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Combining resistance QTLs to *Bremia lactucae* in lettuce and the finemapping of *resistance QTL 1*

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Preface

This is the report of my Major Thesis in plant breeding done at Wageningen University. This thesis is the last part of my study and will finalize 6 years of student life. For this thesis I have chosen for resistance breeding. I have especially chosen for this subject because it is one of the subjects I am interested in. In this thesis resistance QTLs against *Bremia lactucae* were combined in plants and one resistance QTL was finemapped in order to get new insights how to exploit the non-host resistance from *L. saligna* in lettuce (*Lactuca sativa*). I believe that the experiences and knowledge I gained during this thesis will help me in my career as plant breeder.

This thesis project was done in 6.5 months and I have been guided and supervised by Dr. Marieke J.W. Jeuken, Ir. Ningwen Zhang, Dr. Pim Lindhout and Dr. Rients E. Niks.

Summary

CombiBILs were developed in which two *Lactucae saligna* introgressions were combined in *Lactuca sativa* (lettuce). These *L. saligna* introgressions harbor a resistance QTL and originate from different BILs. These combiBILs were developed in order to investigate whether there is an interaction between the different resistance QTLs. In these combiBILs the *L. saligna* introgressions from BIL 2.2 (*rbq5*), BIL 4.2 (*rbq7*), BIL6.3 (*rbq6*) and BIL8.2 (*rbq4*) were combined. Three combiBILs with two *L. saligna* introgressions were already developed. During this thesis three new combiBILs with two *L. saligna* introgressions were developed. The six combiBILs with two different *L. saligna* introgressions, which could be developed from the four BILs, were tested for resistance to *Bremia lactucae* race Bl:14. Based on this disease test an additive effect between *rbq4* and *rbq6* is expected.

RBQ1 is a resistance QTL to *Bremia lactucae* from *L. saligna* and was mapped in the middle part of Chromosome 7. During this thesis *RBQ1* was finemapped based on a recombinant analysis on two lines with a 95% *L. sativa* background. For this finemapping the lines pv02255 and pv02268 which were both segregating for the interval where *RBQ1* was expected were screened for recombinants. In lines pv02255 and pv02268 23 and 22 recombinants were detected respectively. Based on disease test with *Bremia* race Bl:14 and Bl:16 on the recombinants of line pv02268 *RBQ1* might be expected between 44 and 59 cM.

Table of content

Preface ... 3

Summary ... 4

Table of content ... 5

Introduction ... 7

1.1 Lettuce ... 7
1.2 Lettuce downy mildew ... 7
1.3 Non-host resistance ... 9
1.4 Breeding for resistance to Bremia Lactucae ... 10
1.5 The revealing of non-host resistance of L. saligna against B. lactucae; present results 10
1.6 Molecular markers ... 13
1.7 Thesis objectives ... 14

Materials and methods ... 16

2.1 Marker development ... 16
2.2 CombiBILs ... 17
2.3 The finemapping of RBQ1 ... 19
2.4 Bremia disease test on leaf discs ... 20
2.4.1 Leaf disc test on the CombiBILs ... 21
2.4.2 Leaf disc test on plants recombinant for introgression containing RBQ1 ... 21

Results ... 23

3.1 The development of molecular and morphological markers ... 23
3.1.1 Molecular markers ... 23
3.1.2 Morphological markers ... 25
3.2 CombiBILs ... 27
3.2.1 The development of combiBIL 2.2 + 8.2 ... 27
3.2.2 The development of combiBIL 6.3 + 8.2 ... 27
3.2.3 The development of combiBIL 4.2 + 6.3 ... 29
3.2.4 Disease test on the combiBILs ... 29
3.3 The fine mapping of RBQ1 ... 31
3.3.1 Recombinant screening ... 31
3.3.2 Disease test on the recombinants of RBQ1 ... 34

Discussion ... 36

4.1 The development of molecular and morphological markers ... 36

4.1.1 Molecular markers 36
4.1.2 Morphological markers 36
4.2 CombiBIL development and assessment of their resistance level 37
4.3 The finemapping of RBQ1 39
4.4 General 43
4.5 Recommendations: 44

Acknowledgements 46

References 47

Appendices 50

Appendix 1 50
Appendix 2 51
Appendix 3 53
Appendix 4 54
Appendix 5 55
Appendix 6 57

Introduction

1.1 Lettuce

Lettuce, *Lactuca sativa* L. belongs to the genus *Lactuca* L. (Compositae) that consists of about 100 species distributed in temperate and warm regions, mostly in the northern hemisphere (de Vries 1997). *Lactuca sativa* L. is classified in the section *Lactuca,* subsection *Lactuca.*

The centre of origin of cultivated lettuce probably lies in the Kurdistan-Mesopotamia area (de Vries 1997). From South-West Asia cultivated lettuce spread to Egypt, where images of lettuce appeared around 2500 B.C. on wall paintings and reliefs of tomb walls. Lettuce was introduced to Northwest Europe around the Middle Ages. Nowadays lettuce is in general a popular fresh leaf vegetable. Since it is cultivated both inside outside the greenhouse, lettuce can be consumed all year round.

It is still not exactly known which species were involved in the domestication of lettuce. It is certain that *L. serriola* is one of the direct ancestors or the only direct ancestor of lettuce (de Vries 1997). During the domestication of lettuce, humans caused a decrease in some undesired features, like a lower latex content, a less bitter taste, loss of prickles and absence of early bolting.

The haploid genome of *L. sativa* contains nine chromosomes (2n=18). The diploid genome of lettuce contains about $2.6 \cdot 10^9$ bp (Arumuganathan and Earle 1991), which is a relatively large genome, 2.6 and 18 times larger than the tomato and *Arabidopsis* genome, but normal compared to other *Compositae* species (Kesseli and Michelmore 1996).

1.2 Lettuce downy mildew

Downy mildew is caused by *Bremia Lactucae* Regel. *Bremia lactucae* belongs to the Oomycetes, order Peronosporales and family of *Peronosporaceae*. Downy mildew is a destructive disease of lettuce in both commercial fields and glasshouse production systems worldwide. In lettuce cultivation *Bremia* is mainly controlled by genetic resistance and the application of fungicides.

