.0	6.3	5.8	5.0
A40-31	1.0	1.0	1.7	3.0	2.0	4.0	1.0	1.3	3.3
A40-44	0.8	1.0	5.7	1.3	1.0	1.0	1.0	1.0	3.0
A40-51	1.7	6.0	7.0	7.0	2.3	7.0	5.2	4.7	3.5
A40-53	1.0	1.2	6.2	1.8	1.0	1.0	1.0	1.0	1.0
A17-27	1.0	1.0	4.0	4.0	1.0	4.0	1.0	1.0	1.0
487-1	0.2	0.2	4.3	2.0	7.0	0.2	2.5	2.7	3.3
493-7	0.7	2.0	7.0	2.7	4.0	1.3	1.0	1.0	6.0
493-9	0.0	0.3	4.3	0.3	0.3	0.0	7.0	5.3	4.7
543-5	1.0	3.0	3.0	2.0	1.0	1.3	1.0	1.0	1.0
852-5	3.5	3.2	4.0	1.0	1.0	1.0	1.0	1.7	2.0
561-2									
JV12-1017	0.8	1.0	4.3	2.2	6.2	5.7	4.0	4.0	1.0
JV12-1015	1.0	5.0	2.7	4.0	1.7	2.7	1.7	1.0	1.0
JV12-1003									
Desiree	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
A34-41	7.0	6.0	7.0	7.0	1.3	7.0	6.3	6.3	6.7
94-2031	0.5	1.7	4.0	1.0	1.0	5.3	1.7	1.0	3.0
A33-72	2.3	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
A34-62	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
A34-79	5.5	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
A32-66	1.2	5.8	6.0	6.0	2.7	7.0	5.0	5.0	5.0

2. DLA 2 of different *Rpi-chc1* homologs

TE	H3PO4	IPOC	USA 618	NL 7245	Pic 99183	Dinteloord	Katshaar	NL 7379	NL 7135
A32-126	2.0	7.0	7.0	7.0	4.0	7.0	7.0	7.0	7.0
A32-56	5.0	7.0	7.0	7.0	1.7	7.0	7.0	7.0	4.0
A32-78									
A33-112	1.0	4.3	6.0	6.0	1.0	6.0	6.3	7.0	7.0
A33-31	3.3	5.0	7.0	7.0	4.0	7.0	7.0	7.0	7.0
A33-32	5.0	7.0	7.0	7.0	2.3	7.0	7.0	7.0	7.0
A33-33	2.0	2.0	7.0	7.0	2.0	7.0	7.0	7.0	6.0
A33-34	3.7	7.0	7.0	7.0	1.0	7.0	7.0	7.0	7.0
A33-51	2.3	5.0	7.0	7.0	1.0	5.7	7.0	7.0	5.0
A33-52	1.0	5.0	7.0	7.0	1.3	5.3	7.0	7.0	7.0
A33-53	1.0	1.7	3.3	6.3	1.0	7.0	4.0	6.0	6.0
A34-11	6.7	7.0	7.0	7.0	1.0	3.3	7.0	7.0	7.0
A34-13	3.7	7.0	7.0	7.0	1.3	7.0	7.0	7.0	7.0
A35-02	4.3	7.0	7.0	7.0	7.0	6.7	7.0	7.0	7.0
A35-14	3.3	7.0	7.0	7.0	5.3	7.0	7.0	7.0	7.0
A35-86	7.0	7.0	7.0	7.0	5.3	7.0	7.0	7.0	7.0
A35-88	5.7	7.0	7.0	7.0	5.0	7.0	7.0	7.0	6.7
A35-90	7.0	7.0	7.0	7.0	4.7	7.0	7.0	7.0	7.0
A36-13	5.0	7.0	7.0	7.0	5.0	7.0	7.0	7.0	7.0
A36-35	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
A36-74	5.3	7.0	7.0	7.0	4.0	7.0	7.0	7.0	7.0
A36-91	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
A37-23	7.0	7.0	7.0	7.0	3.3	7.0	7.0	7.0	7.0
A37-44	5.0	7.0	7.0	7.0	1.7	7.0	7.0	7.0	6.7
A40-4	2.0	7.0	6.7	7.0	1.0	5.0	7.0	7.0	7.0
A40-28	1.0	2.3	7.0	7.0	1.0	5.0	3.0	4.3	5.7
A40-31	1.0	1.0	1.7	2.3	1.0	1.0	3.0	2.3	1.0

