Towards map based cloning of *Rpi-cap1*

as a tool for cisgenic potato resistance breeding against late blight

Hernawan Rahmadi

Wageningen University & Research

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Hernawan Rahmadi
800719679110

Supervisors:
Dr. Jack Vossen
Ing. Gert van Arkel

Examiners:
Prof. Dr. Ir. Evert Jacobsen
Dr. Ir. Yuling Bai

Breeding for resistance in solanaceae research group
Laboratory of Plant Breeding
Wageningen University & Research

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Abstract

Late blight is a major disease in potato production, while *Solanum capsicibacatum* is one of potato wild relatives with known resistance genes against this disease. Map based cloning approach was used in this study to determine the exact location of these genes and as a tool for cisgenic resistance breeding. Contig from previous studies were sequenced to conceal existing RGA and extend the contigs. However since the recombinant screening was not performed due to seed germination problem, transient complementation and co-expression test in *N. benthamiana* was conducted to identify the putative genes. Four BIBACs construct were agroinfiltrated and challenged with Pi effectors, yet no clear indication of the responsible sub-clone was identified. Positive control with similar insert size to the BIBACs constructs were initially developed and can be use for further co-expression study.

Keywords: potato, *Solanum capsicibacatum,* late blight, map based cloning, transient complementation, co-expression, cisgenic

Contents

Chapter I
General Introduction

Potato is an important crop and a major staple food source after maize, rice and wheat. In 2008, 314 million tones potatoes were produced from 118.2 million ha of field with average yield at 17.3 t/ha (FAOSTAT 2010). Over 150 countries are planting potato and many researches are carried out to improve its performance. This study also has interest in providing knowledge of potential resistance genes from potato wild relatives against late blight. Several aspects which are important in research for late blight resistance are the potatoes genetic backgrounds, late blight characteristics (a-virulence or virulence), and the methods to conduct the research.

Potato origin

Potato was first discovered in 1532 by Pizarro at high Andes of Peru. However, it was not introduced to Europe until 1562, when export of barrels of potato from Gran Canaria to Antwerp in November 1567 was recorded (Hawkes and Francisco-Ortega 1993). According to (Hosaka and Hanneman 1988) the first potato introduced to Europe was *Solanum tuberosum* subspecies *andigena* from the Andes but after the Irish famine it was replaced by *S. tuberosum* subspecies *tuberosum,* which derived from Chile. However, molecular and herbarium studies on early potato shows that crosses between Andean and Chilean *S. tuberosum* plant material, were used already before the late blight epidemic (Jacobs and Van Den Berg 2008; Goverse and Struik 2009).

Potato botany

The first potato publication was performed by Lopez de Gomara in 1552 (Hawkes and Francisco-Ortega 1993) and potato is taxonomically classified as presented in Table 1. Nowadays potato reclassification by morphological support leads to recognition of all landrace populations of cultivated potatoes as a single species, *S. tuberosum*, with the eight cultivar-groups: Ajanhuiri, Andigenum, Chaucha, Chilotanum, Curtilobum, Juzepczukii, Phureja, and Stenotomum (Huaman and Spooner 2002). Each of the cultivar-groups and potato wild relatives require optimum condition in order to thrive. Temperate regions with high humidity are the most suitable condition for potato. Even though its origin is from tropical area in latitude, but the climate is temperate because of the altitude in high Andes.

Temperature and day length are important determinates for potato to form tubers, and it will grow better in light loam soil. The European climates that match these requirements promote the spreading of potato besides its higher productivity compared to other food sources such as

wheat or rye (Agrios 2005). On the other hand, these conditions are also suitable for the existence of fungal-diseases from the same agro-climate with potato in the Andes.

Table 1. The potato classification (USDA):

Classification	Name – (meaning)
Kingdom	*Plantae* – Plants
Subkingdom	*Tracheobionta* – Vascular plants
Superdivision	*Spermatophyta* – Seed plants
Division	*Magnoliophyta* – Flowering plants
Class	*Magnoliopsida* – Dicotyledons
Subclass	*Asteridae*
Order	*Solanales*
Family	*Solanaceae* – Nightshade
Genus	*Solanum* L.
Species	Solanum tuberosum L. – Irish potato

Late blight disease

Potato is subject to several pests and pathogens, and one of the most important diseases in potato is late blight, caused by *Phytoptora infestans*. *P. infestans* (Pi) is an oomycete and can occur in two mating types. It appears like a fungus since it produces mycelium but the cell walls contain cellulose, like plant cell walls. Pi produces sexual oospores as a resting structure and a-sexual zoo-spores that derive from the airborne sporangia (Figure 1); (Agrios 2005). Oospores will enable *P. infestans* to survive outside its host in extended time. Moreover oospores are the result of sexual recombination, thus giving the pathogen higher genetic variations in the population and makes it even more difficult to control (Andersson, Widmark *et al*. 2009).

Current management to deal with late blight is using pesticides and leads to pesticide resistance of *P. infestans* (Van der Vossen E.A.G., Gros J *et al*. 2005). The cost, resistance development and environmental side effects of pesticides encourage other approaches such as breeding for resistant cultivars using *R* genes from related species to overcome late blight (Lokossou, Park *et al*. 2009). Nevertheless, the employment of single *R* gene is not durable because new strains of *P. infestans* will rapidly adapt and the resistance will be easily broken (Pel *et al.,* 2009). Other studies, to introduce partial resistance and QTL based resistance have been carried out (Simko 2002; Bradshaw, Bryan G *et al*. 2006), but still the progress in

obtaining stably resistant cultivars is very slow. There are several obstacles such as linkage drag of undesirable characteristics, an example is the linkage between partial resistance and late maturity (Howard 1970).

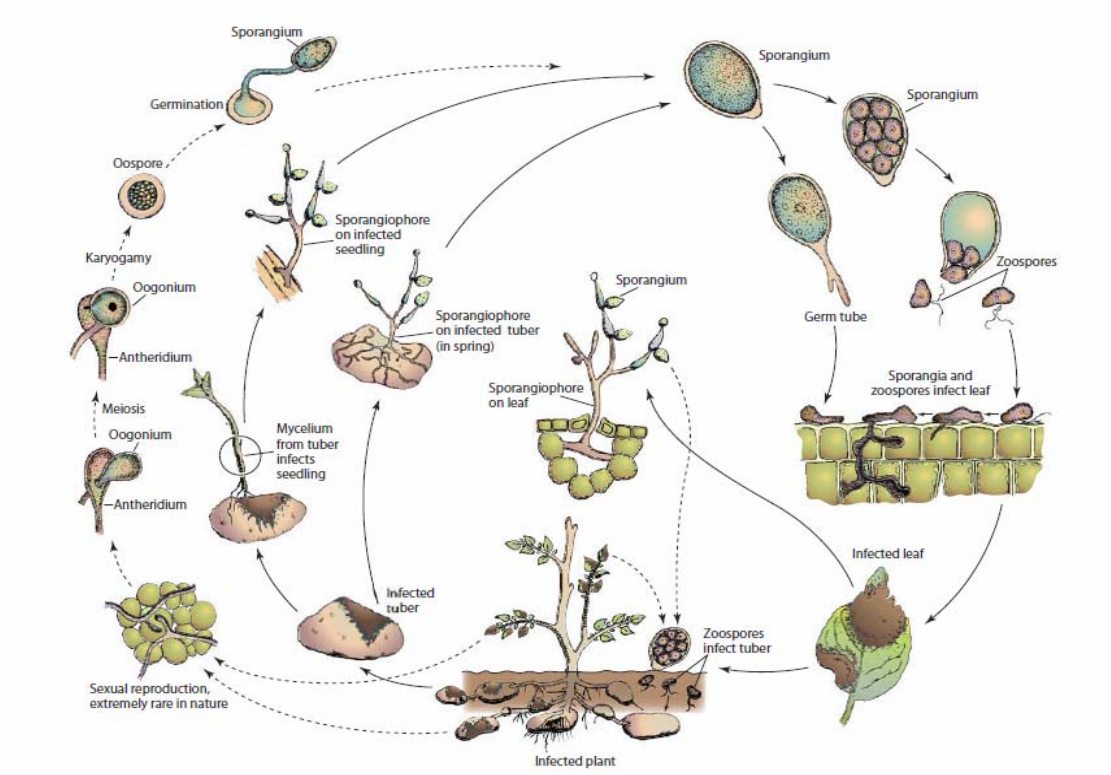

Figure 1. Disease cycle of potato late blight caused by *Phytoptora infestans* (Agrios 2005)