Oomycetes are organisms that show a filamentous growth habit and are often falsely referred to as fungi. Oomycetes are structurally, biochemically and genetically different from all fungi taxa

(Cambell 1993). The cell walls of oomycetes for instance consist mainly of cellulose, while the major compound of the cell wall in fungi is chitin (Cambel 1993).

Bremia is an obligate parasite that grows and sporulates on the leaves of lettuce and several other *lactuca* species (Lebeda and Syrovatko 1988). After germination the germ tube enters an epidermal cell via an appressorium, to form primary and secondary vesicles (Lebeda et al 2001). Penetration through the stomata also occurs, but in a low frequency (Lebeda and Reinink 2001). In susceptible hosts *Bremia* will start colonization of the infected leaf by forming intercellular hyphae and haustoria.

Although *Bremia* can reproduce sexually via oospores, secondary spread of the disease in the field exclusively results from infections by asexually formed sporangia (Wu et al 2000). Since *Bremia* is predominantly heterothallic, sexual reproduction only takes place when isolates of the opposite sexual compatibility type (called B1 and B2) are grown in close proximity in the same tissue (Crute 1992). Sporulation is affected significantly by the temperature, with an optimum at 15ºC and by relative humidity, with sporulation increasing at relative humidity ≥ 90% (Su et al 2004). Sporulation of *Bremia* occurs, dependant on the conditions, 5 to 14 days after infection (Wu et al 2000). Sporangiophores of *Bremia* emerge through the stomata as white tree like structures at the abaxial site of the leaf (Su et al 2004).

Figure 1. The sporulation of *B. lactucae* on the abaxial site of a lettuce leaf.

1.3 Non-host resistance

Plants are continuously exposed to a wide variety of pathogens. Non-host resistance, at species level, is a phenomenon that enables plants to protect themselves against the vast majority of these potentially invasive agents (Holub and Cooper 2004).
A plant species is a non-host to a potential pathogen species, when all genotypes of that plant species are fully resistant to all genotypes of that pathogen species (Heath 1981, Niks 1987). Compared to the defense reactions in host pathogen interactions, not much research has been done on the genetic basis and mechanisms of non-host resistance of plants (Heath 2001, Thordal-Christensen 2003), but non-host resistance is now receiving more interest than in the past. Several different mechanisms have been described which are involved in non-host resistance.
There are various studies, for instance the *INF1* protein in the pathogen *Phytopthora infestans* which elicits cell death in *Nicotiana benthamiana* (Kamoun et al 1998), that demonstrate that major resistance genes in interaction with non-host avirulence genes may explain the resistance of non-host plant species to inappropriate pathogens (Heath 2001). This raises the possibility that non-host resistance involves similar gene-for-gene interactions to those that govern parasite-specific resistance within host species (Heath 2001). In this case host and non-host resistance share the same mechanism.
Another mechanism involved in non-host resistance is a non-specific defense reaction triggered by non-specific stimuli (Heath 2001). These non-specific stimuli are also referred to as "general elicitors" (Thordal-Christensen 2003), a concept which may be on the way to being replaced by the term 'pathogen-associated molecular pattern' PAMP (Reignault and Sancholle 2005). The innate immunity system of plants recognises PAMPs through pattern recognition receptors (PRRs). In this model the inappropriate pathogen is not able to suppress a basal defense system, causing an incompatible reaction. Basal defense is for instance found in barley (*Hordeum vulgare*) against several heterologous rust species (Jafari et al 2006).
In some plant species, resistance to certain plant pathogens may be explained by preformed physical or chemical factors (Heath 1981, Reignault and Sancholle 2005). Such defenses can take place both at anatomical level, like the cuticle or cell wall appositions, or at the biochemical level, like secondary metabolites and antimicrobial proteins (Reignault and Sachnolle 2005). Based on this information it is likely that the phenomenon 'non-host resistance' comprises one or several defense mechanisms.

Non-host resistance is highly effective and durable, which makes is attractive to exploit the mechanism of non-host resistance to generate resistant crop plants (Thordal-Christensen 2003, Holob and Cooper 2004). However it has not been widely used in plant breeding because of the difficulty of selecting plants possessing all the genes for resistance and having good quality traits (Holob and Cooper 2004, Hand et al 2003). The availability of molecular makers nowadays offers the potential for Marker Assisted Selection (MAS) of multigenic traits (Hand et al 2003).

1.4 Breeding for resistance to *Bremia Lactucae*

Lettuce breeders have put a large effort in the breeding of lettuce downy mildew resistance, (Crute 1992, Reinink 1999). The most common exploited resistance is qualitative and is under genetic control of single dominant genes, *Dm* genes (Downy mildew) or *R*-genes that are matched by avirulence genes in *Bremia* in a gene-for-gene interaction associated with a Hypersensitive Response. Unfortunately, *Dm* genes are in most cases quickly overcome by rapid adaptation of the pathogen (Lebeda and Zinkernagel 2003).
Over nineteen *Dm* genes have been introgressed into commercial cultivars from cultivated germplasm sources or closely related species like *L. serriola* (van Ettekoven and van der Arend 1999). Like dominant monogenic resistances in other plant species, the *Dm* genes in lettuce are distributed in clusters over the genome (Kesseli and Michelmore 1996). These three major clusters are located on Chromosome 1, 2 and 4 (Kesseli et al. 1994). However because race specific *Dm* genes are not durable, there is a need for an alternative, race non-specific and durable resistance in lettuce breeding (Jeuken and Lindhout 2003). *Lactuca saligna* is among the *Lactuca* species the best studied non-host to lettuce downy mildew and is crossable with cultivated lettuce (Bonnier et al. 1992). In 1997 M. Jeuken started a study on the resistance of *L. saligna* to *Bremia* to reveal new insights into the ‘non-host’ defense mechanisms of plants.