A40-44	1.0	0.7	1.0	2.0	1.0	4.0	6.3	2.7	4.7
A40-51	1.7	6.0	7.0	7.0	1.7	7.0	7.0	6.7	6.0
A40-53	1.0	1.0	1.7	5.0	1.0	2.3	3.0	4.3	1.0
A17-27	1.0	1.0	1.0	1.7	1.0	1.0	1.0	1.0	1.0
487-1									
493-7	1.0	1.7	7.0	7.0	4.3	5.0	4.0	4.0	3.3
493-9	0.0	3.0	3.7	3.0	3.0	4.7	1.0	1.0	3.0
543-5	5.0	5.0	2.3	2.3	1.0	5.0	1.0	1.0	1.0
852-5	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
561-2									
JV12-1017	1.0	1.0	1.0	2.7	1.0	7.0	5.0	5.0	4.0
JV12-1015	0.7	1.0	1.0	1.0	1.0	1.3	1.0	1.0	1.0
JV12-1003									
Desiree	5.3	7.0	7.0	7.0	1.7	7.0	7.0	7.0	7.0
A34-41	7.0	7.0	7.0	7.0	4.0	7.0	7.0	7.0	7.0
94-2031	0.0	1.3	1.0	1.0	1.0	3.3	3.0	0.3	1.0
A33-72	4.0	7.0	7.0	7.0	5.0	7.0	7.0	7.0	7.0
A34-62	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
A34-79									
A32-66	3.7	7.0	7.0	7.0	7.0	7.0	5.0	5.0	6.3

3. Agro-infiltration 1 of different *Rpi-chc1* homologs

	Avr3b (0.1)	R3b+Avr3b (0.2)	A1-1 (0.1)	B1 (0.1)	C3 (0.1)	B3 (0.1)	C2 (0.05)	C1 (0.1)	A2-2 (0.2)
A32-126	0.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A32-56	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A32-78	0.0	1.6	0.0	0.0	0.0	0.0	0.0	0.1	0.0
A33-112	1.4	1.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A33-31	0.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A33-32	0.0	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A33-33	0.0	1.3	0.0	0.0	0.0	0.1	0.0	0.0	0.0
A33-34	0.2	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A33-51	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A33-52	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A33-53	0.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A34-11	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A34-13	0.0	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A35-02	0.2	1.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A35-14	2.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A35-86	0.2	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A35-88	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A35-90	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A36-13	0.0	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A36-35	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A36-74	0.0	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A36-91	0.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A37-23	0.0	0.9	0.0	0.0	1.3	0.0	0.4	0.0	0.0
A37-44	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A40-4	0.2	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A40-28	0.2	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.5

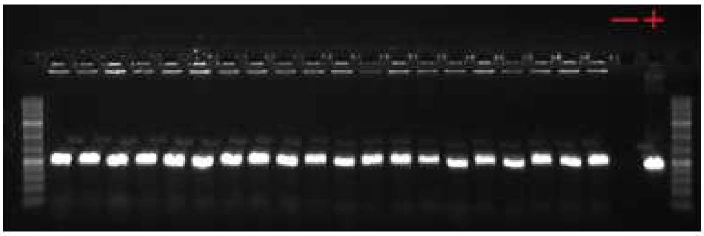
A40-31	0.1	0.6	0.2	0.0	0.0	0.0	0.0	0.1	2.0
A40-44	0.0	0.8	0.1	0.0	0.0	0.0	0.0	0.0	2.0
A40-51	1.2	1.8	0.0	0.0	0.0	0.0	0.0	0.0	1.5
A40-53	0.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0	1.1
A17-27	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	1.6
487-1	1.6	1.3	0.3	1.5	1.6	0.8	1.0	0.8	1.2
493-7									
493-9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
543-5	0.1	0.2	0.3	2.0	2.0	0.8	1.8	0.6	2.0
852-5	0.4	0.5	0.7	0.7	1.0	1.0	0.4	0.4	1.9
561-2									
JV12-1017	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
JV12-1015									
JV12-1003									
Desiree	0.0	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.3
A34-41	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
94-2031	0.0	0.3	0.1	0.0	0.0	0.0	0.3	0.0	0.3
A33-72	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A34-62	0.0	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A34-79	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A32-66	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0

