Characterizing and mapping of qualitative resistance in *Lactuca saligna* to lettuce downy mildew

Thijs Bouten, 2016

Msc. major Thesis (PBR-80436)

WAGENINGEN UR
For quality of life

WAGENINGEN UR

Characterizing and mapping of qualitative resistance in Lactuca saligna to lettuce downy mildew

Thijs Bouten

Msc. Major Thesis
Msc Plant Sciences: specialization Plant Breeding and Genetic Resources

Oktober 2015 – April 2016

Supervisors:
Dr. Marieke J.W. Jeuken
Anne Giesbers Msc.

Examiners:
Dr. Marieke J.W. Jeuken
Anne Giesbers Msc.

WUR registration number: 910306113080
E-mail: thijs.bouten@wur.nl
Date of submission: 26-04-2016
Commisioned by: Laboratory of Plant Breeding, Wageningen UR and Research

WAGENINGEN UR
For quality of life

Acknowledgements

This thesis is part of my master program Plant Sciences with the specialization Plant Breeding and Genetic Resources. I chose this thesis, because I wanted to learn more about genetics and mapping of resistance. At the end this was a good choice, because I learnt a lot and besides the genetic analysis in the lab I didn't lose contact with the crop.

I want to thank especially Marieke Jeuken and Anne Giesbers for guiding me through this thesis. You both really wanted to get the best out of me and although the data analysis in particular was from time to time a difficult process I managed to get good results. Also the help in the lab and providing feedback on my work helped me improving my academic skills. Further I would like to thank the people from the breeders hall which gave me the mental support during my thesis. I hope you will enjoy reading this thesis.

Table of Contents

Abstract

Wild relatives of lettuce (*Lactuca sativa)* are a good source for new resistance against downy mildew (*Bremia lactucae*) the most devastating pathogen in lettuce production. Especially *Lactuca saligna* which is a non-host to downy mildew is an interesting species to investigate. In this thesis the aim is to dissect the resistance against downy mildew in *L. saligna* accessions CGN15726, CGN15699 and CGN13330 by observing the distribution and segregation of resistance levels in BC1 populations. The BC1 populations with *L. saligna* CGN15726 (BC1*sativa*CGN15726) and *L. saligna* CGN13330 (BC1*sativa*CGN13330) were made by crossing the *L. saligna* accession with *L. sativa* cv Olof and backcross it once back to *L. sativa* cv Olof. Furthermore, a BC1*serriola*CGN15699 population was made by crossing *L. saligna* CGN15699 to *L. serriola* CGN04774 and backcrossed it once back to *L. serriola* CGN04774. These backcross populations were tested for disease resistance to Bremia isolate Bl:21 in a leaf disc test. For BC1*sativa*CGN15726 and CGN13330 a continuous segregation over the different infection classes was observed with 9% and 6% of plants being resistant. Based on knowledge of a previous studied population from France BC1*sat*_05271_FR this suggest no monogenic dominant resistance against Bl:21 in these populations, but the resistance might be caused by non-host resistance. BC1*serriola*CGN15699 showed hybrid necrosis which turned out to be based on the same genes as has been studied in *L. saligna* CGN5271 x *L. sativa* cv. Olof. Furthermore there was a continuous segregation over the different infection classes with implications of a possible R-gene. However, in a follow up experiment conducted with a BC2*serriola*CGN15699 population, to verify the results it turned out that the susceptible *L. serriola* parent used in this population showed hardly any infection in the disease tests. Therefore no conclusions could be drawn related to R-gene presence against Bl:21 in this population. A second experiment was also conducted with a BC1*sativa*CGN5947 population were the aim was to find a second dominant monogenic R-gene against Bl:24. One R-gene was already identified. However, there were still a large amount of resistant plants which could not be explained by this R-gene. For this population it was possible to determine a mapping interval for resistance at 102-135 cM at chromosome 4. Using a BC2 population it was possible to verify this mapping interval and even fine-map it to 119.8-126.3 cM (318-340 Mb). This region co-located with MRC4 in *L. sativa.* Finally, as a side experiment, several markers were developed on gaps in the genetic map and at MRC4 to be able to fine map the resistance on that MRC.

Abbreviations and genetic nomenclature

Abbreviations

BC1*sativa*	Population created by crossing a *L. saligna* x *L. sativa* F1 with a *L. sativa*
BC2*sativa*	Population created by crossing a BC1*sat* with a *L. sativa*
BC1*serriola*	Population created by crossing a *L. saligna* x *L. serriola* F1 with a *L. serriola*
BC2*serriola*	Population created by crossing a BC1*serriola* x *L. serriola*
BC1S1*serriola*	Population created by selfing a BC1*serriola*
CGN	Accession ID from the Centre for Genetic Resources in the Netherlands
Bl:	*Bremia lactucae* isolate
EST	Expressed sequence tag
HN	Hybrid necrosis
LG	Linkage groups according to the Lettuce Version 3.2 Database from Lettuce Genome Resource (USDavis)
Chromosome	Chromosome according to the Wageningen genetic map
RIS	Relative infection severity
ADT	Adult plant Disease Test

Genetic nomenclature

a	Homozygous *L. sativa* or homozygous *L. serriola,* depending on the population
b	Homozygous *L. saligna*
h	Heterozygous
c	Genotype b or h
d	Genotype a or h
e	Genotype a or b
u	unknown genotype
n	Negative sample

1. Introduction

Downy mildew caused by the oomycete *Bremia lactucae* has a great scientific interest, because it is one of the most devastating plant pathogens of lettuce (*Lactuca sativa*). Nowadays lettuce varieties suffer from *B.* lactucae because of the rapid development of the pathogen against newly introduced resistance. In this MSc thesis, the focus is on finding new monogenic dominant *R*-genes against *B. lactucae* present in *Lactuca saligna* accessions, a wild relative of *L. sativa.*

1.1 Lettuce characteristics

Cultivated lettuce, *Lactuca sativa,* is one of the most important leafy crops in the world with a production worldwide of 25 million tons in 2013 (FAO 2013). Lettuce is grown in temperate and subtropical regions with major production area's in China, The United States, India and Europe (Figure 1.1). China itself has already more than half of the production of lettuce, mainly for stem production, whereas the western countries produce lettuce as a salad crop. There is a great diversity in colour, shape and leafsize between lettuce cultivars. Six generally recognized types are crisphead, butterhead, romaine, leaf, stem and Latin.

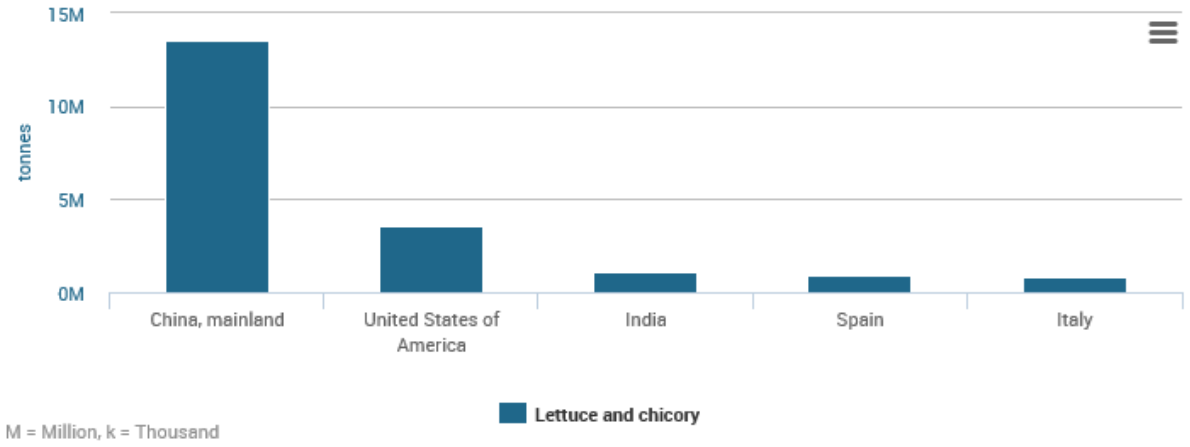


Figure 1.1: Top 5 lettuce producing countries worldwide (FAO, 2013)

Lettuce is a self-pollinating annual plant which is diploid with 18 chromosomes (2n=2x=18). Lettuce cultivars most likely originate from the wild species *Lactuca serriola* (Kesseli et al. 1991). It is thought that the domestication of lettuce started about 4500 years ago in the Middle East, because signs of early cultivation of lettuce have been found in ancient Egyptian tombs with wall paintings of what seems to be lettuce (Lindqvist 1960; De Vries 1997). Within the genus *Lactuca* there are wild species which can be interesting for breeding. *Lactuca* species can be divided into gene pools based on their crossability and fertility after crossing. In the primary gene pool, together with *L. sativa* cultivars and landraces are also *L. aculeata*, *L. altaica*, *L. azerbaijanica*, *L. dregeana*, *L. georgica*, *L.scarioloides* and *L. serriola* (Lebeda et al. 2006). Besides this primary gene pool there is interesting breeding material in the secondary gene pool with *L. saligna* and *L. virosa* as most interesting species because of their potential resistance against diseases like downy mildew. Both *L. saligna* and *L. virosa* can be crossed with *L. sativa*. However, often the offspring is often infertile and the cross *L. saligna* x *L. sativa* is only possible when using *L. saligna* as female parent (de Vries 1990).

Recently, the genome of *L. sativa* cv Salinas (2.7 Gb) has been sequenced (The Lettuce Genome Sequencing Consortium, unpublished data). Version 4 of the draft genome is publicly available (https://lgr.genomecenter.ucdavis.edu/Home.php). Furthermore an ultra-high-density, transcript-based, genetic map of lettuce was developed, consisting of 13.943 markers distributed over nine linkage groups representing the 9 chromosomes of lettuce (Truco et al. 2013)

1.2 *Bremia lactucae* an oomycete pathogen

The oomycete *Bremia lactucae* causes downy mildew in cultivated lettuce and in a wide range of other lettuce species. Susceptibility against downy mildew has been found in primary gene pool species *L. serriola, L. altaica* and *L. aculeata* as well as in *L. virosa* which belongs to the secondary gene pool (Bonnier et al. 1991; Lebeda and Zinkernagel 2003). This pathogen is obligate biotroph and belongs to

the Peronosporales. Oomycetes look like fungi, but they are related to brown algae. A famous other oomycete is *Phytophtora infestans*, the pathogen causing late blight in potato. *Bremia lactucae* is really a specialist which evolves extremely fast in the field.

B. lactucae is a devastating pathogen for lettuce cultivation. This highly specialized oomycete can infect the plant at every growth stage resulting in enormous yield losses. The appearance of new Bremia isolates goes rapidly. In the last 15 years, seven new Bremia isolates have been denominated (International Bremia Evaluation Board 2016). The appearance of new Bremia isolates goes quickly due to the high evolutionary potential of the pathogen. New found monogenic resistance genes in lettuce cultivars can be overcome within a few years after introduction due to both sexual and asexual reproduction, high recombination rate and huge population size (McDonald and Linde 2002). Also the use of new lettuce cultivars is a reason for this new genetic variation. The few isolates that can overcome the resistance grow exponentially and in no time the infection is spread all over the field. Within one field several *B. lactucae* isolates can be present. Even when a resistant cultivar is used in the field, the isolate which it is resistant against can still be present in the field (Van Hese et al. 2015).

Infection of *B. lactucae* can start already at seedling stage of the lettuce plant as a spore lands on the plant epidermis. Spores are dispersed by wind, rain or manually by contact resulting in immediately germination of the asexual spores. The only requirement for the spores to germinate is a humid environment with an optimal temperature between 10 and 20 °C (Su et al. 2004). These spores can directly penetrate the plants epidermis layer and do not need stomata to enter the host. After entering the cell a vesicle is formed followed by a second one. From here the oomycete grows in the intercellular space forming haustoria in the mesophyll and epidermal cells together with hyphae which penetrate the neighbouring cells (Lebeda et al. 2008).The symptoms start with yellow to green lesions on the leaves later resulting in necrotic spots. This whole process repeats itself already within 10 days as the second generation spores is already produced. Yellow/brown discolouring (necrosis) is visible on the upper side of the leave, while sporulation can be seen on the underside of the leave (Figure 1.2).

Figure 1.2: Lesions caused by downy mildew on upper leave (left) and sporulation of downy mildew on the underside of the leave (right) (UC Davis, http://www.ipm.ucdavis.edu/PMG/r441100411.html#SYMPTOMS)

Because of the great impact of downy mildew on lettuce cultivation a lot of research has been performed in the last years with respect to the pathogen. A total of 26.000 ESTs of *B. lactucae* were sequenced (Stassen et al. 2012). Recently the genome of *B. lactucae* has been sequenced using the isolate SF5 (Michelmore et al. 2012). This resulted in over 100 Mb mainly heterozygous genome with about 10.000 predicted gene models. Furthermore, an additional 47 *B. lactucae* isolates were sequenced and compared to the reference SF5 isolate (Gil 2015). In addition to this, next generation sequencing of several important downy mildews have been done (Derevnina 2015). This knowledge can help in understanding the biology of the pathogen and the host-pathogen interactions.

1.3 Plant immune system

First layer of defense

The first layer of defense is a preformed barrier to prevent a pathogen from infecting the plant. There is a morphological type of defense. Examples are leaf shape and size, trichomes on the leaves and stomata structure (Lebeda and Zinkernagel 2003). Furthermore plants can produce anti-microbial products and secondary metabolites to defend themselves against a wide range of pathogens (Figure 1.3).

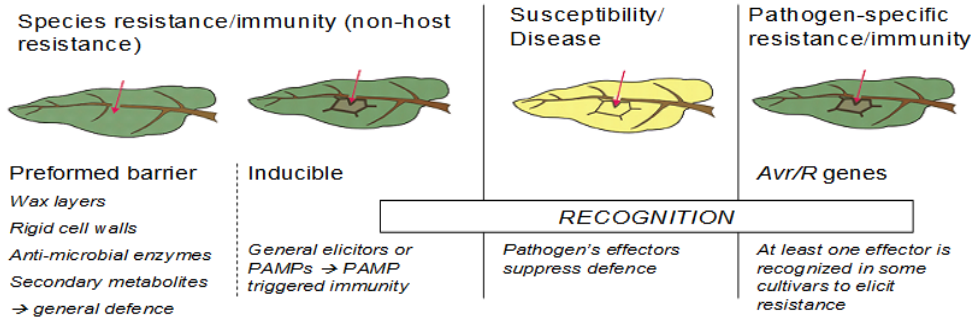


Figure 1.3: Major components of non-host resistance and host resistance (Nürnberger et al. 2004)

Second layer of defense

If pathogens can overcome this first barrier they have to deal with the second layer of defense. This type of defense is based on pathogen recognition which is well explained in the Zig Zag model (Figure 1.4)(Jones and Dangl 2006). First there is the recognition of Pathogen Associated Molecular Patterns (PAMPs) by Pattern Recognition receptors (PRRs). PAMPs are endogenous proteins specific for the type of pathogen. Examples of PAMPs are chitin which are related to fungi (Latgé 2010; Thomma et al. 2011) and bacterial flagellin (Zipfel et al. 2004). Recognition of PAMPS by the plant activates PAMP Triggered Immunity (PTI). Specialized molecules called pathogen effectors suppress these defence reactions and allow a successful infection. Plant proteins that detect and recognize these highly specific effectors are called R proteins. The recognition of these effectors which are then called Avirulence (Avr) proteins will induce effector triggered immunity (ETI) that leads to an hypersensitive response (HR) and thereby prevents further pathogen infection. Direct recognition between R and Avr genes has already been proven, but there are indications for an indirect recognition between R and Avr genes. The 'guard hypothesis' suggests that the R protein guards a pathogen virulence target, so if the effector interacts with this virulence target, the R protein is activated (Van Der Biezen and Jones 1998).

Qualitative resistance is based on the gene-for-gene interaction (Van Der Biezen and Jones 1998) in which one *R*-gene interacts with one Avr protein. This results in race specific resistance when the R-gene can recognize the Avr protein and results in complete susceptibility when there is no recognition between the *R*-gene and Avr protein. The vast majority of R genes encode for intracellular proteins with a nucleotide-binding site which is a highly conserved region and a C-terminal leucine–rich repeat domain, named NLR proteins (Ye and Ting 2008; Glowacki et al. 2011). The LRR domain is the most variable among R-proteins and considered to play a major role in pathogen recognition. There are NLR proteins which contain a Toll-like Interleukin Receptor (TIR) or a Coiled-Coiled (CC) domain (Meyers et al. 2003).

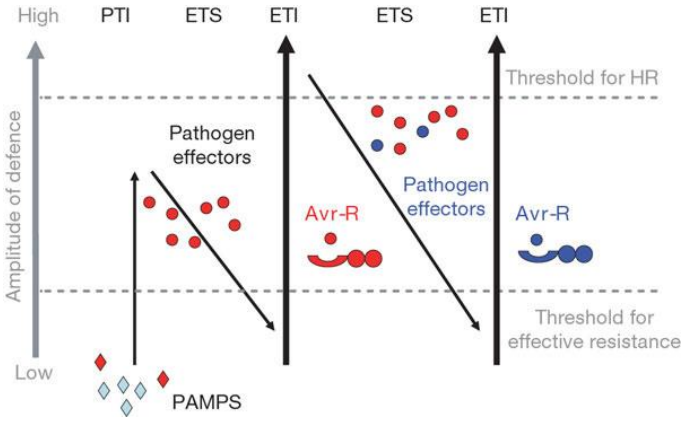


Figure 1.4: Zig-zag model of plant-pathogen interaction (Jones and Dangl 2006)

Non host resistance

Besides host resistance there is also non-host resistance. Non-host resistance means that the complete plant species is resistant to all different races of a pathogen (Niks 1988). This type of resistance is very common among species, because in general a plant is resistant to almost all pathogens. To study the inheritance of non-host resistance it is necessary to make a interspecific hybrid which is often very difficult. A good working example is the *Lactuca-Bremia* interaction (Jeuken et al. 2008). From studies it turned out that the non-host status is polygenic and based on a number of quantitative trait loci (QTLs) with epistatic effects (Jeuken and Lindhout 2002; Zhang et al. 2009; den Boer et al. 2014). So far, attempts to unravel the mechanism behind non-host resistance failed, but it is hypothesised that besides the preformed barrier non-host resistance is based on the same three major components driving host immunity namely, Pattern Recognition receptors (PRRs), NLR proteins and pathogen effectors (Schulze-Lefert and Panstruga 2011). Finding the underlying mechanism(s) would be an enormous step in plant breeding and the fight against pathogens.

1.4 Resistance against *Bremia lactucae*

Terminology	
NLR-gene :	Gene with a nucleotide-binding site and a leucine-rich repeat
R-gene:	Plant gene which gives resistance when recognizing a pathogen *Avr*-gene
Avr-gene:	Pathogen gene which can be recognized by the plant's *R*-gene.
Dm–gene:	resistance gene against downy mildew
MRC:	Major Resistance Cluster

Resistance against *B. lactucae* has been found in both cultivated lettuce as in related *Lactuca* species. The most common type of resistance used in lettuce breeding is based on qualitative resistance. This gene-for-gene based resistance causes a hypersensitive response resulting in resistance which is race specific. This resistance is of short durability due to the use of agricultural methods like monocultures and intensive agriculture. There are already over 30 dominant resistant *Dm* genes identified in lettuce against *B. lactucae* (Michelmore and Wong 2008; Simko et al. 2015).One of these genes, *Dm3* has been cloned and contains a nucleotide-binding site and a leucine-rich repeat domain (Meyers et al. 1998) Recently, potential genes with a NLR coding sequences in the *L. sativa* genome have been identified resulting in a physical map of major resistance clusters (MRCs) of potential (NLR) and already known R-genes (assigned as *Dm*-genes) (Christopoulou, Wo, et al. 2015) (Figure 1.5). Half of all found *Dm* genes are mapped on MRC2. Most of the remaining *Dm* genes have been located at MRCs on chromosome one or four (Christopoulou, McHale, et al. 2015). Lettuce breeders still use *R*-gene based resistance in their programs, but because of the rapid evolution of new *B. lactucae* strains, the need for a more durable type of resistance is desirable.