Resistance genes from wild relatives

Potato wild relatives with different ploidy levels are abundant and their overlapping characteristics trigger long history of debates between potato taxonomist to discriminate their classification (Huaman and Spooner 2002). Several *R* genes have been identified from related diploid *Solanum* species such as: *Rpi-ber* from *S. berthauli* (Ewing and Simko. 2000), *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* from *S. bulbocastanum* (Van der Vossen E.A.G., Gros J *et al*. 2005) and the recent finding of *Rpi-cap1* from *S. capsicibacatum* (Jacobs, B. Vosman *et al*. 2009). Nonetheless, cultivated potato as a tetraploid plant (2n=4x=48) (Huaman 1986), and their heterozigosity are major limitation to breed new cultivar by conventional breeding and it may take 8-15 years from initial breeding until variety release (Spooner and Bamberg 1994). Potato's pollination success rate is just over 30% (Bradshaw, Dale *et al*. 2009), which is adding the difficulty to sexually propagated, therefore potato is prefereably vegetatively propagated.

Cisgenic potato

Potato which is widely grown, receive high amounts of fertilizers and pesticides, and because of the late blight threat, it is potential target crop for genetic modification (O'Brien and Mullins 2009). Nevertheless, the social impacts of its implementation prevent breeding companies to release transgenic potato. The recent approach to overcome late blight is by stacking of several *R* genes into cultivated potato through cisgenesis (Lokossou, Park *et al*. 2009). This principle is based on marker-free transformation and only uses genes from crossable wild potato relatives. In spite of this, there is rejection from organic sector because cisgenesis which is breeding at DNA-level, instead of at whole-plant level, is supposed to violate the integrity of life as described in the concept of naturalness (Lammerts Van Bueren, Tiemens-Hulscher *et al*. 2008). Then again, until now conventional breeding is unable to deliver reliable resistant potato against late blight. Cisgenesis is ethically the most acceptable way to produce resistant cultivar faster, which is a prerequirstie to combat the fast evolving Pi. Cisgenesis is therefore pled to be regarded as a scientific approach which is different from transgenesis and should be regulated differently as it has been done with (induced) mutation breeding and the use of protoplast fusion between crossable species (Haverkort, Boonekamp *et al*. 2008).

Chapter II

Map based cloning of *Rpi-cap1*, *S. capsicibacatum* resistance gene against late blight disease

Introduction

As mentioned in Chapter I, wild relatives of cultivated potato are valuable genetic resources for resistance breeding against late blight. The importance of this germplasm has led to more than 40 prospections by international organizations in the past 40 years (Gopal and Khurana 2006). *S. capsicibacatum* is one of potato wild relatives which is recently identified as having a *R* gene against late blight. The gene is referred to as *Rpi-cap1,* and it was identified using marker CP58 which is located on the long arm of potato chromosome 11 (Jacobs, B. Vosman *et al*. 2009).

The goal of this study is to determine the exact location of *Rpi-cap1* on chromosome 11 and the isolation of the gene by map based cloning. This approach started with screening and isolating clones from BAC library using known molecular markers near the gene. The clones isolated were used to develop markers that co-segregate with the gene and the resistance phenotype. (Martin, Brommonschenkel *et al*. 1993; Andrews and Tommerup 1995).

Rpi-cap1 identification and characterization is important to assure the function and possibility to transfer this gene into modern potato cultivars. The existing contigs of BACs were further characterized and extended to conceal the physical map surrounding the *R* gene. First off, the contigs overhang of previous studies was sequenced by primer walking, successively the sequence information was used to screen for adjacent overhang sequence and isolate individual BAC clones from the library pools.

Material and Methods

Plant Material and recombinant screening

Plant material used in this study was 5000 seeds of *Solanum capsicibacatum* (7358-3b x cap355-10) F2 population. The seeds were soaked for 24 hours in 1000 ppm of Giberelic Acid prior to germination in petridish with wet filter paper (Bamberg 2000). The seedling than transferred to greenhouse and the DNA were isolated for genotyping while the leaves were challenged with late blight for phenotyping. The PCR markers planned to be used to screen for recombinants are: CP058 (F: ATGTATGGTTCGGGATCGG, R: TTAGCACCAACAGCTCCTCT) and T179 (F: CTAGCTCTGTCCCCGTCCAC, R: CCGTGTTTACACCTAACTCAACC).

DNA isolation and Marker Development

Two methods of DNA isolation were carried out in this study. Firstly, NaOH extraction was used if the DNA isolates is needed rapidly and used right away, as described by (Rehman, B. Stodart *et al*. 2007). Secondly, CTAB DNA isolation was performed for long term storage purposes as described by (Borges, S.Rosa. *et al*. 2009). The selected recombinants were conserved with *in vitro* genetic conservation methods described by (Golmirzaie and Toledo 1998).

The F2 population was used to construct a high resolution genetic map of the resistant parent (7358-3b). For this purpose we were using a technique called High-resolution DNA melting curve (HRM) analysis with a Light Scanner (Hi-Res Melting™ system and Idaho's LC Green). This technique will provide post-PCR detection of mutations and SNPs in genomic DNA (Yuan, Haroon *et al*. 2009). Cleaved amplified polymorphism sequence (CAPS) marker was also conducted to obtained better analysis if Light Scanner markers are not sufficient to distinguish between resistant and susceptible genotype (Konieczny and Ausubel 1993).

Nine primer pairs suitable for HRM PCR (Table 3) were developed from adjacent BAC end sequences. Four markers (M33, 19H1T2E16, 825_25a and 825_24b) were developed and the sequences were presented in Table 2. 19H1T2E16 was the only marker that was used in CAPS method. Markers 825_25a and 825_24b were developed from *S. phureja* sequence available from Potato Genome Sequencing Consortium Blast server.

By blasting 1F4-T7 sequence from previous study (presented in Appendix 3) in *S. phureja* sequence, scaffold 825 was obtained, and then from this scaffold primers in the area of 1F4 BAC end were developed. With these primers, the F1 population was amplified and the primers were mapped and positioned with other existing markers which eventually generate markers 825_25a and 825_24b.

Table 2. Markers developed to screen the BAC library with annealing temperature (TM), enzyme for CAPS, and Light Scanner to identify polymorphism.

Marker	Sequence	TM	Enzyme
19H1T2E16	F: gttccctgcttcatccttcc R: tgtcgtttactatcattctttctgtgt	55	E16
825_25a*	F: tattgtgctggcgtttca R: tgggggtgggggtgaggt	60	Light Scanner
825_24b*	F: caggttacgatatgaggggagat R: ccaggatagtttcaggcaggta	60	Light Scanner

* = developed from *S. phureja* sequence scaffold 825 which aligned with 1D9 and 1F4 BAC sequence from *S. capsicibacatum.*

Table 3. PCR primer pairs used to screen BAC library using Light Scanner

Primers	Sequence	TM
19H1INT-7LS	F: ggcagttcccttatcccagagc	60
	R: ttccatataaattaaaatcaaacaa	
1D9T-4LS	F: agacccccaattttcactcct	60
	R: ttagccttccccttgttttctt	
1F4T-3LS	F: tccgatcaacataagaacgaaaaga	60
	R: attgcaacataggtattaaaaggagat	
2P24T1	F: tgccgctccatcaaagaatact	60
	R: aactgccagaatttccacaactac	
2P24T2	F: gtttagccgccataggatagtgc	60
	R: actgcaatataatagatggaggac	
2P24T3	F: cctcgtcgggaaaataagt	60
	R: tttgtaattcgttgtttcaga	
2P24S1	F: cagaaggcgataaaagcacagg	60
	R: tgctgcaatgacgacgagat	
2P24S2	F: tcgtttgtgggtgaagata	60
	R: gaaattctggcagttgtgtaagc	
2P24S3	F: agaatggatccgctgaagt	60
	R: atattatccccctcagtggt	
M33	F: ggcgaagaattgtcatcgccg	55
	R: gtgtaatcccataggtccggg	

Disease test

Phenotyping for resistant recombinants were done with *Phytoptora infestans* strains 90128 (race 1.3a.3b.4.6.7.8.10.11) (Huang, Vleeshouwers et al. 2004). Disease test was conducted as described by (Huang, Vleeshouwers et al. 2005) and (Vleeshouwers, W. van Dooijeweert et al. 1999). Detached leafs of 6 weeks old plant were inoculated with *Phytoptora infestans* and 6 days post inoculation (dpi), the leaves were scored for their resistance according to the symptoms (sporulation) and HR response (necrotic lesions) observed.