4. Agro-infiltration 2 of different *Rpi-chc1* homologs

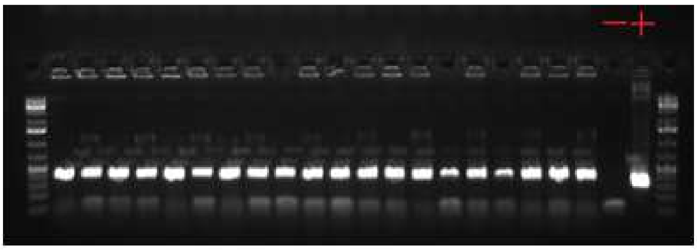
TE	Avr3b (0.1)	R3b+Avr3b (0.2)	A1-1 (0.1)	B1 (0.1)	C3 (0.1)	B3 (0.1)	C2 (0.05)	C1 (0.1)	A2-2 (0.2)
A32-126	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A32-56	0.1	0.8	0.0	0.0	0.0	0.4	0.0	0.0	0.7
A32-78	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A33-112	0.4	1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A33-31	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A33-32	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A33-33	0.4	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.3
A33-34	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A33-51	0.1	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A33-52	0.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A33-53	0.2	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A34-11	0.0	0.3	0.0	0.4	0.0	0.0	0.0	0.0	0.1
A34-13	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A35-02	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A35-14	2.0	2.0	0.0	0.0	0.1	0.1	0.0	0.0	0.0
A35-86	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.2
A35-88	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A35-90	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A36-13	0.0	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A36-35	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A36-74	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A36-91	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.4
A37-23	0.0	0.3	0.0	0.0	0.2	0.0	0.0	0.1	0.2
A37-44	0.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.2
A40-4	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A40-28	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.4
A40-31	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.3	2.0

A40-44	0.0	1.4	0.0	0.0	0.0	0.0	0.0	0.0	1.8
A40-51	0.2	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.8
A40-53	0.0	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.8
A17-27									
487-1	1.6	1.6	0.8	1.0	1.6	1.1	1.4	1.2	1.6
493-7									
493-9	0.2	0.3	0.0	0.0	1.0	0.0	0.3	0.0	0.3
543-5	0.8	1.6	1.2	2.0	2.0	0.5	2.0	0.5	2.0
852-5	0.5	0.8	0.3	1.1	0.4	0.3	0.8	1.3	1.1
561-2									
JV12-1017	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.2
JV12-1015									
JV12-1003	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Desiree	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.1	0.3
A34-41	0.0	0.6	0.0	0.0	0.0	0.0	0.2	0.0	0.0
94-2031	0.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0	1.7
A33-72	0.0	0.1	0.0	0.0	0.0	0.4	0.0	0.0	0.0
A34-62	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A34-79	0.7	0.5	0.0	0.0	0.0	0.0	0.0	0.2	0.2
A32-66	0.1	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0

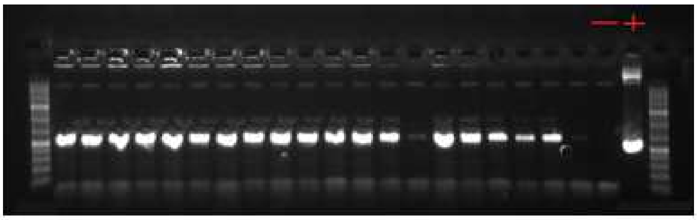
5. Colony PCR check for *Rpi-blb3* (400bp) in construct pBINPLUS-PASSA:blb3



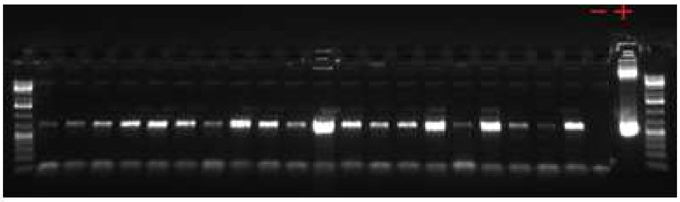
6. Colony PCR check for *Rpi-edn2* (380bp) in construct pBINPLUS-PASSA:edn2



