Characterization of the *Rpi-chc1* family activity and recognition specificity

Daniel Moñino-López

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Supervisor: Dr. Jack Vossen

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Abstract

Late blight, caused by the oomycete *Phytophthora infestans*, is the most devastating disease in potato, leading to a global economic loss of about € 9.4 billion per year. Most of the currently used elite potato cultivars are susceptible to late blight. In order to control *P. infestans*, growers rely on biocide application. However, such chemical control is expensive, time consuming and non-environmentally friendly. As an alternative, the introgression of resistance (*R*) genes from wild potato relatives, e.g. *Solanum chacoense*, is a promising solution, required to reduce the impact of late blight on the potato fields. Most of these *R* genes are part of the Nucleotide-binding – Leucine-rich repeat (NB-LRR) protein family. These receptors recognise pathogen effectors and trigger the plant immune response. However, the molecular mechanisms underlying the receptor activation is poorly understood. This study focuses on the *Rpi-chc1* family, which recognises the RD12 effector family from *P. infestans*. Only 21 single amino acids polymorphisms (SAPs) were identified between the active receptor Rpi-chc1 from *S. chacoense* and the inactive receptor Rpi-tub_D3 from the potato cultivar RH89-39-16. Moreover, a second allele in *S. chacoense*, *Rpi-chc2*, which has a different effector recognition specificity of the RD12 family, showed 17 SAPs when it is compared to the *Rpi-chc1* allele. In this study, we aimed to establish a cloning system to produce plant *(N. benthamiana* and potato) expression vectors in which fragments of different alleles can be efficiently exchanged. Such system is necessary for a better understanding of the perception and activation mechanism of NB-LRR receptors. Ultimately, we want to identify the specific amino acids responsible for the Rpi-tub_D3 inactivity, and the Rpi-chc1 and Rpi-chc2 effector recognition specificity. Decipher the molecular mechanism of the amino acids involved in the receptor activity and recognition specificity is essential for the complete exploitation of the *R* gene engineering for new crop resistances.

Keywords*:* *Phytophthora infestans*, potato late blight, *Solanum chacoense*, *Rpi-chc1* family, NB-LRR receptors, effector recognition.

Introduction

1. Potato late blight disease

Potato (*Solanum tuberosum*) yields nutritionally valuable tubers, which have been spread from their origin in South America to many countries all over the world (Millam, 2006). In terms of global production, potato is the fourth most important crop in the world, after corn, rice and wheat, reaching a total production of 377 million of tones in 2016 (FAO, 2017). However, potato suffers from a very devastating disease which is called potato late blight. Potato late blight, caused by the oomycete *Phytophthora infestans* (*P. infestans*), is the most infamous disease in potato, which causes severe agricultural damage and extensive yield losses (Kamoun et al., 2014). The main symptoms of the infection are dark patches in leaf and stem surrounded by chlorotic tissue in which pathogen spores are visible. The lesions expand rapidly and become necrotic, ultimately destroying the complete plant. Late blight losses are estimated to reach 16% of the potato production, representing a global economic loss of about € 9.4 billion per year (Haverkort et al., 2016). The Netherlands produces over 7 million tons of potato annually, exporting 70% of this production. Only in The Netherlands, the control of late blight costs annually € 125 million (Haverkort et al., 2009). The reason for this is that most of the currently used elite potato cultivars are susceptible to late blight, and the only strategy to control the disease is by spraying very frequently with a mixture of biocides. However, this strategy is expensive and time consuming for growers, especially in developing countries (Foster et al., 2009). Moreover, pesticides have detrimental effects on the plant-mycorrhizae symbiosis and on the environment. For these reasons many biocides are being banned, e.g. copper based biocides, due to their large impact on groundwater that leads to a large ecosystem damage (Sukarno et al., 1996; Gyamfi, 2012; Mathew et al., 2015). In addition, *P. infestans* evolves quickly and becomes resistant to biocides. All these facts reveal the importance of developing novel strategies that allow a more effective control of *P. infestans*, in order to decrease the large impact of this pathogen on the potato fields.

2. *Phytophthora infestans*

P. infestans is a hemibiotroph oomycete with a sort initial biotrophic lifestyle, after which it becomes necrotroph. Furthermore, it has both sexual and asexual life cycle.

The sexual reproduction between the two A1 and A2 mating types determines the resistant oospore formation, which can survive up to 4 years in the ground (Turkensteen, 2000). Additionally, *P. infestans* has a large and fast changing genome, which leads to the constant emergence of new aggressive and pathogenic strains (Akino, Takemoto and Hosaka, 2013). During the asexual stage, *P. infestans* easily wind propagated spores land on the plant surface and germinates after the molecular recognition of the host. The germ tube reaches the inner tissue using injuries, natural openings, i.e. stomata, or via the formation of the appressorium penetration structure (Figure 1A). Subsequently, the hyphae spread intercellularly throughout the plant tissue projecting haustorium structures into the cells (Whisson et al., 2016). Haustoria have been shown to be used by the oomycete to secrete both apoplastic and cytoplasmic effectors (Figure 1B). Effectors are molecules that interact with different host targets to modulate the host physiology and suppress the host defence response (Wang et al., 2017; Haldar et al., 2006).

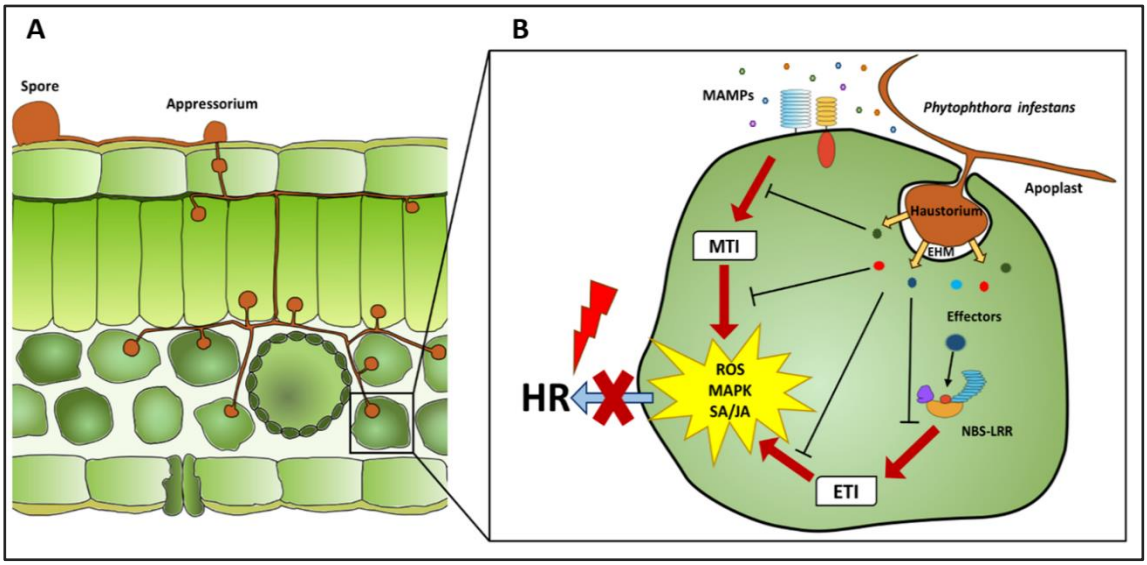

Figure 1. *Phytophthora infestans* infection mechanism. **A**. Colonization process of *P. infestans* in a potato leaf. After the host recognition, the spore germinates and forms the characteristic penetration structure, called appressorium. The hyphae start growing between the cells and projecting haustorium structures into the cells. After this first biotrophic phase, the interaction becomes necrotrophic and devastates the plant tissue. **B**. Zoom into the *P. infestans* cellular infection process. *P. infestans* projects haustorium structures into the plant cell to secrete different kind of effectors. *P. infestans* infection leads to the microbe-associated molecular patterns (MAMPs) recognition by plant transmembrane receptors, which conclude in the activation of the MAMP-triggered immunity (MTI). This immune response is characterised by an initial reactive oxygen species (ROS) burst, followed by a mitogen-activated protein kinase (MAPK) signalling activation which leads to the upregulation of defence hormones like salicylic acid (SA) or jasmonic acid (JA). Effectors can suppress this immune response by interacting with specific intracellular mechanisms. On the other hand, effectors can also be recognised by intracellular plant receptors, e.g. NB-LRR, and prompt the effector-triggered immunity (ETI), concluding in the activation of the hypersensitive response (HR). Some effectors can also suppress ETI, leading to successful pathogen infection [Adapted from Birch et al.., 2009].

During the infection process, *P. infestans* secretes several hundreds of effectors (Wang et al., 2017). Apoplastic effectors target plant extracellular proteins, e.g. papain-like apoplastic proteases (Tian et al., 2006) and endo-ß-1,3-glucanases (Damasceno et al., 2008), to reduce the damage that the primary plant defence response does to the pathogen. Additionally, cytoplasmic effectors have been described to target different subcellular compartments to promote the disease (Whisson et al., 2016). For example, *P. infestans* secretes the extracellular elicitin Infestin1 (INF1), a microbe-associated molecular pattern (MAMP), during the infection process (Kamoun et al., 1998). Upon INF1 recognition by the plant, MAMP-triggered immunity (MTI) is activated. However, *P. infestans* has developed the ability to secrete an additional cytoplasmic RxLR effector Avr3a. This Avr3a is one of the best-studied oomycete effectors, which stabilizes the ubiquitin E3 ligase CMPG1, thereby suppressing the INF1-triggered immunity and the INF1-mediated cell death (Bos et al., 2010). Within the amino acid sequence of *P. infestans* cytoplasmic effectors, a common host-targeting motif Arg-any amino acid-Leu-Arg (RxLR) has been characterized (Whisson et al., 2007). RxLR effectors rapidly evolve by gaining and losing repeated domains, recombining with different paralogs, and being positively selected on point mutations; in order to suppress the plant defence response and modulate the plant physiology to enable colonization (Goss, Press and Grünwald, 2013). In this coevolutionary process, plants have evolved receptors that recognise pathogen effectors that, upon perception of a cognate effector (also referred as Avr), trigger the plant immune response.

3. Potato defence response

Many potato cytoplasmic resistance (R) proteins have been described to recognise *P. infestans* effectors, e.g. R1, R3a, Rpi-blb2 and Rpi-chc1 (Ballvora et al., 2002, El-Kharbotly et al., 1994; Vossen et al., 2005; Vossen et al., 2011). Particularly, the above mentioned Avr3a *P. infestans* effector is recognised by the potato cytoplasmic R protein R3a, which belongs to the Nucleotide-binding – Leucine-rich repeat (NB-LRR or NLR) family (El-Kharbotly et al., 1994; Huang et al., 2004). Some R proteins directly recognize effectors, while others provide indirect recognition. Regarding the indirect recognition, R proteins are able to guard host effector targets by sensing changes in such host protein. Additionally, examples have been described where host proteins in association with R proteins mimic the effector target, a mechanism which is referred to as integrated decoy

model (van der Hoorn and Kamoun, 2008; Cesari et al., 2014). The direct or indirect recognition of the pathogen effector leads to the induction of the effector-triggered immunity (ETI) and the activation of the hypersensitive response (HR). This concludes in the induction of programmed cell death of infected and surrounding cells and the final restriction of the pathogen growth (Lozano et al., 2012). Most of the R proteins present in *P. infestans* resistant potato cultivars, are cytoplasmic receptors from the NB-LRR protein family. NB-LRR proteins are divided in two main subclasses depending on the presence of the Toll-like domain (TIR), called TIR-NB-LRR (TNL), or the presence of the coiled-coil domain (CC), referred as CC-NB-LRR (CNL). More than 82% of the NB-LRR proteins described in the double monoploid *S. tuberosum* DM1-3 516 R44 (DM) belong to the CNL subclass and they are mostly clustered in the chromosomes 4, 9 and 11 (Jupe et al., 2012).