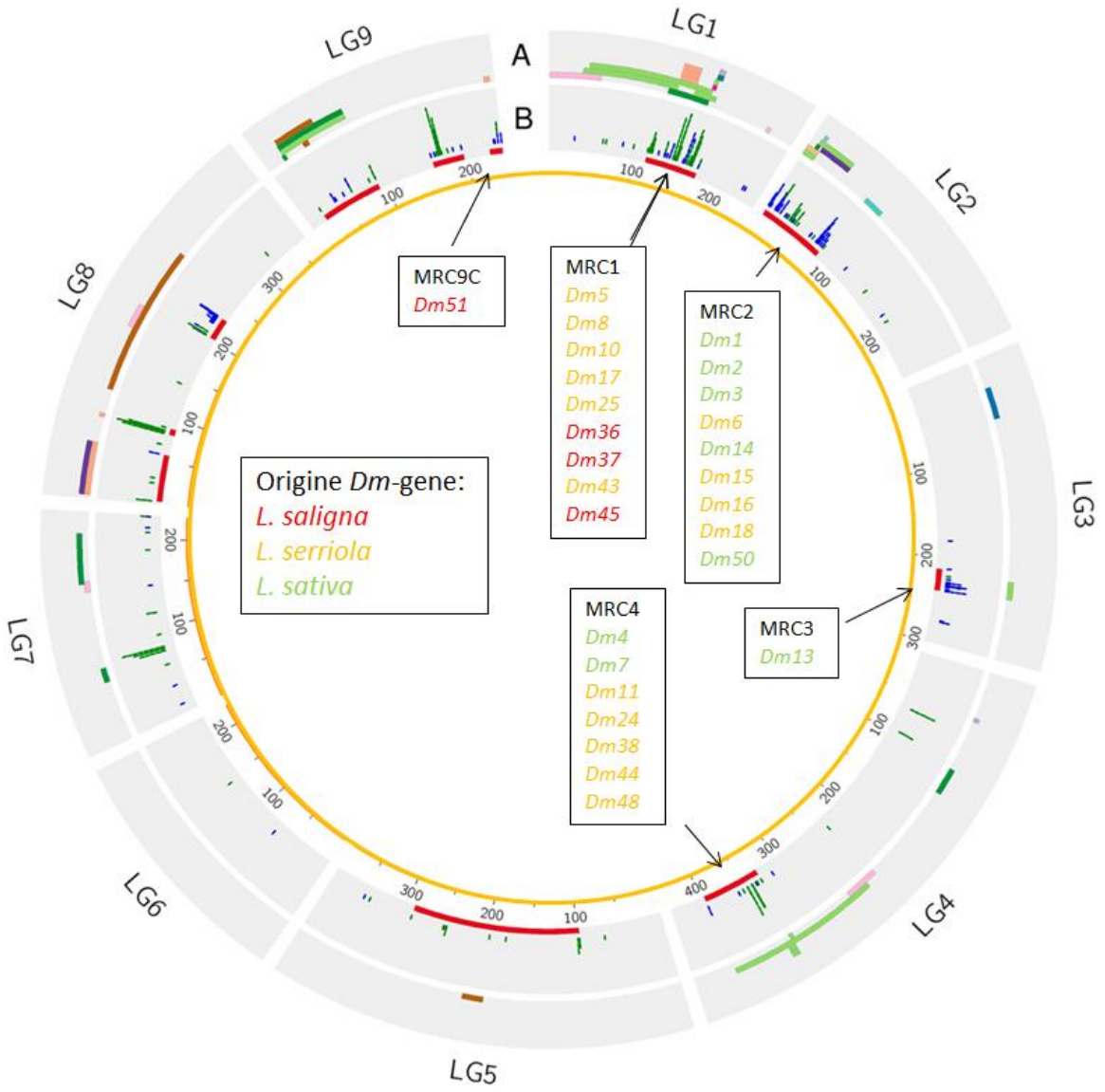


Figure 1.5: Genomic distribution of candidate genes involved in disease resistance for all nine linkage groups in *L. sativa* cv Salinas (assembly version Lsat_1_v6_lg) together with the already identified *Dm*-genes and their origin. Track B contains the NLR-encoding genes. The red bars represent the Major Resistance clusters (MRC). MRC2 contains the cloned *Dm3* gene (Christopoulou, Wo, et al. 2015).

Non-host resistance as mentioned in paragraph 1.3 is promising as a new source of resistance. Different *Lactuca* species were tested for their resistance against downy mildew and it turned out that only *Lactuca saligna* had the non-host status. A total of 52 *L. saligna* accessions gave total resistance when tested to 20 different *B. lactucae* isolates (Bonnier et al. 1991). Additional research was done with more *L. saligna* accessions and it turned out that in a few cases little infection of about 5% was observed in seedling stage to highly virulent isolates of *B. lactucae* (Petrželová et al. 2011). This however disappeared in adult plant stage indicating that the status of non-host is still valid. The last 20 years a lot of research was done in order to find out the mechanism behind this non-host resistance. An interspecific F2 population between *L. saligna* CGN05271 x *L. sativa* cv. Olof was the starting point to study the inheritance of non-host resistance. So far, no functional *Dm* genes are known in cultivar Olof which makes it an ideal candidate for studying the genetic inheritance of non-host resistance. The first genetic map of lettuce with 9 linkage groups was made combining phenotypic and genotypic data from this F2 population. Using this genetic map, the first QTLs for resistance were found (Jeuken and Lindhout 2002). For further investigation of non-host resistance a set of 28 Backcross Inbred Lines (BILs) was developed (Jeuken and Lindhout 2004). These BILs contained a *L. sativa* background with a mostly single homozygous introgression of *L. saligna*. In a few cases a BIL had two *L. saligna* introgressions or a single heterozygous introgression. After a disease test on the F2 population a continuous range of infection

levels was observed and three minor QTLs related to resistance were identified (Jeuken and Lindhout 2002). In 15 of the BIL lines also quantitative resistance was observed against seven Bremia races and assumed to be race non-specific. A total of 16 QTLs related to resistance were found of which 15 originated from *L. saligna* and one from *L. sativa* (Zhang et al. 2009).

Besides non-host resistance another phenomenon was observed when *L. saligna* and *L. sativa* were crossed, namely hybrid necrosis. Hybrid necrosis is a postzygotic genetic incompatibility which can be observed as necrotic lesions on the leafs of a plant. The observed hybrid necrosis in the interspecific cross between *L. saligna* and *L. sativa* turned out to be a digenic interaction between a possible R-gene from cultivar Olof on chromosome 6 and a *Rin4* allele from *L. saligna* CGN05271 on chromosome 9 (Jeuken et al. 2009). Besides the quantitative autoimmunity reaction also race-specific resistance (against Bl:16 and Bl:24) was explained by the same digenic interaction (Table 1.1). Because progenies of interspecific crosses are studied in this thesis, hybrid necrosis is a factor to be aware of.

Table 1.1 hybrid necrosis and race specificity in the cross *L. sativa* cv Olof x *L. saligna* CGN05271. Genotypes are homozygous *L. sativa* (a), homozygous *L. saligna* (b) and heterozygous (h). Hybrid necrosis levels are on macroscopic level.

genotype			Resistance levels			
C6	C9	Hybrid necrosis level	Bl:24	Bl:16	Bl:14	Bl:21
h	h	no	R	R	S	S
a	a	no	S	S	S	S
a	h	severe	R	R	IR	IR
a	b	lethal	n.d.	n.d.	n.d.	n.d.
h	a	no	S	S	S	S
h	b	low	R	R	IR	IR
b	h	no	S	S	S	S
b	a	no	S	S	S	S
b	b	no	S	S	S	S

R=resistant; S=susceptible; IR=intermediate resistant; n.d.= not determined

L. saligna is not only interesting for its non-host status, but it is also a good new source for dominant monogenic R-genes. So far *Dm36* (MRC1), *Dm37* (MRC1),, *Dm45* (MRC1) and *Dm51* (MRC9C) all originated from *L. saligna* accessions (Parra et al. 2016 unpublished manuscript). Because the non-host resistance mechanism is still not unravelled, qualitative resistant genes are still the major source of resistance in lettuce cultivars. In this thesis the aim is to characterize and map qualitative resistance in different *L. saligna* accessions against *B. lactucae*.

1.5 Thesis outline

This thesis is divided in three parts. The first part is about the development of new primers, the second part is the dissection of resistance against downy mildew in three *L. saligna* accessions and the third part is about investigating a possible second *R*-gene in *L. saligna*CGN5947.

Marker development

Terminology	
Primer pair:	Short sequences of DNA that are complementary to a specific DNA sequence . These primer pairs are used in a PCR reaction to copy that specific DNA sequence.
Polymorphism:	The presence of genetic variation within a population.
Marker:	A fragment of DNA that is associated with a certain location within the genome.

The first part is the development of new markers using a physical and genetic map of lettuce. Primers will be tested on a set of reference lines and later on validated and mapped in the F2_1997 genetic map (Jeuken et al. 2001) based on a cross between *L. saligna* CGN05271 x *L. sativa* cv Olof (Figure 1.6). The aim is to create markers which can be integrated into the F2_1997 genetic map. These markers will be developed at loci which co-localize with MRCs in *L. sativa* cv Salinas or at places which have a low marker density at the moment. Some of the markers will be used to map possible R-genes. Furthermore, the newly designed markers can be used in other experiments in the future.

Research questions for this first part of the thesis are:

1. On which part of the genome will the primers be developed?
2. Do the pcr-products show different melting curves for the selected reference lines? YES= polymorphism=marker. NO= no polymorphism=no marker
3. Which markers can be mapped in the F2_1997 genetic map?
4. In which genotypic groups can the F2 population be divided on the HRM analysis for the different markers?
5. On which position on the F2_1997 genetic map are the markers mapped?

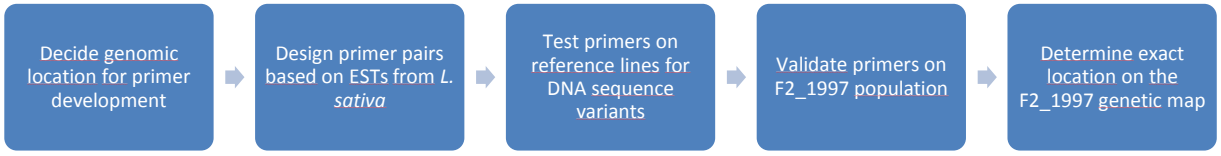


Figure 1.6: General workflow for marker development into the F2_1997 genetic map.

Dissecting the resistance against downy mildew in Lactuca saligna accessions CGN15726, CGN15699 and CGN13330 by observing the distribution and segregation of resistance levels in BC1-populations

In the second experiment the main objective is to identify, characterize resistances in three *L. saligna* accessions, that have not been studied earlier. In general it is expected that there will be non-host resistance present in the *L. saligna* accessions with the possibility to also have host resistance. In order to get insight in the genetics of the resistance, segregating populations were studied. In 2014 crosses between a resistant *L. saligna* accession and a susceptible *L. sativa* accession (or susceptible *L. serriola)* were made by Marieke. F1 plants were backcrossed to the susceptible parent, resulting in three BC1 populations. One population was made by crossing a Russian *L. saligna* accession CGN15726 with *L. sativa* cv. Olof. The second population was made by crossing a Turkish *L. saligna* accession CGN15699 with the *L. serriola* accession CGN04774 and the third BC1 population was made by crossing another Turkish *L. saligna* accession CGN13330 with *L. sativa* cv. Olof (Figure 1.7).

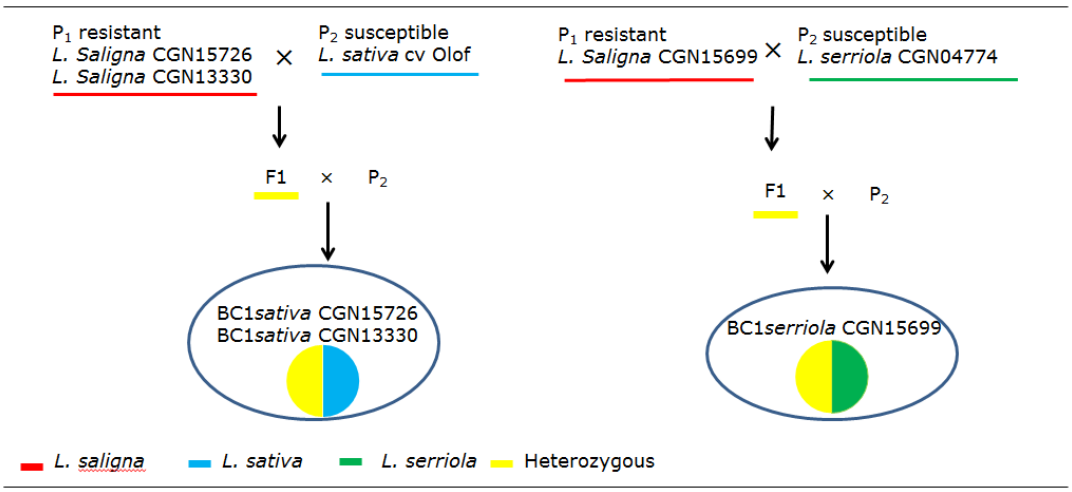


Figure 1.7: Crossing scheme for obtaining BC1 populations. *L. saligna* accessions CGN15726 and CGN13330 are crossed with *L. sativa* cv Olof whereas *L. saligna* accession CGN15699 was crossed with *L. serriola* CGN04774.

Theoretically dominant monogenic R-genes show an expected ratio of 1:1 between resistant vs susceptible in a BC1 population following a normal mendelian segregation. To distinguish between resistance and susceptibility there is an Relative Infection Severity (RIS) threshold of 10%. Plants having a RIS of 10% or lower are called resistant and plants with a RIS higher than 10% are susceptible.

Definition BC1 populations	
BC1*sat*_05271_FR:	BC1 population made from a cross between *L. saligna* accession CGN05271 from France with *L. sativa* cv. Olof which was once backcrosses with *L. sativa* cv. Olof
BC1*sat*_5304_ISR:	BC1 population made from a cross between *L. saligna* accession CGN5304 from Israel with *L. sativa* cv. Olof which was once backcrosses with *L. sativa* cv. Olof
BC1*sat*_15705_GEO:	BC1 population made from a cross between *L. saligna* accession CGN15705 from Georgia with *L. sativa* cv. Olof which was once backcrosses with *L. sativa* cv. Olof

Based on previous research in BC1 populations there are different scenarios which can be expected. The first scenario is highlighted in Figure 1.8A. Resistance of the BC1*sat*_05271_FR plants was tested with Bremia isolate Bl:21. Only 8% of plants are in the resistant class of 10 ISL and the rest of the plants show a continuous range over the other ISL classes suggesting the resistance is not due to a dominant R-gene, but is due to non-host resistance. Genotyping the plants resulted not in an association between a heterozygous genotype and the observed resistant phenotype. Therefore it is suggested that the resistance is based on different QTLs which might represent the non-host resistance.

In Figure 1.8B, BC1*sat*_5304_ISR was tested for resistance against Bl:21. This resulted in 55% of the plants being resistant. The other 45% of the plants showed continuous levels of infection. After genotyping most of the resistant plants (87% of 55%= 48% of total plants) were associated with a heterozygous genotype at one locus. For the remaining 7% plants that were resistant, no association between a heterozygous genotype at a specific locus and the resistant phenotype could be found. These results indicated a dominant resistance locus on top of chromosome 2. The 7% plants without the resistance locus on the top of chromosome 2 were assumed as plants with non-host resistance.

The third scenario is shown in Figure 1.8C where BC1*sat*_15705_GEO was tested for resistance against BL:24. After the disease test, 29% of the plants were resistant. This is unexpected because the BC1 population with only non-host resistance and no functional R-gene showed about 8% resistant plants and the population with a functional R-gene showed a little above 50% resistant plants. After genotyping it turned out that the resistance is based on a dominant R-gene. However, due to distorted segregation of this specific genomic regions the allele of the *L. sativa* parent is overrepresented on the locus were the R-gene is located resulting in less than 50% resistant plants (expected under mendelian segregation). Besides these known possible scenarios there is always a possibility of a new scenario in one of the three populations which will be tested in this thesis.

Several BC1 populations have been tested for disease resistance against Bremia and in populations where no dominant monogenic R-gene was present against a certain isolate the percentage of resistant plants which are presumed to have the non-host status is between 7-13%.

Furthermore, there is a general interest in the phenomenon of hybrid necrosis if this can be observed in the three BC1 populations. If hybrid necrosis is present the aim is to find out which loci and mechanism causes this hybrid necrosis. Is it the same interaction as studied in *L. saligna* CGN05271 or are different genes involved?

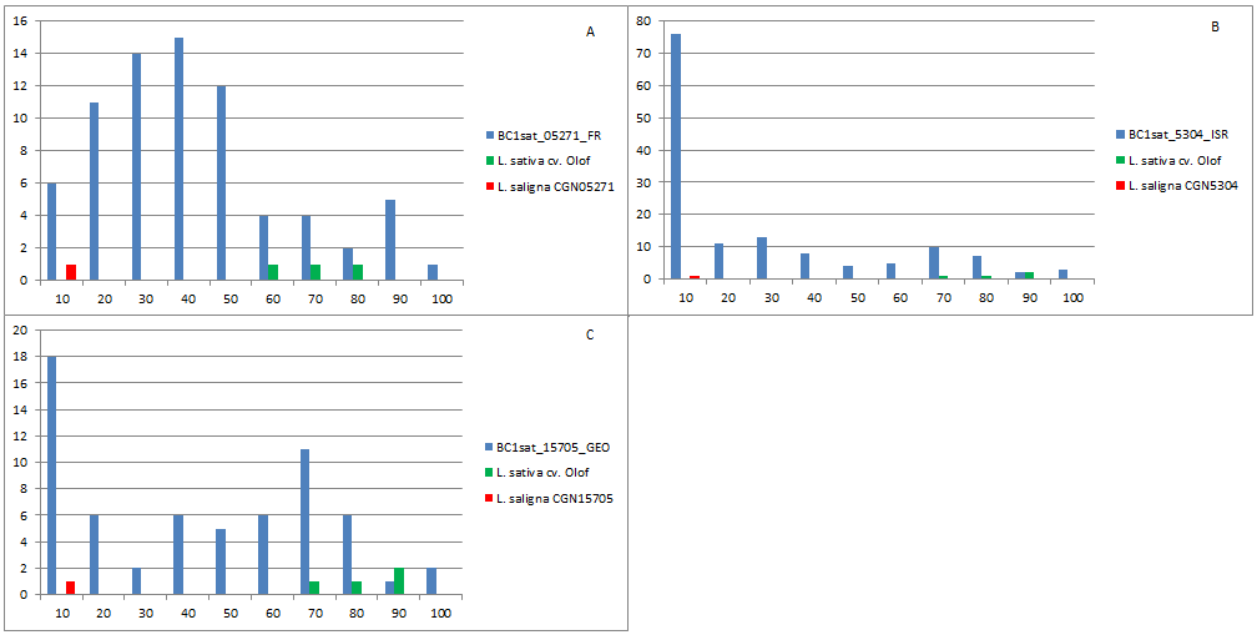


Figure 1.8: Three scenario's where BC1 populations were tested for downy mildew resistance. In all three scenarios the BC1 plants (Blue), the susceptible control *L. sativa* cv Olof (Green) and the resistant *L. saligna* parent are showed (Red). BC1*sat*_05271_FR tested with Bl:21 with non-host resistance (A), BC1*sat*_5304_ISR tested with Bl:21 resulting in host resistance with a mendelian segregation + non-host resistance (B) and BC1*sat*_15705_GEO tested with Bl:24 resulting in host resistance due to distorted segregation of the parents + non-host resistance (C).