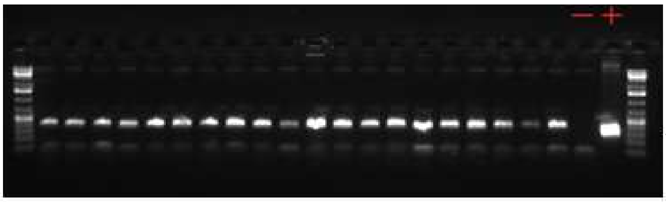
7. Colony PCR check for *Rpi-sto1* (500bp) in construct pBINPLUS-PASSA:sto1



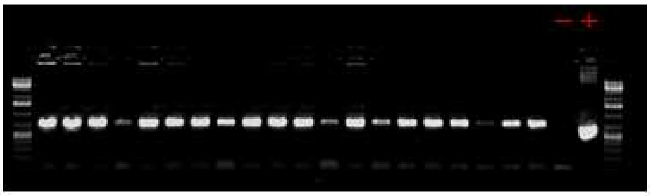
8. A) Colony PCR check for *Rpi-sto1* (500bp) in construct pBINPLUS-PASSA: edn2: sto1



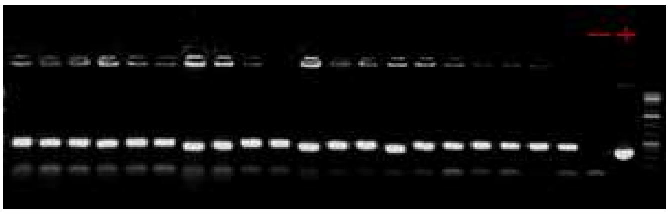
B) Colony PCR check for *Rpi-edn2* (380bp) in construct pBINPLUS-PASSA: edn2: sto1



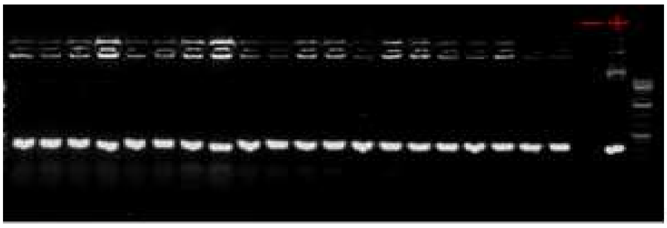
9. A) Colony PCR check for *Rpi-sto1* (500bp) in construct pBINPLUS-PASSA: chc1: sto1



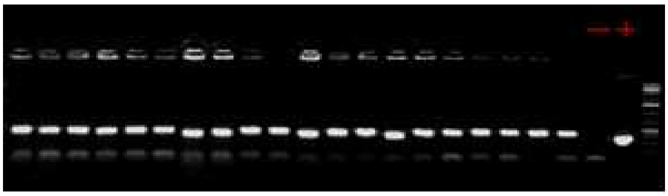
B) Colony PCR check for *Rpi-chc1* (320bp) in construct pBINPLUS-PASSA: chc1: sto1



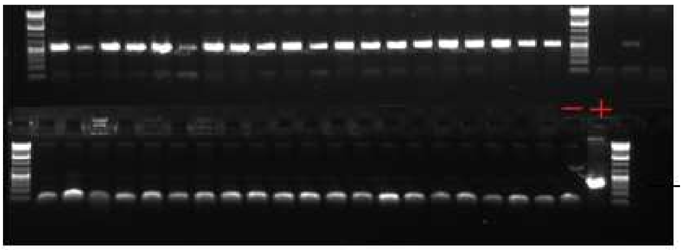
10. A) Colony PCR check for *Rpi-chc1* (320bp) in construct pBINPLUS-PASSA: chc1: edn2



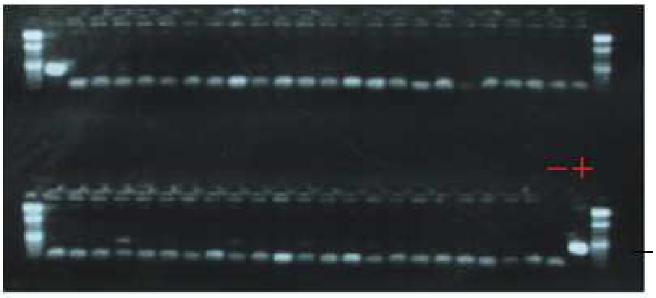
B) Colony PCR check for *Rpi-edn2* (380bp) in construct pBINPLUS-PASSA: chc1: edn2