4. Nucleotide-binding - leucine-reach repeat (NB-LRR)

NB-LRR receptors have been shown to provide resistance against different plant pathogens, e.g. Rxo1 provides resistance to the bacteria *Xanthomonas oryzae* in maize, Sw-5 is associated with resistance against tomato spotted wilt virus (TSWV), and the tomato Mi-1.2 confers resistance to aphids, whiteflies, psyllids, and the root-knot nematode *Meloidogyne incognita*. (Zhao et al., 2005; Gilbert and McGuire, 1956; Rosello et al., 1998; Reinink et al., 1989). NB-LRR are very diverse and fast evolving receptors present in both dicotyledonous and monocotyledonous plants. Furthermore, chromosomal rearrangements like duplications, ectopic recombinations, unequal crossing overs and transpositions have been described to provide the basis for the evolution of NB-LRR gene specificities (Jupe et al., 2012). The sequence conversion rate is higher in LRR domains compared to NB domains. This has been related to the different role among LRR and NB domains. LRR domains play an important role during the effector recognition, whereas NB domains are involved in signal transduction (Sukarta, Slootweg & Goverse, 2016). The fast evolution of LRR domains from the same cluster leads to the recognition of different pathogens by almost identical proteins, e.g. Rx and Gpa2 are homologous protein receptors from potato that recognize a virus and a nematode, respectively (van der Vossen et al., 2000).

The LRR domain consists of repeated ß-strand and ß-turns, which forms the characteristic horseshoe-folded conformation (Jones and Jones, 1997). Moreover, it

presents a C-terminal domain mainly composed by aromatic amino acids and a N-terminal domain with a large amount of positively charged residues. This LRR N-terminal domain interacts intramolecularly with the NB domain, forming a closed conformation that avoids the receptor self-activation in the absence of the pathogen target (Moffett, 2002). The C-terminal domain is exposed on the surface of the protein interacting with the CC domain, concluding in a more compact and stable structure. The LRR C-terminal domain has been hypothesised to be responsible for the ligand perception. The recognition of the ligand leads to a conformational change that exposes different domain binding sites for the interaction with other cellular proteins (Figure 2) (Maekawa et al., 2011). Different protein domains have been reported to be required for NB-LRR downstream signalling, e.g. Rx1 needs the NB domain to activate cell death and the mildew A1 (MLA1) needs a CC domain dimerization to induce the activation of the defence response (Takken and Goverse, 2012).

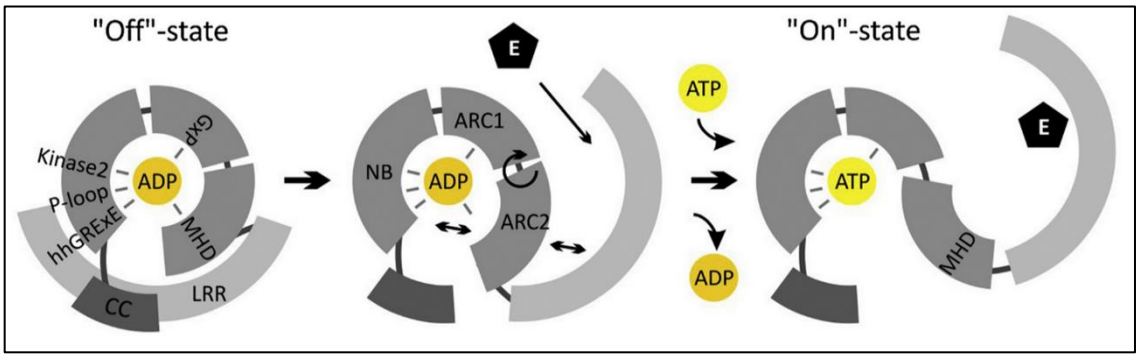

Figure 2. CNL receptor activation process. In a normal physiological Off-state, the CNL is kept in an inactive and compact conformation by interdomain interactions of conserved motifs (including the hhGRExE, P-loop, Kinase2, GxP and MHD motifs) in the NB-ARC subunits (NB, ARC1, ARC2) with ADP. When the effector (E) approximates to the LRR domain, it releases the compact structure (marked by double-headed arrows), allowing the exchange of ADP for ATP. In the active ATP-bound state, the ARC2 is rotated away from the NB-ARC1 subunits, exposing different domains for the interaction with additional proteins from the defence response cascade. Hydrolysis of the ATP molecule by the interaction with other proteins returns the structure to a compact Off-state, preventing unnecessary cell death and allowing the protein to be activated again in the presence of a new effector molecule [Adapted from Sukarta, Slootweg & Goverse, 2016].

5. Potato wild relatives as a source of NB-LRR genes

P. infestans is an aggressive pathogen that has been able to breakdown resistance from potato cultivars like Pentland Dell which contains *R1*, *R2* and *R3* genes from *Solanum demissum* (Malcolmson, 1969). Consequently, a renewed search for broad-spectrum and durable resistances that can combat new pathogen isolates was initiated (Haverkort et al., 2016). Potato wild relatives are an important source of dominant *R*

genes (Malcolmson and Black, 1966). Until now, different *R* genes have been mapped and cloned from potato wild relative species, e.g. *R1*, *R2*, *R3a*, *R3b*, *R8* and *R9a* from *S. demissum* (Ballvora et al., 2002; Lokossou et al., 2009; Armstrong et al., 2005; Li et al., 2011; Vossen et al., 2016; Jo et al., 2015); *Rpi-blb1* and *Rpi-blb2* from *Solanum bulbocastanum* (Song et al., 2003; van der Vossen et al., 2005); *Rpi-chc1* from *Solanum chacoense* (Vossen et al., 2011); or *Rpi-vnt1* from *Solanum venturii* (Pel et al., 2009). However, the introgression of single *R* genes from *S. demissum* in different potato varieties resulted in a rapid resistance breakdown. The introgression of multiple *R* genes in one cultivar, referred as pyramiding or stacking, can be the solution to produce more durable resistant potato cultivars. A cultivar with multiple *R* genes that recognise several unrelated effectors from the pathogen has a lower chance to be overcome. This is because, it is unlikely the accumulation of multiple independent mutations in a single spore that can overcome all the introgressed *R* genes (Jo et al., 2016). This approach would conclude in both durable and broad-spectrum resistant cultivars. However, the introgression of the single *Rpi-blb2* gene in the potato varieties Bionica and Toluca took 46 years. The stacking of multiple *R* genes could be even more difficult and slower (Haverkort et al., 2009). For this reason, new techniques such as cisgenesis have being developed to accelerate the gene introgression into the currently used potato cultivars.

6. Late blight resistance genes from *Solanum chacoense*

Solanum chacoense (*S. chacoense*) is a wild relative diploid potato from South America. Several plant accessions of this species have been tested against different *P. infestans* isolates, and some of them remained unaffected. Using a mapping population, an R locus was mapped in chromosome 10, and within this locus, the *Rpi-chc1* gene was described to be the origin of the resistance. *Rpi-chc1* encodes for a 1302 amino acids NB-LRR protein with 29 imperfect leucine-rich repeats (Vossen et al., 2011). Using a locus directed profiling technique, it was founded that *Rpi-chc1* is an allelic variant of the previously described *Rpi-ber* (Rauscher et al., 2006). Both are located in the long arm of the chromosome 10, region where also the tomato Ph-2 QTL from *Solanum pimpineliifolium* against *P. infestans* was mapped (Moreau et al., 1998). The *P. infestans* isolate T30-4 is unable to infect plants expressing the *Rpi-chc1* allele; therefore, the recognised molecule must be present in this isolate. A collection of T30-4 effectors has been cloned into the pGR106 plant expression vector, which are referred to as the PEX

set (Vleeshouwers et al., 2008). This set was co-agroinfiltrated with *Rpi-chc1* in *N. benthamiana*. Only the co-infiltration with the specific RD12 effector family induced the plant defence response and the final HR. The RD12 effector family is composed by 19 members, which have been classified into A, B, C and D clades. Additionally, the A clade can be divided into A1 and A2 sub-clades (Figure 3A).

A germplasm of 225 *Solanaceae* species, including the susceptible RH89-39-16 (RH) potato cultivar, and the two potato wild relatives *S. berthaultii* and *S. tarijense*, was analysed for the presence of *Rpi-chc1* homolog genes. Among all the related species, 23 major homolog sequences were identified. All the *Rpi-chc1* homologs were analysed and classified into 1, 2, 3 and 4 clades (Figure 3B). Most of the homologs were cloned and tested for their biological activity to recognize different *P. infestans* RD12 family members and the IPO-C isolate (Figure 4). *Rpi-chc1* belongs to the clade 3, which recognises the RD12 clade A. In the clade 1, different active receptors have been described to recognise both B and C RD12 clades. In this clade 1, an alternative allele from *S. chacoense*, *Rpi-chc2*, was described among the active alleles to have a broad spectrum recognition activity. The clades 2 and 4 contain receptors that were not able to trigger the plant immune response in the presence of any member of the RD12 effector family. Therefore, the genes from these clades, including *Rpi-tub_D3* from the RH cultivar, were considered inactive (Vossen J.H. personal communication).

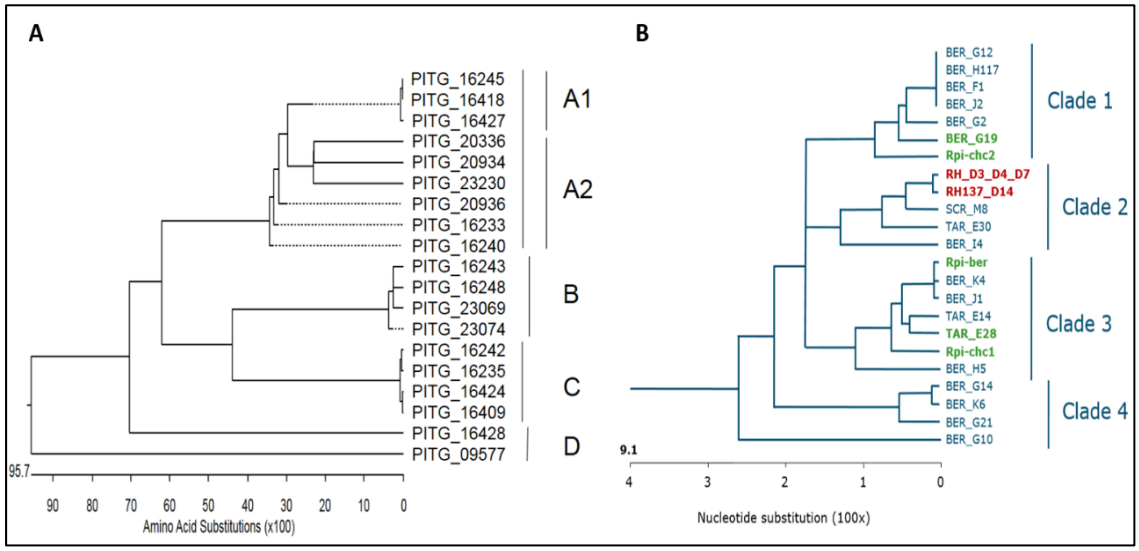

Figure 3. A. Phylogenetic tree of the *P. infestans* RD12 effector family based on the amino acid homology (x100). The RD12 effector family were classified in four A, B, C and D clades. A was additionally divided in two A1 and A2 sub-clades. **B**. Phylogenetic tree of the *Rpi-chc1* homologs based on the DNA sequence. The *Rpi-chc1* homologs were classified into 1, 2, 3 and 4 clades base on the nucleotide homology. In the clade 1 and 3 we can distinguish the active copies in green of *Rpi-chc1*, *Rpi-chc2* and *Rpi-ber*. In the clade 2 we can distinguish the inactive alleles from RH in red (Vossen J.H. personal communication).