1.5 The revealing of non-host resistance of L. saligna against B. lactucae; present results

To unveil the non-host resistance of *L. saligna* two different strategies were used (Jeuken et al 2001). Strategy A was the classical F_2 mapping strategy and strategy B was using Backcross Inbred Lines (BILs) (Fig 1).

For strategy A a F_2 population was derived from a cross between the non-host *L. saligna* CGN5271 and the susceptible *L. sativa* cultivar 'Olof' (Jeuken et al 2001). The F_2 population consisted of 126 plants and was used in for the construction of an interspecific AFLP map (Jeuken et al 2001). The 126 plants of the F_2 population were genotyped using this AFLP map and tested for resistance to two *Bremia* races Bl:14 and Bl:16 (Jeuken and Lindhout 2002). QTL mapping revealed three QTLs, which were designated *RBQ1, RBQ2* and *RBQ3* (*Resistance to Bremia QTL*) (Jeuken and Lindhout 2002).

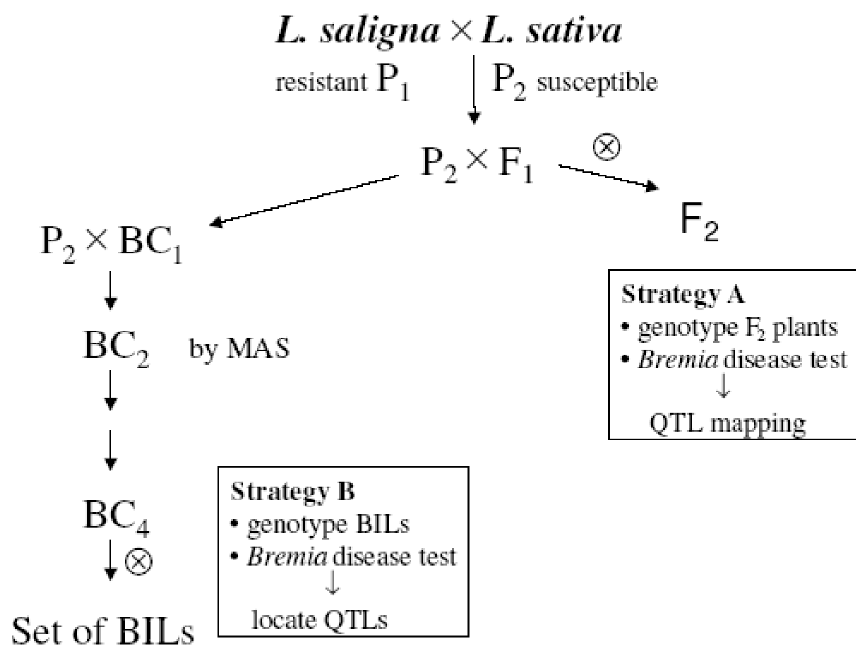

Figure 2. The two strategies that were used for the genetical dissection of the resistance of *L. saligna* to downy mildew.
⊗ = selfing, MAS = Marker Assisted Selection

For strategy B Backcross Inbred Lines (BILs) were developed in which chromosome segments of *L. saligna* were introgressed into *L. sativa* cultivar 'Olof' (Jeuken and Lindhout 2003). These lines were developed by four to five backcrosses with the recurrent parent (*L. sativa*) and one generation of selfing (Jeuken and Lindhout 2004). For the development of the BILs, MAS was used from BC_4 generation and continued until the final set of BILs was reached (Jeuken and Lindhout 2003). A set of 28 lines was developed (Figure 2) that together contained 96% of the *L. saligna* genome (Jeuken and Lindhout 2003, Jeuken and Lindhout 2004). Using the BIL strategy four new QTLs were identified (Jeuken and Lindhout 2003, Jeuken et al 2005).