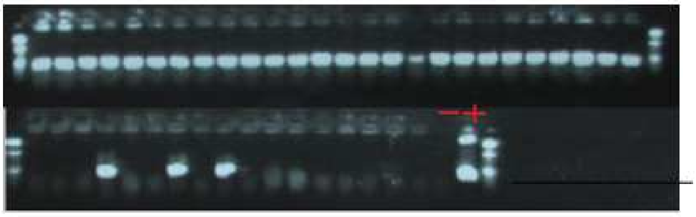
11. A) Colony PCR check for *Rpi-blb3* (400bp) in construct pBINPLUS-PASSA: blb3:edn2



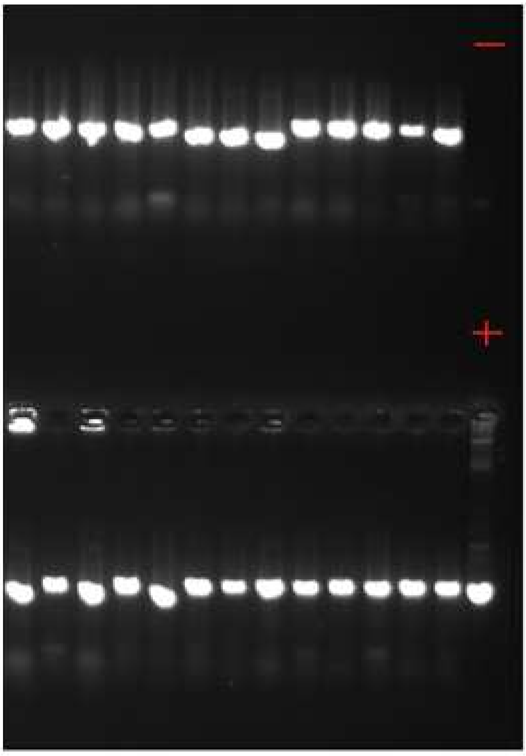
B) Colony PCR check for *Rpi-edn2* (380bp) in construct pBINPLUS-PASSA: blb3:edn2



12. A) Colony PCR check for *Rpi-chc1* (320bp) in construct pBINPLUS-PASSA: blb3:chc1



B) Colony PCR check for *Rpi-blb3* (400bp) in construct pBINPLUS-PASSA: blb3:chc1



13. DLA 1 of *R* genes in cisgenic Desiree plants (A26 plants harboring *Rpi blb3-Rpi sto1*)

TE	IPO-C	IPO-O
A03-142	7.0	1.0
A09-277	1.5	1.2
A10-43	1.0	1.0
A14-16	1.0	1.0
Desiree	6.5	4.5
A26-1023	2.3	1.1
A26-1065	1.7	1.1
A26-1172	7.0	1.0
A26-1206	1.0	1.3
A26-1263	7.0	4.8
A26-1369	7.0	2.2
A26-1389	3.3	2.3
A26-1554	1.1	1.0
A26-1679	1.0	1.0
A26-1735	3.3	1.0
A26-1985	5.3	1.3
A26-2351	6.8	3.1
A26-2371		
A26-528	1.0	1.0
A26-767	1.0	1.0
A26-768	7.0	1.6
A26-871	1.3	1.6
A26-882	2.1	1.2
A26-915	2.5	0.6
A26-941	6.8	2.1
A26-946	7.0	1.5
A26-947	7.0	3.6
A26-948	6.8	1.8
A26-97	1.0	1.0
A26-1048		
A26-1386	7.0	6.5
A26-1018	7.0	1.1
A09-268	1.0	1.0
A09-283	1.0	1.0