BAC library

From previous studies a BAC library derived from 7358-3b was available and consisted of 482 pools of 500 clones. In this study two library batches (3b-4 and 3b-5) were screened with primers developed from *S. capsicibacatum* sequence. 3b-4 library consists of 47 pools while 3b-5 library consists of 98 pools. On the other hand marker 825_25a and 825_24b developed from *S. phureja* were used to screen all 482 pools from 3b-1 until 3b-5 library batches. The BAC library was kept in Luria Bertani (LB) medium containing 18% glycerol and chloramphenicol at -80^{o}C. Physical and genetic maps of *Rpi-cap1* were already generated and

4 BAC clones were positioned in the vicinity of this gene (Figure 2). Nevertheless, a gap is still present between these BACs.

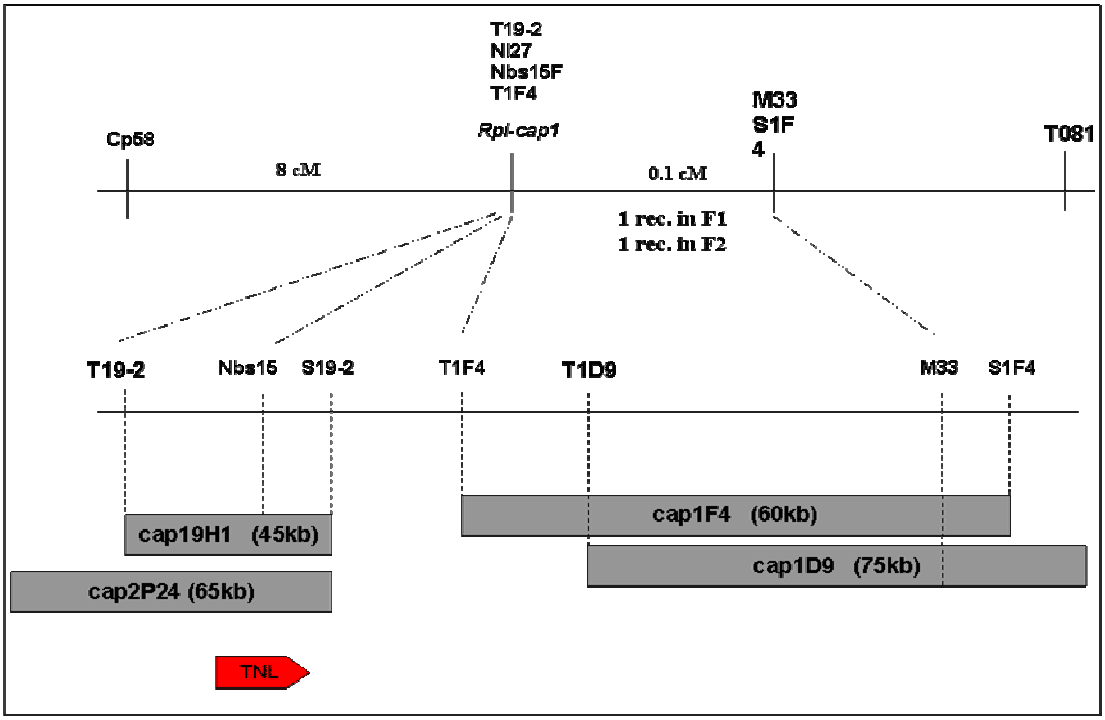

Figure 2. Genetic and physical map of *Rpi-cap1* in *S. capsicibacatum* at the beginning of this study.

Pulsed field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) was used to determine the insert size of the BAC screened from the library. Pulsed field gel electrophoresis (PFGE) is a technique enabling separation of DNA molecules in agarose gel using two alternating electric fields, with vectors directed to each other at different angle. This technique is able to separate large molecule of DNA more than 20 kb which can not be done in regular gel electrophoresis. This study used Bio-Rad CHEF (contour-clamped homogeneous electric field) to obtain straight lanes and stable DNA separation (Nassonova 2008). Cold TBA (4^{o}C) was used as buffer with instrument setting: 200 volt and 5 – 15 seconds pulse for 14 hours 12 minutes. After 14 hours the gel was soaked in TBE and ethidium bromide for 20 minutes in a shaker, which then the lanes were documented in Octopus F.

Result

Recombinant screening

The seeds from 7358-3b x cap 355-10 F2 population (seed batch 09-3094) was germinated three times from late January until April 2010, with 4 weeks interval of each germination. Each germination attempt 2 petridishes of 30 seeds were germinated and kept in room temperature for five days. The seeds were kept in two different storage temperature (4ºC and room temperature) and prior germination test, 8% Giberelic Acid (GA) in sterile water was added to the germination dish. Nevertheless no germination was observed in either treated seeds with GA or the control. Since the seed in this study was not germinating, we were not able to perform recombinant screening needed to zoom in the genetic map of *Rpi-cap1* gene.

BAC library screening to fill the gap

Since the first approach by development of markers in 2P24 BAC end sequences and successive screening of the existing BAC library did not give any positive BACs as a candidate to fill in the gap, then the 1F4 and 1D9 contig were aligned to *S. phureja* sequence (Figure 3). From the ORF in the sequence which adjacent to 1F4, several markers were developed to screen the BAC library as described by (Rouppe van der Voort, J. K. Kanyuka *et al*. 1999). Each primer combination from the sequence was first tested in the F1 population to confirm the marker map position in *S. capsicibacatum*.

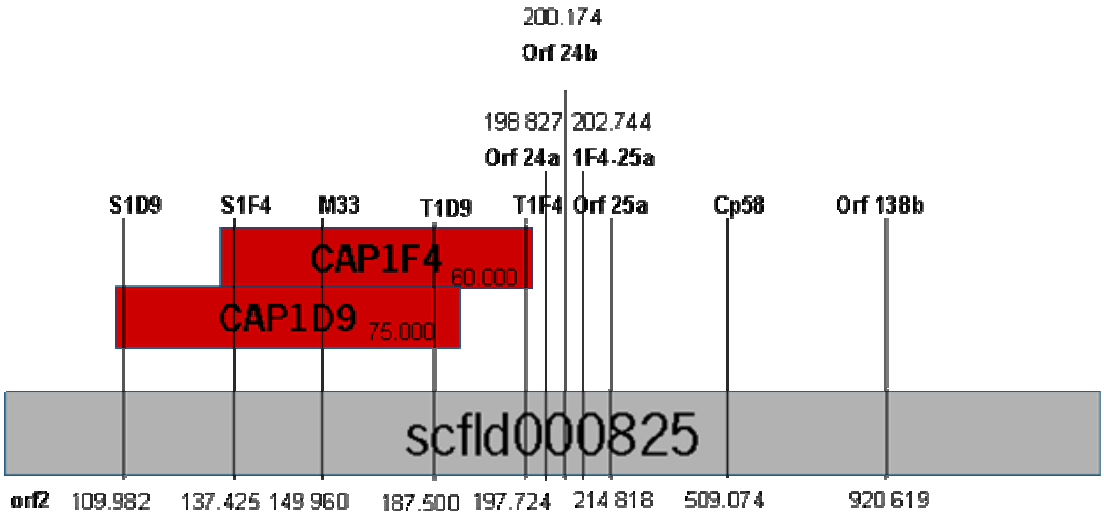

Figure 3. Marker development by aligning 1F4 and 1D9 contig to *S. phureja* sequence scaffold 825. Markers are described in Table 3

Marker screenings were done by isolating plasmid DNA from each pool and positive pools were identified using PCR. Bacteria from positive pools were diluted and plated on LB agar with chloramphenicol (12.5 µg/ml). Individual colonies were picked into 384-well microtiter and single positive BAC clone was also identified using PCR. Markers and primers used to screen the BAC library are provided in Table 2 and 3.