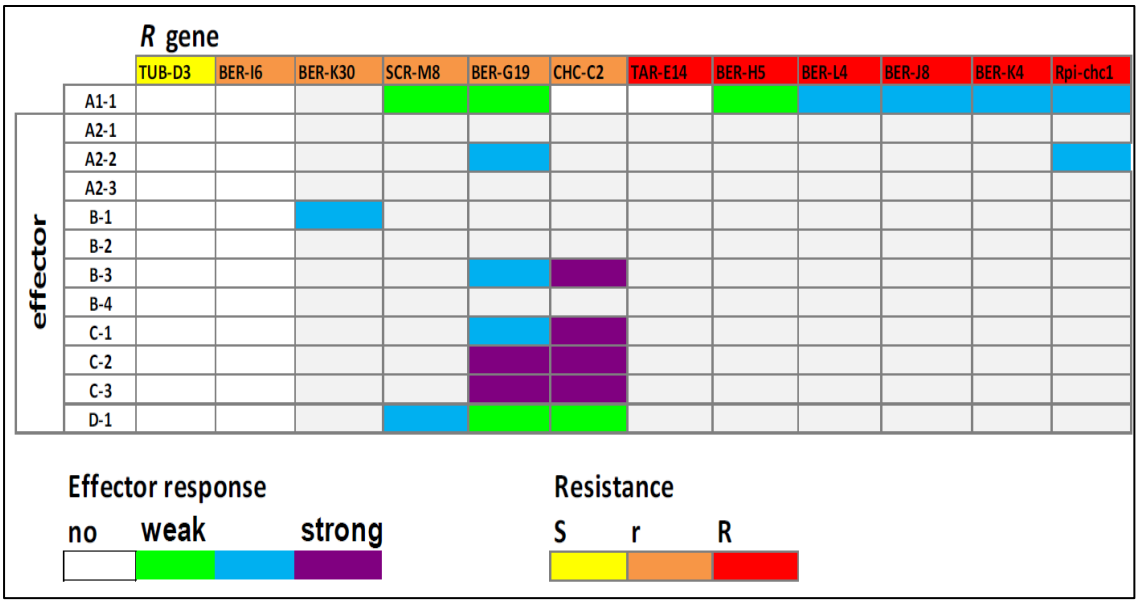

Figure 4. Summary table of the *Rpi-chc1* homologs recognition spectrum of the RD12 effectors in *N. benthamiana*. After the co-infiltration of the different receptors and the RD12 effectors, the plant response was evaluated for the area with an induced HR. The response was graded from weak (green) to strong (purple). Plants expressing the different receptors were also challenged with the *P. infestans* isolate IPO-C. The resistance to IPO-C was evaluated from susceptible (yellow) to fully resistant (red) (Vossen J.H. personal communication).

Hypothesis and Objectives

The main objective of this project is to identify the amino acids responsible for the receptor activity and effector recognition specificity. The fact that only 21 SAPs are the major difference between an active and an inactive receptor, makes us hypothesize that a single or a couple amino acids are responsible for the Rpi-tub-D3 receptor inactivity and the final RH susceptibility to *P. infestans*. Additionally, we are also interested in the identification of the amino acids that are responsible for the receptor recognition specificity of the RD12 effectors between Rpi-chc1 and Rpi-chc2. The high homology between these sequences makes the *Rpi-chc1* alleles a nice starting material to investigate the amino acids responsible for the *Rpi-chc1* homologs activity and recognition specificity. The specific objectives are:

1. Establishing a cloning system of the active and inactive alleles in a plant expression vector under the *Rpi-ber* promoter and terminator.
2. Studying the capacity of *Rpi-ber* promoter and terminator to drive the transient expression of the *Rpi-chc1* homolog genes in *N. benthamiana*.
3. Performing domain exchange between the different *Rpi-chc1* homologs, creating different chimeric proteins from the three Rpi-tub_D3, Rpi-chc1 and Rpi-chc2 selected receptors.
4. Testing the different chimeric receptors in the model plant *N. benthamiana*. These chimeric proteins will allow us to reduce the number of SAPs required for the activation of the *Rpi-tub_D3* allele and the modification of the effector recognition specificity of the Rpi-chc1 and Rpi-chc2 receptors.

Materials and Methods

1. Establishment of the plant expression system

For the initial design of the plant expression system of the *Rpi-chc1* homologs, we decided to use the *Rpi-ber* promoter and terminator sequences to drive the expression of the different active and inactive *Rpi-chc1* homologs. The two active homologs, *Rpi-chc1* and *Rpi-chc2*; and the inactive allele, *Rpi-tub-D3* from RH were respectively amplified from the previously amp166, amp167 and 168amp vectors. The *Rpi-ber* promoter and terminator were amplified from the 290_pBINPLUS-PASSA-ber vector. Additionally, the *uidA* gene that encodes for the β-D-glucuronoside (GUS) was amplified from the pENTR-GUS (Invitrogen) to be introduced under the same *Rpi-ber* regulatory sequences. This construct was used as a negative control in the agroinfiltration experiments.

1.1. Zero Blunt™ PCR Cloning Kit

The active/inactive alleles and the *Rpi-ber* regulatory elements were amplified using Phusion High-Fidelity DNA Polymerase (New England BioLabs), in order to minimize amplification mistakes. The amplification with Phusion polymerase leads to PCR products with blunt ends. Therefore, we decided to use the Zero Blunt Cloning Kit (Invitrogen) to introduce the PCR fragments into the pCR™-Blunt II-TOPO® vector. This vector is a linearized vector that contains a Vaccinia virus DNA topoisomerase I covalently bound to the 3´ end of each DNA strand. When the vector is incubated with the PCR fragment, the topoisomerase ligates the fragments to the vector, circularising and disrupting the lacZα-ccdB gene fusion. Therefore, cells that contain non-recombinant vector are killed upon plating. The protocol was strictly followed from the manual Zero Blunt Cloning Kit from Invitrogen.

1.2. Gibson Assembly

The amplification of the active/inactive alleles and the *Rpi-ber* regulatory elements was performed using Phusion High-Fidelity DNA Polymerase (New England BioLabs), in order to avoid amplification mistakes. In this strategy, the pBINPLUS-PASSA vector was used as backbone for the new plant expression system. This backbone was amplified from the previously designed vector 290_pBINPLUS-PASSA-ber, which includes the *Rpi-ber* promoter and terminator. This technique allows the one-step cloning of multiple fragments regardless of fragment length or end compatibility. After the PCR

amplification of the required fragments, they were mixed and incubated with the Gibson Assembly buffer during 1 hour at 50 °C. In this buffer, an exonuclease created single stranded 3'-overhangs that facilitated the annealing with the complementary single stranded sequence of the contiguous fragment. Afterwards, a DNA polymerase filled-in the gaps and a ligase sealed the nicks. The result was a final circularised double stranded DNA molecule ready to be transformed in *Escherichia coli* (*E. coli*). The protocol was strictly followed from the Gibson Assembly manual from New England BioLabs.

2. Bacterial transformation

Due to the large size of the final vector (~20Kb), electroporation was the selected method for bacterial transformation. The TOP10 electrocompetent cells (Invitrogen) were used to transfer the new plasmids into *E. coli*. After the electrical pulse at 1800V, 200Ω, 25µF in a 1 mm electroporation cuvette, the cells were incubated 1 hour at 37 °C in SOC medium. Cells were plated in LB-agar plates with 50 µg/mL of kanamycin and incubated at 37 °C O/N. The colonies were analysed for the desired recombinant vector by colony PCR, restriction digestion and sequencing.

Once the analysis of *E. coli* colonies confirmed the correct recombination, the corresponding plasmids were isolated for *Agrobacterium tumefaciens* transformation. *A. tumefaciens* strain Agl1 + VirG electrocompetent cells were used as a host to direct plant transformation. The electrical pulse was performed at 1800V, 200Ω, 25µF in a 1 mm electroporation cuvette. After the electrical pulse the cells were incubated 2 hours at 28 °C in SOC medium. Cells were plated in LB-agar plates with 50 µg/mL of kanamycin, to select for the binary vector, 100 µg/mL of carbenicillin and 25 µg/mL of chloramphenicol, to retain the VirG helper plasmid. Plates were incubated at 28 °C O/N, and a colony PCR was performed to prove the presence of the correct plasmid. One colony per construct was selected and a plasmid stability test was performed. In this test, a liquid culture from a single colony was streaked in a LB plate with 100 µg/mL of carbenicillin, 25 µg/mL of chloramphenicol and 50 µg/mL of kanamycin, and incubated O/N. The next day, a colony PCR was performed in 25 independent colonies from each plate. In this way, we can check if the constructed plasmid is stable in Agl1 + VirG Agrobacterium strain.

3. *R* genes and effectors

Most of the *R* genes and effectors used in this study were already available in the *A. tumefaciens* strain Agl1 in different plant expression vectors (Table 1). All these constructs were used in the two agroinfiltration experiments in order to be able to evaluate the activity of the recently cloned receptors.

Table 1. Summary table of the available *R* genes and effectors, including vector and antibiotic resistance.

Gene	Vector	Antibiotic Resistance
Rpi-chc1	pDEST	Spectinomycin
Rpi-chc2	pDEST	Spectinomycin
Rpi-ber_G19	pDEST	Spectinomycin
Rpi-tub_D3	pDEST	Spectinomycin
R3a	pBINPLUS	Kanamycin
Avr3a	pK7WG2	Spectinomycin
PITG-16245 (RD12-A1-1)	pGR106_GW	Kanamycin
PITG-20336 (RD12-A2-1)	pK7WG2	Spectinomycin
PITG-20934 (RD12-A2-2)	pK7WG2	Spectinomycin
PITG-20934 (RD12-A2-2)	pGR106_GW	Kanamycin
PITG-23230 (RD12-A2-3)	pK7WG2	Spectinomycin
PITG-16243 (RD12-B1)	pK7WG2	Spectinomycin
PITG-16248 (RD12-B2)	pK7WG2	Spectinomycin
PITG-23069 (RD12-B3)	pK7WG2	Spectinomycin
PITG-23069 (RD12-B3)	pGR106_GW	Kanamycin
PITG-23074 (RD12-B4)	pK7WG2	Spectinomycin
PITG-16242 (RD12-C1)	pK7WG2	Spectinomycin
PITG-16235 (RD12-C2)	pK7WG2	Spectinomycin
PITG-16424 (RD12-C3)	pK7WG2	Spectinomycin
PITG-16428 (RD12-D1)	pK7WG2	Spectinomycin

4. Agroinfiltration

The protocol started the first day with the inoculation of the different constructs from glycerol stocks in 3 mL of LB medium with the corresponding antibiotics. The culture grew O/N at 28 °C and was shaken at 200 rpm. At the end of the second day, 400 µL of the O/N culture were inoculated in 15 mL of YEB (5 g/L beef extract, 1 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose and 2 mM $MgSO_4$) with 1.5 µL of 200 mM acetosyringone, 150 µL of MES 1M and the corresponding antibiotics. The bacteria grew

O/N at 28 °C and were shaken at 200 rpm. The third day, the bacterial cells were pelleted at 4000 rpm for 10 min and the supernatant was discarded. The pellet was resuspended in MMA (20 g/L of sucrose, 5 g/L of MS salts without vitamins, 1.95 g/L of MES and 1 mL/L of acetosyringone 200 mM; pH = 5.6 by adding 1M NaOH). The optical density (OD) at 600nm was measured using a BioPhotometer (Eppendorf). The OD_{600} was annotated and the cultures were diluted to OD_{600} = 1. The cultures were incubated for 2-3 hours in the dark at room temperature for acclimatization. After the acclimatization the cultures were mixed in a 1:1 ratio after which they were infiltrated with a syringe on the abaxial-side of a 4-week-old *N. benthamiana* plant. A maximum of two leaves per plant were infiltrated.

The infiltrated leaves were scored for their ability to induce HR 3 days post inoculation (dpi). The plant defence response was evaluated absent, weak, medium or strong depending on the intensity of the plant immune response. In all the agroinfiltration experiments, the well characterised recognition of Avr3a by R3a and the subsequent fast HR induction, was used as a positive control of the agroinfiltration. Negative controls with the independent infiltration of the different receptors and RD12 effectors were performed, to prove that the plant response was only caused by receptor-effector interaction. Two biological replicates per construct were included in each experiment.

Results

1. Rd12 recognition specificity by *Rpi-chc1* alleles

As previously mentioned, the *Rpi-chc1* alleles have been identified to recognise different *P. infestans* RD12 family members (Vossen J.H. personal communication). In this study, we first would like to repeat the previous biological activity test performed in our group to the different *Rpi-chc1* homologs for their RD12 recognition specificity. For this purpose, we used the *Rpi-chc1*, *Rpi-chc2* and *Rpi-tub_D3* alleles cloned in the pDEST plant expression vector under the 0.9 Kb *Rpi-chc1* promoter. These receptors were co-infiltrated with the *P. infestans* RD12 A1-1, A2-2, B3 and C2 effectors in *N. benthamiana* leaves. After 3 days, the leaves were evaluated for the HR induction. As previously described, Rpi-chc1 was only able to induce HR in the presence of A1-1 and A2-2 effectors from the RD12 family (Figure 5A). As we can observe in the Figure 5A, the HR response induced by Rpi-chc1 in the presence of RD12 A2-2 was less intense compared to A1-1. Rpi-chc2 was able to strongly trigger the immune response in the presence of the B3 and C2 RD12 effectors (Figure 5B). Rpi-ber_G19 was also able to induce the plant response only in the presence of B3 and C2 effectors from the RD12 family (Figure 5C), but in this case, the recognition of RD12 B3 was less intense compared to C2. Rpi-tub_D3 was not able to trigger the HR in the presence of any RD12 family member (Figure 5D). The agroinfiltration results from this experiments were summarized in the Table 2. Additionally, we could observe in each leaf the positive control activation of the plant immune response when the R3a receptor and the matching Avr3a effector were co-infiltrated. The negative controls with the infiltrations of the separated *Rpi-chc1*, *Rpi-chc2* and *Rpi-tub_D3*, and the *P. infestans* RD12 A1-1, A2-2, B3 and C2 effectors did not trigger the HR. Moreover, we performed an infiltration test to evaluate the effect of the addition of the p19 silencing suppressor protein during the RD12 effector recognition by the *Rpi-chc1* homologs (Figure 5E). The presence of the p19 protein could only intensify the activation of the plant immune response. This result proved that p19 can be used for a better identification of the *Rpi-chc1* homologs-RD12 effectors recognition without having any detrimental effect on the effector recognition. All the infiltration results were summarized in Table 2.