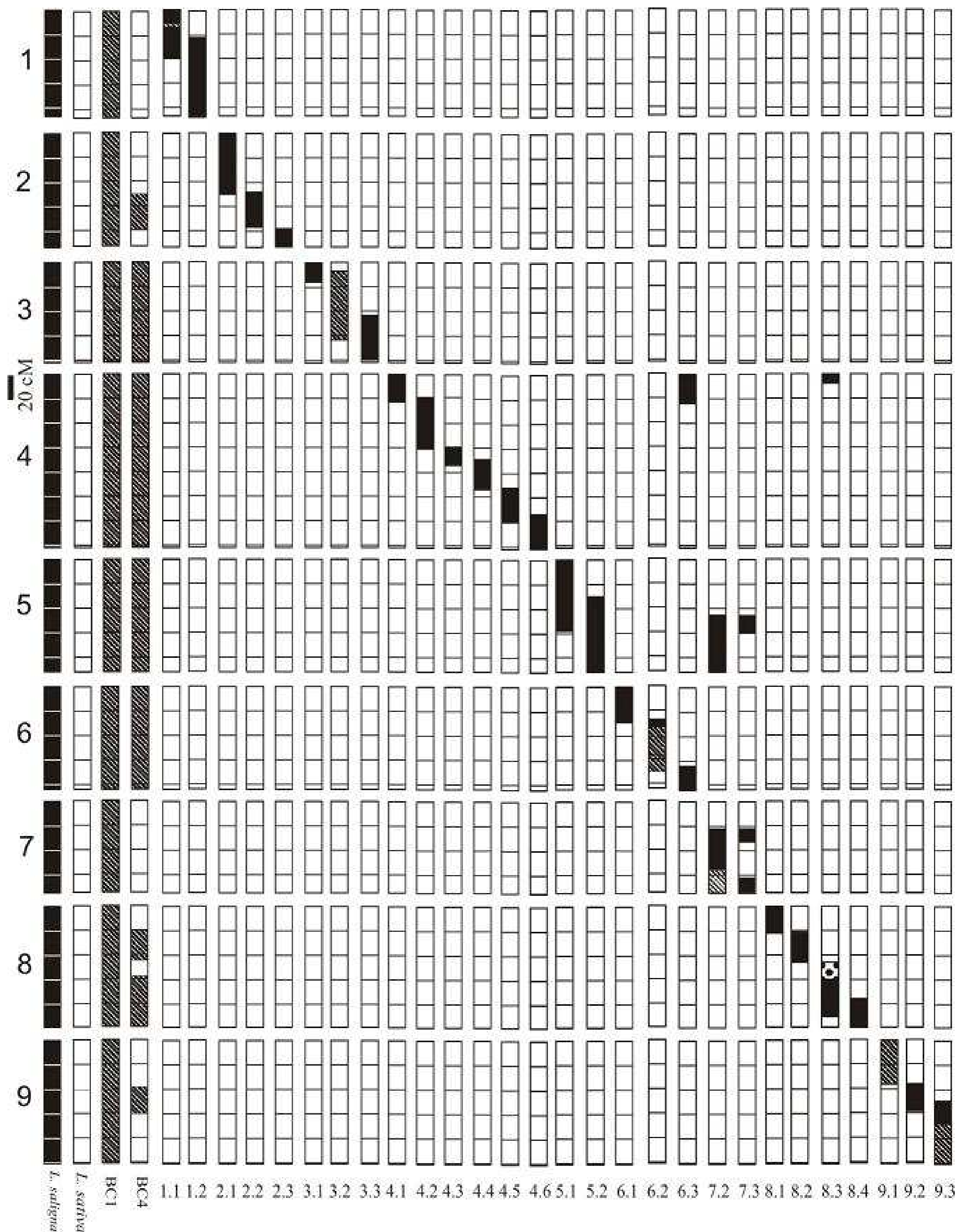

Figure 3. Genome coverage of the BC1 and BC4 generations and the genotypes of 28 back-cross lines (*lines1.1-9.3*) that cover 96% of the *L.saligna* genome. *Vertical bars* represent the nine chromosomes of lettuce. The chromosomes are segmented in 20 cM intervals that are delimited by horizontal lines. The genomes of the BC1 and BC4 populations and of the 28 backcross lines are indicated in black, white and diagonal stripes: white homozygous *L.sativa*, black homozygous *L.saligna*, diagonal stripes heterozygous. *Dot* indicates an unknown genotype as not enough markers were analyzed in that region (Jeuken and Lindhout 2004).

1.6 Molecular markers

DNA marker technologies offer great possibilities for plant research and plant breeding. They are used for marker assisted selection (MAS) and for the construction of high density maps. MAS can be used in early stages of breeding programs to avoid disease tests and to pyramid resistance genes (Reinink 1999). The development of high-density molecular maps offers opportunities for the (fine) mapping and tagging of traits of interest.

Most of the markers developed and used in the past were developed from the genomic DNA and could therefore belong to either the transcribed region or to the non-transcribed region of the genome (Gupta and Rustgi 2004). Since these DNA-based markers, like AFLP markers, can be derived from any region of the genome, they have been described as random DNA markers (RDMs) (Andersen and Lubberstedt 2003).

In recent years there was a shift in the interest from the RDMs to gene targeted markers (GTMs), representing the transcribed regions of the genome (Gupta and Rustgi 2004). GTMs are more informative and wider applicable, since transcriptome based markers could detect both length and sequence polymorphism in the expressed region of the genome (Gupta and Rustgi 2004). This shift has become possible due to the availability of a large number of cDNA clones in a variety of plant systems and due to the accumulation of large numbers of expressed sequence tags (ESTs) in public databases (Gupta and Rustgi 2004). These databases are used as a supplier of GTMs which are used for the construction of genetic linkage maps. For lettuce there is a large database of EST sequences available on University of California at Davis (http://cgpdb.ucdavis.edu). This database contains lettuce cDNA contigs which are based on *Lactuca sativa* cv Salinas and the wild lettuce *Lactuca serriola.*

The genetic linkage map of lettuce that was developed for this project consisted in the first stage of AFLP-markers (Jeuken et al 2001). Nowadays GTMs like Sequence Characterized Amplified Regions (SCAR), Cleaved Amplified Polymorphic regions (CAPs) (both Gupta and Rustgi 2004) and derived Cleaved Amplified Polymorphic regions (dCAPs) (Neff et al 1998, Ilic et al 2004) are added to this genetic linkage map. SCAR marker shows a length polymorphism between the amplification products. When the amplified fragments show a polymorphism after cleavage with a suitable restriction enzyme, this marker is called a CAPs marker. For a dCAPs marker a primer is developed with one or two nucleotide mismatches just before the single nucleotide polymorphism, creating a restriction site for a suitable restriction enzyme in the amplification product of one of the plants. These markers are in general codominant which makes them more informative than the dominant AFLP markers.

1.7 Thesis objectives

This thesis presents a study on two subjects. The first subject is about the development of lettuce plants containing two resistance QTLs against *Bremia* (combiBILs) and the assessment of the resistance level of these plants. The second subject reports the finemapping of *RBQ1* based on a recombinant analysis.

To accomplish these two subjects it was necessary to develop new EST-markers. These new EST-markers were developed on the *L. saligna* introgressions of BIL 7.2, BIL 8.2 and BIL 9.2, where *RBQ1, rbq4 and RBQ3* are located (Jeuken and Lindhout 2002, Jeuken et al 2005), since not many EST-markers have been developed for these regions. When an EST-marker is developed, which is located on an interesting position and no other EST-markers were developed in that region, this EST markers will be used for the development of the combiBILs and the search for recombinants for *RBQ1*. When a codominant EST-marker was developed based, it was mapped on F_2 population.

One of the final goals of this research is to implement durable resistance into commercial cultivars. In order to obtain this durable resistance it is important to investigate whether this is possible by combining different resistance QTL from *L. saligna* in a plant. This is accomplished by combining *L. saligna* introgressions from the BILs, in which a resistance QTL was detected, in a plant (combiBIL). The combiBILs were developed in order to investigate whether there is an interaction between the QTL or not and if there is an interaction to research whether this interaction is additive or epistatic.