Two types of polymerase were used in this BAC screening. At the beginning of this study DreamTaq™ from Fermentas was used, while at the end Phire® Hot Start DNA Polymerase from Finnzymes was used. Phire® amplifications were better for Light Scanner with small amount of DNA compared to DreamTaq™ amplifications.

In total 13 markers and primers were used to screen the BAC library and two positive BAC clones were found in this study (Table 4). No positive BAC pools were identified from 3b-4 library with six primer pairs of 2P24 BAC end sequence (3 T and 3 S sides). 3b-4 library which consist of 47 BAC pools were screened with 19H1T2 marker and two pools (306 and 316) were found positive with this marker. Then the PCR products in these pools were digested with E16 enzyme to identify the polymorphism and compared with 19H1 PCR product as control. Pool 306 and not pool 316 gave the same pattern as the 19H1 control, which is known to be in coupling phase. Therefore only pool 306 was selected to isolate the individual positive BAC clone from.

398 colonies were tested from pool 306 and two were positive with 19H1T2 marker (306A and 306B BAC clones). Afterward both colonies were purified to extract the plasmid using Midiprep Qiagen tip-100. By fingerprinting with Hind III it was recognized that both colonies are the same and similar to 2P24, although not identical. The BAC ends from one positive colony were sequenced, and Pulsed Field Gel Electrophoresis (PFGE) was used to determine the insert size. From the BAC end sequence and PFGE size determination, it was known that BAC clone, 306A was mapped inside the 2P24 contig, hence there was no additional information gathered from this BAC (Table 5).

Markers 825_24b and 825_25a, which has 14.6 kb distance in *S. phureja* sequence were able to amplify bands from several pools but only one pool (374) was amplified by both marker and this pool also amplified by T1F4 primer which is 2.5 kb away from 825_24b. Since 825_25a and T1F4 amplicon pattern in Light Scanner was less obvious than 825_24b amplicon, only marker 825_24b was used to isolate the individual BAC clone.

Three individual colonies were identified from 796 colonies of pool 374 screening. One of the colonies is K18 which located in the gap between contig 2P24 and 1F4. The K18 pccF BAC end is present in 1F4 sequence and positioned at 5.6 kb from 1F4 T end (Figure 4). Due to

limited time in this study the insert size and sequence of K18 BAC clone was not yet discovered.

Table 4. Screening result of several BAC library batches using markers developed in this study. 306 (306A clone) and 374 (K-18 clone) are the positive BAC pools screened using different markers, while library batches which were not gave clear positive pools are indicated with x.

Markers	BAC library				
	3b-1	3b-2	3b-3	3b-4	3b-5
19h1T2E16				306	
19H1INT-7LS				x	x
1D9T-4LS				x	
1F4T-3LS				x	374
2P24F1				x	
2P24F2				x	
2P24F3				x	
2P24R1				306	x
2P24R2				x	x
2P24R3				x	
M33				x	
825_25a	x	x	x	x	374
825_24b	x	x	x	x	374

Table 5. Individual BAC clone screened from the BAC library using several primers and markers.

BAC	size	phase	position	markers	methods
306A	42 kb	Coupling	inside contig 2P24	19H1T2E16	CAPS
K-18	?	Repulsion	1F4 overhang	825_24b	Light Scanner

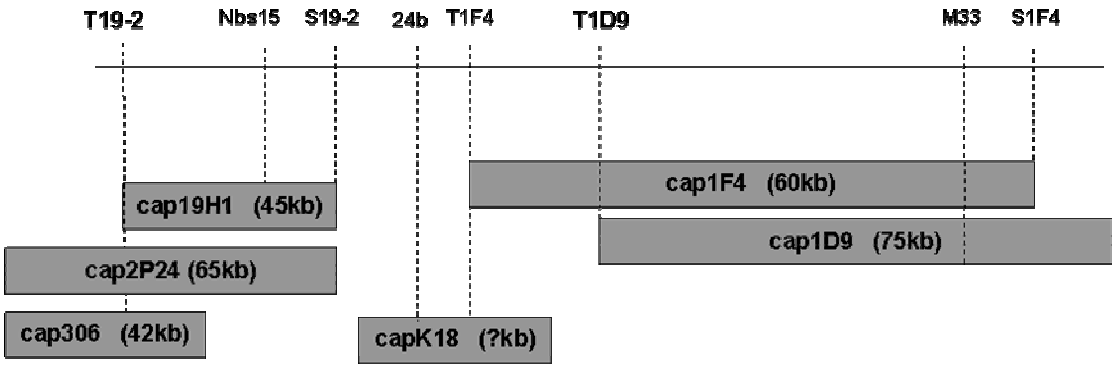

Figure 4. BAC 306A (screened with 19H1T2E16) and K18 (screened with 825_24b) position in the physical map.

Coupling or repulsion phase identification

Even though Light Scanner method is faster and easier than CAPS, the coupling or repulsion phase determination of the positive clone identified is more complicated and difficult. In order to identify the coupling or repulsion phase, the PCR products of the positive BAC need to be sequenced and compared with the susceptible and resistant F1 population alleles. To separate two alleles in the F1 plants, the PCR products were sub cloned into pGEM®-T easy Vector and transformed to DH5α *E. coli* competent cells using chemical transformation methods. With this method individual allele could be picked from single colony (Kobs 1997). Four susceptible and resistance F1 DNA were amplified using 825_24b marker and four colonies of each amplicon were sequenced at Greenomics.

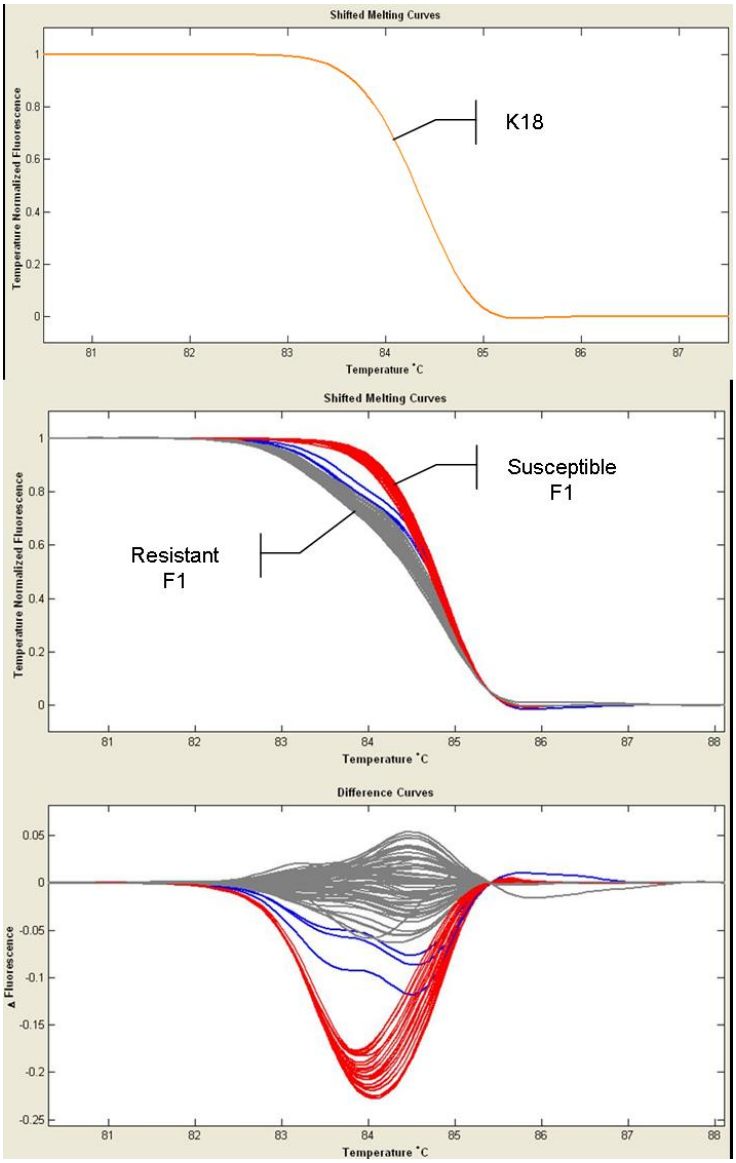

Figure 5. High resolution melting curves of *S. capsicibacatum* F1 population (susceptible and resistant) and K18 BAC clone amplicon, amplified with 825_24b marker.