Research questions for this part of the thesis are:

1. How does the population BC1*sativa*_CGN15726, BC1*sativa*_CGN13330 and BC1*serriola*_CGN15699 segregate for the different infection classes?
2. Does the segregation coincide with one of the known scenarios described in Figure 7?
3. What underlying resistance type might explain the segregation of the different BC1 populations?
4. Is it possible to determine a mapping interval for an identified monogenic resistance and what is this mapping interval?
5. Where on the genome is this resistance mapping interval located and does this co-locate with one of the known Major Resistance Clusters in *L. sativa*?
6. If hybrid necrosis is observed in the BC1 populations: Are the same genes involved as has been studied in *L. saligna*_CGN5271 x *L. sativa* cv. Olof?
7. If this is not the same interaction: on which part of the genome can this than be mapped?

The general workflow for this experiment is to first do an adult disease test for downy mildew resistance on the BC1 populations, characterize the segregation in the different infection classes and if possible link phenotype to genotype and create a mapping interval. For a BC1 population without a dominant monogenic R-gene this is the end step. For a BC1 population with a possible dominant monogenic R-gen the second step is to validate the previously found mapping interval by using a BC2 population. If this mapping interval can be confirmed in the BC2 population the last step is to fine-map the resistance by using BC2 recombinants in the mapping interval (Figure 1.9). In order to make the mapping interval smaller, new markers will be developed within that mapping interval.

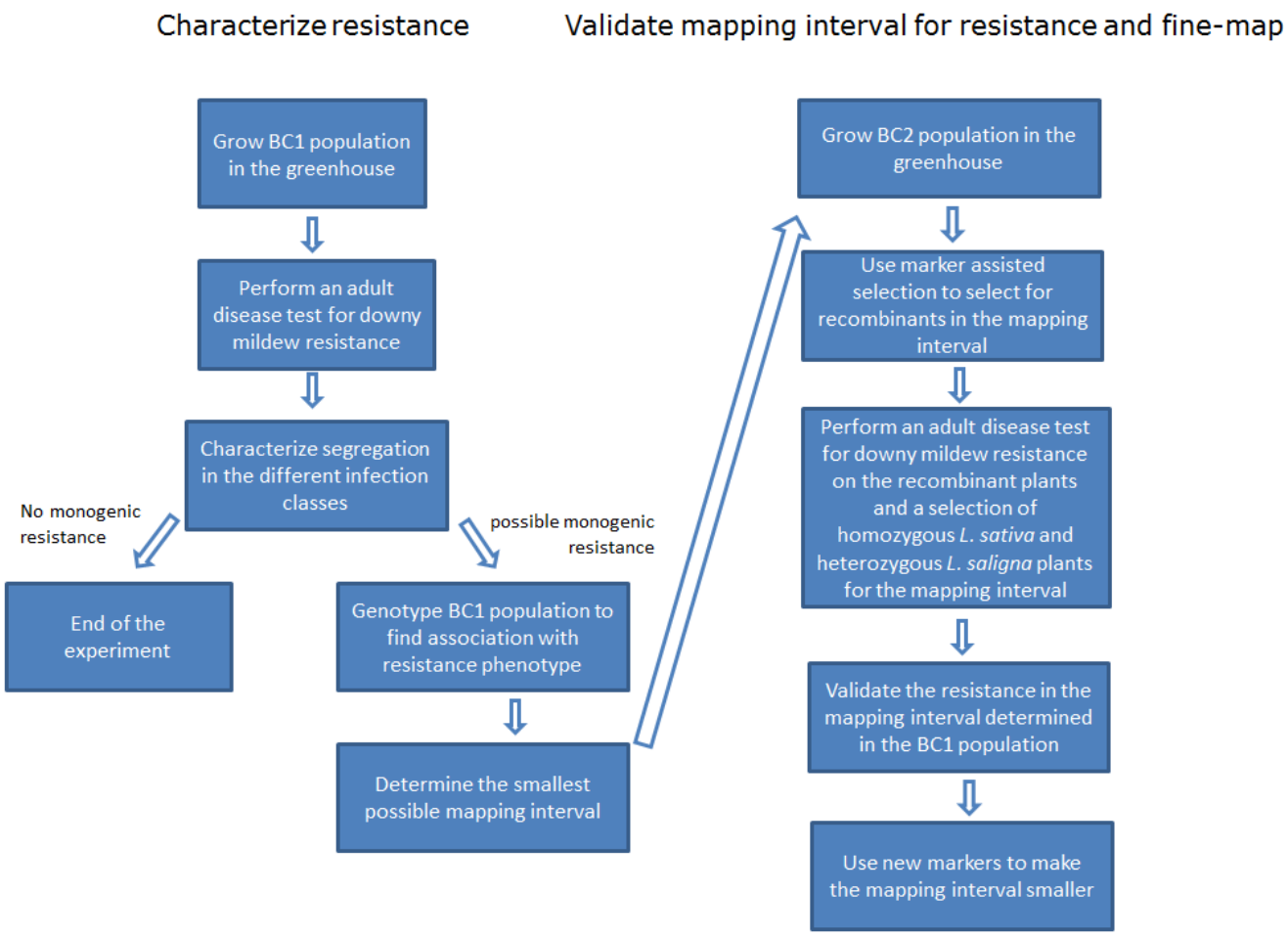


Figure 1.9: Workflow to characterize (Left) and validate (Right) resistance in BC1sativa_CGN15726, BC1sativa_CGN13330 and BC1serriola_CGN15699.

Investigate possible second dominant R-gene in BC1sativa_CGN5947

The last part of this thesis is further investigation of resistance in BC1*sativa*_CGN5947. This population was used in a effector response experiment resulting in 73% of the plants being resistant. 44% could be explained by the BLR31 effector response which was linked to a monogenic dominant R-gene on C2. This leaves 29% unexplained. Like in other BC1*sativa* populations, non-host resistance could also explain the resistant plants. Based on the BC1*sat*_05271_FR population the percentage of BC1-plants containing non-host resistance is around 8%, which is much lower than the 29% of plants which are resistant in this population. Therefore, the hypothesis is that there is a second monogenic dominant resistance gene responsible for the observed resistance. The aim of this experiment is to investigate whether there is a second dominant resistance gene responsible for the observed resistance in the 29% which don't have the R-gene on C2 and where this R-gene is located on the genome (Figure 1.10)? The workflow is similar to the previous experiment (Figure 1.9), although the disease test already has been performed.

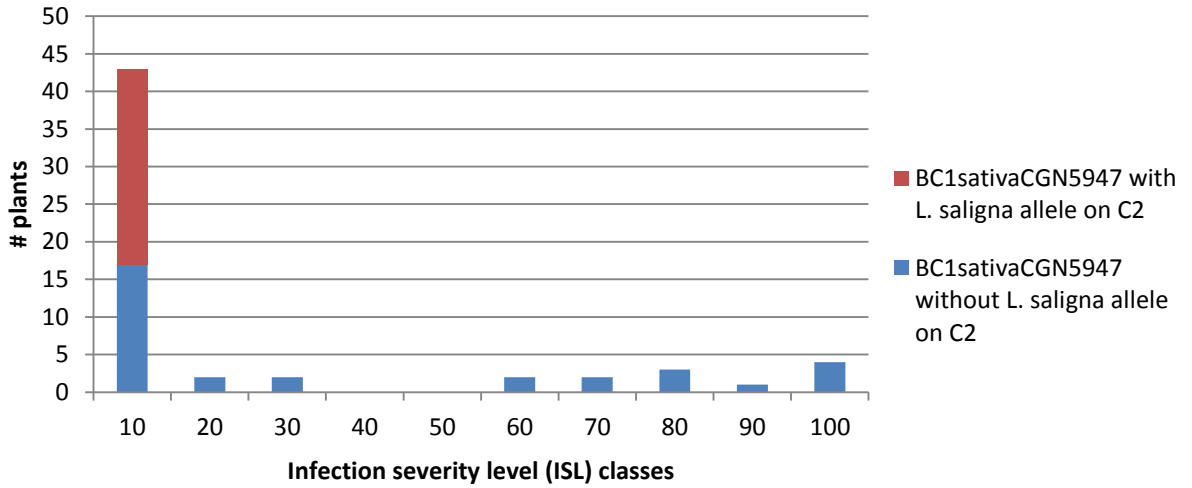


Figure 1.10: Segregation in infection classes of the BC1*sativa*_CGN5947 population separated in plants carrying a *L. saligna* allele on chromosome 2 (C2) and plants without a *L. saligna* allele on C2 .

The research question for this experiment is:

1. Is it possible to determine a mapping interval for monogenic resistance for the plants which don't have the resistance on chromosome 2 and what is this mapping interval?
2. does the mapping interval co-locate with one of the known Major Resistance Clusters in *L. sativa*?

2. Material and Methods

2.1 Marker development

2.1.1 Primer selection

Primer pairs were designed on Expressed Sequence Tags (EST) at chromosome 7 of the *L. sativa* reference genome. These ESTs were picked randomly around 50, 135 and 225 Mb of chromosome 7 using EST data from Marieke Jeuken and per EST two primer pairs were designed. The Lettuce GBrowse database which is normally used to pick primers was offline at that moment and could not be used. EST names were provided by Marieke Jeuken and NCBI was used to find the corresponding sequence of the ESTs selected. The sequence found in NCBI was used in the Primer3Plus tool (Untergasser et al. 2007) to design the primers within the EST. The product size range was set to 200-300 bp in the Primer3Plus tool together with the standard settings.

A second set of primers was designed on ESTs at 300, 313, 330 and 350 Mb on chromosome 4 of the *L. sativa* reference genome. This time the GBrowse database was back online and the primers were designed at exons with an intron in between with a product size of 300-500 bp. Two primer pairs were designed per EST with a total of four ESTs for each genomic position.

2.1.2 High Resolution Melting analysis

High Resolution Melting curve (HRM) analysis was used to test the designed primers. HRM analysis is based on the binding of a fluorescent dye to double-stranded DNA. During a PCR reaction the fluorescent dye binds to the double-stranded DNA and after amplification there is a high level of fluorescence in the sample. In the lightscanner, the samples are heated and the two strands of the DNA will be separated and loose there fluorescence. DNA sequence variants between different samples will be visible with this technique, because the melting curve is different when there is variation in the DNA sequence. In this case LCGreen plus has been used as fluorescent dye. If there are DNA sequence variants between genotypes this will become visible after the HRM analysis (Figure 2.1).

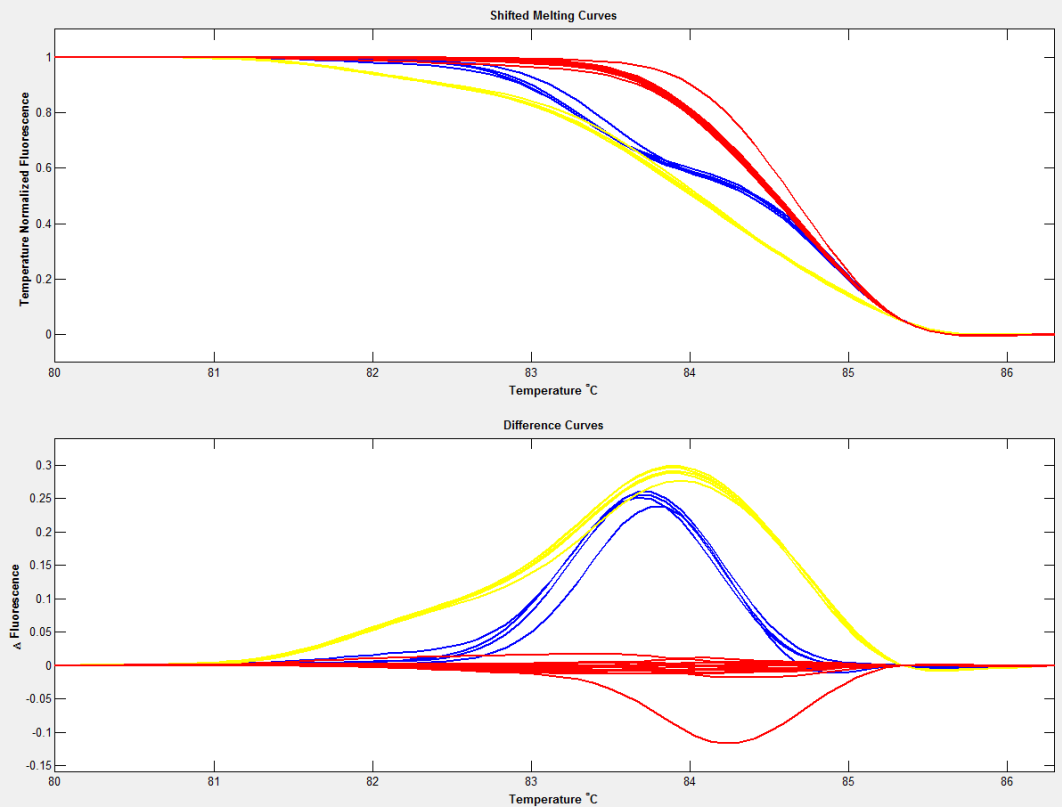


Figure 2.1:example of a light scanner result where the blue and red lines are the two homozygous genotypes and the yellow line is the heterozygous genotype.

2.1.3 Primer testing

The designed primers were tested for DNA sequence variants on several reference lines. *L. sativa* cv Olof with four biological replicates and *L. saligna* accessions CGN05271, CGN15705, CGN15726, CGN20697 and CGN05310 with each three biological replicates. Furthermore, hybrid F1_5271 with one biological replicate and hybrids F1_15705 and F1_5304 with two biological replicates were added to the reference lines.

Testing of the primers was done by first preparing the mastermix containing the fluorescent dye LC Green Plus to bind at the double-stranded DNA and amplifying the DNA (Table 2.1). After amplification, a HRM analysis with the LightScanner® System (Idoha Technology, Salt Lake City, UT, USA) was used to visualize whether there were DNA sequence variants between the different genotypes. If the PCR products showed different melting curves there was a sequence variant between the samples and this primer pair was further tested on the F2_1997_FR population. If the PCR products showed no different melting curves it means there were no sequence variants between de different samples so the designed primer was not useful and was discarded.

The next step was to test if the primers could be mapped in a segregating population. If this was indeed possible the primer pair could be used as a marker. The F2_1997_FR population made from a cross between *L. saligna* CGN05271 x *L. sativa* cv Olof was used to map newly designed markers. This F2_1997_FR population consist of 126 lines and the designed primers which were selected for mapping were again tested with the HRM analysis, but this time on the 126 lines of the F2 population. As references in the HRM analysis *L. saligna* CGN05271, *L. sativa* cv Olof and the F1 of these lines were used.

Mapping of the new markers on the genetic map was done by hand. The genotype of all the 126 lines for the markers was compared with the with the already existing genotypes of previous mapped markers. The mapped position was the position were the smallest amount of recombination occurred between the new marker and the flanking old markers.

Table 2.1: Mastermix and PCR program

Mastermix per sample		**PCR program**		
Components	Volume (µl)	Temperature (°C)	Time (s)	
MilliQ	5.45	98.0°C	30	
5x reaction buffer	2	98.0°C	10	40x
dNTP 5 mM	0.4	60.0°C	10	
Forward primer 100 mM	0.025	72.0°C	30	
Reverse primer 100 mM	0.025	72.0°C	30	
10x LC Green™ Plus+	1	94.0°C	30	
Phire® Hot Start II	0.1	25.0°C	30	
DNA 10 ng[a]	1	10.0°C	∞	
Total	10			

2.2 Resistance in *L. saligna* CGN15726, *L. saligna* CGN15699 and *L. saligna* CGN13330

2.2.1 Plant material and growing conditions

In order to investigate host and non-host resistance, three BC1 populations were made. The population of BC1sativa_CGN15726 consisted of 85 seeds from five plants. The second population BC1serriola_CGN15699 consisted of 72 seeds obtained from two plants. The third population, BC1sativa_CGN13330 had a total of 88 seeds obtained from three plants. In addition the two parental lines were included for each population together with several controls. *L. sativa* cv. Olof and *L. sativa* cv. Cobham Green were used as susceptible controls, dBIL468 and *L. sativa* cv. Iceberg were partial resistance controls with low infection and BIL8.2 was the partial resistance control with intermediate infection (Table 2.2).

Table 2.2: Number of seeds available for the three BC1 populations together with the control lines used in the experiments and their expected ISL values.

population	# seeds	accession	control	Expected ISL*
BC1*sativa*_CGN15726	85	*L. sativa* cv. Olof	susceptible	51-92%
BC1*serriola*_CGN15699	72	*L. serriola* parent	susceptible	Unknown
BC1*sativa*_CGN13330	88	*L. sativa* cv. Cobham Green	susceptible	38-93%
		BIL8.2	partial resistance with intermediate infection	10-70%
		L. sativa cv. Iceberg	partial resistance with low infection	0-45%
		dBIL468	partial resistance with low infection	0-35%
		L. saligna parent	resistant	0%

*Based on collected data from adult disease tests by Erik den Boer (Boer 2014).

Seeds were sown in 40 well trays and stored for 2 days in a cold room at 4 °C. For all controls 2 seeds per pot were used with for each control 4 pots. After two days the pots were translocated to a greenhouse where the seeds germinated and were growing in a controlled greenhouse with a day/night temperature of 18/15°C and 16 hours of light. Leaf samples from the first leaf were obtained in duplo around 14 days after sowing and stored at -80°C for further analysis after the disease test. The plants were transplanted in 14 cm pots 17 days after sowing and stayed in the greenhouse till after the disease test was performed.

2.2.2 Phenotyping and disease test

An Adult disease test (ADT) was performed on 35 days old plants of populations BC1sativa_CGN15726 and BC1serriola_CGN15699 according to the General protocol for disease test with downy mildew on leaf pieces of lettuce (appendix 1). The experimental design consisted of 4 replicates of each plant divided over 5 boxes with space for 130 leaf pieces. Four leaf pieces of each control were also added to each box. Furthermore, a similar ADT with the same replicate number for the population and the controls was performed on BC1sativa_CGN13330. The experimental design consisted now on four boxes with a total space of 108 samples. *B. lactucae* isolate Bl:21 was used and the leaf pieces were inoculated with a concentration of 2 to $4x10^5$ spores/ml. After inoculation the boxes were placed in the climate room at 15°C and 16/8 day and night rhythm. Leaf pieces were scored for infection at 9 and 10 days post inoculation (dpi). After performing the disease test the data was analysed.