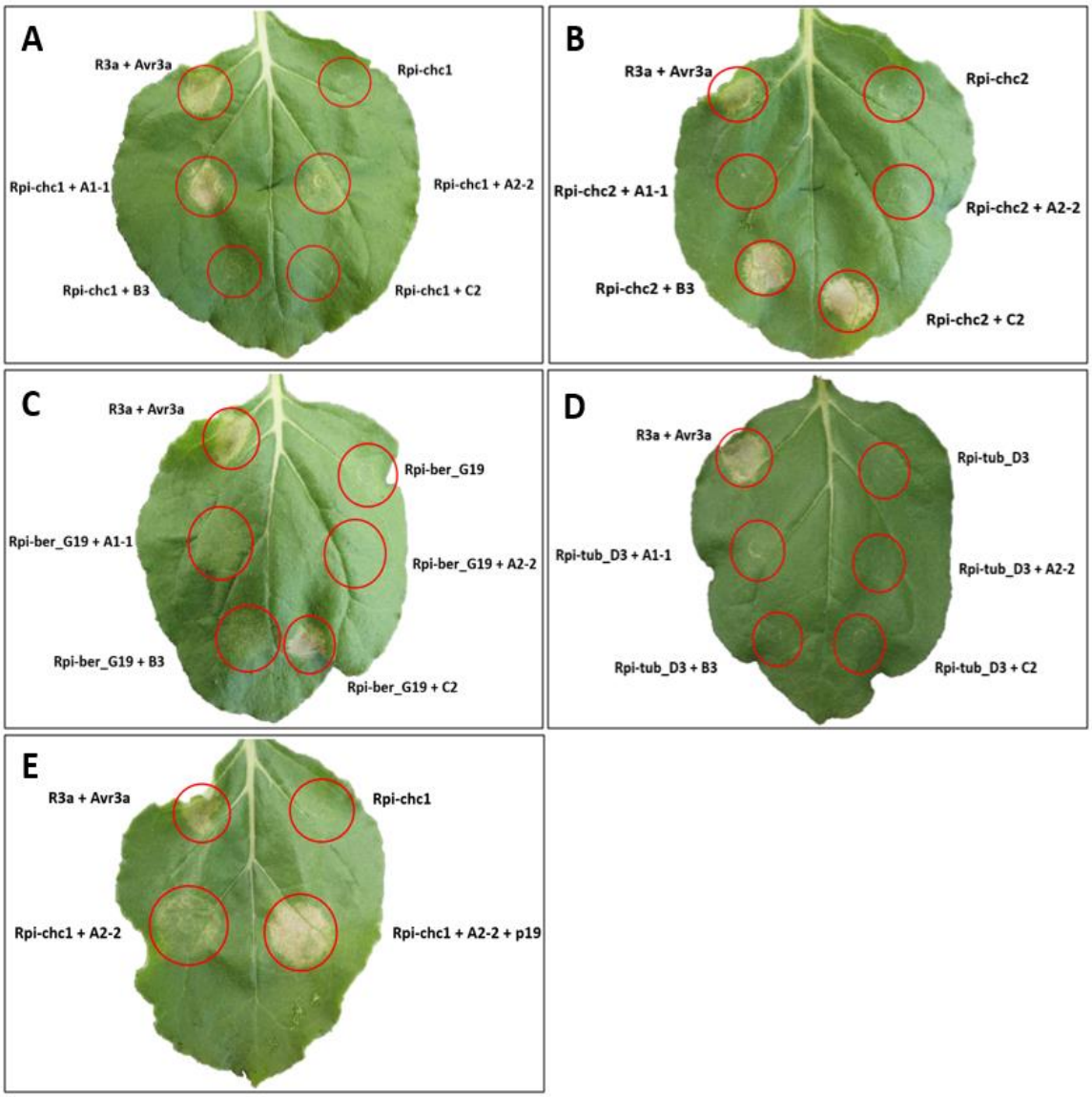

Figure 5. Co-infiltration of the Rpi-chc1, Rpi-chc2, Rpi-tub_D3 receptors and the different RD12 effector family members. **A**. Rpi-chc1 (in pDEST) response in the presence of A1-1, A2-2, B3 and C2 effectors after 3 days. **B**. Rpi-chc2 (in pDEST) response in the presence of A1-1, A2-2, B3 and C2 effectors after 3 days. **C**. Rpi-ber_G19 (in pDEST) response in the presence of A1-1, A2-2, B3 and C2 effectors after 3 days. **D**. Rpi-tub_D3 (in pDEST) response in the presence of A1-1, A2-2, B3 and C2 effectors after 3 days. **E**. Rpi-chc1 (in pDEST) response in the presence of the A2-2 with or without the p19 silencing suppressor.

Table 2. Summary table of the RD12 effector response observed in the *N. benthamiana* agroinfiltration experiment in the presence of the different *Rpi-chc1* homologs. The effector response was classified absent, weak, medium or strong.

		Rpi-chc1 in pDEST	Rpi-chc2 in pDEST	Rpi-ber_G19 in pDEST	Rpi-chc1_RH in pDEST
RD12 effectors	A1-1				
	A2-2				
	B3				
	C2				

Effector response			
No	Weak	Medium	Strong

2. *Rpi-chc1* alleles effector recognition specificity

The alignment between the protein sequences of the active Rpi-chc1, and the inactive Rpi-tub-D3 from RH, showed 97% identity of the amino acid sequence. Among the 43 single amino acid polymorphisms (SAPs), 21 lead to a relevant modification of the amino acid properties, i.e. hydrophaty index, charge and size (Table 3A). As we can observe in the figure 6, 19 of these amino acid changes are located in the LRR domain, which has been previously described to be involved in the effector recognition and the interdomain interactions. Based on the foregoing, it was hypothesized that some of these amino acid changes are responsible for the inactivity of the Rpi-tub-D3 homolog in the susceptible potato cultivar RH. The additional alignment between Rpi-chc1 and Rpi-chc2 shows also 97% identity of the amino acid sequence; indicating that also a small number of SAPs is responsible for the effector recognition specificity. Among the 40 SAPs found between Rpi-chc1 and Rpi-chc2, 24 lead to a relevant change in the amino acid properties (Table 3B).

Table 3. Most relevant amino acid substitutions which cause a significant change in the amino acid properties. **A**. Twenty-one most relevant amino acid changes between Rpi-chc1 and Rpi-tub_D3. **B**. Twenty-four most relevant amino acid substitutions between Rpi-chc1 and Rpi-chc2.

A

Rpi-chc1 Vs. Rpi-tub_D3					
1	K26I	**9**	T938R	**17**	D1158N
2	G335R	**10**	R960S	**18**	E1161K
3	K647E	**11**	N988E	**19**	T1175R
4	S816R	**12**	E1032I	**20**	V1188E
5	K886E	**13**	C1035F	**21**	R1211S
6	K890E	**14**	V1057E		
7	Q934E	**15**	V1084E		
8	K936E	**16**	K1096E		

B

Rpi-chc1 Vs. Rpi-chc2					
1	R738G	**9**	C1035F	**17**	E1139A
2	F864L	**10**	H1037Y	**18**	D1158N
3	K886E	**11**	V1057E	**19**	T1175R
4	K890E	**12**	N1060H	**20**	V1188E
5	R893H	**13**	L1080F	**21**	R1211S
6	Q934E	**14**	L1084D	**22**	H1235Q
7	K936E	**15**	S1086R	**23**	H1242L
8	K965N	**16**	R1107S	**24**	R1259G

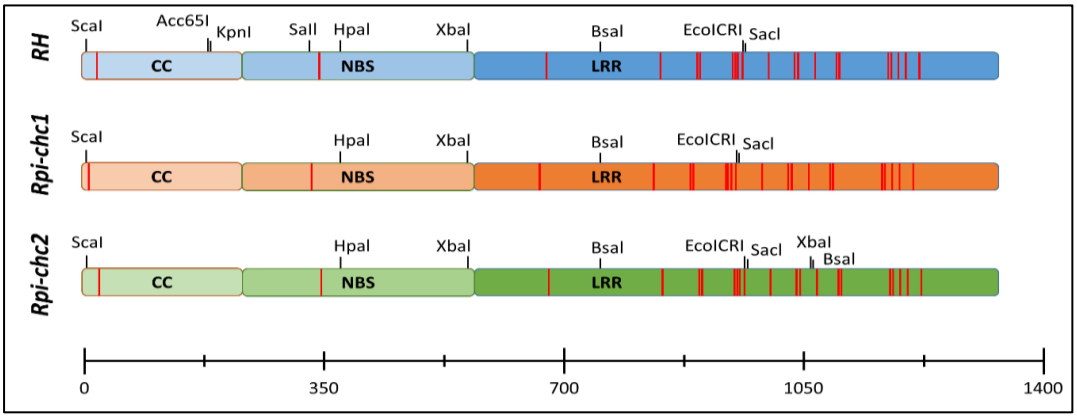

Figure 6. Schematic representation of the inactive *Rpi-chc1* homologs present in RH and the active alleles *Rpi-chc1* and *Rpi-chc2* from *S. chacoense*. Most of the enzyme restriction sites are conserved along the three homologs. The red vertical lines in the different protein domains represent the 21 most relevant amino acid substitutions. 19 of these 21 SAPs are accumulated in the LRR domain.

3. Cloning of the *Rpi-chc1* alleles

To be able to identify the amino acids involved in the receptor activity and recognition specificity, we proposed a domain exchange between the different active and inactive *Rpi-chc1* homologs. In this domain swap, the 21 SAPs between the active *Rpi-chc1* and the inactive *Rpi-tub_D3* can be exchanged to identify the minimum amount of active sequence required for the activation of the inactive allele. Additionally, the domain exchange between Rpi-chc1 and Rpi-chc2 can be used for the identification of the amino acids responsible for the different RD12 recognition pattern observed between these two receptors. In order to do so, first we need to establish an effective transient expression system in which *Rpi-chc1* alleles (*Rpi-chc1*, *Rpi-chc2* and *Rpi-tub_D3*) could be cloned for *P. infestans* effector recognition studies in *N. benthamiana*.

In previous studies, it was found that the promoter that drives the expression of the different *R* genes is crucial for the identification of the effector recognition. In previous experiments, late blight resistant stable transformants could be obtained using a *Rpi-chc1* 3.3 Kb promoter. Unfortunately, the 3.3 Kb promoter was not capable to drive the transient expression of *Rpi-chc1* homologs. However, the 0.9 Kb smaller version of the same promoter from *Rpi-chc1* could only drive the *Rpi-chc1* transient expression in *N. benthamiana*. Alternatively, *Rpi-ber* was successfully used in stable potato transformation under the control of its own 1.6 Kb promoter and 1.2 Kb terminator in the pBINPLUS-PASSA vector. This experiment proved that this promoter can efficiently drive the stable expression of *Rpi-chc1* alleles in potato plants. If the *Rpi-ber* 1.6 Kb promoter could also drive the transient expression of *Rpi-chc1* alleles in *N. benthamiana*, we could develop a plant expression system that can be used for stable and transient expression of the *Rpi-chc1* alleles in both *N. benthamiana* and potato plants. For this reason, we decided to use the same 1.6 Kb *Rpi-ber* promoter and the 1.2 Kb *Rpi-ber* terminator to test the transient expression of the active *Rpi-chc1* alleles in *N. benthamiana* using the same pBINPLUS-PASSA backbone. Additionally, we included the *uidA* gene (GUS) in pBINPLUS-PASSA as a negative control for the agroinfiltration experiments.