There are three SNP's found from the sequence alignment of 825_24b amplicons from F1 resistant population and K18 BAC clone (Appendix 1 and 2). While in the F1 susceptible population and K-18 alignment, no SNP's were identified. This finding might explained the similarity of HRM curves from K18 with the susceptible plants (Figure 5) and coupling and repulsion phase determination of the positive clone can be easily done with light scanner.

Sequencing 2P24 BAC

The overhang of BAC 2P24 (Figure 2) size at the beginning was predicted to be 6 kbp long, than it was sequenced using primer walking strategy (1000 by 1000 bp). With primer walking methods 4000 bp sequence was obtained at the forward side of the 2P24 overhang and 1000 bp at the reverse side. Later from the PFGE size determination it was discovered that the overhang is larger than expected (20 kb. From this finding it was decided to use another method, 454 high throughput sequencing to sequence the whole 2P24 contig. Subsequently. The 454 sequencing produced 8 contigs in the 2P24 BAC clone. These contigs can be aligned with 19H1 contig and the primer walking sequence. Nevertheless there are still gaps between 2P24 contigs in the overhang area (Figure 6). To fill these gaps a second round of primer walking was conducted. From the primer walking it was known that between contig 1 and contig 5 there are 1165 bp sequence. Contig 3 (663 bp) was not aligned with 19H1 contig nor contig 1-contig 5 primer walking sequence. The primers developed from contig 3 were also unable to be sequenced. These are the reason why contig 3 was not included in the complete sequence. The entire sequence of 2P24 BAC was compiled in one fasta file (2P24 complete sequence.fas).

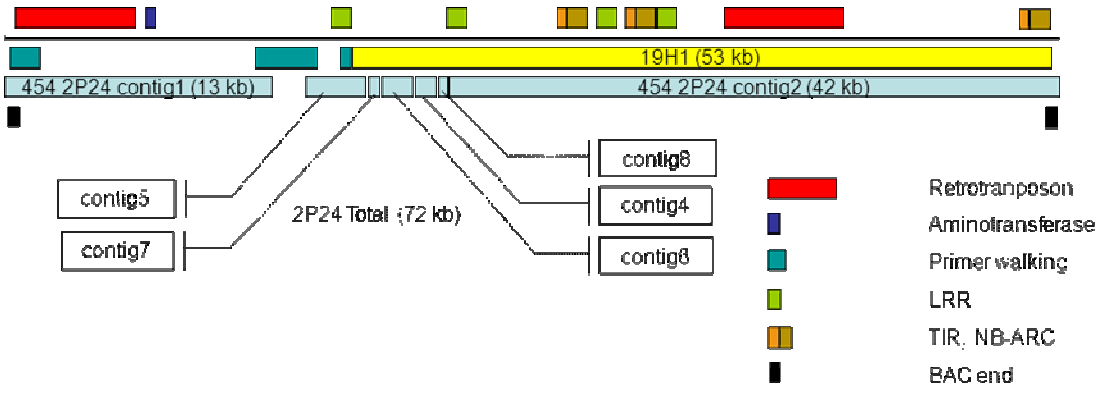

Figure 6. 2P24 454 sequencing and several genes found in this area.

Approaches to extend the BAC contig

The existing contig were extended to conceal *R* gene that may present as functional resistance gene homologs (RGH). The existing putative gene in the 2P24 overhang sequence was predicted using FGenesh and InterProScan. FGenesh algorithm from the Softberry website was used to find genes within the 2P24 sequence and InterProScan was used to identify the gene families. From this blasting we found retrotansposons and aminotransferase and we did not find any additional *R* gene homologs in the overhang sequence. The *R* gene cluster was found only in the overlap between 2P24 and 19H1 sequence. With retrotransposons in contig 2P24 overhang and RGA in contig 19H1 end it is difficult to develop markers which could be use to extend the BAC contig. Therefore synthenous sequence from *S. capsicibacatum* relatives was used to develop markers.

Discussion

Germination failure

The F2 seeds were produced in spring 2009. Seeds storage duration is supposed to affect the germination since some of potato seeds is known to have dormancy periods (Simmonds 1963). Thus longer storage time is better for several potato seeds germination. The temperature of storage was known to have an affect on the germination (Simmonds 1963; Lam 1968), but it seems that even though the seeds was kept in warmer temperature for 2 months (room temperature, not 4^{o}C), still no germination occured. Overnight GA soaking also did not induce seed germination. After several attempts and failure to germinate the seed, it was concluded that the recombinant screening is not possible to be executed in this study.

BAC screening and contig extension

The T region of 2P24 contig contains retrotransposons (Figure 6) that hindered the BAC screening. Retrotransposons as transposable elements are frequently found in plant genome (Wessler 1996). Consequently primers from this region will amplify bands from almost all of the BAC pools available, for this reason it is impossible to screen for BACs flanking 2P24 with markers located at the ends. BAC screening with 2P24 S primers was not successful also, because in this region contains an resistance gene analogs (RGA). Because of those difficulties the synthenous *S. phureja* sequence was used to develop markers. A huge gap between 1F4 and 2P24 is suggested since different DM scaffolds are aligned with 1F4 and 2P24 sequence . Because of the small genetic distance observed between the BAC contigs, it is postulated that the RGA from 2P24 and 19H1are newly integrated into this genomic region in

S. capsicibacatum. Another reason probably due to chromosomal rearrangement between different potato species. Chromosomal rearrangement was observed in potato and tomato short arm chromosome 6 using cross species multi color BAC-FISH method (Xiaomin Tang, Dora Szinay et al. 2008), this phenomenon might occur also in chromosome 11 where the *Rpi-cap1* gene cluster is located.

Repulsion and coupling phase

HRM distinguishes alleles and measures differential expression of alleles between different organs, storage treatments, stage of tubers, and between varieties (Yuan, Haroon et al. 2009). While the HRM sensitivity of heterozygote scanning approaches 100% (Wittwer 2009). The difficulties to determine the coupling or repulsion phase using HRM, caused by lack of information in the alleles involved which determine the pattern observed. Therefore the alleles from resistant and susceptible F1 population need to be separated and sequenced to identify the SNP's responsible for the different pattern in the Light Scanner. Then, the plasmid sequence can be compared to the susceptible and resitance alleles to determine the coupling and repulsion phase.

Single pattern in susceptible plants and large diversity observed in resistant plants HRM curve differences (Figure 5) indicate that the susceptible plants are homozygous while the resistant plants are heterozygous (Figure 7). The resistance alleles sequencing resulted in two different consensuses with three SNP's observed. While from the susceptible alleles only one consensus observed and the K18 amplicon is similar to this consensus. It is concluded that K18 BAC clone screened with 825_24b using Light Scanner was in repulsion phase, therefore the putative R gene was not in this plasmid. This finding suggested that in some cases (solid and different curve of resistant and susceptible plants) the HRM can be used to distinguish between coupling or repulsion phase of screened clones.

Since this study was not able to perform recombinant screening, the exact location of the *Rpi-cap1* was still not identified. However, from the 2P24 overhang sequencing we found out that there was no additional R gene identified (Figure 6). To identify the *Rpi-cap1* position without recombinant screening, additional research was conducted (Chapter III). For further study, the K18 BAC end can be used to screen other BAC pools in coupling phase. The BAC end sequence of K18 is more informative then the *S. phureja* sequence, since the R-gene cluster in *S. capsicibacatum* might be newly integrated in the *S. capsicibacatum* hence the R-gene cluster was not available in *S. phureja* sequence. Alternatively, the rest of the BAC library can be screened using the melting curve of 825_24b marker on K18 BAC as control for the repulsion phase.