Absolute infection scores were transformed into Relative infection scores (RIS). This was done by using *L. sativa* cv. Olof or *L. serriola* CGN04774 as reference and set this to 100%. The rest of the scores were adjusted to this new standard resulting in a better estimation of resistance. In order to give reasonable interpretation for the infection levels in the BC1 plants, the several control accessions are added. In the disease tests the ISL of susceptible accession *L. sativa* cv. Olof should be between 51-92%, for *L. sativa* cv Cobham Green this is more a less the same with 38-93. Furthermore, the ISL of accession BIL8.2 which is partial resistant with intermediate infection severity should be in the interval of 10-70%. The

two controls which are partial resistant with low ISL are *L. sativa* cv Olof and dBIL468 with an ISL of 0-45% and 0-35% respectively. Lastly, the resistant. *L. saligna* parent should be completely resistant. It is preferred that BC1 plants with a ISL lower than the controls *L. sativa* cv. Iceberg and dBIL468 are selected. This comes down to a RIS<10%. Therefore, plants with a RIS<10% were called resistant and plant with a RIS>10% were considered as nonresistant and could be susceptible but with varying levels.

2.2.3 DNA-isolation

For DNA isolation of the collected leaf samples, a NaOH method was used (Wang et al. 1993). This is a quick method to isolate DNA from leaf samples, but the DNA will degrade after time. In this method, 20 µl NaOH 0,5M was added to 0.5 cm^2 leaf samples and then shaked for 5 minutes with a Tissue striker apparatus (KisanBiotech) at full speed. Than 20 µl Tris 100mM was added to the samples and this was spinned down. Finally, 5 µl was pipetted to another tube with 200 µl Tris 100mM and mixed.

2.2.4 Genotyping

Starting genotyping was based on the outcome of the RIS spectrum of each population. Using the three previous explained scenarios (Figure 1.8) it was decided whether or not monogenic resistance could be present in the population or not. Only if the population had indications for dominant monogenic resistance, the population was genotyped. This genotyping was done with the High Resolution Melting analysis which is the same method used in paragraph 2.1. BC1serriola_CGN15699 was genotyped for markers which were also used to map hybrid necrosis in the research of Marieke Jeuken (Jeuken et al. 2009) Furthermore this population was genotyped with markers based on known MRCs in *L. sativa* cv Salinas (Christopoulou, Wo, et al. 2015). Depending on the size of a MRC, two or three markers were selected to cover the whole MRC.

2.2.5 Data analysis

Based on the segregation of genotypes in the resistant (RIS < 10%) and susceptible (RIS > 10%) group of plants it was determined whether or not a marker was associated with resistance. For marker at the genetic location where there was association between genotype and phenotype, more markers were tested to come to a mapping interval for this resistance. This mapping interval was based on recombinant plants in the susceptible group. In BC1serriola_CGN15699 not only resistance based on a monogenic dominant R-gene was analyzed, but also the digenic interaction causing hybrid necrosis was analyzed. Both the marker results for resistant as the combined genotype causing hybrid necrosis were compared with the phenotypic result to determine whether the resistance is caused by a monogenic dominant R-gene or caused by Race specific resistant due to hybrid incompatibility.

2.2.6 Validation resistance

In order to validate the resistance in BC1*serriola*CGN15699, two different populations were made. A BC1S1*serriola*CGN15699 population was made by selfing a BC1*serriola*CGN15699 plant with a C6hC9h genotype for hybrid necrosis and heterozygous (h) genotype for the determined mapping interval. This new population was used to test whether or not the resistance is based on race specificity caused by hybrid incompatibility or not. A BC2*serriola*CGN15699 population was made from resistant plants with the complete heterozygous introgression in the mapping interval to validate and fine-map the determined mapping interval found in the BC1*serriola*CGN15699 population if it turned out that the resistance was not involved in hybrid incompatibility.

140 seeds from the new BC1S1*serriola*CGN15699 population and 80 BC2*serriola*CGN15699 seeds were sown and grown as described in paragraph 2.2.1. Leaf samples were taken 17 and 18 days after sowing for the BC2*serriola*CGN15699 and BC1S1*serriola*CGN15699 populations respectively. DNA isolation from these leaf samples was done using the NaOH method described in paragraph 2.2.3. The next step was to do marker assisted selection on the plants to select plant with the desired genotype. BC1S1*serriola*CGN15699 were genotyped with the same markers used to genotype hybrid necrosis in the BC1*serriola*CGN15699 population. From each genotype, three plants were selected if possible. Furthermore, markers within the mapping interval were used to screen for recombinants and these plants were also selected. The BC2*serriola*CGN15699 population was also genotyped to select only plants without hybrid necrosis and plants with recombination in the determined mapping interval in the BC1*serriola*CGN15699.

With the selected plants from populations BC1S1serriola_CGN15699 and BC2serriolaCGN15699 an ADT was performed with Bl:21 on 31 days old plants according to the General protocol for disease test with downy mildew on leaf pieces of lettuce (appendix 1). Infection was scored at 8, 9, 10 and 14 dpi.

2.3 Resistance in *L. saligna* CGN5947

2.3.1 Plant material

Plants of this experiment were from a cross between *L. saligna* CGN5947 and *L. sativa* cv Olof which was then backcrossed to *L. sativa* cv Olof to obtain a BC1 population. An effector response experiment was done on this population by Anne Giesbers resulting in the detection of a monogenic dominant R-gene at chromosome 2. The plants without the effector response/R-gene were genotyped more extensively in this experiment. A disease test was already performed with Bl:24 according to the general protocol for disease test with downy mildew on leaf pieces of lettuce by Anne Giesbers.

2.3.2 Genotyping

The genotyping was done with markers located in/bordering MRCs in *L. sativa* cv Salinas ((Christopoulou, Wo, et al. 2015)). Depending on the size of a MRC, two or three markers were selected to cover the whole MRC. Testing of the markers was done by first performing a PCR in the same way as described in Table 2.1. After amplifying the DNA a High Resolution Melting analysis was performed to visualize the genetic content of all the plants for a certain marker.

2.3.3 Data analysis

Based on the segregation of genotypes in the resistant (RIS < 10%) and susceptible (RIS > 10%) group of plants it was determined whether or not a marker was associated with resistance. For marker at the genetic location where there was association between heterozygous genotype and resistant phenotype, more markers were tested to come to a mapping interval for this resistance. This mapping interval was based on recombinant plants in the susceptible group.

2.3.4 Validation resistance

In order to validate the resistance mapping interval found in BC1*sativa*CGN5947, resistant plants with an heterozygous introgression in the mapping interval were selected and backcrossed to the susceptible *L. sativa* cv Olof to obtain a BC2*sativa*CGN5947 population. Because plants were used with a heterozygous introgression, the expectation is that resistance is inherited to the BC2*sativa*CGN5947 population in 50% of the plants based on normal mendelian segregation. From the two week old plants, leaf samples were taken and DNA was isolated according to the NaOH protocol in paragraph 2.2.3. Plants were genotyped for the flanking markers in the mapping interval and the middle marker to find recombinants. All recombinant plants were selected together with 6 plants with a homozygous *L. sativa* introgression and 6 plants with a heterozygous introgression in the mapping interval. An ADT was performed with Bl:24 on these plants. Phenotypic data obtained from the disease test was compared with the genotypic data obtained from the marker assisted selection to validate the resistance locus/interval. The next step was to test more markers for the plants with recombination in the mapping interval to fine-map the region and finally this region was compared with the known MRCs to see if they co-locate.

3. Results

3.1 Marker development

Chromosome 4

A total of 32 primer pairs were designed on 16 different ESTs at linkage group 4. From these 32 primer pairs only 15 PCR products showed different melting curves for the selected reference lines and therefore had the potential to be used as a marker. For each genomic position only one marker needed to be designed at 300, 313, 330 and 350 Mb on chromosome 4 of the *L. sativa* reference genome. A total of six primer pairs were selected and tested on the F2_1997_FR population resulting in four primer pairs which could be mapped, one on each position (Table 3.1). The markers CLSM5677.1, CLS_S3_Contig9679.2, CLS_S3_Contig5545.2 and CLSM3095.2 on linkage group four were all scored as co-dominant on the reference lines and on the F2 population. Furthermore, the genetic position was determined (Table 3.2)

Chromosome 7

For linkage group 7 a total of 24 primer pairs on 12 different ESTs were designed. A total of 5 out of 24 primers gave PCR products which showed differences in melting curves for the selected reference lines. This gave a success rate of 21%. These five primer pairs were then tested on the F2_1997_FR population resulting in 4 primer pairs which could be mapped (Table 3.1). For these five primers a HRM analysis was performed on the F2_1997_FR population consisting of 126 plants. Primer QGB15O22.1 and CLSM8124.2 were scored as a dominant marker, primer QGJ3F10.2 was partly scored as dominant and partly scored as co-dominant and primer CLSS12441.2 gave no different melting curve on the HRM analysis so it was impossible to make groups. The next step was to map the primers into the F2_1997_FR genetic map. For QGB15O22.1 this corresponds with a genetic location between 11.7 and 16.6 cM, for CLSM8124.2 between 16.6 and18.6 cM and for CLSS12441.2 between 58.6 and 59.7 Cm. QGJ3F10.2 was mapped between 62.2 and 62.6 cM (Table 3.2). At this time the Lettuce GBrowse database was back online and the EST names could be searched for QGB15O22.1, CLSM8124.2, CLSS12441.2.

Table 3.1: Overview of the success rate for the tested primer pairs per linkage group for the reference lines and the F2_1997_FR population.

		Reference lines			F2_1997_FR population		
LG	nr of ESTs	primer pairs	# succes	succes (%)	primer pairs	# succes	succes (%)
4[a]	16	32	15	47	6	4	75
7[b]	12	24	5	21	5	4	80

[a] ESTs designed using the sequence from the Lettuce GBrowse database containing introns and exons
[b] ESTs designed using the sequence from NCBI containing only the exon sequence

Table 3.2 : Results of allele specificity for the newly developed markers and mapping position on the F2_1997_FR genetic map. a = *L. sativa*; b = *L. saligna*; h = heterozygous; d = a or h; e = a or b; LG = linkage group.

marker	EST_ID	Reference lines	F2 population	position on F2_1997_FR genetic map		
		HRM analysis	HRM analysis	LG	Cm	Mb
QGB15O22.1	QGB15O22.yg.ab1	Dominant, score h and e	Dominant, score h and e	7	11.7-16.6	38
CLSM8124.2	CLSM8124.b1_G15.ab1	Dominant, score h and e	Dominant, score h and e	7	16.6-18.6	73
QGJ3F10.2	QGJ3F10.yg.ab1	Dominant, score b and d	co-dominant / dominant, Partly d and b and partly score a, b and h	7	62.2-62.6	-
CLSS12441.2	CLSS12441.b1_B15.ab1	Dominant, score h and e	Co-dominant, score a, b and h	7	58.6-59.7	200
CLSS13106.1	CLSS13106.b1_C13.ab1	Co-dominant, score a, b and h	no result	7	-	-
CLSM5677.1	CLSM5677.b1_J04.ab1	Co-dominant, score a, b and h	Co-dominant, score a, b and h	4	109-110	301
CLS_S3_Contig9679.2	CLS_S3_Contig9679	Co-dominant, score a, b and h	Co-dominant, score a, b and h	4	116.6-116.9	314
CLS_S3_Contig5545.2	CLS_S3_Contig5545	Co-dominant, score a, b and h	Co-dominant, score a, b and h	4	124.4-124.7	330
CLSM3095.2	CLSM3095.b1_M05.ab1	Co-dominant, score a, b and h	Co-dominant, score a, b and h	4	132.6-134.4	352

3.2 Observe resistance segregation and distribution over the different infection classes in BC1*sativa*CGN15726, BC1*serriola*CGN15699 and BC1*sativa*CGN13330

3.2.1 Disease test and phenotyping hybrid necrosis

To compare development of infection process between two disease tests, the control lines of disease test with BC1*sativa*CGN15726 and BC1*serriola*CGN15699 were compared with the disease test of BC1*sativa*CGN13330 for the time points 9, 10 and 13 dpi (Figure 3.1). For disease test BC1*sativa*CGN15726 and BC1*serriola*CGN15699 the highest absolute infection for all controls was scored at 10 dpi with an absolute infection score of 20% for *L.* serriola CGN04774 and 66% for *L. sativa* cv Olof (both susceptible parental lines). For the disease test with BC1sativaCGN13330 The control lines had the highest absolute infection score at 13 dpi with an absolute infection score of 46% for *L. sativa* cv Olof. The two time points with the highest absolute infection score for the susceptible controls were chosen for further analysis of the results.

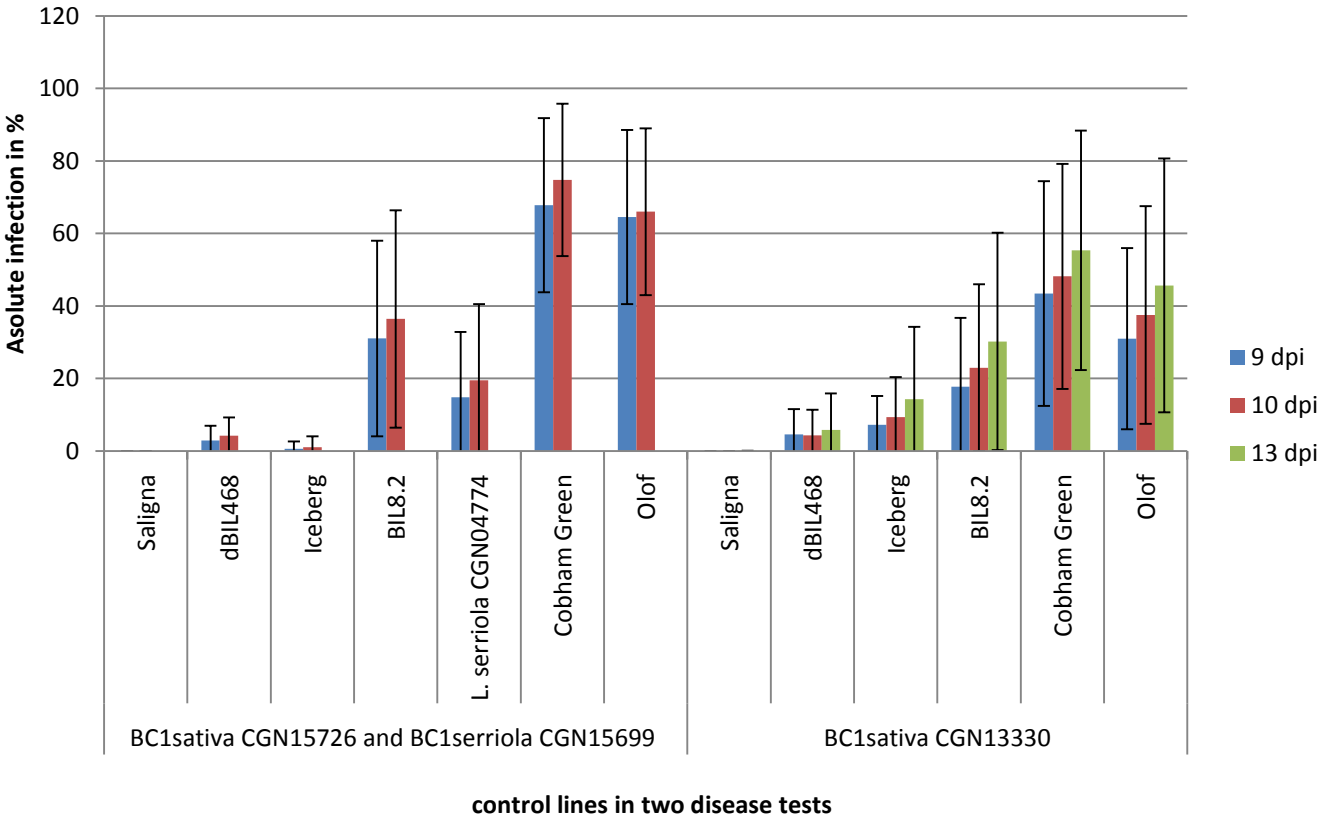


Figure 3.1: Absolute infection scored as the percentage of leaf area covered with sporulation for the control lines from disease test 1 with BC1*sativa*CGN15726 together with BC1*serriola*CGN15699 and disease test 2 with BC1*sativa*CGN13330. Disease tests are performed with Bl:21 and scored at different time points.

The absolute infection data of 68 BC1 plants of population BC1*sativa*CGN15726 were transformed to relative infection levels (RIS) by setting *L. sativa* cv Olof at 100%, which showed an average absolute infection level of 66% (based on 4 plants and 5 replications per plant) in the disease test. This disease test on the BC1*sativa*CGN15726 plants resulted in a continuous distribution over all the relative infection classes (Figure 3.2). There are 6 plants (9%) with a RIS <10% for this population. Both the continuous distribution and the 9% plants with RIS<10 was comparable with the scenario of BC1*sat*_05271_FR, indicating the presence of non-host resistance and absence of a dominant monogenic R-gene against Bl:21. Therefore no genetic analysis was performed for this population.

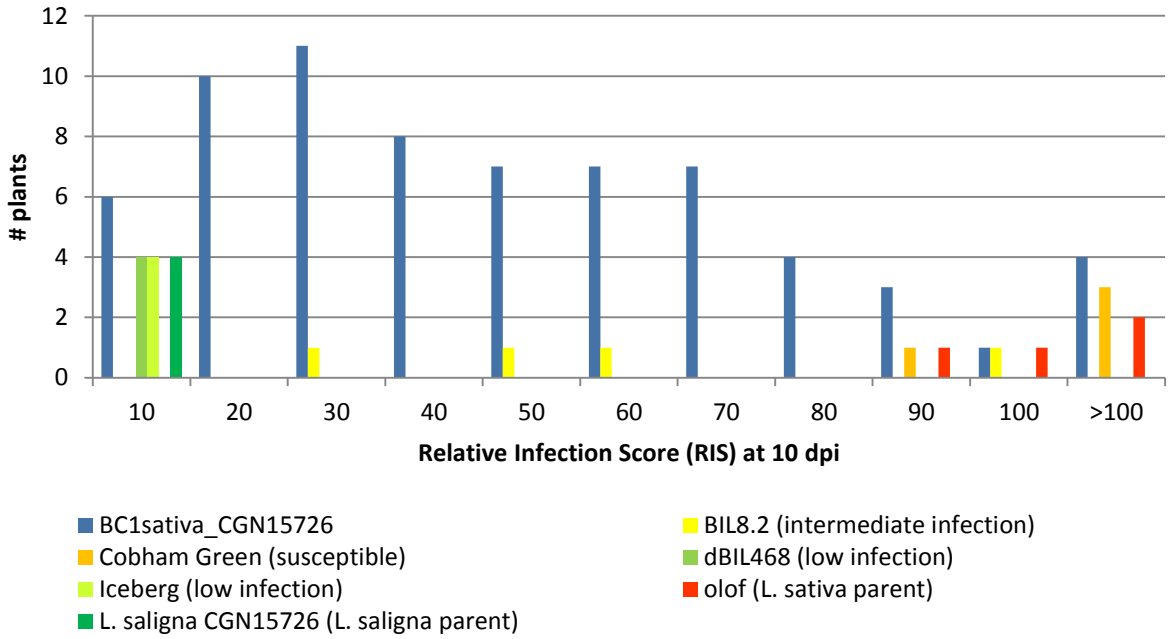


Figure 3.2: Segregation of BC1*sativa*_CGN15726 plants and several control lines for the different relative infection score classes to Bl:21 at 10 dpi. N=68 for BC1*sativa*_CGN15726. Relative infection score is based on *L. sativa* cv Olof and each BC1 plant has four technical replicates. Disease test is performed with Bl:21.