14. DLA 2 of *R* genes in cisgenic Desiree plants (A26 plants harboring *Rpi blb3-Rpi sto1*)

TE	IPO-C	IPO-O
A03-142	7.0	0.4
A09-277	2.8	0.6
A10-43	1.0	0.5
A14-16	0.8	0.0
Desiree	6.7	3.5
A26-1023	4.3	0.4
A26-1065	7.0	0.7
A26-1172	7.0	0.6
A26-1206	2.1	1.0
A26-1263	7.0	1.0
A26-1369	7.0	0.9
A26-1389	4.4	1.3
A26-1554	2.7	0.9
A26-1679	1.1	0.8
A26-1735	5.4	0.8
A26-1985		
A26-2351	7.0	1.0
A26-2371		
A26-528	1.0	0.7
A26-767	1.0	1.0
A26-768	7.0	0.8
A26-871	1.3	1.0
A26-882	4.1	1.0
A26-915	2.5	0.9
A26-941	7.0	1.3
A26-946	6.5	1.2
A26-947	7.0	1.5
A26-948	7.0	1.9
A26-97	1.0	0.3
A26-1048		
A26-1386		
A26-1018		
A09-268	1.0	1.0
A09-283	1.0	0.8

15. Agro-infiltration 1 of *R* genes in cisgenic Desiree plants (A26 plants harboring *Rpi blb3-Rpi sto1*)

Transgenic event	Avr3b (0.1)	R3b+Avr3b (0.2)	Avr2 (0.1)	Avr2 (0.05)	IPI-O1 (0.1)	IPI-O1 (0.05)
A03-142	0.0	1.0	1.6	0.9	0.0	0.0
A09-277	0.0	0.1	0.0	0.0	1.1	0.4
A10-43	0.0	0.7	0.8	0.7	1.5	1.0
A14-16	0.0	0.4	1.4	1.9	1.8	1.4
Desiree	0.0	1.2	0.0	0.0	0.0	0.0
A26-1023	0.0	0.9	0.0	0.0	0.6	0.1
A26-1065	0.0	0.3	0.0	0.0	0.9	0.1
A26-1172	0.0	0.8	0.0	0.0	0.0	0.0
A26-1206	0.0	0.2	0.0	0.0	1.3	0.7
A26-1263	0.0	0.1	0.0	0.0	0.0	0.0
A26-1369	0.0	0.3	0.0	0.0	0.0	0.0
A26-1389	0.0	0.2	0.0	0.0	0.6	0.1
A26-1554	0.0	0.4	0.1	0.0	0.9	0.5
A26-1679	0.0	0.3	0.0	0.0	1.6	0.7
A26-1735	0.0	0.4	0.0	0.0	0.3	0.0
A26-1985						
A26-2351	0.0	0.2	0.0	0.0	0.0	0.0
A26-2371						
A26-528	0.0	0.4	0.0	0.0	1.7	1.4
A26-767	0.0	0.6	0.0	0.0	1.4	1.1
A26-768	0.0	0.2	0.0	0.0	0.1	0.0
A26-871	0.0	0.4	0.0	0.0	1.4	1.0
A26-882	0.0	0.7	0.0	0.0	1.0	0.1
A26-915	0.0	0.4	0.0	0.0	0.7	0.4
A26-941	0.0	0.8	0.0	0.0	0.0	0.0
A26-946	0.0	0.4	0.0	0.0	0.0	0.0
A26-947	0.0	0.8	0.0	0.0	0.0	0.0
A26-948	0.0	0.5	0.0	0.0	0.0	0.0
A26-97	0.0	0.4	1.8	1.7	1.8	1.8
A26-1048						
A26-1386						
A26-1018						
A09-268	0.0	0.7	0.0	0.0	1.6	0.9
A09-283	0.1	0.7	0.9	0.8	1.6	1.3