Due to the absence of restriction site in-between promoter-CDS and CDS-terminator we decided to add them by designing the primers with the restriction site as an overhang (Figure 7).

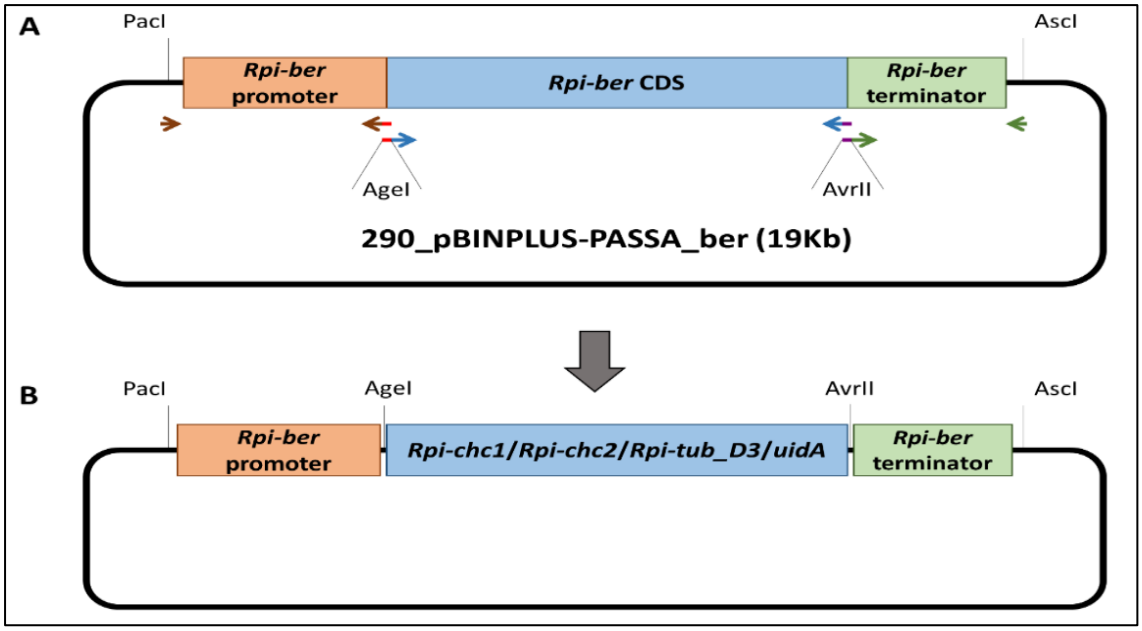

Figure 7. Representation of the our designed cloning strategy. **A**. We used 290_pBINPLUS-PASSA as a vector backbone, and to amplify the *Rpi-ber* promoter and terminator. In the primers we included, as an overhang, the AgeI and AvrII restriction sites. These sites were used to ligate the CDS insert and circularize the vector. **B**. Final vector representation after including the AgeI and AvrII restriction sites, and the *Rpi-chc1*, *Rpi-chc2*, *Rpi-tub_D3* or *uidA* (GUS) genes of interest.

3.1. Zero Blunt™ PCR Cloning Kit

The ligation of four large fragments (backbone, promoter, CDS and terminator) direct after PCR can be very inefficient. For this reason, we decided to subclone the PCR amplified *Rpi-chc1*, *Rpi-chc2*, *Rpi-tub_D3*, *uidA*, and *Rpi-ber* promoter and terminator, in an intermediate vector. In this vector the fragments can be cloned and sequenced to make sure that the insertion sequences are correct. Once the inserts have been checked, they can be digested and ligated with a higher efficiency as compared to digestion/ligation of PCR products. All the fragments were amplified using Phusion polymerase, which creates amplicons with blunt ends. The different *Rpi-ber* promoter and terminator, *Rpi-chc1*, *Rpi-chc2*, *Rpi-tub_D3* and *uidA* (GUS) were successfully amplified from previously built plasmids using specific forward and reverse primers (Figure 8; primer sequences in Appendix 1).

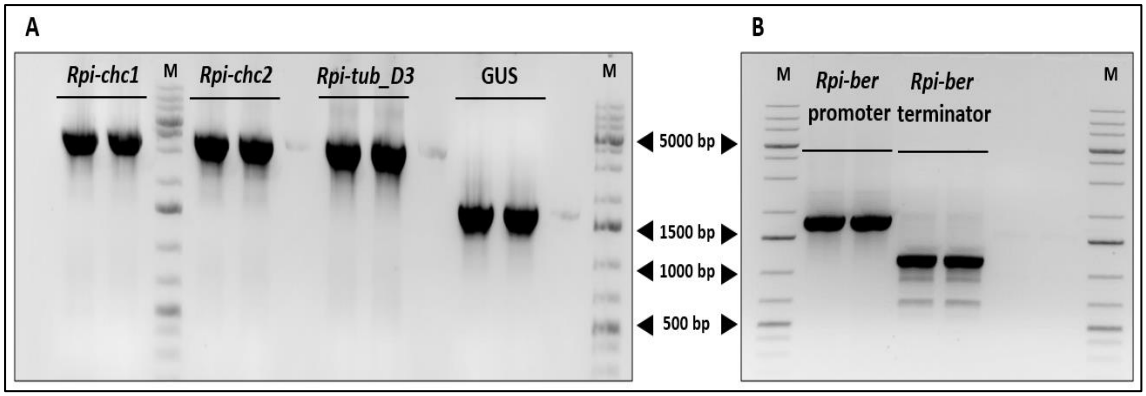

Figure 8. PCR amplified genes, promoter and terminator. M, represents the 1Kb Plus DNA ladder with 5000 bp, 1500 bp and 500 bp as intense bands. **A**. Gel with the amplified *Rpi-chc1*, *Rpi-chc2*, *Rpi-tub_D3*, *uidA* (GUS) of 3909 bp, 3912 bp, 3912 bp and 1812 bp, respectively. **B**. Gel with the amplified *Rpi-ber* promoter and terminator of 1609 bp and 1192 bp, respectively.

As suggested in the company protocol, 8 colonies per gene were used for colony PCR with three different primer combinations: forward and reverse primers from the CDS (CDSFw + CDSRv), forward primer from the CDS and reverse primer in the vector backbone (CDSFw + M13Rv), and forward primer in the CDS and reverse primer in the vector backbone (CDSFw + M13Fw). These three combinations were performed in order to identify the orientation of the inserted sequence. None of the colony PCRs gave the expected amplification band (Figure 9A). The remaining bacterial culture was used to isolate the plasmid (Miniprep) and perform a restriction digestion using EcoRI. EcoRI has two restriction sites in the pCR™-Blunt II-TOPO® vector, one in each side of the inserted fragment. Therefore, the digestion with EcoRI leads to the liberation of the inserted fragment, resulting in a band of 3 Kb from the pCR vector backbone plus the band from the inserted sequence (*Rpi-chc1* homologs around 4Kb and the *uidA* gene of 1.9 Kb). Additionally, the *Rpi-chc1* homologs contain two EcoRI recognition sites in the sequence, concluding in the 4 Kb fragmentation into two 1.6 Kb bands and one 820 bp band. The *uidA* and the *Rpi-ber* promoter and terminator sequences do not have any EcoRI recognition site, concluding in the non-fragmented 2 Kb, 1.6 Kb and 1.2 Kb, respectively. All the extracted vectors were digested with EcoRI, but none of the digested vectors contained the desired inserted sequence (Figure 9B). In most of the digestions, only the band of 3 Kb from the pCR™-Blunt II-TOPO® vector could be observed, confirming that the cloning was unsuccessful.

After the negative result, eight additional colonies from each reaction were used for colony PCR, plasmid isolations and EcoRI restriction digestions. Once again, none of the colonies contained the correct plasmid. Considering that the ligation reaction could have been unsuccessful, we decided to repeat the ligation reaction with new amplified sequences and the pCR™-Blunt II-TOPO® vector. The complete cloning process, including the analysis of 16 colonies per insert with the three different colony PCRs and EcoRI restriction digestion, was repeated again without a successful result.

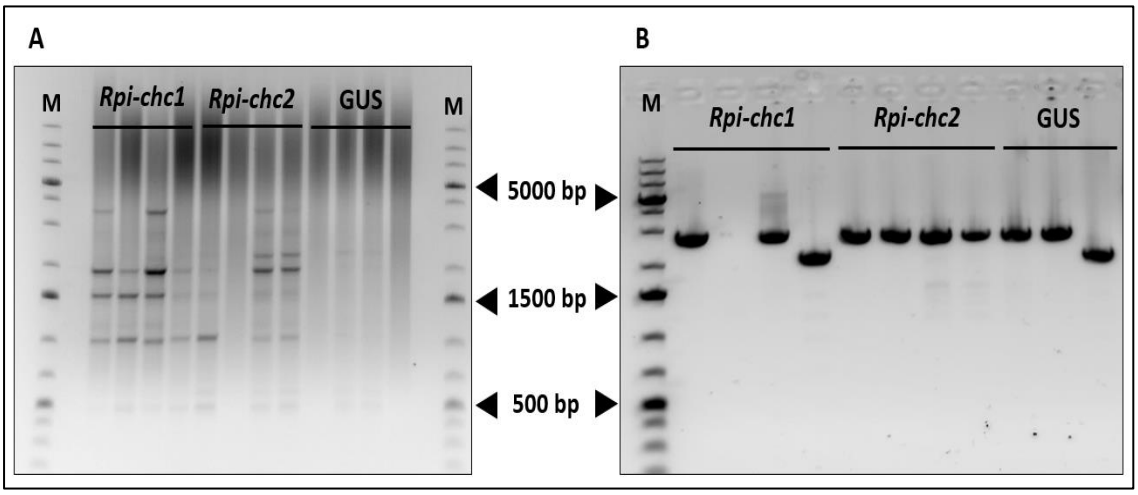

Figure 9. Colony PCR and restriction digestion gels of the different genes. M, represents the 1Kb Plus DNA ladder with 5000 bp, 1500 bp and 500 bp as intense bands. **A**. Colony PCR using the CDSFw + CDSRv, CDSFw + M13Rv and CDSFw + M13Fw primer combinations to amplify *Rpi-chc1, Rpi-chc2* (both of 4 Kb) and GUS (2 Kb). No bands of the expected size could be observed. **B**. Gel with the isolated and digested plasmids with EcoRI. Only the vector backbone of 3 Kb can be observed. For the *Rpi-chc1* and *Rpi-chc2* genes, two fragments of 1.6 Kb and the one of 820 bp were expected, but not observed. Neither, the *uidA*(GUS) insert of 2 Kb could be observed.

3.2. Gibson assembly

After the unsuccessful attempt to clone the genes into the pCR™-Blunt II-TOPO® vector, we decided to search for an alternative cloning strategy. This alternative is Gibson assembly. In our design, the fragments were: the pBINPLUS-PASSA backbone, the *Rpi-chc1* CDS, the *Rpi-chc2* CDS, the *Rpi-tub_D3* CDS, the *uidA* CDS, and the *Rpi-ber* promoter and terminator. The pBINPLUS-PASSA vector backbone is more than 15 Kb; therefore, we decided to divide the amplification of this fragment into two smaller fragments (F1 and F2) to facilitate the amplification process (Figure 10). The F1 fragment was successfully amplified but the band was extremely faint; the F2 fragment could not be amplified under these conditions (Figure 11A). In order to perform an efficient Gibson

assembly reaction, the concentration of the fragment after gel extraction should be at least 80 ng/µL. Therefore, we needed to optimise the PCRs to obtain a very intense and single band in the gel. For the amplification of the F1 and F2 fragments, different parameters were tested (Table 4). The F1 fragment amplification was successfully optimised using Phusion GC buffer and 6% of DMSO in the PCR mix (Figure 11B). Unfortunately, the F2 fragment of about 7 Kb could not be amplified under any of the tested PCR conditions or modified parameters (Table 4). Therefore, we decided to divide the F2 fragment into two smaller fragments, F2-1 (4.8 Kb) and F2-2 (2.4 Kb), to facilitate the amplification process (Figure 10).