For the development of the combiBILs the QTLs *rbq4, rbq5, rbq6 and rbq7* which were detected in BIL 8.2, BIL 2.2, BIL 6.3 and BIL 4.2 respectively (Jeuken and Lindhout 2003, Jeuken and Lindhout 2004, Jeuken et al manuscript in preparation) will be used. The *L. saligna* introgressions of BIL 2.2, BIL 4.2, BIL 6.3 and BIL 8.2 will be referred to as *L. saligna* introgression 2.2, 4.2, 6.3 and 8.2 in this report. The combiBILs 2.2 + 4.2, 2.2 + 6.3 and 4.2 + 8.2 are already developed in the past. For this thesis the combiBILs 2.2 + 8.2, 4.2 + 6.3 and 6.3 + 8.2 will be developed. In the future also combiBILs with three and four resistance QTLs will be developed using these four QTLs.

RBQ1 was detected in the F_2 population on Chromosome 7 and is expected between 50 and 59 cM on (Jeuken et al manuscript in preparation). From the three QTLs detected in the F_2 population this QTL showed the largest effect (Jeuken and Lindhout 2002). This QTL is confirmed in preBIL7.2 for three out of five leaf disc disease tests (Marieke Jeuken, personnel communication). A preBIL is a line with a heterozygous introgression, from which a BIL is developed. Allelic test are

performed at the moment for preBIL7.2 to confirm the dominance of RBQ1. Disease tests for this QTL are complicated, since preBIL7.2 plants carrying this QTL show different leaf morphology, early bolting and a decreased vitality. These phenotypes seem to be closely linked to this QTL (Marieke Jeuken, personnel communication).

The aim of this experiment was to find recombinants with a smaller *L. saligna* introgression, which still contains the resistance QTL, but lost the genes which encode for the unattractive features. Another aim of this experiment is to asses the effect of *RBQ1* on the resistance level against *Bremia* of lettuce plants with a *L. sativa* background.

Materials and methods

2.1 Marker development

In the first part of this thesis codominant EST-markers were developed on three different *L. saligna* introgression regions, since the intensity of EST-markers in these introgressions is poor. These new EST-markers were developed on the lower part of Chromosome 7 where *RBQ1* is located (Jeuken and Lindhout 2003), on the introgression of BIL 8.2 (Jeuken and Lindhout 2004) where *RBQ4* is located and on the middle part of Chromosome 9 where *RBQ3* is located (Jeuken and Lindhout 2003).
The CGPDB database of Michelmore from the University of California at Davis consists of EST-sequences of *L. sativa* and *L. serriola,* which are assembled in contigs. Using these sequences markers were developed, which were mapped on the progeny of a *L. sativa* X *L. serriola* cross. Additional to the EST-sequence, information about the marker type and in some cases the primer sequences are given. Most markers in this database are SSCP (Single-strand Conformational Polymorphism) markers. The CGPDB database, which is frequently updated, was screened for new EST-markers and a selection was made for candidate markers. For the three regions of our interest in total 21 new candidate EST-markers were selected. If the primer sequence of a candidate EST-marker was not available or the primers of Michelmore did not work, new primer sequences were developed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).
These candidate EST-markers were tested on *L. sativa* cv Olof and *L. saligna* CGN 5271, using the PCR reaction as described in appendix 1. The amplification products were resolved by electrophoresis in a 2% agarose gel in 0.5 x TBE buffer and stained with ethidium bromide.
The candidate EST-markers that gave an amplification product of equal size were sequenced, by Greenomics (business unit PRI, Wageningen). The sequences were analyzed, using the SeqMan software package (DNASTAR Inc., Madison, WI, U.S.A.).
When a deletion was found in one of the amplification products, new primers were designed in order to develop a SCAR marker. If a single nucleotide polymorphism was detected between the amplification products of *L. sativa* cv Olof and *L. saligna* CGN 5271 Clone Manager 6 (version 6.00; Scientific and Educational software, Durham, NC) was used to search for an appropriate enzyme which could cleave the amplification product of one of these plants. An EST-marker where

the amplified fragments show a polymorphism after cleavage with a suitable restriction enzyme is called a CAPs marker.

If on a certain polymorphism no appropriate enzyme could be detected, a dCAPs marker was developed using http://helix.wustl.edu/dcaps/dcaps.html. For a dCAPs marker a primer is developed with one or two nucleotide mismatches just before the polymorphism. Using this method a polymorphism can be created in the amplification product, which can be cut with a suitable restriction enzyme

The newly developed EST-markers were mapped based on manual fitting this EST-marker on the F_2 population (Jeuken et al 2001). When new EST-markers were mapped on a position of interest and no previously developed EST-markers were available for this position, they were used for the selection of plants with the desired genotype.

2.2 CombiBILs

For the development of the combiBILs crosses were made in the past. BIL 2.2 was crossed with BIL 6.3 (+4.1) and BIL 4.2 was crossed with BIL 8.2. The F_1 from these crosses were selfed. From these F_2 plants combiBIL 2.2 + 6.3 and combiBIL 4.2 + 8.2 were selected. These combiBILs were crossed, followed by one round of selfing. The plants from this F_2 population were genotyped and the progeny of some of these plants were used for the development of a certain combiBIL.

The lines that were used for the development of the combiBILs are presented in table 1. These lines were obtained by self pollination of the parental plant. The number of plants that should be tested with a 95% confidence of finding a plant with the desired genotype was calculated using the formula:

P(# plants with desired genotype $\geq$1) = 1 – P(# plants with desired genotype = 0)

Table 1. The genotypes of the parental lines, from which the combiBILs 2.2 + 8.2, 6.3 + 8.2 and 4.2 + 6.3 were developed. The BILs from which these introgressions originate (Jeuken and Lindhout 2004) are presented under genotype parent as: a homozygous *L. sativa*, b homozygous *L. saligna,* h heterozygous. In the column indicated with n the number of plants is presented that should be tested with a 95% confidence of finding the desired genotype. The last column presents the number of plants that were tested.