The absolute infection data of 78 BC1 plants of population BC1*sativa*CGN13330 were transformed to RIS by setting *L. sativa* cv Olof at 100% which showed an average absolute infection level of 46% (based on 4 plants and 4 replications per plant) in the disease test. Because of this low infection score for the susceptible control *L. sativa* cv Olof both the RIS (Figure 3.3A) and the absolute infection scores (Figure 3.3B) of the controls and the BC1 plants were visualized. Based on RIS levels at 13 dpi there were three plants in the lowest class (RIS <10%) which is 4% of the total plants. Furthermore, all RIS classes were represented, but because of the low absolute infection score for *L.* sativa cv Olof the number of plants with a RIS>100% is very high with 37 plants. Looking at the absolute values of the BC1*sativa*CGN13330 plants there were five plants (6%) of the plants in the lowest infection class (RIS<10%) and the rest of the plants were distributed over the other infection classes. Both the continuous distribution and the 4% resistant plants based on RIS and the 6% resistant plants based on absolute infection indicating the presence of non-host resistance and the absence of a dominant monogenic R-gene against Bl:21. Therefore this is the end of this experiment and there will be no genotyping.

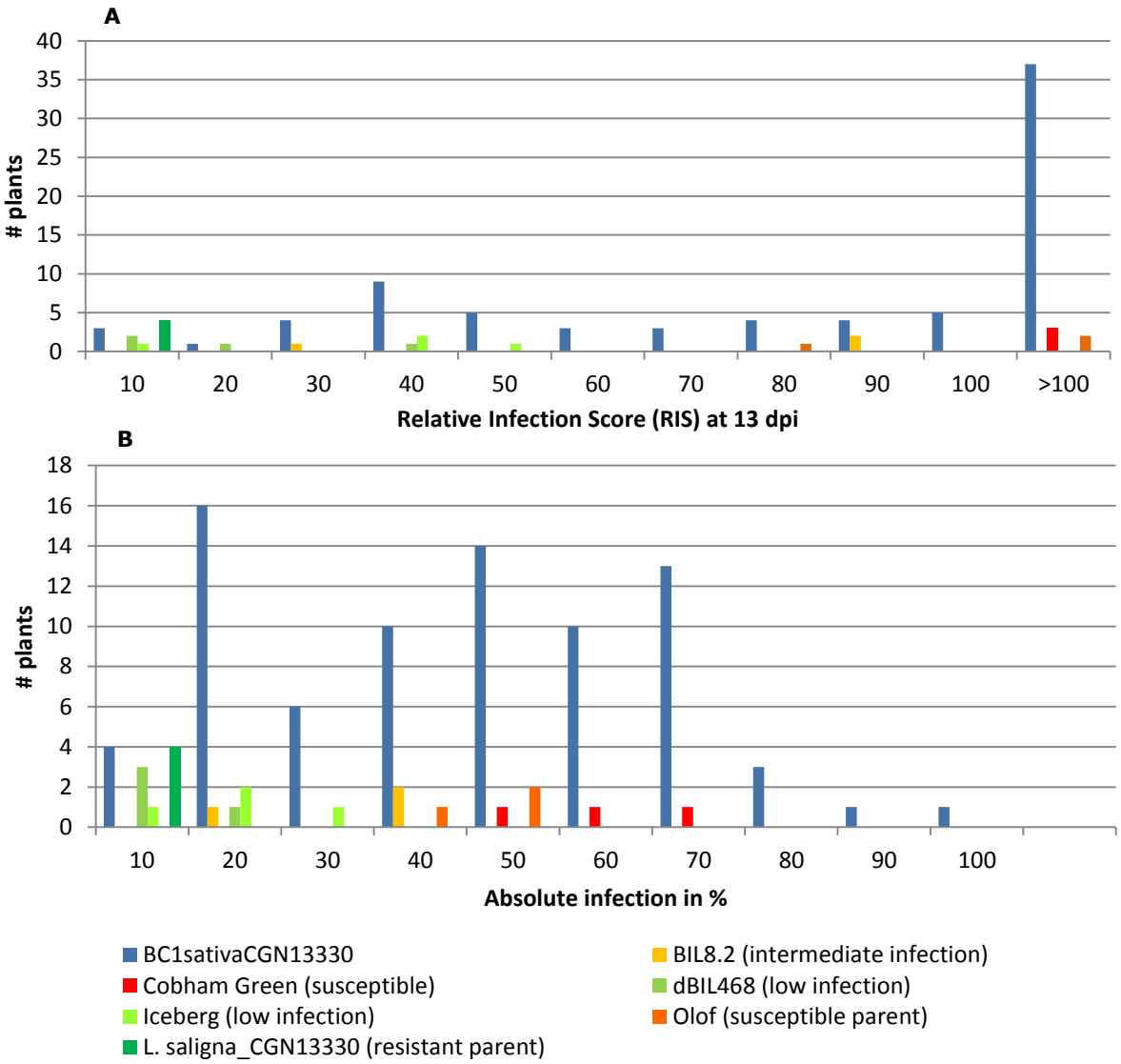


Figure 3.3: Segregation of BC1*sativa*_CGN13330 plants and several control lines for the different relative infection scores (A) and absolute infection scores (B)to Bl:21 at 13 dpi. N=78 for BC1*sativa*_CGN13330. Relative infection score is based on the infection of 4 *L. sativa* cv Olof plants and each BC1 plant has four technical replicates.

In population BC1*serriola*CGN15699 hybrid necrosis was observed in 11 out of 52 plants (21%). Necrotic spots were mostly seen in the older leaves of the plant. Symptoms are necrotic areas around the main vein of the leaf and necrotic spots covering the whole leaf (Figure 3.4). The plants with hybrid necrosis are expected to be resistant due to hybrid incompatibility based on previous research in a population of *L. saligna* CGN5271 and *L. sativa* cv Olof (Jeuken et al. 2009) and therefore separated from the plants without hybrid necrosis in the segregation for the different infection classes.

Figure 3.4: Observed hybrid necrosis symptoms in a 38 days old plant from the BC1*serriola*CGN15699 population. A: whole plant with necrotic spots on the lower leafs. B: Leaf with necrotic area around the main vein. C: Leaf covered by necrotic spots.

The absolute infection score for the susceptible controls *L. serriola* CGN04774 and *L. sativa* cv Olof were 20% and 66% respectively. This is unexpected, because in previous leaf disc tests with the same bremia isolate, *L. serriola* CGN04774 had the same RIS levels as *L. sativa* cv Olof. For both controls, the relative infection score for the BC1*serriola*CGN15699 plants were calculated and divided over the different infection classes (Figure 3.5A and 3.5B) at 10 dpi. For RIS based on *L. serriola* CGN04774 (Figure 3.5A) there were 5 plants (12%) in the resistant group (RIS<10%). Furthermore, a large part of 17 plants ended up in the group with RIS>100%. The rest of the plants were distributed over the other RIS classes. For RIS based on *L. sativa* cv Olof there were 13 plants (32%) with a RIS<10% and only 1 plant with a RIS>100%. The rest of the plants were distributed over the other RIS classes. Calculating RIS with *L. serriola* CGN04774 would suggest a scenario like the BC1*sat*_05271_FR population. However, Figure 3.5B with RIS based on *L. sativa* cv Olof can be interpreted as the scenario from BC1*sat*_15705_GEO were an R-gene has been found in a distorted segregation region. Additional research was performed based on RIS Olof. This was risky, because the *L. serriola* CGN04774 RIS level was so low.

If there were indications for a dominant monogenic R-gene the next step in the workflow was the genotype these plants in order to confirm these indications and if possible to map the location of the resistance locus. Based on the phenotypic results of all three BC1 populations, further research by genotyping was only interesting for population BC1*serriola*CGN15699. Both the hybrid necrosis and the resistance was investigated further in the next paragraph. From the populations BC1*sativa*CGN15726 and BC1*sativa*CGN13330 the resistant plants were collected for further research to non-host resistance and were not used in this thesis anymore.

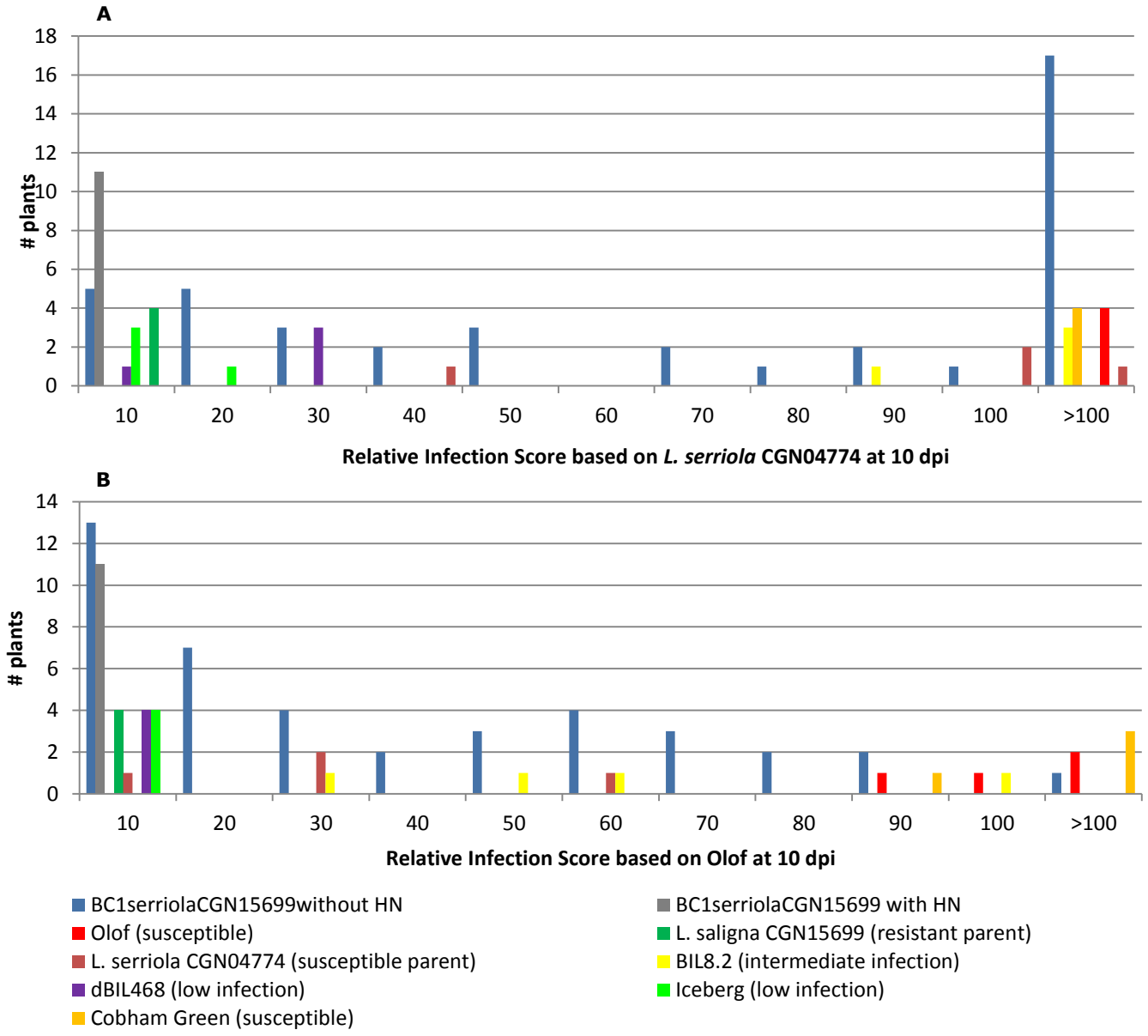


Figure 3.5: Segregation of BC1*serriola*_CGN15699 plants with and without Hybrid necrosis (HN) and several control lines for the different relative infection score classes to Bl:21 at 10 dpi (A) and 13 dpi (B). N=41 for BC1*serriola*_CGN15699 without HN and N=11 for BC1*serriola*_CGN15699 with HN. Relative infection score is based on *L. sativa* cv Olof and each each BC1 plant has four technical replicates.

3.2.2 Genotyping hybrid necrosis in BC1*serriola*CGN15699

It has been found that hybrid necrosis in a cross between *L. saligna* CGN5271 and *L.* sativa cv Olof could be explained by an interaction of a *L. saligna RIN4* allele on chromosome 9 and a *L.* sativa allele on chromosome 6 (Jeuken et al. 2009). In order to find out if the same interaction is causing hybrid necrosis in BC1*serriola*CGN15699, the BC1*serriola*CGN15699 plants were genotyped for marker QGB24E10 at 31.3 cM on chromosome 6 and marker Rin4 at 6.7 cM on chromosome 9 (Table 3.3). All 11 plants with a hybrid necrosis phenotype were homozygous *L. sativa* on chromosome 6 and heterozygous on chromosome 9. Non hybrid necrosis plants were divided over all genotypes. There was one plant with the same genotype as the plants showing hybrid necrosis, 19 plants with on both chromosomes homozygous *L. sativa*, 12 plants with a heterozygous genotype on chromosome 6 and homozygous *L. sativa* on chromosome 9 and nine plants with both a heterozygous genotype on chromosome 6 and 9.

Table 3.3: Number of BC1*serriola*_CGN15699 plants with or without hybrid necrosis linked to the genotype for marker QGB24E10 (31.3 cM) on chromosome 6 and marker Rin4_snp4 (6.7 cM) on chromosome 9. a = *L. serriola*; h = heterozygous.

Genotype		Phenotype	
Chromosome 6 (31.3 cM)	Chromosome 9 (6.7 cM)	hybrid necrosis	no hybrid necrosis
a	**h**	**11**	**1**
a	a	0	19
h	a	0	12
h	h	0	9
	total:	11	41

3.2.3 Genotyping and determination of mapping interval for resistance in BC1*serriola*CGN15699

The outcome of the disease test was sorted based on absolute infection and all the BC1*serriola*CGN15699 lines were genotyped with several markers within major resistant gene clusters on LG 1, 3, 6, 7 and 9 (Table 3.4). Both the RIS based on *L. serriola* CGN04774 as the RIS based on *L.* sativa cv Olof were used in combination with the genotypic data obtained from the markers. This was done because of the low absolute infection of the susceptible parent *L. serriola* CGN04774. To find association the plants were divided into a resistant and susceptible group . Plants with a RIS <10% were called resistant and plants with a RIS>10% were called susceptible. For RIS based on *L. sativa* cv Olof there were 13 resistant and 28 susceptible plants. For RIS based on *L. serriola* CGN04774 there were 5 resistant and 36 susceptible plants.

Plants with hybrid necrosis were not used for genotyping, because it cannot be said whether this resistance is caused by an possible R-gene or hybrid incompatibility. The remaining 41 plants were genotyped with markers to find association between heterozygous genotype and resistant phenotype (RIS<10%). In a scenario were a monogenic dominant R-gene is present the expected percentages of genotypes in the resistant group (RIS<10%) is 87-93% heterozygous ("h") genotype and 7-13% homozygous *L. sativa* ("a") genotype. The 7-13% homozygous *L. sativa* can be explained by complete non-host resistance as has been stated in paragraph 1.5. The 87-93% heterozygous genotype can be explained by 100% - 7-13% non-host resistance. For the susceptible group (RIS>10%) the expected percentages are 0% heterozygous ("h") genotype and 100% homozygous *L. sativa* ("a") genotype. Because non-host resistance is still present in the population, the most reliable plants are the plants in the susceptible group and therefore focus was more on the susceptible group when comparing the genotype and phenotype.

In both the RIS based on *L. sativa* cv Olof as *L. serriola* CGN04774 the best associated marker with the expected genotype percentages in the susceptible group was marker LE1109 which was located at 0.1 cM on chromosome 9 (Table 3.5). Also the other markers at chromosome 9, NL0126 at 0.3 cM, Rin4 at 6.7 cM, NL0919 at 8.5 cM and LE0361 at 26.6 cM gave a high association with the susceptible phenotype indicating a possible location for the monogenic dominant R-gene.

Table 3.4: Disease assay data performed with Bl:21 for all BC1*serriola*_CGN15699 plants sorted on RIS values together with the marker results. Genotype "a" = homozygous *L. serriola,* genotype "b" = homozygous *L. saligna* and genotype "h" = heterozygous. R= resistant (RIS <10%) and S = susceptible (RIS > 10%).

				accession	L. saligna CGN15699	*L. serriola* CGN04774	pv15261.01	pv15261.06	pv15261.09	pv15261.15	pv15261.17	pv15262.09	pv15262.14	pv15262.15	pv15262.17	pv15262.23	pv15262.28	pv15261.16	pv15262.18	pv15261.04	pv15262.06	pv15262.20	pv15262.19	pv15262.02	pv15261.02	pv15262.12	pv15262.05	pv15261.19	pv15261.14	pv15261.18
				Absolute infection	0	20	0	0	0	0	0	0	0	0	0	0	0	0	1	1	2	2	2	3	3	3	4	4	4	5
				RIS ser04774	0	100	0	0	0	0	0	0	0	0	0	0	0	0	5	6	8	9	10	15	17	17	18	21	22	23
				RIS Olof	0	30	0	0	0	0	0	0	0	0	0	0	0	0	2	2	2	3	3	5	5	5	5	6	6	7
				hybrid necrosis			x	x	x	x	x	x	x	x	x	x	x													
				Infection class based on RIS *L. serriola* CGN04774	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	S	S	S
				Infection class based on RIS *L. sativa* cv Olof	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
LG physical map	**Chrom WUR**	**marker**	**cM**	**Mb**																										
hybrid necrosis																														
8	6_R	QGB24E10	31.3		b	a	a	a	a	a	a	a	a	a	a	a	a	h	h	h	h	h	a	a	h	a	h	h	h	h
9	9	RIN4	6.7		b	a	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	a	a	a	h	a	h	a	h	a
Possible R-gene																														
1	1_R	QGD7M24	27.3	173	b	a	a	h	a	a	a	a	a	a	h	a	n	u	a	a	a	h	a	a	a	a	a	h	h	h
1	1_R	NL0460	58.8		b	a	a	h	a	a	h	a	a	h	h	a	a	h	a	h	a	h	a	a	a	a	a	h	a	a
6	3	CLSX3965	89.7	234	b	a	a	h	a	a	h	h	a	h	n	a	a	a	h	a	a	a	a	h	h	h	a	a	a	a
7	7	LK1504	5.3		b	a	h	h	a	h	h	h	a	a	a	h	h	h	h	h	h	h	a	a	h	h	a	h	h	h
7	7	CLS_S3_11143	20-25	101	b	a	a	h	h	h	h	a	h	h	a	h	a	h	h	h	h	a	a	a	h	h	u	a	a	h
3	8_R	QGG16B23 tophit2_2	0		b	a	a	h	a	h	h	h	h	a	h	a	h	h	h	h	a	a	h	h	h	h	h	h	a	h
3	8_R	LE1065	11.6		b	a	a	a	a	h	h	h	h	a	h	a	u	u	h	h	a	a	h	h	h	u	u	u	a	h
3	8_R	QGG32A02	19.4		b	a	n	a	a	u	u	n	h	a	n	a	n	n	n	n	h	h	n	n	n	h	h	h	a	u
9	9	LE1109	0.1		b	a	n	n	h	h	h	a	h	h	n	n	h	h	h	h	h	a	a	a	n	n	h	a	h	a
9	9	NL0126	0.3		b	a	h	h	h	h	h	a	h	h	h	h	h	h	h	h	h	a	a	a	h	a	h	a	h	a
9	9	RIN4	6.7		b	a	h	h	h	h	h	h	h	h	h	h	h	h	h	h	h	a	a	a	h	a	h	a	h	a
9	9	NL0919	8.5		b	a	h	h	a	h	h	h	h	h	h	h	h	h	h	h	h	a	a	a	h	a	h	a	h	a
9	9	LE0361	26.6		b	a	a	h	a	h	h	h	h	h	h	u	h	h	h	h	h	a	a	h	h	a	h	a	h	h
9	9	NL1302	42.8		b	a	a	h	a	a	h	h	h	h	h	h	h	h	h	h	h	a	h	h	h	a	h	h	h	h
9	9	CLLX1765	75-85	159	b	a	a	h	a	a	h	h	h	h	a	a	a	a	a	h	a	a	a	h	h	a	h	h	h	a
9	9	CLX12996	95.1		b	a	a	h	a	h	h	h	h	a	a	a	a	a	a	h	a	a	h	h	h	a	a	h	a	a
9	9	CLR_S1_contig4210	112.1	241	b	a	a	h	a	h	n	h	n	n	n	a	n	a	a	h	a	a	h	h	h	a	n	n	a	n

Table 3.4: continued.