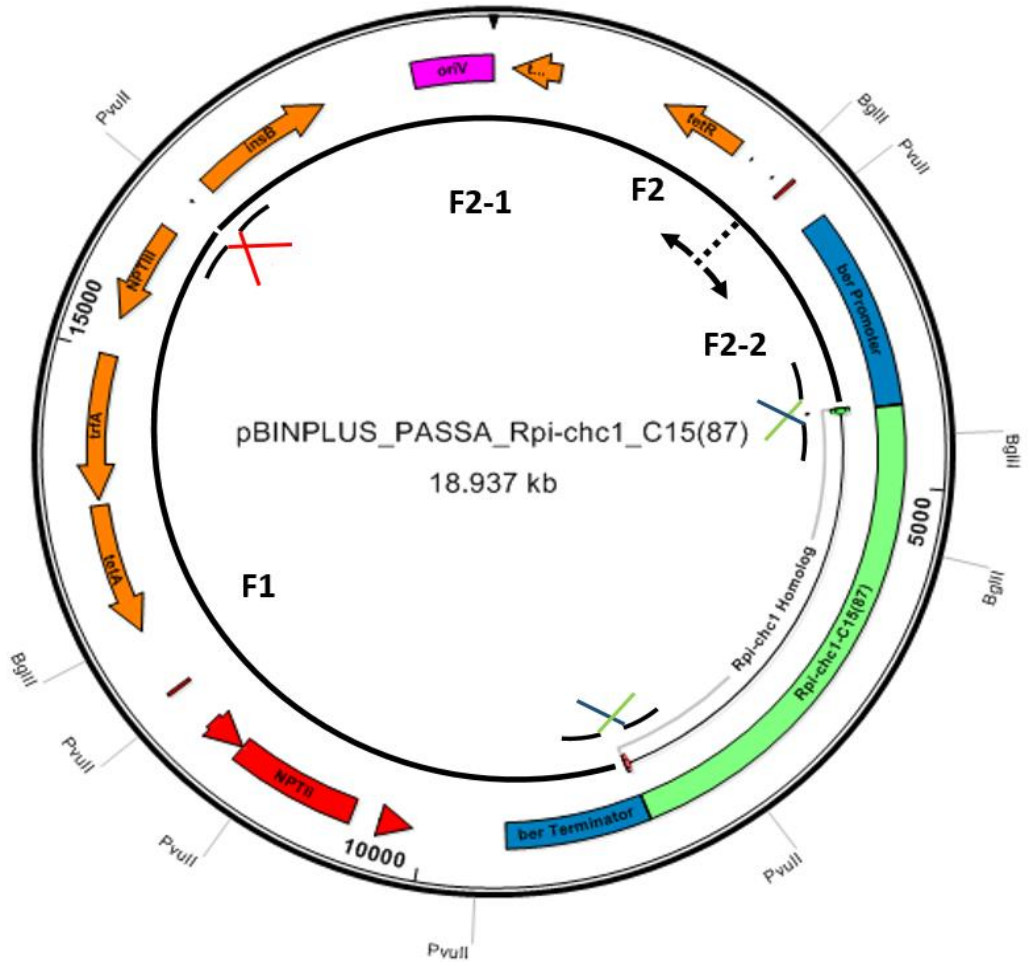

Figure 10. Gibson assembly cloning strategy. In this picture we can observe the division of the vector backbone into fragment 1 (F1) of 8.1 Kb, fragment 2 (F2) of 7 Kb, and the gene of interest. The primers to amplify each fragment have an overhang complementary sequence to the adjacent fragment. The F2 fragment was, at a later stage, divided in two different fragments: F2-1 (4.7 Kb) and F2-2 (2.3 Kb).

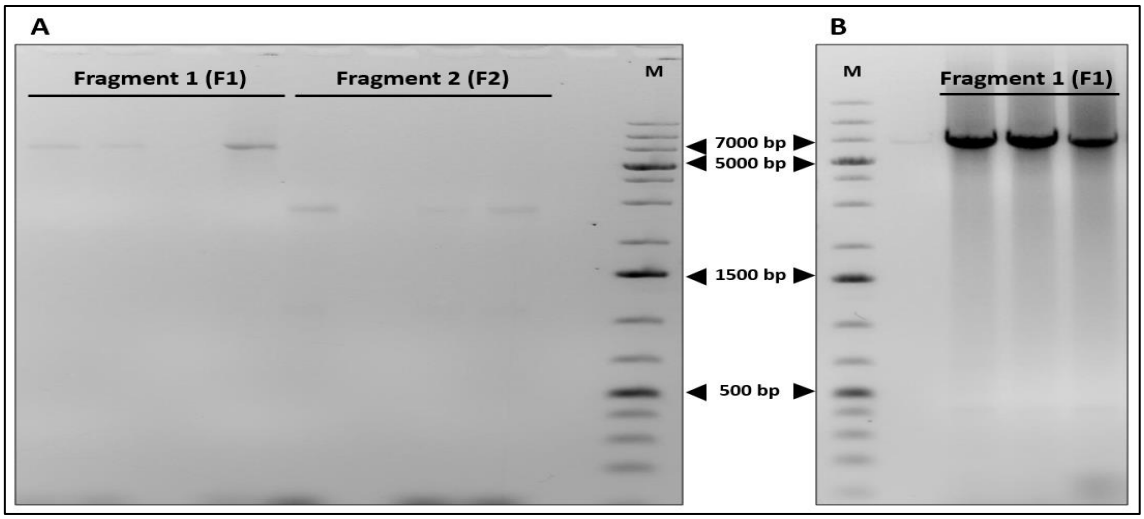

Figure 11. F1 and F2 fragment amplification. M, represents the 1Kb Plus DNA ladder with 5000 bp, 1500 bp and 500 bp as intense bands. **A**. The fragment F1 gave a thin and faint band of about 8 Kb as it was expected. The PCR of the fragment F2, failed to give a band of 7 Kb. **B**. F1 fragment optimised amplification using Phusion GC buffer, 6% of DMSO and 1.5 units of polymerase/50µL of PCR reaction.

Table 4. Different parameters and PCR conditions used for the optimization of the F1 and F2 amplification.

Optimized parameters	**Fragment 1 (F1)**	**Fragment 2 (F2)**
Phusion HF reaction buffer Vs. GC reaction buffer	GC buffer	No 7Kb band
0%, 3%, 6% and 9% of DMSO	6% of DMSO	No 7Kb band
Initial denaturation time: 30 sec - 3 min	1 min	No 7Kb band
Denaturation time within the cycles: 10 sec-30 sec	15 sec	No 7Kb band
Annealing time: 30 sec and 35 sec	30 sec	No 7Kb band
Annealing temperature from 47 °C to 67 °C	61 °C	No 7Kb band
Amplification time from 15 sec/Kb until 1min/Kb	1min/Kb	No 7Kb band
Amplification temperature: 65 °C, 70 °C and 72 °C	72 °C	No 7Kb band
Polymerase concentration: 1 and 1.5 units/50uL	1.5 units/50uL	No 7Kb band

The PCR for the amplification of the F2-2 fragment was successful but faint. However, the F2-1 fragment could not be amplified. Therefore, the PCR was optimised again for the amplification of both F2-1 and F2-2 fragments, using the previously tested parameters (Table 4). The F2-2 fragment could be successfully amplified at 61 °C using the Phusion HF reaction buffer and 3% DMSO, but the F2-1 fragment could not be amplified under any of the tested conditions (Figure 12). Considering alternative strategies to obtain the F2-1 fragment, we realized that the fragment was flanked by a PvuII and a BglII restriction enzyme sites, as can be observed in the Figure 9. Therefore,

the F2-1 fragment was extracted from the PvuII and BglII digested 290_pBINPLUS-PASSA_ber. Fortunately, this approach was successful and the F2-1 fragment could be obtained (Appendix 2).

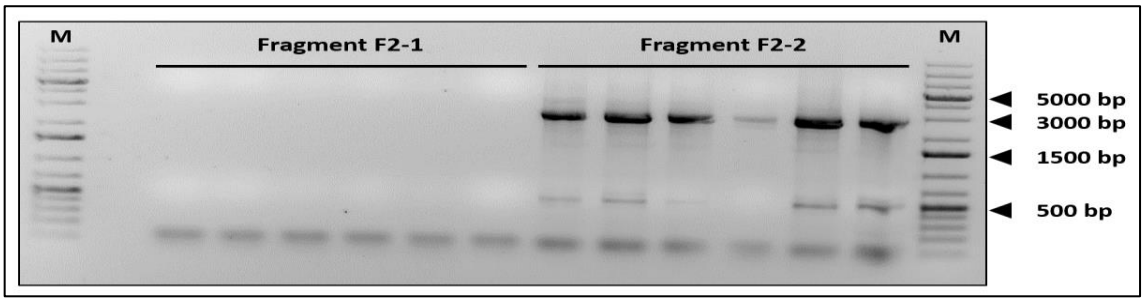

Figure 12. F2-1 and F2-2 fragment amplification. The fragment F2-2 (2.3 Kb) was amplified at 61 °C using the standard PCR conditions with the Phusion HF Buffer. Unfortunately, the F2-1 fragment (4.7 Kb) could not be amplified under in any of the tested conditions. M, represents the 1Kb Plus DNA ladder with 5000 bp, 1500 bp and 500 bp as intense bands.

Now that we obtained all the fragments of interest with a concentration of at least 80 ng/µL; we incubated the fragments with the Gibson assembly reaction mix. The plasmid from 3 colonies per construct were isolated and digested with PvuII. Two colonies with the proper restriction pattern were found with *Rpi-chc1*, one positive colony with *Rpi-chc2*, one positive colony with *Rpi-tub_D3* and three positive colonies with GUS (Figure 13). Since most the fragments have been amplified using PCR, we decided to sequence from right border (RB) to left border (LB), to make sure that the complete sequence that is going to be introduced in the plant is correct. One colony per construct was sequenced and all the sequences were correct, confirming the absence of any mutation.

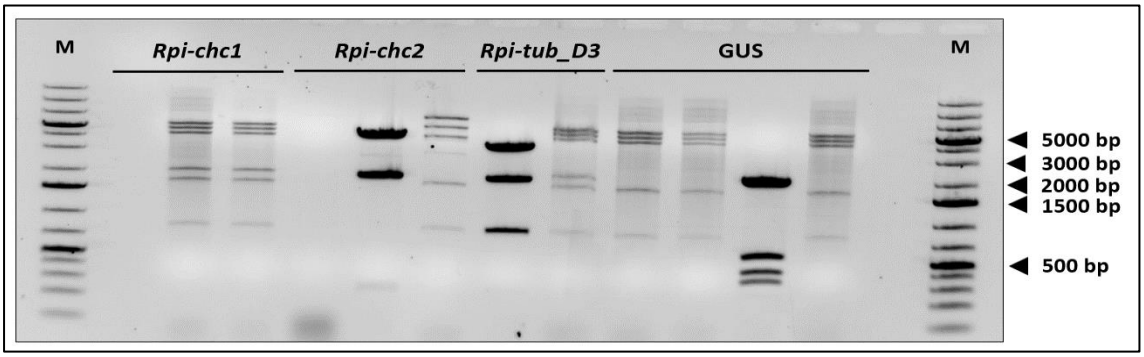

Figure 13. Restriction digestion with PvuII of the different *Rpi-chc1*, *Rpi-chc2*, *Rpi-tub_D3* and GUS constructed plasmids. Two colonies with the proper restriction pattern were found with *Rpi-chc1*, with 5309 bp, 4859 bp, 4218 bp, 2040 bp, 1699 bp and 821 bp fragments. One positive colony with the proper restriction pattern were found with *Rpi*-chc2, with 6903 bp, 5309 bp, 4218 bp, 1699 bp and 821 bp fragments. One positive colony with the proper restriction pattern were found with *Rpi-tub_D3*, with 5309 bp, 4862 bp, 4218 bp, 2041 bp, 1699 bp and 821 bp fragments. Three positive colonies with the proper restriction pattern were found with *uidA* (GUS), with 5309 bp, 4802 bp, 4218 bp, 1699 bp and 821 bp fragments. M, represents the 1Kb Plus DNA ladder with 5000 bp, 1500 bp and 500 bp as intense bands.