Parental plant (F_2)	line	Genotype parent (BIL introgression)					desired genotype	n	# sown
		2.2	4.1	4.2	6.3	8.2			
pv04181_7	pv05115	h	0-25 h	a	a	b	2.2+8.2	11	20
pv04181_1	pv05109	a	0 h 18-22 b	a	h	h	6.3+8.2	45	100
pv04181_2	pv05110	a	0-25 h	h	h	h	4.2+6.3+(4.1)	200	240

Since distorted segregations have been found for introgression 6.3 (favouring the *L. sativa* alleles) and for introgression 8.2 (favouring the heterozygous alleles) in the F_2 population (Jeuken et al 2001) more seeds were sown for the lines which were segregating for these introgressions than calculated.

Leaf material was collected from seven to ten week old plants. DNA was extracted according to the CTAB method as described in appendix 2. The PCRs were performed according to appendix 1 and the amplification products were resolved by electrophoresis in a 2% agarose gel in 0.5 x TBE buffer and stained with ethidium bromide.

The candidate plants for combiBIL 2.2 + 8.2 were genotyped for *L. saligna* introgression 2.2 and 4.1 using the EST-markers presented in Figure 3. The homozygous *L. saligna* introgression 8.2 was confirmed using EST-marker LE1111 (Figure 3). The first genotyping experiment was repeated for selected target combiBIL plants in order to confirm the genotypes of these plants.

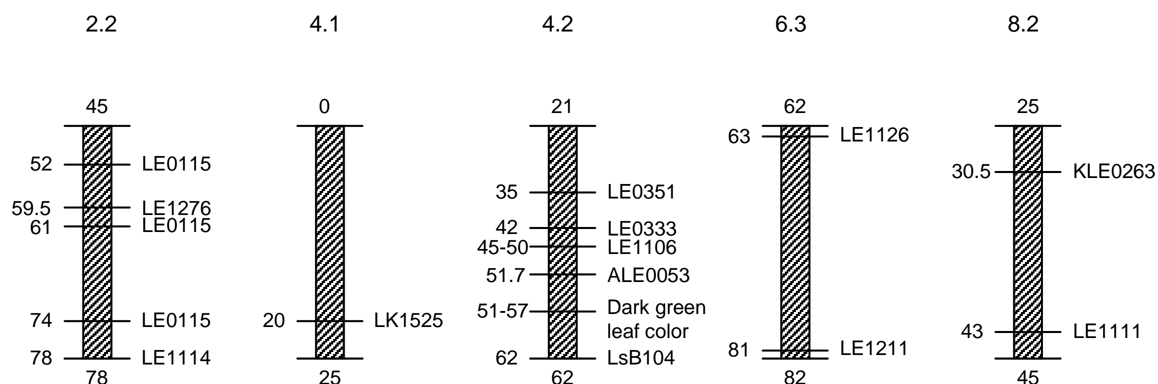

Figure 4. The vertical bars represent the *L. saligna* introgressions of BIL2.2, BIL4.2, BIL6.3 and BIL8.2 containing *RBQ5, RBQ7, RBQ6 and RBQ4* respectively, which were used for the combiBIL development. In some parental lines the undesired introgression 4.1 was present. The EST-markers that were used for the selection of these introgressions and their positions are also included in this figure. The distances are in cM.

The candidate plants for combiBIL 6.3 + 8.2 were genotyped for *L. saligna* introgression 6.3 and 8.2 using the EST-markers presented in Figure 3.

The candidate plants for combiBIL 4.2 + 6.3 were first tested for introgression 6.3, since this introgression showed a distorted segregation favouring *L. sativa* alleles. The plants which were homozygous *L. saligna* for introgression 6.3 were genotyped for the other segregating introgressions. For this analysis the EST-markers LE1126 and LE1211 were used. The plants that were homozygous *L. saligna* for introgression 6.3 were selected. From the selected plants the DNA isolation was repeated and these plants were tested for the *L. saligna* introgression 4.2 and 8.2 using the EST-markers presented in Figure 3. For the genotyping of introgression 4.2 also the

phenotypic marker dark green leaf color, which was located between 50.6 and 56.7 cM (Virginie Portemer, personnel communication) was used. Introgression 6.3 was confirmed using LE1126 and LE1211.

The resistance level of the developed combiBILs will be assessed in a leaf disc test. This leaf disc test is described in chapter 2.4.

2.3 The finemapping of *RBQ1*

For the finemapping of *RBQ1* new EST-markers were developed on Chromosome 7, tested on the F2 population and analyzed for linkage on Chromosome 7, using JOINMAP 3.0 software (Van Ooijen and Voorips 2001).

For the finemapping of *RBQ1* two lines were screened for recombinants. Line pv02268 which is developed by self pollinating plant pv02172.06 and line pv02255 which is developed by self pollinating plant pv02173.8 (Figure 4). Line pv02173.8 is heterozygous between 49 and 73 cM on Chromosome 7 and line pv02172.6 is heterozygous between 40 and 65 cM on Chromosome 7. Line pv02173.8 has also two homozygous *L. saligna* introgressions on Chromosomes 5 and 7. Based on the LOD profile of previously performed disease tests on the F2 population RBQ1 is expected between 50 and 59 cM on Chromosome 7 (Jeuken et al manuscript in preparation).

In earlier performed recombinant screenings in preBIL 2.2 and preBIL 4.2 a 10 times suppression of recombination was found (Jeuken et al 2005). Since the recombination could be suppressed 10 times and the interval where RBQ1 was estimated (9 cM), it was calculated that 600 plants should be tested to find 5 recombinant plants. From the lines pv02255 and pv02268 240 and 320 plants were screened for recombinants respectively.

Leaf material was sampled from five to eight week old plants. DNA was isolated according to the NaOH method as described in appendix 3. The PCRs were performed (protocol, Appendix 1) and the amplification products were resolved by electrophoresis in a 2% agarose gel in 0.5 x TBE buffer and stained with ethidium bromide. The plants from line pv02172.8 were screened with the EST-markers M2395 and LE9018 (Figure4). The plants of line pv02173.8 were screened for recombinants with the EST-markers LE1263, M2395 and LK1513 (Figure 4). The positions of the EST-markers in figure 4 were estimated, based on manual ordering these markers on the F_2 population (Jeuken et al 2001).