					L. saligna CGN15699	*L. serriola* CGN04774	pv15261.12	pv15262.24	pv15261.07	pv15261.10	pv15262.04	pv15261.05	pv15262.21	pv15261.03	pv15261.20	pv15261.22	pv15261.11	pv15262.27	pv15262.10	pv15261.13	pv15262.25	pv15262.29	pv15262.13	pv15261.21	pv15262.26	pv15262.22	pv15262.08	pv15262.03	pv15262.16	pv15261.01	pv15261.23	pv15262.07	pv15262.11	pv15261.08
			accession																														
			Absolute infection		0	20	8	8	10	10	10	13	13	15	16	18	19	21	21	31	32	32	35	36	36	38	42	45	45	50	50	54	54	69
			RIS ser04774		0	100	38	38	49	49	49	65	65	74	83	90	96	105	109	158	162	164	179	186	186	196	215	228	228	256	258	276	278	353
			RIS Olof		0	30	11	11	14	14	14	19	19	22	25	27	28	31	32	47	48	48	53	55	55	58	64	67	67	76	76	81	82	104
			hybrid necrosis																														
			Infection class based on RIS *L. serriola* CGN04774		R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
			Infection class based on RIS *L. sativa* cv Olof		R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
LG physical map	**Chrom WUR**	**marker**	**cM**	**Mb**																													
hybrid necrosis																																	
8	6_R	QGB24E10	31.3		b	a	h	h	h	a	a	a	h	a	a	a	a	h	h	a	a	h	a	a	a	h	h	h	a	h	a	a	a	a
9	9	RIN4	6.7		b	a	h	a	h	h	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Possible R-gene																																	
1	1_R	QGD7M24	27.3	173	b	a	a	n	h	n	h	a	a	a	h	h	h	a	h	h	h	h	a	h	h	h	h	h	h	a	a	a	a	h
1	1_R	NL0460	58.8		b	a	a	h	a	u	h	h	a	h	h	h	a	a	a	a	a	a	a	h	a	a	h	h	a	h	a	h	h	h
6	3	CLSX3965	89.7	234	b	a	h	h	u	h	a	h	a	a	a	a	a	h	a	a	h	a	a	a	a	h	a	h	a	a	h	a	h	h
7	7	LK1504	5.3		b	a	h	a	h	h	h	a	a	h	h	a	a	h	h	a	h	a	a	h	a	h	a	h	a	a	a	h	a	a	a	h
7	7	CLS_S3_11143	20-25	101	b	a	a	a	h	h	h	a	a	h	h	a	a	a	a	a	h	h	a	h	h	h	a	a	a	h	a	a	a	h
3	8_R	QGG16B23 tophit2_2	0		b	a	h	h	a	a	h	a	a	h	a	a	h	h	h	h	h	a	a	h	h	a	h	a	h	h	h	h	h	a
3	8_R	LE1065	11.6		b	a	a	u	a	u	h	a	h	h	h	a	h	h	h	a	h	a	a	u	h	u	h	a	a	h	h	h	h	u
3	8_R	QGG32A02	19.4		b	a	h	n	n	n	h	n	h	h	h	a	h	u	n	a	n	a	h	h	n	h	h	n	h	n	h	a	n	n
9	9	LE1109	0.1		b	a	n	a	n	n	a	a	a	n	a	a	a	a	a	a	a	a	a	a	u	n	a	a	n	a	a	a	u	n
9	9	NL0126	0.3		b	a	a	a	h	h	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
9	9	RIN4	6.7		b	a	h	a	h	h	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
9	9	NL0919	8.5		b	a	h	a	h	h	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
9	9	LE0361	26.6		b	a	h	a	h	u	h	a	a	a	a	h	a	a	a	a	a	a	a	a	a	u	a	a	a	a	a	a	a	a
9	9	NL1302	42.8		b	a	h	a	h	h	h	a	a	h	a	h	a	a	h	a	a	a	a	a	a	h	a	a	a	a	a	a	h	a
9	9	CLLX1765	75-85	159	b	a	a	a	h	a	h	a	h	h	a	a	a	a	h	a	a	a	a	a	a	n	h	a	a	h	h	h	h	a
9	9	CLX12996	95.1		b	a	h	a	h	h	a	a	h	a	h	a	a	a	h	a	a	h	a	h	a	a	h	a	a	h	h	a	a	a
9	9	CLR_S1_contig4210	112.1	241	b	a	h	a	a	h	a	a	h	a	n	a	a	a	h	a	n	h	a	h	a	a	h	a	n	h	h	a	n	a

Table 3.5: Markers tested on BC1*serriola*_CGN15699 on different genomic locations. The percentage plants with genotype "a" (homozygous *L. serriola*) and "h" (heterozygous) for a certain marker are represented for the susceptible group (RIS< 10%) and resistant group (RIS>10%). This is done for RIS to Bl:21 based on *L. sativa* cv Olof and for *L. serriola*_CGN04774 at 10dpi. Plants with hybrid necrosis are not included in this table. High percentage of a genotype is green and a low percentage of a genotype is red.

					RIS based on	*L. sativa* cv Olof				*L. serriola* CGN04774			
					Infection class	RIS< 10 (N=13)		RIS > 10 (N=28)		RIS< 10 (N=5)		RIS > 10 (N=36)	
					Genotype:	"h"	"a"	"h"	"a"	"h"	"a"	"h"	"a"
					Expected percentages of "a" and "h" when a R gene is present:	87-93	7-13 [a]	0	100	87-93	7-13	0	100
LG physical map	Chr WUR	marker	cM	Mb		%	%	%	%	%	%	%	%
1	1_R	QGD7M24	27.3	173		33	67	62	38	25	75	56	44
1	1_R	NL0460	58.8			31	69	48	52	60	40	40	60
6	3	CLSX3965	89.7	234		31	69	41	59	20	80	40	60
7	7	LK1504	5.3			77	23	46	54	100	0	50	50
7	7	CLS_S3_11143	20-25	101		58	42	43	57	80	20	43	57
3	8_R	QGG16B23 tophit2_2	0			83	17	61	39	60	40	69	31
3	8_R	LE1065	11.6			75	25	61	39	50	50	67	33
3	8_R	QGG32A02	19.4			100	0	75	25	100	0	79	21
9	9	LE1109 [b]	0.1			55	45	0	100	80	20	8	92
9	9	NL0126	0.3			54	46	7	93	80	20	14	86
9	9	Rin4_snp4	6.7			54	46	11	89	80	20	17	83
9	9	NL0919	8.5			54	46	11	89	80	20	17	83
9	9	LE0361	26.6			69	31	15	85	80	20	26	74
9	9	NL1302	42.8			85	15	32	68	80	20	44	56
9	9	CLLX1765	75-85	159		46	54	37	63	20	80	43	57
9	9	CLX12996	95.1			38	62	39	61	20	80	42	58
9	9	CLR_S1_contig4210	112.1	241		40	60	38	63	20	80	41	59

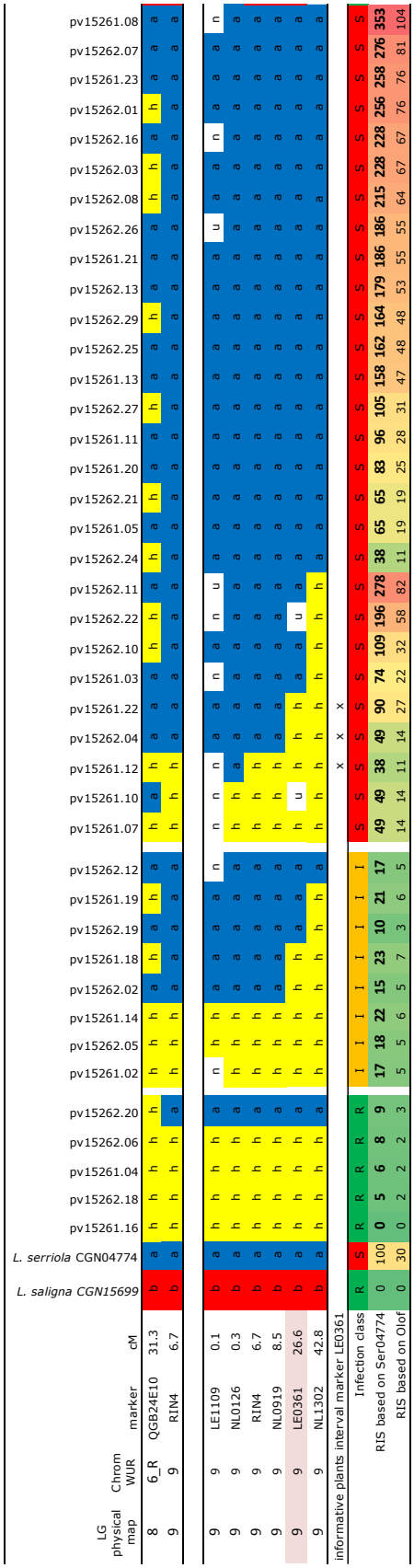
[a] Percentage of non-host plants in the group RIS<10

[b] Most associated marker with susceptibility

Based on the association with phenotype and genotype, the focus was on chromosome 9. The BC1*serriola*CGN15699 plants were divided in three groups. The resistant group were the plants with a RIS<10% for both *L. sativa* cv Olof as *L. serriola* CGN04774. The intermediate group were plants with a RIS<10% when RIS was based on *L. sativa* cv Olof, but a RIS <10% when RIS was based on *L. serriola* CGN04774. Finally, the resistant group were the plants with a RIS >10% for both *L. sativa* cv Olof as *L. serriola* CGN04774. Within these three groups the plants were sorted for recombinations between marker LE1109 at 0.1 cM and marker NL1302 at 42.8 cM. Within the resistant group there were no recombinations within the 0.1-42.8 interval. Within the susceptible group there were 3 plants with a recombination between NL0919 at 8.5 cM and LE0361 at 26.6 cM. These recombinations were confirmed by marker NL1302 at 42.8 cM. These three informative plants were used for determining the bottom marker of the mapping interval (Table 3.6). For the top marker there is not enough data due to negative samples and a lack of recombination and therefore no marker, but the top of chromosome 9 was chosen as start of the mapping interval.

Another observation is the association between resistant plants and the hybrid necrosis phenotype. Plants with a heterozygous introgression on chromosome 9 always have a heterozygous genotype for marker QGB24E10 at chromosome 6 (31.3 cM). This can indicate another type of resistance explained by the two genes involved with hybrid necrosis. In previous research on hybrid material of *L. saligna* CGN05271 x Olof, the double heterozygous genotype shows no hybrid necrosis phenotype but a race specific resistance effective against Bremia races Bl:16 and Bl:24, but not to Bl:14 and Bl:21 (Table 1.1).

Table 3.6: Genotype and RIS values to Bl:21 for the plants without hybrid necrosis. Markers for hybrid necrosis and for the 0.1-42.8 cM interval on chromosome 9 are visualised. The bottom marker for the mapping interval is highlighted in pink and is based on three informative recombinants in the susceptible group. The top of the interval is the beginning of chromosome 9. Genotypes are: a = *L. serriola*; h = heterozygous; n = negative sample; u = unknown.



3.2.4 Validating resistance

To validate findings in BC1*serriola*CGN15699, a disease test was performed with Bl:21 for BC1S1*serriola*CGN15699 and BC2*serriola*CGN15699. The plants were scored for absolute infection at 8,9,10 and 14 dpi. In Figure 3.6 the absolute infection of the control lines are visualized at these time points. In both disease tests the susceptible parent *L. serriola* CGN04774 showed very low infection. The *L. serriola* CGN04774 parent had an absolute infection of 9.8% in the BC1S1*serriola*CGN15699 disease test and 8.8% in the BC2*serriola*TB disease test at 14 dpi. For the other susceptible controls Cobham Green and Olof the absolute infection was within the expected range. Cobham Green had an average absolute infection of 56.3% and 42.5% and for Olof this was 47.5% and 62.5%. For the BC1S1 population there were four plants with an absolute infection over 10% and for the BC2 population this was only one plant with an absolute infection over 10%. Because of the low infection scores it was not possible to distinguish between resistance and susceptibility and therefore is was not useful to do a genotypic analysis.

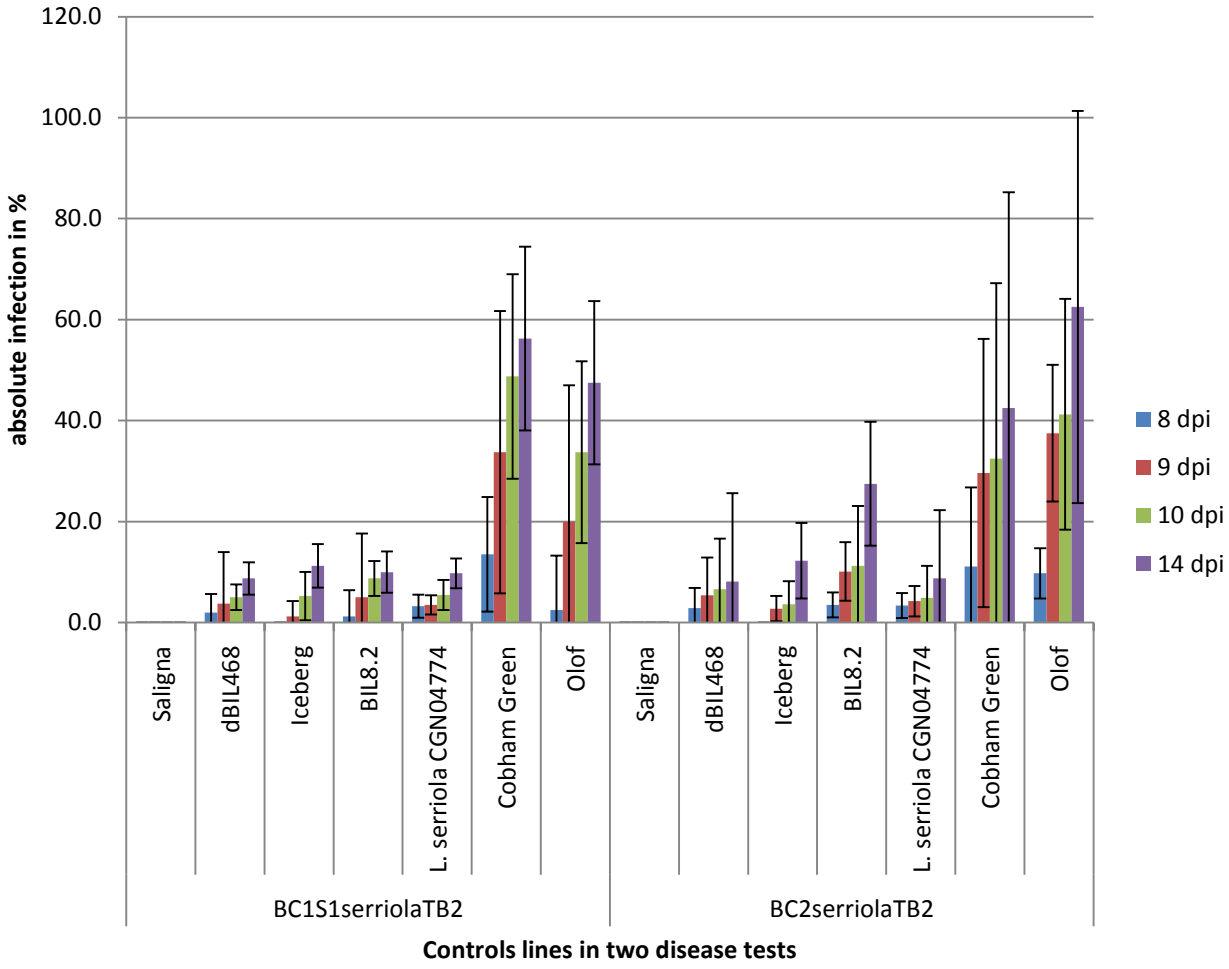


Figure 3.6: Absolute infection and standard deviation scored as the percentage of leaf area covered with sporulation for the control lines from two different disease tests. Disease tests are performed with Bl:21 and scored at different time points.

3.3 Resistance in BC1*sat*iva_CGN5947

3.3.1 Genotyping and determination of mapping interval for resistance

An adult disease test was performed on the BC1*sativa*CGN5947 plants by Anne Giesbers in a previous experiment. The outcome of the disease test was sorted based on absolute infection and all the BC1*sativa*CGN5947 plants were genotyped with several markers within major resistant gene clusters on LG 1, 2, 3, 4, 8 and 9. Plants with a RIS <10% were called resistant and plants with a RIS>10% were called susceptible. There were 17 plants in the resistant group and 16 plants in the susceptible groups.

The percentages of genotypes within the resistant and susceptible were compared with the scenario when a monogenic dominant R-gene is present (Table 3.7). In a scenario were a monogenic dominant R-gene is present the expected percentages of genotypes in the resistant group (RIS<10%) is 87-93% heterozygous ("h") genotype and 7-13% homozygous *L. sativa* ("a") genotype. The 7-13% homozygous *L. sativa* can be explained by complete non-host resistance based on previous experiments with BC1 populations. For the susceptible group (RIS>10%) the expected percentages are 0% heterozygous ("h") genotype and 100% homozygous *L. sativa* ("a") genotype. Because non-host resistance is still present in the population, the most reliable plants are the plants in the susceptible group and therefore focus was more on the susceptible group to find association between the homozygous *L. sativa* genotype and the susceptible phenotype (RIS<10%)

Highest associated markers for homozygous *L. sativa* genotype and the susceptible phenotype (RIS<10%) in the susceptible group compared to the expected percentages are CLS_S1_1938 at 112.8 cM and CLS_S3_2729 at 119.8 cM, both with 81% of the plants having a homozygous *L. sativa* genotype and 19% heterozygous genotype in the susceptible group. The next step was to test more markers in the C4 region and to determine a mapping interval.

Table 3.7: Markers tested on BC1*sat*iva_CGN05947 plants for different genomic locations. The percentage plants with genotype "a" (homozygous *L. sativa*) and "h" (heterozygous) for a certain marker are represented for the susceptible group (RIS>10%; N=16) and resistant group (RIS<10%; N=17). This is done for RIS to Bl:24 based on *L. sativa* cv Olof. Plants with the R-gene on chromosome 2 are not included in the calculations. High percentage of a genotype is green and a low percentage of a genotype is red.