4. Activity testing of the *Rpi-chc1* alleles

Once we obtained all the vectors with the *Rpi-chc1*, *Rpi-chc2*, *Rpi-tub_D3*, *uidA* (GUS) genes under the control of the *Rpi-ber* promoter and terminator, we proceed to analyse if the expression system is able to drive the expression of the receptors and the HR is triggered in the presence of the complementary RD12 effectors from *P. infestans*. In order to do so, first we introduced the vectors in the Agl1 *Agrobacterium tumefaciens* strain. After the transformation, a stability test was performed for each construct, in which at least a 92% of stability could be observed for every construct. This result indicates that the constructions are stable and allows us to proceed with the plant expression experiments.

In order to test if the receptors were efficiently expressed in the new designed plant expression system, we decided to co-infiltrate them in *N. benthamiana* with different *P. infestans* effectors from the RD12 family. In this agroinfiltration experiment, we tested the recently cloned receptors Rpi-chc1, Rpi-chc2, Rpi-tub_D3 in pBINPLUS-PASSA with all the available *P. infestans* effectors from the RD12 family (A1-1, A2-1, A2-2, A2-3, B1, B2, B3, B4, C3 and D1). Due to the p19 result from the previous agroinfiltration experiment, we included the p19 silencing suppressor protein in every infiltration, in order to intensify the HR response after the effector recognition. After 3 days, none of the receptors were able to trigger the plant immune response in the presence of any member of the RD12 family (Figure 13A, C and E). This result was unexpected since the agroinfiltration of Rpi-chc2 could trigger the plant immune response in the presence of the B3 and C2 RD12 effectors after 3 days (Figure 13G). This failure was not due to the delay in the HR response since observation after 5 days did not reveal an HR. After 6 days, HR responses were observed, but only in the effectors that were cloned in the pGR106 vector (Figure 13B, D and F). This was however due to the overexpression of the effector, since HR could also be observed in the absence of the resistance gene. Additionally, it was unlikely that the failure was due to the presence of the p19 silencing suppressor, since in the previous agroinfiltration experiment proved that it can only enhance the activation of the plant defence response.

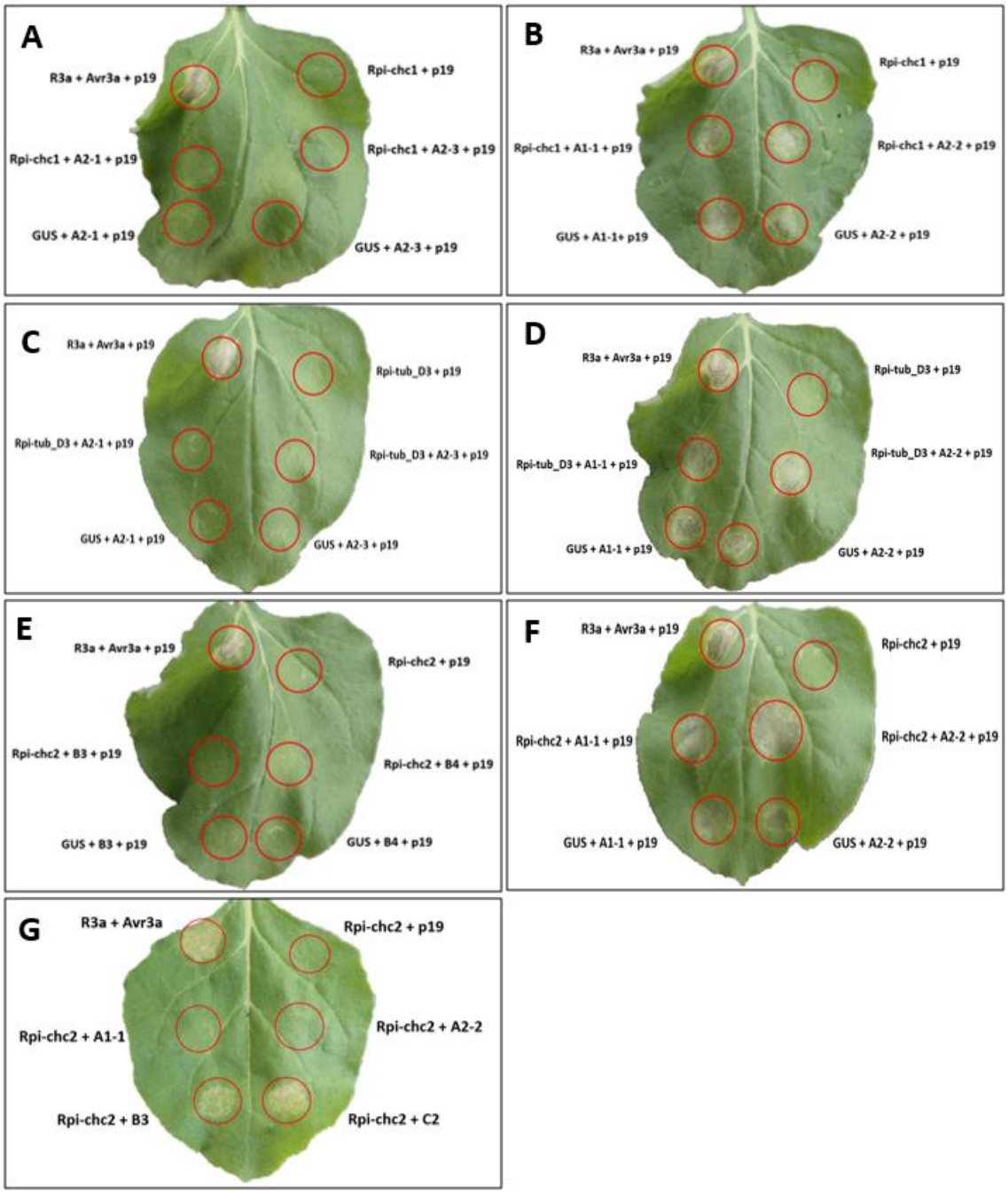

Figure 13. Plant defence activation in the presence of the Rpi-chc1, Rpi-chc2, Rpi-tub_D3 receptors and the different RD12 effector family members. The R3A and Avr3a interaction was used as a positive control of the infiltration in the top left of every leaf. **A**. Rpi-chc1 response in the presence of the A2-1 and A2-3 effectors after 3 days. **B**. Rpi-chc1 response in the presence of the A1-1 and A2-2 effectors after 6 days. **C**. Rpi-tub_D3 response in the presence of the A2-1 and A2-3 effectors after 3 days. **D**. Rpi-tub_D3 response in the presence of the A1-1 and A2-2 effectors after 6 days. **E**. Rpi-chc2 response in the presence of the B3 and B4 effectors after 3 days. **F**. Rpi-chc2 response in the presence of the A1-1 and A2-2 effectors after 6 days.

Discussion

In this study we could reproduce the previous results obtained in our group in which the *Rpi-chc1* family is responsible for the *P. infestans* RD12 effector family recognition. Specifically, Rpi-chc1 recognised class A RD12 effectors, while Rpi-chc2 had a broader recognition spectrum being able to recognise B and C RD12 effectors. The Rpi-ber receptor was also able to recognise class B and C of the RD12 effectors. However, even though Rpi-ber and Rpi-chc2 belong to the same clade 1, we saw that Rpi-ber recognition of the B class is weaker. Therefore, we hypothesize that the some of the SAPs between the two receptors are involved in the effector recognition and their mutation leads to the partial loss of the class B recognition. The alignment and analysis of Rpi-chc2 and Rpi-ber amino acid sequences concludes in the presence of only 10 relevant SAPs between the two receptors; among which the amino acids responsible for a weaker B recognition can be found. This case help us to identify the amino acids responsible for the effector recognition in the different Rpi-chc1 alleles. Moreover, we could also observe that the Rpi-tub_D3 allele from the potato cultivar RH was unable to induce HR in the presence of none of the members of the RD12 effector family.

In this study we aimed to establish a plant expression system that can be used for *Rpi-chc1* alleles transient and stable expression in both *N. benthamiana* and potato. *R* gene activity is determined by the ability to recognise pathogen effectors and the posterior induction of the plant defence response. It has been described that not only the amino acid sequence, but also the expression levels of *R* genes influence the pathogen effector recognition. When chimeric NB-LRR receptors were expressed under the constitutive 35S promoter, an expression threshold for autoactivity was reached and the immune response was triggered without effector. When the R protein levels are reduced by introducing an out-of-frame start codon before the original start codon, the receptor autoactivation disappeared (Slootweg et al., 2017). In previous and in the present study, the original 0.9 Kb promoter from *Rpi-chc1* was successfully used for *R* gene transient expression in *N. benthamiana* using the pDEST vector; but this promoter was unable to drive the expression of stable transformed *R* genes in potato. Therefore, we search for an alternative promoter and terminator sequences that can drive the transient and stable expression both in *N. benthamiana* and potato plants. We decided to use the promoter and terminator sequences from *Rpi-ber*, a gene from the same *Rpi-chc1* family, which were

previously used to stably express *Rpi-chc1* homologs in *N. benthamiana*, using the pBINPLUS-PASSA plant expression vector. Unfortunately, our designed plant expression system in pBINPLUS-PASSA with the *Rpi-ber* promoter and terminator sequences driving the R gene expression, were not able to properly drive the expression of the different *Rpi-chc1* homologs. Consequently, the receptors were not able to trigger the plant immune response in the presence of the matching RD12 effectors. The pBINPLUS plant expression vector is an Agrobacterium low copy compared to the pDEST vector. The low number of transferred T-DNAs containing the R gene of interest, could be the reason for which the plant defence response cannot be triggered. R genes are relatively lowly expressed in the plant cells, and their expression is strictly controlled by different transcriptional and translational mechanisms (Frazier et al., 2016). This could also explain the different promoter activity between the Rpi-chc1 3.3 Kb and the 0.9 Kb promoters. The presence of transcription enhancer or repressors in the promoter sequence can lead to the proper or insufficient R gene expression, which leads to the consequent proper or insufficient effector recognition. In our case we hypothesize that a transcription repressor sequence is present in the promoter after the 0.9 Kb. Due to high similarities between the *Rpi-chc1* and the *Rpi-ber* promoter, we suggest that this transcription suppressor is still present in the 1.6 Kb promoter version. Therefore, we suggest that this is the reason for which our promoter was not able to drive the *R* genes expression. A shorter version of the promoter might be able to drive the proper expression of the different *Rpi-chc1* alleles. Additionally, in our designed plant expression vector we included two restriction sites between promoter-gene and between gene-terminator. This fact can also influence the expression of the *Rpi-chc1* alleles avoiding the final effector recognition. For all these reasons, we can conclude that the *Rpi-chc1* alleles expression is very complex which include many variables such as plant expression vector or promoter sequence and length. Therefore, the establishment of a plant expression system that can work for transient and stable transformations in both *N. benthamiana* and potato can be enormously challenging.

In this project, the main challenge was the cloning of the *Rpi-chc1* homolog sequences. Even though the reason for the negative cloning results is completely unknown, we suggest that the sequence length could have been one of the most important limitations. The Zero Blunt™ PCR Cloning strategy is sequence dependent; therefore, after different unsuccessful results, we decided to try to build the different plasmids using

Gibson assembly. This alternative approach is fast, highly efficient and not fragment length dependent. After the assembly of all the fragments, the different vectors were PvuII digested. This enzyme was chosen due to the presence of at least six restriction sites, both inside and in-between the different assembled fragments, concluding in a complex restriction pattern that minimize the selection of false positives. Due to the amplification of the fragments by PCR and the probability of having amplification mistakes, we decided to sequence the vector from right border (RB) to left border (LB), which is the sequence that is going to be transferred to the plant. As the F2 fragment was introduced from a sequenced digested plasmid, the only sequence left without sequencing in the new vector was a backbone fragment of 4Kb long. We did not consider essential to sequence this 4 Kb fragment, because only the bacterial *nptII* and the *tetA* resistance genes are present in this sequence. If any of the resistance genes would have been mutated, the bacteria would not have been resistant and they would have died.

Despite the large amount of the described NSB-LRR receptors, very little is known about the effector recognition and the defence activation mechanisms. In the DM potato clone, more than 700 NB-LRR receptors have been identified; this together with the high levels of sequence polymorphisms observed in different potato haplotypes, it is likely that the currently used tetraploid potato varieties contain as many as 1.600 different NB-LRRs in their genome (Jupe et al., 2012). NB-LRR receptors are one of the most rapidly evolving genes in the plant genome; particularly, some amino acids from the LRR predicted to be solvent exposed are hypervariable and subjected to a high evolutionary positive selection. These results have been interpreted as strong evidences that these solvent exposed amino acids, which have a consensus xxLxLxxxx motif sequence, are involved in the pathogen effector recognition (Jones and Jones 1997; Mondragon-Palomino, 2002).