The genotype of selected recombinant plants was tested again after a second DNA isolation according to the CTAB method as described in appendix 2. This time the CTAB method was used, since the number of plants that had to be tested was much smaller and the isolated DNA can be

stored much longer using this method compared to the NaOH method. The recombinant plants were tested with the four EST-markers presented in Figure 4. The recombinant plants were also tested with three AFLP-markers by Koen Pelgrom and Marieke Jeuken. These AFLP-markers were mapped using the BIL population on Chrom 7 positions 50, 55 and 70 cM. During the second DNA-isolation also the plants of preBIL 7.2 were screened, since these plants were segregating for Chromosome 7 between 49 and 74 cM.

In the lines pv02268 and pv02255 two phenotypic markers were observed and mapped using JOINMAP 3.0 software (Van Ooijen and Voorips 2001).

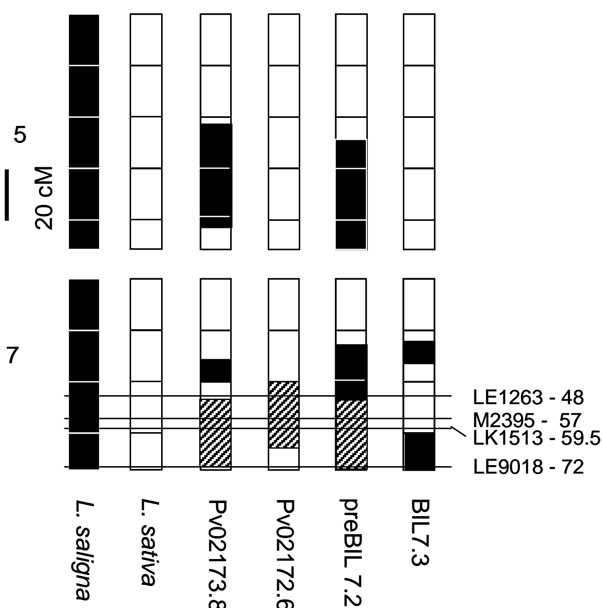

Figure 5. A genotype overview of the two parental plants pv02172.6 and pv2173.8 of which the progeny were used for the finemapping of *RBQ1* and the four control lines, L. sativa, L. saligna, preBIL7.2 and BIL7.3 The EST markers that were used for the screening for recombinants and their estimated positions are presented. The *vertical bars* represent the chromosomes 5 and 7 of lettuce. The chromosomes are segmented in 20-cM intervals that are delimited by *horizontal lines*. The genomes of the lines are indicated in *black, white* and *diagonal stripes: white=* homozygous *L. sativa, black=* homozygous *L. saligna* and *diagonal stripes=* heterozygous. The lines are homozygous *L. sativa* for the chromosomes which are not included in this figure.

2.4 *Bremia* disease test on leaf discs

Bremia was maintained in plastic boxes on seedlings of susceptible cultivars. The inoculum was prepared by washing sporulating seedlings in fresh water. The leaf disc test was performed in plastic boxes of 40x25x8 cm, which were closed by bright plastic covers. The growing conditions were: a photo active period of 16 hours and a constant temperature of 15°C.

Leaf discs of 1.7 cm diameter were cut from fully expanded healthy leaves and laid upside down on filterpaper moistened with tap water. The leaf discs were inoculated directly after cutting. After inoculation a dark period of sixteen hours was given. To minimize the risk of escapes, 24 hours after the first inoculation a second inoculation was applied. Seven and eight days after the first inoculation the leaf disks ware assessed for sporulation.

2.4.1 Leaf disc test on the CombiBILs

The developed combiBILs, in combination with the single BILs, the combiBILs that were already developed and the control plants, were placed randomly in the greenhouse during the growth period. The leaf disc test was performed on twelve week old plants, using *Bremia* race Bl14. The spore concentration was for the first and second inoculation adjusted to 2 x 10^5 spores per ml. Each line was tested using five plants, taking four leaf discs from one leaf per plant. When less than five plants per line were available, more than four leaf discs per plant were taken, in order to obtain 20 leaf discs per combiBIL. The four leaf discs of each plant were placed per pair randomly in two replicates in the box.
As controls five plants of the susceptible parent *L. sativa* cv Olof and five plants of the resistant parent *L. saligna* CGN 5271 were included. As susceptible controls also BIL 4.4 and BIL 8.4 were included, since these lines have proven to be fully susceptible in previous performed disease tests (Jeuken et al manuscript in preparation). The single BILs 2.2, 4.2 6.3 and 8.2 were included in this disease test in order to estimate the effect of single resistance QTLs compared to the effect of combined QTLs.
These scores were arcsine square root transformed in order to improve homogeneity of residual variance. A Tukey HSD test and a Dunett test were performed to compare the averages of the BILs and lines.

2.4.2 Leaf disc test on plants recombinant for introgression containing RBQ1

The leaf disc test was performed on the plants, which were recombinant for the region where *RBQ1* is mapped. The plants were twelve weeks old. From each plant two leaves were taken, since leaves of different stadiums were tested in this way. Per leaf two leaf discs were taken. Two samples from different leaves from one plant were placed as pair randomly in the box.
For this test the *Bremia* races Bl:14 and Bl:16 were used. The spore concentration for the first and second inoculation with Bl14 were to 3 x 10^5 and 2· 10^5 spores per ml respectively. For Bl16

the spore concentration was adjusted to $2.5 \cdot 10^4$ and $2 \cdot 10^4$ spores per ml for the first and second inoculation respectively.
As controls five plants of the susceptible parent *L. sativa* cv Olof and five plants of the lines preBIL7.2 and BIL7.3 (Figure 4) were used.
These scores were arcsine square root transformed in order to improve homogeneity of residual variance. The data were analyzed per line and per *Bremia* race. For the analysis the recombinants per line were grouped based on their recombinant genotype. The infection severities of these groups were compared to Olof using a Dunnett test. The recombinant genotypes for which one or two plants were detected were excluded from the statistical analysis.