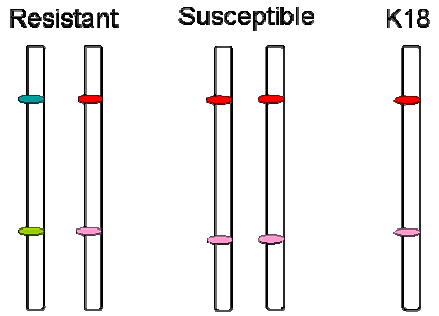

Figure 7. K18 BAC clone in repulsion phase

Chapter III

Agroinfiltration to identify *Rpi-cap1,* *S. capsicibacatum* resistance gene against late blight

Introduction

Since the recombinant screening to zoom in on the genetic map was not performed in this study (Chapter II), another approach was conducted in order to locate the active gene (s). The method to do this is agroinfiltration of the whole BAC or parts thereof into *N. benthamiana*. Successively, the agroinfiltrated leafs are challenged with late blight. Alternatively, the genes present on the BAC can be co-expressed with late blight effectors corresponding to *Rpi-cap1*.

Agroinfiltration is an *A. tumefaciens*-based method *in planta*, with high throughput capacity for transient expression of genes of interest (Lokossou, Park *et al*. 2009; Oh, Young *et al*. 2009; Vleeshouwers and Rietman 2009). *A. tumefaciens* strains that contain the *Avr* gene are coinfiltrated with *A. tumefaciens* strains that contain the *R* gene. As a result both genes are co-expressed in the infiltrated leaf and a HR will be visible (Vleeshouwers and Rietman 2009).

Avr proteins from oomycete plant pathogens carry an N-terminal type II secretion signal peptide followed by a conserved RXLR motif. Next to RXLR motif, there aminoacid sequences which are referred to as effector and are sometimes required for virulence. RXLR motif functions in translocating these effectors into host cells. A defense response is induced which leads to cell death and is visible as HR, when effectors in host cells are recognized directly or indirectly by the R gene (Vleeshouwers, Rietman *et al*. 2008).

The aim of this study is to locate and identify *Rpi-cap1* responsible for *S. capsicibacatum* resistance against late blight by agroinfiltration and R-Avr interaction (effector based identification). The knowledge to locate and identify the putative genes is important for further study to clone and introgress them into cultivated potatoes according to the cisgenesis principle.

Material and Methods

BIBAC construction

Both the BAC clones and the BIBAC were digested with NotI enzyme at 37^{o}C for 4 hours to release the insert and to open the BIBAC. Since there is one NotI site inside the 1F4 BAC clone, the insert was divided in to two parts; 1F4-7 (cosegregates with M33 marker) and 1F4-14 (cosegregate with T1F4 marker).

TSAP was added to the digested BIBAC to prevent recirculation of the vector by incubation in 37°C for 15 minutes. The insert from BAC clones were ligated with digested BIBAC using T4 ligase M0202T enzyme with incubation at 16°C for 24 hours. Prior to transformation the ligation mixes were drop dialyzed against 50% TE buffer for 1 hour. The ligation mixes were transformed to DH10B *E. coli* competent cells by electroporation, plated on LB plates containing kanamycin, and incubated at 37°C for 18 hours.

A. tumefaciens *transformation*

E. coli colonies with BIBAC and insert were selected; plasmid was purified and transformed by electroporation to COR308 *A. tumafaciens* competent cells. The transformed cells were plated on LB plates containing kanamycin and tetracycline, followed by incubation at 30°C for 48 hours. In order to verify the integrity of the plasmid in the COR 308 transformants, plasmids were isolated and transformed back to DH5alfa *E. coli*. Plasmids from the resulting colonies was isolated and fingerprinted with Hind III and compared to the original plasmid.

Transient complementation in N. benthamiana

Infiltrations of the *A. tumefaciens* containing BIBACs were performed into *N. benthamiana*. The method to prepare A. tumefaciens prior to infiltration was adapted from (Lokossou, Park et al. 2009; Vleeshouwers and Rietman 2009). The leafs of four to five weeks old seedlings were infiltrated with A. *tumefaciens* culture at OD_{600} =0.2. Two days later the leafs were challenged with 10 ul zoospore suspension (10.000/ml) from *P. infestans* strains 90128 or IPO-C. At 4 till 8 dpi the leaves were scored for HR and sporulation.

Coexpression in N. benthamiana

The effectors used in this study were kindly provided by Hendrik Rietman. *A. tumefaciens* carrying two effectors (PITG_16705 and PITG_148848) and one empty vector pMDC32 (negative control) were mixed with *A. tumefaciens* carrying putative *R* genes in 7 weeks old *N. benthamiana*. Preparation of *A. tumefaciens* for coexpression followed the same procedure as described above. The *A. tumefaciens* strains were mixed with R:Avr ratios as follows: OD_{600}=2.0:0.25; 2.0:0.5; 2.0:1.0 and 2.0:2.0. As a positive control, *A. tumefaciens* carrying *R3a* and *Avr3a was* used at OD_{600} ratios 0.5:0.5. The HR was observed 5 dpi.

Attempt to develop positive control

Individual clones from BAC library of *S. capsicibacatum* were isolated by Miniprep Qiagen. The sizes of the clones were determined by PFGE as described in Chapter II. R-gene from *S. venturi* (*Rpi-vnt1*) was planned to be inserted in the individual BAC clones. Restriction enzymes (Kas I: G*GCGCC), which located in pCC1BAC™ vector between Not I sites (2 and 631 bp) and absent in *Rpi-vnt1* sequence was used to cut the clone. Primers matching the *Rpi-vnt1*

start and stop codon regions and containing Kas I adaptors were developed. The *Rpi-vnt1* amplified with this primer was ligated using T4 enzyme and transformed into DH5α competent cells.

Result

BIBAC construction and A. tumefaciens *transformation*

To eliminate tedious subcloning steps needed for gene verification, a binary-BAC (BIBAC) vector was developed from existing BAC clone in the *Rpi-cap1* area. The BIBAC vector can be used for plant transformation with large insert (Hamilton 1997).

Four inserts from three pccBAC™ clones (19H1, 2P24 and 1F4) identified in the area of *Rpi-cap1* (Figure 2, Chapter I) were used as DNA sources for the subcloning into BIBAC. One BIBAC without insert was developed as negative control. The BIBAC construction method was described by (Hamilton 1997). Insert sizes in the BIBAC are: 19H1 (53 kb), 2P24 (60 kb), 1F4-7 (23 kb) and 1F4-14 (43 kb).

Only 1F4-7 and 1F4-14 were able to be identified using DNA fingerprinting of the colony PCR products of the transformed COR308 cells, while other construct were not digested nor amplified. Both 1F4-7 and 1F4-14 were stable in *A. tumefaciens* and identical to the DNA source. However all of the *A. tumefaciens* were used in the transient complementation and co-expression assay in *N. benthamiana*.

Transient complementation in N. benthamiana

In total 40 plants were used in this study, ten plants for each construct and three leafs were infiltrated in each plants. After two days the leafs were challenged with 90128 and IPO-C. Four days post inoculation only few sporulation occurred and no difference was found between BIBAC construct. The HR expected from the 19H1 and 2P24 subclones containing the RGA were not observed. In the other hand at the 8 dpi observation, both 1F4-7 and 1F4-14 isolates had lower sporulation percentages than the other constructs (Table 6)..

The difference in virulence of the two isolate was obviously seen even in 4 dpi observation and this result was constantly seen in multiple leafs. IPO-C isolate is more virulent than 90128. Both 90128 and IPO-C are however a-virulent on *S. capsicibacatum* plants and should therefore express Avr-cap1

Table 6. Percentage of leafs with sporulation in *N. benthamiana* agroinfiltrated with 4 BIBAC constructs and challenged with two *Phytophthora* isolates (90128 and Ipo-C).