In this project we focused on the *Rpi-chc1* gene family due to the small differences between the amino acid sequences between the active and the inactive alleles. The alignment of the active *Rpi-chc1* and the inactive *Rpi-tub_D3* alleles indicated that there are only 21 SAPs that lead to a significant change into the amino acid properties. Nineteen of these SAPs are located in the LRR protein domain, previously described as the domain responsible for the effector recognition. If we additionally assume that the inactivity of the RH allele is due to the inability of the receptor to recognise the effector, we can limit

the SAPs to those located in the xxLxLxxxx motif. Subsequently, only 14 SAPs are hypothesised to be located on the receptor surface, and therefore expected to be involved in the effector recognition. Additionally, small amino acid changes are also responsible for the receptor specificity. Aligning Rpi-chc1 and Rpi-chc2, we observed 23 SAPs, out of which 17 are part of the xxLxLxxxx motif. We hypothesized that within these amino acids, we could find those responsible for the effector recognition. Therefore, these genes present an excellent study platform to reach the main objective of this project: identify the amino acids responsible for the Rpi-chc1 receptor family activity and recognition specificity.

Moreover, the inactivity of an *R* gene is not only a consequence of the solvent exposed amino acids that are responsible for the effector recognition. It is also possible that the inactive Rpi-tub_D3 receptor does not have a proper intradomain or interdomain interaction, which can lead to the inactivity of the receptor, even though the amino acids responsible for the effector recognition are intact. A nice example for this case are the two potato paralog CNL proteins, Gpa2 and Rx1, which share 88% identity in the amino acid sequence. Gpa2 confer resistance to the potato cyst nematode *Globodera pallida*, while Rx1 confers resistance to the *Potato virus X* (PVX) (Bendahmane et al., 1999; van der Vossen et al., 2000). As a result of the high similarity of these two R protein sequences, they provide an excellent system to investigate the evolution of *R* genes; specially the development of new recognition specificities, and the relevance of intradomain and interdomain interactions during the receptor activation. Particularly, the fusion of the CC-NB domains of Rx1 with the LRR domain of Gpa2 and vice versa, resulted in aberrant proteins that were not functional (Rairdan and Moffett, 2006). The exchange of polymorphic residues between the two receptors resulted in the identification a 68 amino acid fragment responsible for the interaction between the first LRR repeats and the ARC2 subdomain. The amino acid interactions in this fragment determines the final receptor conformation, leading to normal, closed unresponsive or autoactivated receptor conformations (Slootweg et al., 2013).

In the same way as potato *Rpi-chc1* homologs differ in the RD12 family member recognition, researchers have found Pik pair receptors in rice to differ in the effector recognition. Pik pair comprises two CNLs, Pik-1 and Pik-2, sensor and helper receptors, respectively. This complex recognizes the AVR-Pik effector family. Two allele variants,

Pikp (with Pikp-1 and Pikp-2) and Pikm (with Pikm-1 and Pikm-2) are almost identical except for the HMA domain (between the CC and the NB domain) in which 30 amino acids are different. While pikp is only able to recognise AVR-PikD, pikm is able to recognise AVR-PikD, AVR-PikE and AVR-PikA (De la Concepcion et al., 2018). This example supports the idea that the LRR domain is responsible for not only the effector activity and specificity, but also the intradomain and interdomain interactions. The aforementioned interactions play an essential role in the recognition specificity and the posterior defence activation. Additionally, both Rpi-chc1 and Pik have the potential to be genome edited in susceptible potato and rice cultivars, respectively. If only a few amino acids are the responsible for the inactivity or the narrow effector recognition of certain *R* genes, these amino acids are promising targets for genome editing tools like CRISPR-Cas9. The edition of these amino acids could activate and extend the effector recognition specificity of R proteins. Therefore, the genome edition of *R* genes represents a promising strategy for targeted mutagenesis and artificial evolution that may lead to the fast development of durable and broad-spectrum resistances against major pathogens.

Conclusions and Further recommendations

In this project, we aimed to identify the essential amino acids involved in the Rpi-chc1 receptor family activity and recognition specificity. Specially, we focussed on the Rpi-chc1, Rpi-chc2 and Rpi-tub_D3 receptors. Our main research question was which are the amino acids responsible for the inactivity of Rpi-tub_D3 and the amino acids that lead to the difference in effector recognition between Rpi-chc1 and Rpi-chc2. In order to answer these questions, we first needed to establish a proper plant expression system that is able to drive the transient and stable expression of *Rpi-chc1* homologs both in *N. benthamiana* and potato. We decided to use the pBINPLUS vector backbone and the *Rpi-ber* promoter and terminator to control the receptor expression. Based on our results, we concluded that the *Rpi-ber* promoter and terminator are not able to properly drive the expression of the different Rpi-chc1, Rpi-chc2 and Rpi-tub_D3 receptors. For this reason, we recommend to use an alternative promoter and terminator to properly express the different *Rpi-chc1* homologs for the co-infiltration with the different *P. infestans* RD12 effectors. This result was considered from the beginning of the project. For this reason, two restriction sites were added flaking both promoter and terminator sequences. Therefore, the plasmids only have to be digested and ligated with the new amplified

promoter and terminator sequences. Additionally, we corroborated the ability of Rpi-chc1 to recognise RD12 A1 and A2, while Rpi-chc2 is able to recognise RD12 B and C subclasses. Additionally, a different vector backbone with higher Agrobacterium copy number such as pCAMBIA can be used to try to elucidate if the T-DNA copy number is the reason for which our designed pBINPLUS-PASSA plant expression system cannot be used for R gene transient expression in *N. benthamiana*.

For future experiments, once the expression system is established, we recommend to perform the planed domain exchange between the different receptors. In this way, the number of SAPs required for the activation of the Rpi-tub_D3 can be reduced. This number can be further lowered with the creation of additional chimeric proteins with individual and combinations of the remaining SAPs. In this way we could identify the amino acids responsible for the inactivity of Rpi-tub_D3. This strategy would also help us to elucidate the amino acids responsible for the effector recognition in Rpi-chc1 and Rpi-chc2.

Once these amino acids have been identified, genome editing could be used to modify these amino acids and change the activity and the recognition specificity of different *Rpi-chc1* homologs. For instance, the inactive allele Rpi-tub_D3 from the susceptible potato cultivar RH, could be targeted with CRISPR-Cas9 to edit the essential amino acids responsible for the receptor activity. If the promoter of *Rpi-tub_D3* is still active, the edited Rpi-tub_D3 receptor will lead to resistant RH potato plants to *P. infestans*. This will open a new era in plant breeding with crop genome engineering for the development of new and beneficious varieties, which will conclude in the tremendous increase of the quality of our crops.

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Appendix

Appendix 1. Primer list with name and sequence (5' – 3') of the used primers.

	Name	Sequence (5' – 3')
1	BER_Prom_PacI_Fw	ccagtgaattgttaattaaatagg
2	BER_Prom_AgeI_Rv	ctacataccggtatacaatcattcaaacagtaataaaa
3	BER_Termin_AvrII_Fw	ctacatcctagggtcgcttgcatttttaattag
4	BER_Termin_AscI_Rv	gaaacagctatgaccatgattac
5	Rpi-chc1_AgeI_Fw	ctacataccggtatgaattattgtcttccttcg
6	Rpi-chc1_AvrII_Rv	ctacatcctaggtcagaaagtgaaagagaaacc
7	Rpi-chc2_AgeI_Fw	ctacataccggtatgaattattgtcttccttcg
8	Rpi-chc2_AvrII_Rv	ctacatcctaggtcagaaagtgaaagagaaacc
9	Rpi-chc_RH_AgeI_Fw	ctacataccggtatgaattattgtcttccttcg
10	Rpi-chc_RH_AvrII_Rv	ctacatcctaggtcagaaagtgaaagagaaacc
11	GUS_AgeI_Fw	ctacataccggtatggtccgtcctgtagaaac
12	GUS_AvrII_Rv	ctacatcctaggttattgtttgcctccctg
13	101_Rpi-chc_Seq	ccaaatctctaccgacaatatcag
14	102_Rpi-chc_Seq	cctcggtctacaatcactcatgg
15	103_Rpi-chc_Seq	ggattttctttatgctattttctcc
16	104_Rpi-chc_Seq	gtgccaaggtctaccgttggc
17	105_Rpi-chc_Seq	ggaagttcctggagtgaaaagc
18	106_Rpi-chc_Seq	ccaaaatcgggaagctgatatac
19	107_Rpi-chc_Seq	ccaatgcattccaactcatgg
20	108_Rpi-chc_Seq	gaagagtcgctaccaaatttgg
21	109_Rpi-chc_Seq	agaatcccagtgcccacgtcc
22	110_Rpi-chc_Seq	ggtcctttctcagagatggtgg
23	111_A_HiFiGibson_Rpi-chc_homo	tttattactgtttgaatgattgtataccggtatgaattattgtcttccttcgag
24	112_B_HiFiGibson_Rpi-chc_homo	ttggaactcgtggaaaataacctaggtcagaaagtgaaagagaaacc
25	113_C_HiFiGibson_Backbone	cctaggttattttccacgagttcca
26	114_D_HiFiGibson_Backbone	gtcagtagctgaacaggag
27	115_E_HiFiGibson_Backbone	ctcctgttcagctactgac
28	116_F_HiFiGibson_Backbone	accggtatacaatcattcaaacagtaataaa
29	117_A_HiFiGibson_GUS	tttattactgtttgaatgattgtataccggtatggtccgtcctgtagaaac
30	118_B_HiFiGibson_GUS	ttggaactcgtggaaaataacctaggttattgtttgcctccctg
31	119_G_HiFiGibson_Backbone	ctgatcgtaattctgagcac
32	120_H_HiFiGibson_Backbone	gtgctcagaattacgatcag
33	121_F_HiFiGibson_Backbone	accggtatacaatcattcaaacagta
34	122_F_HiFiGibson_Backbone	accggtatacaatcattcaaacag

35	123_A_HiFiGibson_Rpi-chc_homo	gattgtatatgaattattgtcttcc
36	124_F_HiFiGibson_Rpi-chc_homo	ggaagacaataattcatatacaatc
37	125_A_HiFiGibson_GUS	tactgtttgaatgattgtatatggtccgtcctgtagaaac
38	126_F_HiFiGibson_GUS	atacaatcattcaaacagt
39	127_G_HiFiGibson_Backbone	atcttgctcgtctcgct
40	128_Backbone_Seq	agcttggcgtaatcatg
41	129_Backbone_Seq	gatctcgtcgtgaccca
42	130_Backbone_Seq	ctcttcagcaatatcacgg
43	131_Backbone_Seq	gcctgtatcgagtggtgat
44	132_Backbone_Seq	cgtcgttgtggtatatctagtc
45	133_Backbone_Seq	gactagatataccacaacgacg
46	134_GUS_Seq	gcaagactgtaaccacgc
47	135_Backbone_Seq	tatacaagttttatagagaggc
48	136_Backbone_Seq	ctatctataactcacacctctc
49	137_GUS_Seq	ctttaatcgcctgtaagtgc
50	138_Backbone_Seq	gcctctctataaaacttgtata
51	139_BER-Fw	gaatgattgtataccggtataggagaaacatagagaaggtgat
52	140_BER-Rv	cgtggaaaataacctaggtaagatgaacgtatactgctaaggac
53	141_Rpi-chc-C15	tgacttccctcgaattcttttg
54	142_Rpi-chc-C2	tgacttccctcgaattctttta
55	143_Rpi-chc-D7	tgacttccctcatattcttttt
56	144_Rpi-ber_2ndSTART	gaatgattgtataccggtactgcagaggaagaggaag

Appendix 2. Digestion of the pBINPLUS-PASSA-ber vector with PvuII and BglII restriction enzymes. The digestion with these two enzymes lead to the 18950 bp fragmentation into 10 fragments of 4950 bp, 3599 bp, 2103 bp, 2059 bp, 1898 bp, 1699 bp, 843 bp, 821 bp, 619 bp, and 359 bp. The F2-1 fragment of interest is the top band of 4950 bp. M, represents the 1Kb Plus DNA ladder with 5000 bp, 1500 bp and 500 bp as intense bands.