					Infection class:	RIS< 10%		RIS > 10%	
					Genotype:	"h"	"a"	"h"	"a"
		Expected percentages of "a" and "h" when a R gene is present:				87 - 93	7-13[a]	0	100
LG physical map	Chrom WUR	marker	cM	Mb		%	%	%	%
1	1_R	QGD7M24	27.3	173		41	59	81	19
1	1_R	NL0460	58.8			75	25	63	38
2	2	LE1276	66.2			53	47	81	19
4	4	NL1337	87.9			65	35	63	38
4	4	LE1233	102.0			71	29	27	73
4	4	CLS_S1_1938[b]	112.8	308		88	12	19	81
4	4	CLS_S3_2729[b]	119.8	318		94	6	19	81
4	4	CLS_S3_655	126.3	340		100	0	20	80
4	4	CLS_S3_6943	~135	360		82	18	44	56
4	4	LE1162	136.5			80	20	44	56
8	6_R	CLX_S3_1404	42.4			29	71	56	44
3	8	QGG32A02	19.4			59	41	69	31
9	9	CLLX1765	~80	159		31	69	36	64
9	9	CLR_S1_contig4210	112.1	241		18	82	25	75

[a] Percentage of non-host plants in the resistant group

[b] Highest associated marker with susceptibility

Plants with recombination within all the tested markers on chromosome 4 for the BC1*sativa*CGN5947 population were sorted within their infection group (Table 3.8). Infection groups were divided based on RIS values. The resistant group were plants with a RIS < 10%, the intermediate group were plants with a 10% < RIS < 30% and the susceptible group were plants with a RIS > 30%. The top marker of the mapping interval was based on five plants with a recombination between marker LE1233 (102 cM) and marker CLS_S1_1938 (112.8) in the resistant group. The marker CLS_S1_1938 with two recombinant plants in the resistant group and CLS_S3_2729 with one recombinant plant in the resistant group could also be used as top marker, but due to possible non-host resistance effects it is chosen to go for a more reliable top marker. The bottom marker of the mapping interval was based on three plants with a recombination between marker CLS_S3_655 (126.3)and marker CLS_S3_6943 (+/-135 cM) in the resistant group and 4 plants with a recombination between the same markers in the susceptible group. This results in a mapping interval between 102 and 135 cM on Chromosome 4.

Table 3.8: Genotype and RIS values for the resistant plants (RIS < 10%), intermediate plants (10%< RIS <30%) and the susceptible plants (RIS > 30%). with recombination in the 87.9-136.5 cM interval on chromosome 4. The mapping interval is visualized in pink and is based on the number of informative recombinants. The genotypes are: a = *L. serriola*; h = heterozygous; n = negative sample; u = unknown. Isolate BI:24 is used.

LG physical map	Chrom WUR	marker	cM	Mb	pv15091.01	pv15092.19	pv15092.17	pv15092.02	pv15094.11	pv15094.20	pv15092.07	pv15093.02	pv15092.23	pv15092.05	pv15092.12	pv15092.24	pv15093.01	pv15094.12	pv15092.18	pv15092.04	pv15094.08	pv15092.16	pv15094.05	pv15092.03	pv15092.01	pv15093.05	pv15092.20
4	4	NL1337	87.9		h	h	h	h	h	a	a	a	a	a	a	a	h	h	h	h	h	h	h	h	h	h	a
4	4	LE1233	102.0		h	h	h	h	a	a	a	a	a	h	h	h	h	h	h	a	a	a	a	a	a	a	a
4	4	CLS_S1_1938	112.8	308	h	h	h	h	a	a	h	h	h	h	h	h	h	h	a	a	a	a	a	a	a	a	a
4	4	CLS_S3_2729	119.8	318	h	h	h	h	h	a	h	h	h	h	h	h	h	h	a	a	a	a	a	a	a	a	a
4	4	CLS_S3_655	126.3	340	n	h	h	h	h	h	h	h	h	n	h	h	h	h	a	a	a	a	a	a	a	a	a
4	4	CLS_S3_6943	~ 135	360	h	a	a	a	h	h	h	h	h	h	h	h	h	h	a	a	a	a	a	h	h	h	h
4	4	LE1162	136.5		a	n	a	a	h	h	h	h	h	h	h	h	h	h	a	a	a	a	a	h	h	h	h
2	2	CLSM16923_F2	6.1		a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
informative plants interval marker LE1233									x	x	x	x	x														
informative plants interval marker CLS_S3_6943						x	x	x																x	x	x	x
Resistance (R), Intermediate (I) or Susceptible (S)					R	R	R	R	R	R	R	R	R	R	R	I	I	I	I	S	S	S	S	S	S	S	S
Relative infection score (RIS)					0	3	0	1	5	1	0	9	0	4	1	27	15	26	22	69	77	93	76	85	99	99	55

3.3.2 Validate mapping interval for resistance and fine-map

The BC2*sativa*CGN5947 plants were genotyped with markers based on the mapping interval found in the BC1 population (Table 3.8). The markers used to screen for recombinants were LE1233 (102 cM), CLS_S3_2729 (119.8 cM) and CLS_S3_6943 (+/-135 cM). This resulted in four plants with a recombination between these markers. Plants with pv number 16023 were showing in some cases also a homozygous *L. saligna* genotype for the three markers which is not possible in a BC2*sativa* population. This indicates selfing instead of backcrossing and therefore these plants were not used in further research. The recombinant plants together with six plants with a homozygous *L. sativa* introgression and six plants with a heterozygous introgression were selected for the disease test performed with BI:24.

A disease test was performed with the selected BC2*sativa*CGN5947 plants and 10 dpi was chosen to be the best time point of scoring infection according to the controls (Figure 3.7). At 10 dpi the susceptible parental control *L. sativa* cv Olof had the highest average absolute infection score with 78%. The other susceptible control Cobham Green had an absolute infection of 36.3%. the controls dBIL468, Iceberg and BIL8.2 had an absolute infection of 3.8, 17.3 and 17.5 respectively. The resistant *L. saligna* parent showed no infection. The absolute infection score of the selected BC2*sativa*CGN5947 were transformed to a RIS score using *L. sativa* cv Olof as reference.

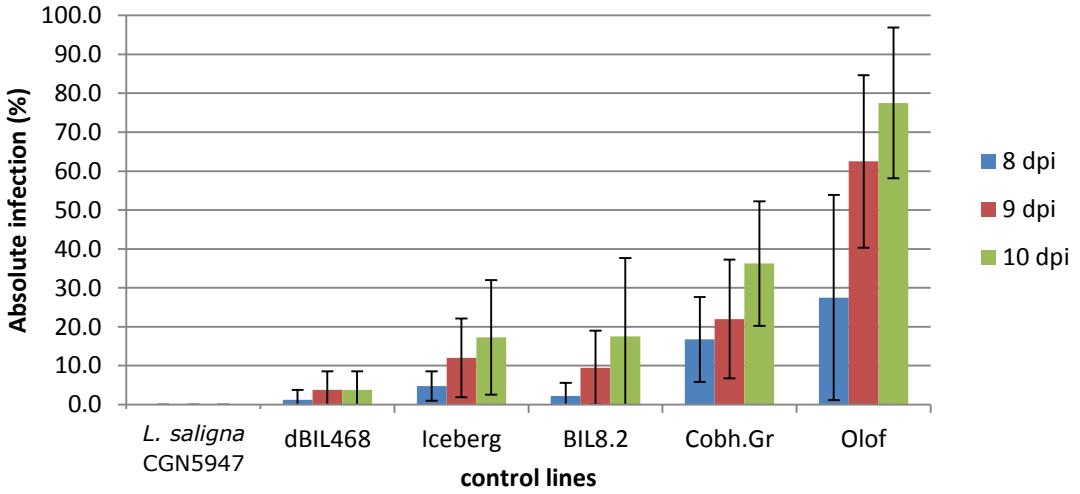


Figure 3.7: Absolute infection with standard deviation at different time points scored as the percentage of leaf area covered with sporulation for the control lines in the disease tests performed with Bl:24. Four plants were used per control with one leaf disc per plant.

In the next step, the RIS was compared with the genotypic data (Table 3.9). The plants were sorted on their RIS values from low to high and divided in three different infection classes. These infection classes were based on the same criteria as has been used in the BC1*sativa*CGN5947 population. RIS<10% was the resistant class, 10%<RIS<30% was the intermediate resistant class and RIS>30% was the susceptible class. All plants with a homozygous *L. sativa* genotype in the mapping interval were assigned to the susceptible group. plants with a heterozygous introgression in the mapping interval were either assigned to the resistant group or to the intermediate group with a maximum RIS of 18%. Three of the recombinants were resistant and one of the recombinants was susceptible and with a RIS of 116% the plant with the highest infection score.

Table 3.9: Sorted RIS of the BC2*sativa*CGN5947 plants and their associated genotypes for markers LE1233, CLS_S3_2729 and CLS_S3_6943. Infection classes are R= Resistant (RIS <10%), I = intermediate resistant (10%<RIS<30%) and S = Susceptible (RIS > 30%). Orange BC1 plants are the recombinants.

				L. saligna CGN05947	*L. sativa* cv Olof	pv16024.2	pv16024.10	pv16024.29	pv16024.18	pv16024.30	pv16024.38	pv16024.32	pv16024.26	pv16024.11	pv16025.1	pv16024.3	pv16024.36	pv16024.47	pv16024.34	pv16024.41	pv16024.37
			RIS 10 dpi	0	100	0.3	0.6	0.6	1	1.3	1.6	2.3	12	18	63	68	77	85	90	92	116
			Infection class	R	S	R	R	R	R	R	R	R	I	I	S	S	S	S	S	S	S
Chr	marker	cm	Mb																		
4	LE1233	102		b	a	a	h	h	h	h	h	h	h	h	a	a	a	a	a	a	h
4	CLS_S3_2729	119.8	318	b	a	h	h	h	h	h	h	h	h	h	a	a	a	a	a	a	h
4	CLS_S3_6943	~135	360	b	a	h	h	h	h	h	a	a	h	h	a	a	a	a	a	a	a

The recombinant plants were genotyped with newly developed markers CLSM5677 (301 Mb), CLS_S1_1938 (308 Mb), CLS_S3_contig9679 (314 Mb), CLS_S3_contig5545 (330 Mb), CLS_S3_655 (340 Mb) and CLSM3095 (352 Mb) (Table 3.10). With this information a new mapping interval for resistance was determined. The top marker for the interval was based on plant Pv1602.37 which was the plant with the highest infection score in the susceptible group. Between marker CLS_S3_2729 (318 Mb) and CLS_S3_contig5545 (330 Mb) there was a recombination for that plant. Therefore marker CLS_S3_2729 (318 Mb) was chosen as the top marker for the mapping interval. Marker CLS_S3_655 (340 Mb) was chosen as the bottom marker for the mapping interval based on a recombination event between this marker and marker CLSM3095 (352 Mb) for plant pv16024.32 in the resistant group. The new mapping interval was determined now between 318 and 340 Mb. Comparing this new mapping

interval with the mapping interval found in the BC1*sativa*CGN5947 confirms the resistance present at chromosome 4.

Table 3.10: Sorted RIS of the BC2*sativa*CGN5947 plants and their associated genotypes. Infection classes are R= Resistant (RIS <10%), I = intermediate resistant (10%<RIS<30%) and S = Susceptible (RIS > 30%). The top marker and the plant which it is based on are highlighted in pink and the bottom marker together with the plant which it is based on are highlighted in green.

				L. saligna CGN05947	*L. sativa* cv. Olof	pv16024.2	pv16024.10	pv16024.29	pv16024.18	pv16024.30	pv16024.38	pv16024.32		pv16024.26	pv16024.11		pv16025.1	pv16024.3	pv16024.36	pv16024.47	pv16024.34	pv16024.41	pv16024.37
		RIS 10 dpi		0	100	0.3	0.6	0.6	1	1.3	1.6	2.3		12	18		63	68	77	85	90	92	116
		Infection score		R	S	R	R	R	R	R	R	R		I	I		S	S	S	S	S	S	S
LG	marker	cm	Mb																				
4	LE1233	102		b	a	a	h	h	h	h	h	h		h	h		a	a	a	a	a	a	h
4	CLSM5677		301	b	a	a					h	h											h
4	CLR_S1_1938	112.8	308	b	a	h					h	h											h
4	CLS_S3_Contig9679		314	b	a	h					h	h											h
4	CLS_S3_2729	119.8	318	b	a	h	h	h	h	h	h	h		h	h		a	a	a	a	a	a	h
4	CLS_S3_Contig5545		330	b	a	h					h	h											a
4	CLS_S3_655	126.3	340	b	a	h					h	a											a
4	CLSM3095		352	b	a	h					a	a											a
4	CLS_S3_6943	~135	360	b	a	h	h	h	h	h	a	a		h	h		a	a	a	a	a	a	a

Finally, the found mapping interval of 318-340 Mb on chromosome was compared with the MRC on chromosome 4 in *L. sativa.* MRC4 start around 330 Mb and ends around 400 Mb. Part of the mapping interval found in the BC2*sativa*CGN5947 population co-locates with MRC4.

4. Discussion

In this thesis a second monogenic dominant R-gene has been identified in *L. saligna* CGN5947. This resistance gene is located between 318 and 340 Mb on chromosome 4 which partly co-locates with major resistance gene cluster 4 in *L. sativa.* No monogenic dominant R-gene have been identified in *L. saligna* CGN15726, CGN15699 and CGN13330. For CGN15726 and CGN13330 the segregation pattern of the BC1 population resembles the segregation pattern of the BC1*sat*_05271_FR population and therefore it is supposed that the resistance is caused by non-host resistance and not by a monogenic dominant R-gene. BC1*serriola*CGN15699 showed hybrid necrosis and resistance. The genotyping of plants with hybrid necrosis confirms that this phenotype is based on the same genes as has been studied in *L. saligna*_CGN5271 x *L. sativa* cv. Olof (Jeuken et al. 2009). In the same population the segregation of plants in the different infection classes suggested a possible monogenic dominant R-gene gene, but could not be verified in other backcross populations, because the *L. serriola* accession turned out to be partial resistant with low infection levels. Furthermore, markers were successfully developed at chromosome 4 and 7 in the F2_1997_FR genetic map.

Resistance and hybrid incompatibility in BC1*serriola*CGN15699

Hybrid necrosis was observed in 21 % of the BC1*serriola*CGN15699 plants. This hybrid necrosis was based on the same loci and possibly the same mechanism as has been identified for material from the *L. saligna*_CGN5271 x *L. sativa* cv. Olof cross (Jeuken et al. 2009). In the research of Jeuken et al. it has been found that the Rin4 allele from *L. saligna* on chromosome 9 interacts with a possible R-gene from *L. sativa* on chromosome 6. Tested the same markers on the BC1*serriola*CGN15699 population resulted in the same outcome. All plants with a hybrid necrosis phenotype were homozygous *L. serriola* on chromosome 6 and heterozygous for Rin4 on chromosome 9. Hybrid necrosis has been found earlier in some crosses between *L. saligna accessions* and *L. sativa*, but this is the first time this phenomenon has been observed in a cross between *L. saligna* and *L. serriola*.

The disease test with Bl:21 resulted in an absolute infection of 20% for the susceptible parent *L. serriola* CGN04774 at 10 dpi. Because of this low infection score, both the infection score for *L. serriola* as the infection score of the susceptible control *L. sativa* cv Olof (66%) were used to transform all the infection values to RIS. This resulted in 5 resistant plants based on RIS *L. serriola* and 13 resistant plants based on RIS *L. sativa* cv Olof. Plants with hybrid necrosis were excluded in this analysis, because these plants were all resistant because of hybrid incompatibility and not because of R-gene based resistance. Association between genotype and phenotype for both the resistant and susceptible group was found at the top of chromosome 9 with a mapping interval starting at the top of chromosome 9 till marker LE0361 at 26.6 cM. the bottom marker was based on three susceptible plants having a recombination between this marker and the NL0919 marker at 8.5 cM. It was not possible to find a top marker, therefore the beginning of the chromosome was used as the top of the mapping interval.

During the analysis of the results the question raised if this resistance was caused by a monogenic dominant R-gene or if something else was causing the plants being resistant. Remarkable was that almost all resistant plant had a heterozygous genotype for the markers used to test hybrid necrosis. Interestingly this phenomenon was seen before. The *Rin4* gene can also cause race-specific resistance in an interspecific lettuce hybrid (Jeuken et al. 2009). The interaction between the *L. saligna Rin4* allele and a sativa allele at C6 caused race-specific resistance to BL:15, Bl:16, BL:17, Bl:18, BL:20, Bl:22, Bl:24-Bl:28, but not to Bl:10, Bl:14 and Bl:21. This research was done with *L. sativa* as susceptible parent and in this research *L. serriola* has been used as susceptible parent. This can cause the difference in race-specificity.

In order to find out if this resistance was indeed caused by the *Rin4* interaction a new population had to be made with also homozygous *L. saligna* genotype at chromosome 6 and 9. Therefore a resistant plant with a heterozygous genotype at chromosome 6 (31.3 cM) and chromosome 9 (6.7 cM) and a heterozygous introgression at chromosome 9 was selfed to obtain a BC1S1 population which was used to test the hypothesis. Besides this BC1S1, several BC1 plants with a heterozygous introgression at chromosome 9 were backcrossed to *L. serriola* CGN04774 to obtain a BC2 population for validating the possible resistance locus at chromosome 9. Unfortunately the infection of the controls in the disease test were very low and the BC1S1 and BC2 plants hardly showed infection. *L.* Especially the *L. serriola* parent

didn't show a high percentage of infection with average 9.8% and 8.8% in both disease tests. This indicates that the *L. serriola* CGN04774 accession is possibly not as susceptible as has been though before starting the experiment. Therefore the observed resistance cannot be determined to either race-specific resistance caused by the *Rin4* interaction or by a dominant monogenic R-gene locus at chromosome 9. To determine whether or not there is a resistant gene present in *L. saligna* CGN15699 a new Bc1 population can be made with *L. sativa* cv Olof.

Resistance found in *L. sativa* CGN5947

For the BC1*sativa*CGN5947 population the disease test was already performed which led to the hypothesis that a second dominant R-gene was present besides the R-gene /effector-reponse locus at C2. Genotyping was done with markers in MRCs of *L. sativa*. As can be seen in Figure 1.5 of the introduction, all *Dm* genes are found in these MRCs, which makes the chance highest to find an association with heterozygous genotype and disease resistance in one of the MRCs. There was a clear association between heterozygous genotype and resistant phenotype, but also between homozygous *L. sativa* genotype and susceptible phenotype at chromosome 4. This resulted in a mapping interval of102-136.5 cM. This association was confirmed with a BC2 population created by crossing resistant BC1 plants, with a complete heterozygous introgression in the mapping interval, with *L. sativa* cv. Olof. At the same mapping interval there was a clear division between resistant and susceptible genotype linked to the corresponding phenotype. After the design of new markers in the mapping interval of the BC1*sativa*CGN5947 population it was possible to narrow down the resistance mapping interval to 119.8-126.3 cM and 318-340 Mb on chromosome 4. In a BC2 population, the resistance due to non-host is less as in a BC1 population which makes the results based on this BC2*sativa*CGN5947 more reliable than only using the BC1*sativa*CGN5947 results.

So far more dominant monogenic R-genes have been found in MRC4. The approximately 25cM large cluster consists of a lot of genes with a NLR motif from which four downy mildew resistance loci have been identified (*Dm4, Dm7, Dm11 and Dm44*) (Christopoulou, McHale, et al. 2015; McHale et al. 2009). None of these genes have been identified in *L. saligna* accessions, so this is the first identified resistance loci for downy mildew located at chromosome 4 from a *L. saligna* accession. Other *Dm* genes identified from *L. saligna* are located at MRC1, MRC8c and MRC9C (Parra et al. 2016 unpublished manuscript). The recently dissected MRC4 gives an insight in how the genes having a NLR motif are distributed over the MRC. Most NLR genes are located between 330 and 360 Mb with two clusters at 340 and 345 Mb (Christopoulou, McHale, et al. 2015). Our current resistance interval of 318-340Mb partly overlaps withMRC4. It is possible that one of the genes in the cluster around 340 Mb is the causal gene for resistance.