BIBAC	Sporulation (Percent)*			
	90128		Ipo-C	
	4 dpi	8dpi	4 dpi ns	8 dpi
2 (19H1)	17	30	27	33
6 (2P24)	13	33	33	50
7 (1F4-7)	7	10	17	30
11 (1F4-14)	0	13	7	13
Control(empty)	0	0	0	0

* = The average number of leafs agroinfiltrated in each treatments are 30

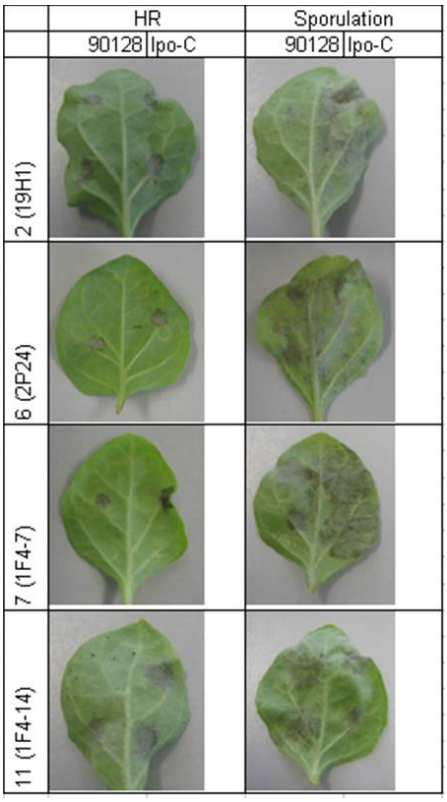

Figure 8. HR and sporulation observed 8 days post inoculation of *N. benthamiana* agroinfiltrated with 4 different BIBAC (19H1, 2P24, 1F4-7 and 1F4-14) construct with two *P. infestans* isolate (90128 and IPO-C).

Most of the treatments have more sporulation as compared to the control (empty BIBAC) (Table 6). HR observed in 1F4-7 might have been an indication that the gene responsible for the resistance is located in this construct (Figure 8). However, the small amount of leafs with sporulation in this study is possibly caused by the *N. benthamiana* plants age not due to the coexpression. Non-host resistance against late blight might have occurred in older *N.*

benthamiana plants which indicated in the control sporulation percentage where the leaf used in this treatment are larger and older compare to other treatments. The HR observed in 1F4-7 construct also not clarified since no repeats were performed in this experiment.

The cisgenisis approach avoids using antibiotic markers and relies on the *in planta* test and stable transformation. To identify *R* genes usually have difficulties to determined the border between susceptible and resistance. In this experiment non-host resistant of older *N. benthamiana* against late blight more or less effecting the identification of the *R* genes and therefore adding the difficulties to determine the susceptible and resistance.

Coexpression in N. benthamiana

Twenty five plants were used in this assay and three leaves in each plant were agroinfiltrated. The experiment then repeated twice, the first repeat with 20 plants and the last repeat with 12 plants. At the last repeat only 1F4-7 construct was challenged with the effectors. The HR observed from the positive control (*R3A* + *Avr3A-KI*) were very clear and distinguishable. Interestingly, only one co-expression gave HR in this study.

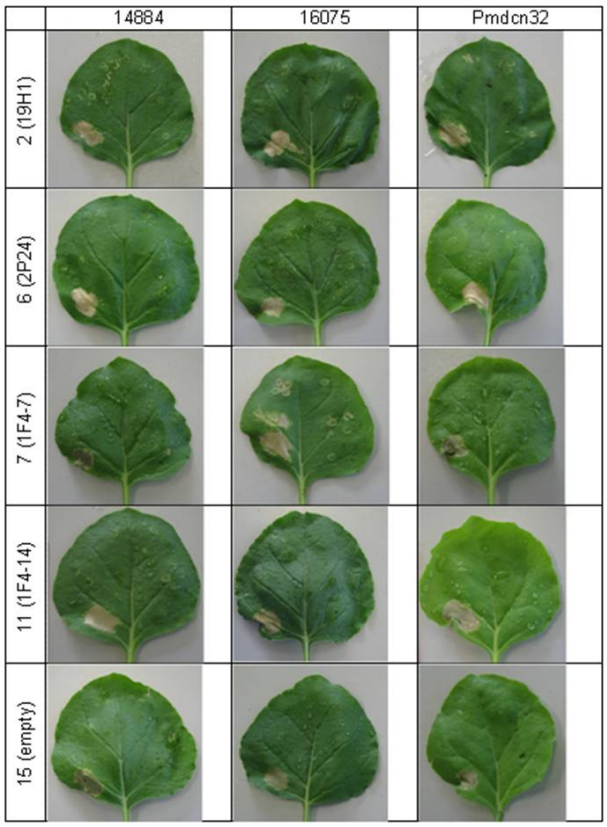

Figure 9. Five dpi *N. benthamiana* leaves co-infiltrated with *A. tumefaciens* carrying BiBAC constructs (19H1, 2P24, 1F4-7, 1F4-14 and empty as negative control) from *S. capsicibacatum* and effectors (14884, 16075 and Pmdcn 32 as negative control) from *P. infestans.*

1F4-7 with PITG 16075 co-infiltration in *N. benthamiana* leaves promote HR in two out of three leaves in the first assay, yet again the response is not as clear and obvious as the positive control (Figure 9). This finding is in parallel with the transient complementation assay observation which observed that the 1F4-7 construct is more resistant compared to other construct. However this finding was not repeatable in the second experiment. This difference was thought to be human error, therefore in the third experiment only 1F4-7 construct was used. Nevertheless, also in the third experiment with six plants challenged with PITG 16075, none of the leaf indicates any HR.

Development of a positive control BiBAC

To develop a positive control BIBAC, firstly the existing BACs with certain insert size were isolated, afterwards the *Rpi-vent1* gene was added to the insert and finally convert the insert into BIBAC. In order to select BACs with different insert sizes, 23 colonies that were isolated from BAC library were screened and four clones were chosen according to their sizes. From the PFGE, the sizes of these clones were identified as: 54 kb, 59 kb, 60 kb and 67 kb. The clone names and sizes were listed in table 7.

The *Rpi-vnt1* gene was amplified using *Rpi-vnt1*:KasI primers (F: CGCGGCGCCCT TTGAAAAGAGGCTTCATA, R: GGCGGCGCCGAATTCACTAGTGATTAGTTA) with reading proof DNA polymerase enzyme Phusion. The four ligations were transformed successfully into DH5α competent cells. Only one colony contained the *Rpi-vnt1* as apparent from the amplification by *Rpi-vnt1*: KasI primers in a colony PCR. The insert was integrated into the 54 kb BAC resulting in a 58 kb (54 kb + 4 kb) plasmid.

Table 7. Individual BAC clones uses as positive control with *Rpi-vnt1* gene

Clone	Size (kb)
306_12	54
306_15	60
306_16	67
306_19	59

Discussion

Transient complementation in N. benthamiana

Hind III fingerprinting shows only 1F4-7 and 1F4-14 with 23 and 43 kb was found to be stable in *A. tumefaciens*. 19H1 and 2P24 with 53 and 72 kb insert was not stable in *A. tumafaciens.* The HR and inhibitions of sporulation observed in 19H1 and 2P24 construct largely due to non-host resistant of *N. benthamiana* against late blight. Insert larger than 20 kb tends to be more difficult to transformed *in planta*, even though larger insert was reported successful in rice, Arabidopsis and maize (Li, Uhm et al. 2007; Vega, Yu et al. 2008; Wang, Wu et al.). Therefore other approach to identify and determine which construct is responsible for the resistance can be done by coexpression assay between BIBAC construct hauling the putative gene and the Pi effectors.

Coexpression in N. benthamiana

Coexpression assay is a high troughput system that accelerates the identification of R genes (Vleeshouwers, Rietman *et al*. 2008; Lokossou, Park *et al*. 2009; Oh, Young *et al*. 2009; Vleeshouwers and Rietman 2009) and BIBAC vector will accommodate larger insert size to overcome high resolution mapping difficulties in this study. However, the HR observed in the coexpression of 1F4-7 BIBAC sub clone with 16705 effectors, was not clearly designate that the gene of interest is in this subclone since the HR was not repeatable. The attempts to develop positive control carrying known R gene in BIBAC with similar size (20-70 kb) might be very helpful to determine the maximum insert size of *A. tumafaciens* based agroinfiltration, hence reconfirm the possibility of agroinfiltration with large insert size. Without this positive control it was difficult to state that the lack of HR observed in this assay was due to the absence of a putative R gene. Positive control developments was successful only until BAC clone isolation, R-gen ligation and transformation in competent cells, therefore it was not yet used in this study.