16. Agro-infiltration 2 of *R* genes in cisgenic Desiree plants (A26 plants harboring *Rpi blb3-Rpi sto1*)

	Avr3b (0.1)	R3b+Avr3b (0.2)	Avr2 (0.2)	Avr2 (0.1)	IPI-O1 (0.1)	IPI-O1 (0.05)
A03-142	0.0	0.0	1.6	1.5	0.0	0.0
A09-277	0.0	0.3	0.1	0.0	0.7	0.5
A10-43	0.0	0.3	1.1	1.1	1.4	0.8
A14-16	0.0	0.0	1.9	2.0	1.9	1.1
Desiree	0.0	0.1	0.0	0.0	0.0	0.0
A26-1023	0.0	0.0	0.0	0.0	1.1	0.3
A26-1065	0.0	0.0	0.0	0.0	1.4	0.7
A26-1172	0.0	0.3	0.4	0.2	0.0	0.0
A26-1206	0.0	0.1	0.0	0.0	1.7	1.1
A26-1263	0.0	0.0	0.0	0.0	0.0	0.0
A26-1369	0.0	0.1	0.0	0.0	0.0	0.0
A26-1389	0.0	0.0	0.1	0.0	1.2	0.7
A26-1554	0.0	0.0	0.1	0.2	1.1	0.5
A26-1679	0.0	0.1	0.0	0.0	1.5	1.1
A26-1735	0.0	0.0	0.1	0.3	1.9	0.7
A26-1985						
A26-2351	0.0	0.0	0.0	0.0	0.0	0.2
A26-2371						
A26-528	0.0	0.0	0.0	0.0	1.7	1.6
A26-767	0.0	0.0	0.0	0.0	1.3	0.9
A26-768	0.0	0.0	0.0	0.0	0.0	0.0
A26-871	0.0	0.0	0.0	0.0	1.6	0.9
A26-882	0.0	0.0	0.0	0.0	0.9	0.9
A26-915	0.0	0.0	0.2	0.0	1.6	1.4
A26-941	0.0	0.2	0.0	0.0	0.0	0.0
A26-946	0.0	0.0	0.0	0.0	0.0	0.0
A26-947	0.0	0.0	0.0	0.0	0.0	0.0
A26-948	0.0	0.0	0.0	0.0	0.0	0.0
A26-97	0.0	0.3	1.8	2.0	1.9	1.8
A26-1048						
A26-1386						
A26-1018						
A09-268	0.0	0.0	0.1	0.0	1.6	1.2
A09-283	0.0	0.0	0.0	0.0	0.8	0.5

17. Agro-infiltration 1 of *R* genes in cisgenic Desiree plants (A19 plants harboring *Rpi chc1-Rpi vnt1*)

Transgenic event	Avr3b (0.1)	R3b+Avr3b (0.1)	A2-2 (0.1)	A2-2 (0.05)	Avr-vnt1 (0.1)	Avr-vnt1 (0.05)
A19-2	0.0	0.0	0.0	0.3	2.0	1.3
A19-4	0.0	0.6	0.0	0.1	1.1	0.0
A19-5	0.3	0.0	0.1	0.3	2.0	1.3
A19-23	0.3	0.2	1.0	0.6	2.0	1.8
A19-35	0.0	0.1	0.3	0.0	2.0	1.7
A19-46	0.1	0.1	0.0	0.1	1.7	2.0
A19-63	0.1	0.2	0.0	0.0	2.0	1.0
A19-66	0.1	0.4	0.3	0.0	1.8	1.4
A19-67	0.0	0.2	0.0	0.0	2.0	1.1
A19-73	1.2	0.4	0.0	0.0	1.5	0.5
A19-77	0.0	0.7	0.1	0.5	1.5	1.6
A19-94	0.1	0.1	0.0	0.0	2.0	1.9
A19-95	0.0	0.2	0.0	0.0	2.0	1.8
A19-96	0.0	0.0	0.2	0.2	2.0	1.1
A19-99	0.0	0.1	0.0	0.4	2.0	1.2
A19-105	0.0	0.1	0.0	0.0	1.9	1.8
A19-115	0.0	0.1	0.1	0.1	1.7	1.3
A19-118	0.0	0.0	0.0	0.3	1.2	0.6
A19-120	0.0	0.1	0.0	0.0	1.9	1.8
A17-27	0.0	0.0	0.2	0.2	0.0	0.0
A13-13	0.0	0.6	0.0	0.1	2.0	1.5
Desiree	0.0	0.1	0.0	0.0	0.0	0.0
A09-268	0.0	0.1	0.0	0.0	0.1	0.4
A09-07	0.0	0.1	0.0	0.0	0.0	0.0
A09-277	0.0	0.1	0.0	0.0	0.0	0.0