Further fine-mapping and finding of the resistance gene in *L. saligna* CGN5947 will be the next step in this research. Obtaining a BC3 population from the resistant plants and genotype for new recombinant would be the first step. These recombinants than have to be phenotyped for disease resistance to narrow down the mapping interval. The BC3 population would give even a more reliable mapping interval due to loss of non-host alleles. Furthermore new markers can be developed between the genes with a NLR motif to possible exclude the clustered genes at 340 Mb. This is possible thanks to the physical map with the NLR genes recently published by Christoupoulou et al. If it is possible to narrow down the candidate NLR genes attempts to knock out these genes can be made. This is possible by random mutagenesis like EMS or with RNAi techniques. In order to make this founding more interesting for breeding companies, more Bremia isolates need to be tested for this resistance, so the resistance spectrum is known.

Finding the causal resistance genes will have benefits for breeding. This allows breeders to develop molecular markers in or close to the gene of interest which will not recombine in the next generation. This gives more reliable results and can help with pyramiding resistance genes. This pyramiding of resistance genes will eventually led to a more durable resistance against downy mildew, but also other diseases (Michelmore et al. 2013).

5. Conclusion

Marker development

Markers are developed on chromosome 4 at 301, 314, 330 and 352 Mb and on chromosome 7 at 38, 73 and 200 Mb. From one marker the physical position could not be determined, but the genetic position was between 62.2 and 62.6 cM. For the tested primers on chromosome 4, 47% showed different melting curves for the selected lines. For the primers tested on chromosome 7 this was 21%. The markers on chromosome 4 were al co-dominant on the HRM analysis when tested for the F2_1997_FR population. Two markers on chromosome 7 were dominant, one markers was partly dominant, partly co-dominant and one marker was co-dominant for the F2-1997_FR population.

Resistance in *L. saligna* CGN15726, *L. saligna* CGN13330

BC1*sativa*CGN15726 and BC1*sativa*CGN13330 both have a continuous segregation over the different infection classes with 9% and 6% resistant plants respectively. In both populations this is the same kind of segregation as the BC1*sat*_05271_FR population which implicates that the resistance in these populations is based on non-host resistance.

Resistance in *L. saligna* CGN15699

Hybrid necrosis was observed in BC1*serriola*CGN15699 which was based on the same loci as has been studied in *L. saligna*_CGN5271 x *L. sativa* cv. Olof. There was a continuous segregation over the different infection classes with implications for a possible R-gene present. However, it turned out that the susceptible parent used in this population was not very susceptible in the disease test. Therefore it could not be determined if a monogenic dominant R-gene was present in *L. saligna* CGN15699.

Resistance in *L. saligna* CGN5947

It was possible to determine a mapping interval for resistance in the BC1*sativa*CGN5947 population. This interval was located between 102 and 135 cM at chromosome 4. Using a BC2 population this interval was fine-mapped to 119.8 and 126.3 cM (318-340 Mb). The mapping interval co-locates partly with MRC4 in *L. sativa* which starts around 330 M.

References

Boer, E. d. 2014. *Genetic investigation of the nonhost resistance of wild lettuce, Lactuca saligna, to lettuce downy mildew, Bremia lactucae*, Wageningen University, Wageningen.

Bonnier, F. J. M., K. Reinink, and R. Groenwold. 1991. New sources of major gene resistance in Lactuca to Bremia lactucae. *Euphytica* 61 (3): 203-211. http://dx.doi.org/10.1007/bf00039659.

Christopoulou, M., L. K. McHale, A. Kozik, S. R. C. Wo, T. Wroblewski, and R. W. Michelmore. 2015. Dissection of two complex clusters of resistance genes in lettuce (Lactuca sativa). *Molecular Plant-Microbe Interactions* 28 (7): 751-765. http://dx.doi.org/10.1094/mpmi-06-14-0175-r.

Christopoulou, M., S. R.-C. Wo, A. Kozik, L. K. McHale, M.-J. Truco, T. Wroblewski, and R. W. Michelmore. 2015. Genome-Wide Architecture of Disease Resistance Genes in Lettuce. *G3: Genes|Genomes|Genetics* 5 (12): 2655-2669. http://dx.doi.org/10.1534/g3.115.020818.

de Vries, I. M. 1990. Crossing experiments of lettuce cultivars and species (Lactuca sect. Lactuca, Compositae). *Plant Systematics and Evolution* 171 (1-4): 233-248. http://dx.doi.org/10.1007/bf00940608.

———. 1997. Origin and domestication of Lactuca sativa L. *Genetic Resources and Crop Evolution* 44 (2): 165-164. http://dx.doi.org/10.1023/a:1008611200727.

den Boer, E., K. T. B. Pelgrom, N. W. Zhang, R. G. F. Visser, R. E. Niks, and M. J. W. Jeuken. 2014. Effects of stacked quantitative resistances to downy mildew in lettuce do not simply add up. *Theoretical and Applied Genetics* 127 (8): 1805-1816. http://dx.doi.org/10.1007/s00122-014-2342-7.

Derevnina, L. *The Downy Mildews: So Many Genomes, so Little Time*, 13-1-2015 2015 [cited 20-1-2016. Available from https://pag.confex.com/pag/xxiii/webprogram/Paper17147.html.

FAO. *Food And Agricultural Organisation of the United Nations (FAO)* 2013 [cited 2015-10-22. Available from http://faostat3.fao.org.

Gil, R. J. *Comparative Genomics of Bremia lactucae* 12-1-2015 2015 [cited 8-12-2015. Available from https://pag.confex.com/pag/xxiii/webprogram/Paper16520.html.

Glowacki, S., V. K. Macioszek, and A. K. Kononowicz. 2011. R proteins as fundamentals of plant innate immunity. *Cellular and Molecular Biology Letters* 16 (1): 1-24. http://dx.doi.org/10.2478/s11658-010-0024-2.

International Bremia Evaluation Board. 2016. *Race denomination of new bremia isolates* 2016 [cited 18 April 2016]. Available from http://www.worldseed.org/our-work/plant-health/other-initiatives/ibeb/.

Jeuken, M., and P. Lindhout. 2002. Lactuca saligna, a non-host for lettuce downy mildew (Bremia lactucae), harbors a new race-specific Dm gene and three QTLs for resistance. *Theoretical and Applied Genetics* 105 (2-3): 384-391. http://dx.doi.org/10.1007/s00122-002-0943-z.

Jeuken, M., R. Van Wijk, J. Peleman, and P. Lindhout. 2001. An integrated interspecific AFLP map of lettuce (Lactuca) based on two L. sativa × L. saligna F2 populations. *Theoretical and Applied Genetics* 103 (4): 638-647. http://dx.doi.org/10.1007/s001220100657.

Jeuken, M. J. W., and P. Lindhout. 2004. The development of lettuce backcross inbred lines (BILs) for exploitation of the Lactuca saligna (wild lettuce) germplasm. *Theoretical and Applied Genetics* 109 (2): 394-401.

Jeuken, M. J. W., K. Pelgrom, P. Stam, and P. Lindhout. 2008. Efficient QTL detection for nonhost resistance in wild lettuce: Backcross inbred lines versus F2 population. *Theoretical and Applied Genetics* 116 (6): 845-857. http://dx.doi.org/10.1007/s00122-008-0718-2.

Jeuken, M. J. W., N. W. Zhang, L. K. McHale, K. Pelgrom, E. Den Boer, P. Lindhout, R. W. Michelmore, R. G. F. Visser, and R. E. Niks. 2009. Rin4 causes hybrid necrosis and race-specific resistance in an interspecific lettuce hybrid. *Plant Cell* 21 (10): 3368-3378. http://dx.doi.org/10.1105/tpc.109.070334.

Jones, J. D. G., and J. L. Dangl. 2006. The plant immune system. *Nature* 444 (7117): 323-329. http://dx.doi.org/10.1038/nature05286.

Kesseli, R., O. Ochoa, and R. Michelmore. 1991. Variation at RFLP loci in Lactuca spp. and origin of cultivated lettuce (L. sativa). *Genome* 34 (3): 430-436.

Latgé, J. P. 2010. Tasting the fungal cell wall. *Cellular Microbiology* 12 (7): 863-872. http://dx.doi.org/10.1111/j.1462-5822.2010.01474.x.

Lebeda, A., E. J. Ryder, R. Grube, I. Dole_alová, and E. K. ístková. 2006. "Lettuce (Asteraceae; Lactuca spp.)." In *Genetic Resources, Chromosome Engineering, and Crop Improvement*, 377-472. CRC Press.

Lebeda, A., M. Sedlářová, M. Petřivalský, and J. Prokopová. 2008. Diversity of defence mechanisms in plant-oomycete interactions: A case study of Lactuca spp. and Bremia lactucae. *European Journal of Plant Pathology* 122 (1): 71-89. http://dx.doi.org/10.1007/s10658-008-9292-3.

Lebeda, A., and V. Zinkernagel. 2003. Characterization of new highly virulent german isolates of Bremia lactucae and efficiency of resistance in wild Lactuca spp. germplasm. *Journal of Phytopathology* 151 (5): 274-282.

Lindqvist, K. 1960. ON THE ORIGIN OF CULTIVATED LETTUCE. *Hereditas* 46 (3-4): 319-350. http://dx.doi.org/10.1111/j.1601-5223.1960.tb03091.x.

McDonald, B. A., and C. Linde. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. In *Annual Review of Phytopathology*.

McHale, L. K., M. J. Truco, A. Kozik, T. Wroblewski, O. E. Ochoa, K. A. Lahre, S. J. Knapp, and R. W. Michelmore. 2009. The genomic architecture of disease resistance in lettuce. *Theoretical and Applied Genetics* 118 (3): 565-580. http://dx.doi.org/10.1007/s00122-008-0921-1.

Meyers, B. C., D. B. Chin, K. A. Shen, S. Sivaramakrishnan, D. O. Lavelle, Z. Zhang, and R. W. Michelmore. 1998. The Major Resistance Gene Cluster in Lettuce Is Highly Duplicated and Spans Several Megabases. *The Plant Cell* 10 (11): 1817-1832. http://dx.doi.org/10.1105/tpc.10.11.1817.

Meyers, B. C., A. Kozik, A. Griego, H. Kuang, and R. W. Michelmore. 2003. Genome-wide analysis of NBS-LRR-encoding genes in Arabidopsis. *Plant Cell* 15 (4): 809-834. http://dx.doi.org/10.1105/tpc.009308.

Michelmore, R., and J. Wong. 2008. Classical and molecular genetics of Bremia lactucae, cause of lettuce downy mildew. *European Journal of Plant Pathology* 122 (1): 19-30. http://dx.doi.org/10.1007/s10658-008-9305-2.
Michelmore, R., J. Wong, R. J. Gil, and K. I. 2012. Sequencing the Obligate Oomycete Plant Pathogen, Bremia lactucae. In *International Plant and Animal Genome Conference XX* San Diego, California.
Michelmore, R. W., M. Christopoulou, and K. S. Caldwell. 2013. Impacts of resistance gene genetics, function, and evolution on a durable future. In *Annual Review of Phytopathology*.
Niks, R. E. 1988. Nonhost plant species as donors for resistance to pathogens with narrow host range. II. Concepts and evidence on the genetic basis of nonhost resistance. *Euphytica* 37 (1): 89-99. http://dx.doi.org/10.1007/bf00037229.
Parra, L., B. Maisonneuve, A. Lebeda, J. Schut, M. Christopoulou, M. Jeuken, L. McHale, et al. 2016 unpublished manuscript. Rationalization of Genes for Resistance to Bremia lactucae in Lettuce. *unpublished manuscript*.
Petrželová, I., A. Lebeda, and A. Beharav. 2011. Resistance to Bremia lactucae in natural populations of Lactuca saligna from some Middle Eastern countries and France. *Annals of Applied Biology* 159 (3): 442-455. http://dx.doi.org/10.1111/j.1744-7348.2011.00507.x.
Schulze-Lefert, P., and R. Panstruga. 2011. A molecular evolutionary concept connecting nonhost resistance, pathogen host range, and pathogen speciation. *Trends in Plant Science* 16 (3): 117-125. http://dx.doi.org/10.1016/j.tplants.2011.01.001.
Simko, I., O. E. Ochoa, M. A. Pel, C. Tsuchida, C. Font I Forcada, R. J. Hayes, M. J. Truco, et al. 2015. Resistance to downy mildew in lettuce 'La Brillante' is conferred by Dm50 gene and multiple QTL. *Phytopathology* 105 (9): 1220-1228. http://dx.doi.org/10.1094/phyto-02-15-0057-r.
Stassen, J. H. M., M. F. Seidl, P. W. J. Vergeer, I. J. Nijman, B. Snel, E. Cuppen, and G. Van Den Ackerveken. 2012. Effector identification in the lettuce downy mildew Bremia lactucae by massively parallel transcriptome sequencing. *Molecular Plant Pathology* 13 (7): 719-731. http://dx.doi.org/10.1111/j.1364-3703.2011.00780.x.
Su, H., A. H. C. Van Bruggen, K. V. Subbarao, and H. Scherm. 2004. Sporulation of Bremia lactucae affected by temperature, relative humidity, and wind in controlled conditions. *Phytopathology* 94 (4): 396-401.
Thomma, B. P. H. J., T. Nürnberger, and M. H. A. J. Joosten. 2011. Of PAMPs and effectors: The blurred PTI-ETI dichotomy. *Plant Cell* 23 (1): 4-15. http://dx.doi.org/10.1105/tpc.110.082602.
Truco, M. J., H. Ashrafi, A. Kozik, H. van Leeuwen, J. Bowers, S. Reyes Chin Wo, K. Stoffel, et al. 2013. An Ultra High-Density, Transcript-Based, Genetic Map of Lettuce. *G3 (Bethesda)*. http://dx.doi.org/10.1534/g3.112.004929.
Untergasser, A., H. Nijveen, X. Rao, T. Bisseling, R. Geurts, and J. A. M. Leunissen. 2007. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research* 35 (SUPPL.2): W71-W74. http://dx.doi.org/10.1093/nar/gkm306.
Van Der Biezen, E. A., and J. D. G. Jones. 1998. Plant disease-resistance proteins and the gene-for-gene concept. *Trends in Biochemical Sciences* 23 (12): 454-456. http://dx.doi.org/10.1016/s0968-0004(98)01311-5.
Van Hese, N., C.-J. Huang, D. De Vleesschauwer, I. Delaere, E. Pauwelyn, P. Bleyaert, and M. Höfte. 2015. Evolution and distribution of virulence characteristics of Belgian Bremia lactucae populations between 2008 and 2013. *European Journal of Plant Pathology*: 1-11. http://dx.doi.org/10.1007/s10658-015-0779-4.
Wang, H., M. Qi, and A. J. Cutler. 1993. A simple method of preparing plant samples for PCR. *Nucleic Acids Research* 21 (17): 4153-4154.
Ye, Z., and J. P. Y. Ting. 2008. NLR, the nucleotide-binding domain leucine-rich repeat containing gene family. *Current Opinion in Immunology* 20 (1): 3-9. http://dx.doi.org/10.1016/j.coi.2008.01.003.
Zhang, N. W., P. Lindhout, R. E. Niks, and M. J. W. Jeuken. 2009. Genetic dissection of Lactuca saligna nonhost resistance to downy mildew at various lettuce developmental stages. *Plant Pathology* 58 (5): 923-932. http://dx.doi.org/10.1111/j.1365-3059.2009.02066.x.
Zipfel, C., S. Robatzek, L. Navarro, E. J. Oakeley, J. D. G. Jones, G. Felix, and T. Boller. 2004. Bacterial disease resistance in Arabidopsis through flagellin perception. *Nature* 428 (6984): 764-767. http://dx.doi.org/10.1038/nature02485.

Appendix 1: General protocol for disease test with downy mildew on leaf discs of lettuce

by Marieke Jeuken, Wageningen University, last update 2013

The aim of the experiment is to estimate the quantitative/partial resistance to *Bremia* on leaf discs of lettuce plants. Quantitative differences in susceptibility can be observed on leaf discs of adult plants, and usually cannot be observed on the cotyledons of seedlings. Disease tests on seedlings are mostly used to screen qualitative resistance (=completely resistant versus completely susceptible).

Description of *Bremia* disease test on leaf discs of lettuce

Plant conditions

Disease tests are performed on mature plants in the tenth to twelfth leaf stage (7 to 10 weeks old plants depending on the season). The plants should be in perfect condition: no damage by insects/parasites and not sprayed with fungicides. Before your leaf disc sampling, water the plants to have good strong erect leaves. This will longen the quality of your leaf disc during the experiment.

Collect leaf discs

Four leaf discs of 17 mm in diameter (or leaf pieces by scissors) are taken from full-grown leaves of each plant and placed upside down on a filter paper-cotton wool combination (top and bottom) moistened with water in a plastic box of 40×25×8 cm. A specified number of leaf discs of each plant are placed randomly in blocks (number of blocks are defined in each experiment separately, depending on plant material and experiment size). Plants of diverse control lines are included (susceptible, resistant, partial resistant , parental controls). Each box can contain about 150- 200 positions for leaf discs.

Preparing inoculum

Bremia is maintained on seedlings of susceptible cultivars grown in plastic boxes or on young plants in large cages in the greenhouse. Use fresh infected leaves or deeply-frozen infected leaf material (-80° C, up to 4-5 months storage) to prepare inoculum. Wash sporulating seedlings/leaves in tap water. The spore suspension is filtrated with cheesecloth to get rid of dirt or plant particles. Measure the spore concentration by the use of a haemocytometer. A concentration of 2-4 $\times$ 10^5 spores per ml is recommended. (Spore counting in a haemocytometer, see below)

Inoculation of leaf discs and conditions during the infection period

The leaf discs are inoculated by spraying with a spore suspension. To spray a spore suspension we use a very fine sprayer and we do **not** use a pipette making droplets! To minimize the risk of escapes, a second inoculation can be performed the day after the first inoculation (recently we only inoculate once). Directly after inoculation the boxes with leaf discs are enclosed in a plastic transparent bag to keep humidity high. The boxes are placed in the climate cell or conditioned greenhouse compartment and incubated in the dark for at least 12 hours (maximally 16 hours) by covering the top of the box or switch off the light. Germination of spores is very sensitive to high radiation of light. It can be slowed down or totally stopped. During the first days when the spores will germinate and penetrate the leaf discs a high humidity in the box high is optimal. Check regularly if the filter paper is still wet and moisten to keep the conditions in the box at high humidity.

Climate cell conditions

The boxes with leaf discs are placed on a table in a climate cell. Growth conditions are a photoactive period of 16 hours and a constant temperature of 15° C, light conditions for leaf discs is 20 W/m^2 (= 101 micromole m^{-2} s^{-1}; only light between 400 and 700 nm are measured for light intensity measurements, between 80 and 100 micromole are good light conditions!).

Assessment of resistance

Assessment of resistance is best observed daily from seven to ten days after inoculation depending on the virulence of the Bremia isolate. The first sporulation usually appears after 6 days and 7 days for Bl:16/Bl:21 and Bl:14 respectively. Decide to assess the disease test based on the results of the susceptible controls. Their leaf discs should be covered with sporangiophores for 75 and 100% of the surface. *Bremia* infection severity is scored on leaf discs distributed as the percentage of leaf area covered with sporulation (**No** qualitative scoring in classes like 0, 1,2 3, and 4!). See Figure 1 and pictures at the end of the document