Another approach to reconfirm the possibilities of the RGA responsible for resistance of *S. capsicibacatum* against late blight is to test the recombinant with related effectors. Two effectors (16075 and 14884) which gave HR in parental resistant plants need to be challenged with the recombinant plants to test the possibility of two closely linked genes recognizing a different effector. If different recombinants indicate different interaction paterns with the effectors, this shows that there are two genes responsible for the resistance. One gene interact with 14884 which located in the left side of 1F4, while the other gene interact with 16075 which located in 1F4-7.

R gene in the 1F4-7

If the HR observed in transient complementation and coexpression assays in *N. benthamiana* are in fact caused by an R gene in 1F4-7, then it is a new R gene class because no NB-LRR, nor RLP, nor RLK class was found in this area (Figure 10). 1F4-7 only contains zinc finger, phosphopanteteine and NADH ubiquinone oxydoreductase.

Zinc finger contributes to enhanced cold tolerance in plants as well as in bacteria (Jin, Sun *et al*. 2010; Park, Kwak *et al*. 2010) and can be used to construct DNA-binding protein for specific intervention in gene expression(Klug 2010; Weinthal, Tovkach *et al*. 2010).

The phosphopantheteine over expression in Arabidopsis enhanced vegetative and reproductive growth and salt/osmotic stress resistance, while this gene knockdown will severely impaired in plant growth and seed production (Rubio, Whitehead *et al*. 2008). NADH ubiquinone oxydoreductase or Complex I gene also play role in cold and NaCl tolerance in Arabidopsis (Lee, Lee *et al*. 2002).

A relation between salt and Pi tolerance was found in a report about a UND/PUB/ARM repeat type gene, which is associated with late blight resistance and NaCl stress. An UND/PUB/ARM repeat type gene (StPUB17) silenced plants exhibited more susceptibility to the infection of *P. infestans* and more sensitivity to the stress of NaCl (Ni, Tian *et al*. 2010). In previous studies genes found in 1F4-7 was known responsible for salt tolerance, but from this study, genes in this sub clone might also responsible for R gene against late blight.

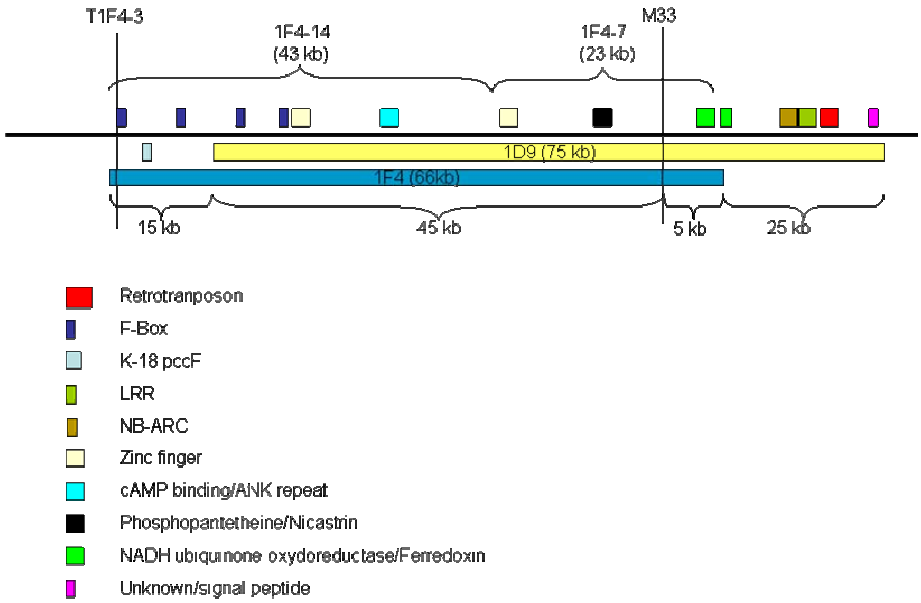

Figure 10. Physical map and predicted genes in 1F4 and 1D9 BAC clone.

According to these findings, it migt be possible that the *Rpi-cap1* genes responsible for *S. capsicibacatum* resistance against *P. infestatnt* is in the 1F4-7 sub clone. Even though the resistance to late blight in *S. capsicibacatum* is known as monogenic and conferred by one dominant gene located on chromosome 11(Verzaux 2010), this study did not rule out the possibility of more than one gene in very close area are responsible for the resistance. For further study susceptible cultivated potatoes can be transformed using this sub clone and tested for resistance against *P. infestans.* Resistance induced by stable transformation will confirm the possibility to use 1F4-7 as R genes source for cisgenic potato breeding against late blight.

Acknowledgement

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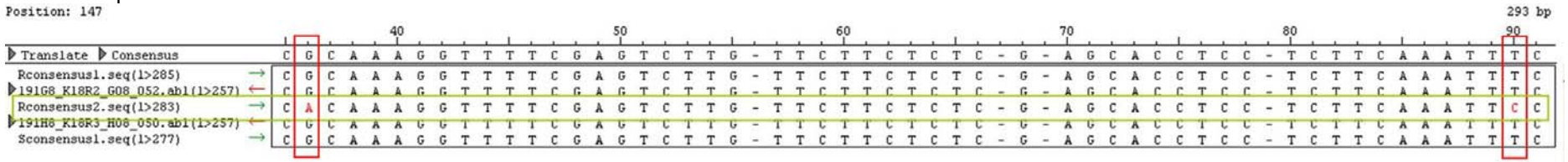
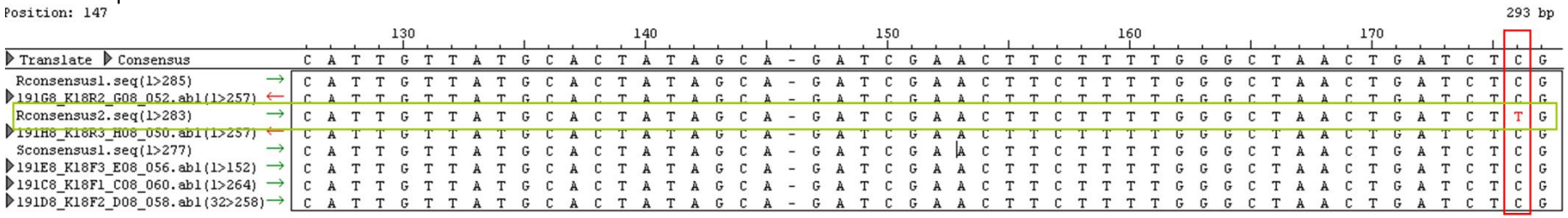
Appendices

Appendix 1. Two SNP's (red words) observed in F1 resistant population 825_24b amplicon sequences aligned with K18 BAC clone 825_24b amplicon sequences.



Appendix 2. One SNP's (red word) observed in F1 resistant population 825_24b amplicon sequences aligned with K18 BAC clone 825_24b amplicon sequences



Appendix 3. 1F47-T sequence used in *S. phureja* blasting to develop markers 825_25a and 825_24b.

GCATCAGAATGACATCGGATATATATCAGGAAGGTAGCTAAGAACGTCAGGAAGTAAACTTCGACA
ATGCTTTCTGCCTTTAGGAGGCATCATATATCAAAAATCACCAAGAACCTGTAGAAAATAAGCAATA
ATACAAAGTAAGGATCTCCAAATAATGTGGATGGAATTTCTATACACATTTTCCGATCAACATAAGA
ACGAAAAGAACACTTACAATACTAATCACTAAAATTCTGTAGTGATAAACAATAATATGAAGTAAGG
ACTCTCAAACTATCTGAATAGTACATCTATTCACATGGTTTTTATTAACATAACAAATTAAGGTGCAT
TCTTGTTGAAATCAGTCAACTATCTCCTTTTAATACCTATGTTGCAATTTGCCTTGAGATACCCATAT
TGAACGACAGGATATTAGCTTGGGTATTTCGTGCAGATTCTTCAAGAAACACCAAAAATCAGAAGG
AAAAATACACTTGACATACCCAGACTGAGTATGTACCAGTAAGGAAATCCATGTAACATAGCTTAAT
ATTCATTACTTGCATTCTCACAGAATGAACACCTTCCATTAAGGGTGTGTTTGGTATGTAGTTTCCA
ATTTTCTCCTGTTAAATTGGTCAAAAGTTTGGAAAACATTTTCTCTAGTGAAACATAAAATTGAGGA.