18. Agro-infiltration 2 of *R* genes in cisgenic Desiree plants (A19 plants harboring *Rpi chc1-Rpi vnt1*)

	Avr3b (0.1)	R3b+Avr3b (0.2)	A2-2 (0.2)	A2-2 (0.1)	Avr-vnt1 (0.1)	Avr-vnt1 (0.05)
A19-2	0.0	0.5	0.0	0.0	1.8	1.6
A19-4	0.1	1.2	0.0	0.0	0.2	0.0
A19-5	0.0	0.5	0.0	0.0	2.0	1.7
A19-23	0.0	0.9	1.7	1.3	2.0	1.8
A19-35	0.0	0.4	0.0	0.0	1.7	0.7
A19-46	0.0	1.1	0.4	0.0	2.0	2.0
A19-63	0.0	0.6	0.1	0.0	2.0	1.5
A19-66	0.1	1.2	0.0	0.6	2.0	2.0
A19-67	0.0	0.7	0.2	0.0	2.0	1.3
A19-73	1.2	1.3	0.0	0.0	1.7	0.1
A19-77	0.0	0.0	0.0	0.0	1.6	0.4
A19-94	0.0	0.5	0.0	0.2	1.9	1.3
A19-95	0.0	0.1	0.0	0.2	2.0	1.4
A19-96	0.2	0.8	0.2	0.0	2.0	1.6
A19-99	0.0	0.6	0.0	0.0	2.0	1.6
A19-105	0.0	0.7	0.0	0.0	2.0	1.6
A19-115	0.0	0.9	0.4	0.1	2.0	1.4
A19-118	0.0	0.0	0.0	0.0	0.9	0.2
A19-120	0.0	0.1	0.0	0.0	2.0	1.1
A17-27	0.1	0.4	1.6	1.4	0.0	0.0
A13-13	0.0	0.8	0.0	0.0	1.8	1.0
Desiree	0.3	0.5	0.0	0.0	0.0	0.0

19. Co-infiltration of newly cloned double and triple *R* genes with their corresponding *Avr* effectors

	0.5 (0.25 each)	0.5 (0.25 each)	0.5 (0.25 each)	0.5 (0.25 each)	0.25	0.25	0.25
pBIN+PASSA vnt1-sto1	construct + avrsto1	const + avrvnt1	vnt1 + avrvnt1	sto1 + avrsto1	construct	Avr-vnt1	Avr-sto1
	0.4	0.6	0.0	0.0	0.9	0.0	0.0
pBIN+PASSA vnt1-chc1	construct + avrvnt1	const + avrchc1	vnt1 + avrvnt1	chc1 + avrchc1	construct	avr-vnt1	avr-chc1
	0.3	0.2	0.1	0.0	0.4	0.0	0.0
pBINAW vnt1-chc1	construct + avrvnt1	const + avrchc1	vnt1 + avrvnt1	chc1 + avrchc1	construct	avr-vnt1	avr-chc1
	0.0	0.0	0.0	0.8	0.0	0.0	0.0
pBIN+PASSA blb3-vnt1	construct + avrblb3	const + avrvnt1	vnt1 + avrvnt1	sto1 + avrblb3	construct	Avr-blb3	Avr-vnt1
	0.0	0.1	0.0	0.0	0.1	0.0	0.0
pBIN+PASSA edn2-chc1	construct + avrchc1	const + avredn2	chc1 + avrchc1	edn2 + avredn2	construct	Avr-chc1	Avr-edn2
	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pBIN+PASSA sto1-chc1	construct + avrsto1	const+ avrchc1	sto1 + avrsto1	chc1 + avrchc1	construct	Avr-sto1	Avr-chc1
	0.1	0.0	0.0	0.0	0.0	0.0	0.0
pBIN+PASSA vnt1-edn2	construct + avrvnt1	const+ avredn2	vnt1 + avrvnt1	edn2 + avredn2	construct	avr-vnt1	avr-edn2
	0.0	0.0	0.0	0.0	0.0	0.0	0.0

	0.5 (0.25 each)	0.5 (0.25 each)	0.5 (0.25 each)	0.5 (0.25 each)	0.5 (0.25 each)	0.25	0.25	0.25	0.25	0.5 (0.25 each)
pBIN+PASSA vnt1-blb3-chc1	construct + avrvnt1	const + avrvblb3	const+ avrchc1	vnt1 + avrvnt1	blb3 + avrblb3	construct	Avr-vnt1	Avr-blb3	Avr-chc1	chc1 +avrchc1
	0.4	1.4	1.8	0.1	0.0	2.0	0.0	0	0	0
pBINAW vnt1-blb3-chc1	construct + avrvnt1	construct + avrvblb3	const + avrchc1	vnt1 + avrvnt1	blb3 + avrblb3	construct	Avr-vnt1	Avr-blb3	Avr-chc1	chc1 +avrchc1
	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	0	1.25