Results

3.1 The development of molecular and morphological markers

3.1.1 Molecular markers

For the development of new EST-markers 21 candidate EST-markers were tested on *L. sativa* cv Olof and *L. saligna* CGN 5271. Twelve EST-markers gave an amplification product of the same size, eight EST-markers gave no amplification product and one EST-marker gave multiple amplification products. The candidate EST-markers which gave no or multiple amplification products were not further used for the development of new codominant EST-markers. The twelve candidates which gave an amplification product of the same size were sequenced. Based on this sequence it was decided whether a SCAR, a CAPs or a dCAPs was developed.
The new markers were tested on *L. saligna* and *L. sativa* cv Olof and when a polymorphism was detected, the marker position of the EST-marker was estimated, based on a comparison of the scores of this marker on the F_2 population (Jeuken et al 2001) to the scores of existing markers on the F_2 population. Eight new primers were developed of which 1 SCAR marker, 3 CAPs marker and 4 dCAPs marker (Table 2).

Table 2. The new codominant EST-markers that were developed and their estimated position on the genetic map. Furthermore the type of marker and the restriction enzymes for the CAPs and dCAPs markers are presented.

Merker	chrom	Estimated position	type	enzym
LK1513*	7	59	CAPs	Eco52I (XmaIII)
LE1120**	7	60	CAPs	MboII
LK1426	7	64	dCAPs	MnII
KLK1473_850	2	51-55	dCAPs	HhaI
LK1444	8	44-46	CAPs	EcoRI
M431	9	17	dCAPs	DdeI
KLK1115	9	15	dCAPs	HindIII
LK1330	9	33-37	SCAR	

* A dominant marker
** A difficult marker

The EST-marker KLK1473_850 was based on our data mapped on Chromosome 2 (Table 2), whereas Michelmore mapped this marker on Chromosome 8. The new EST-markers which were developed on Chrom 8 and 9, except LK1330, were mapped on chromosome regions for which already EST-markers were developed. EST-marker LK1330 is estimated on Chromosome 9, in the interval where *RBQ3* is expected.

For Chromosome 7 a new linkage map was calculated, since some new markers were developed on this chromosome and some new data were generated from three EST-markers on the F_2 population (Figure 6). Based on this extra data a better estimation could be made on the position of the markers.

Chromosome 7

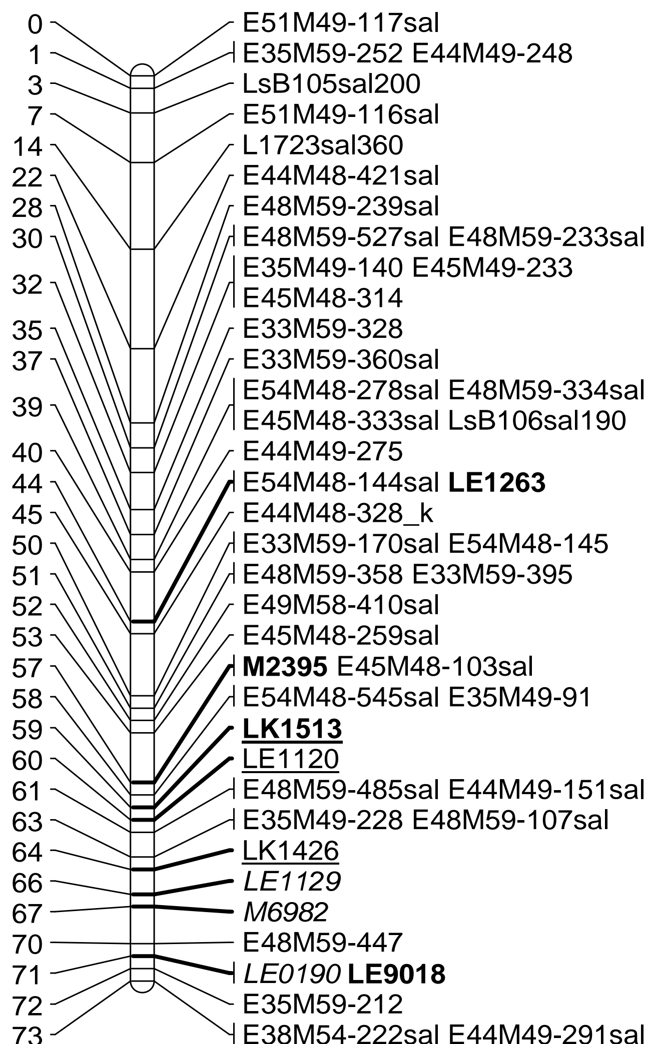

Figure 6. The new integrated map of Chromosome 7 based on an interspecific F_2 population between *L. saligna* and *L. sativa* (Jeuken et al 2001). This map consists of both AFLP and EST-markers. The underlined markers are new developed codominant EST-markers. The EST-markers from which new data are generated on the F_2 population are presented italic. Also two new AFLP primer combinations were added to this linkage map. The markers which are presented bold were used for the recombinant screening of *RBQ1*.

3.1.2 Morphological markers

The progeny (n=320) of line pv0172.6 was segregating for the morphological characteristic “early bolting”. These plants were scored for early bolting and these data were compared with the data of molecular markers. A small linkage map was constructed of the introgression for which line pv02268 was segregating. Based on this map early bolting is associated with *L. saligna* alleles between the EST-markers M2395 and LK1513 (Figure 7a). Early bolting is a dominant trait as it was associated with genotypes that are homozygous *L. saligna* and heterozygous for the introgression on Chrom 7.

The morphological traits “early bolting” and “pustules” were segregating within the progeny of line pv02173.8 (n=250). Pustules are small swollen spots that appear at the abaxial site of mature leaves (Figure 7b).
Based on the molecular and morphological data a small linkage map was constructed for the segregating introgression in Chromosome 7. Based on the data of line pv02255 early bolting is associated with the *L. saligna* alleles between the EST-markers LK1513 and LE9018 (Figure 7b). The morphological trait pustules is associated with *L. saligna* alleles above EST-marker LE9018 (Figure 7b). These traits are considered as dominant, since these traits were associated to genotypes that are homozygous *L. saligna* and heterozygous for the introgression on Chrom 7.

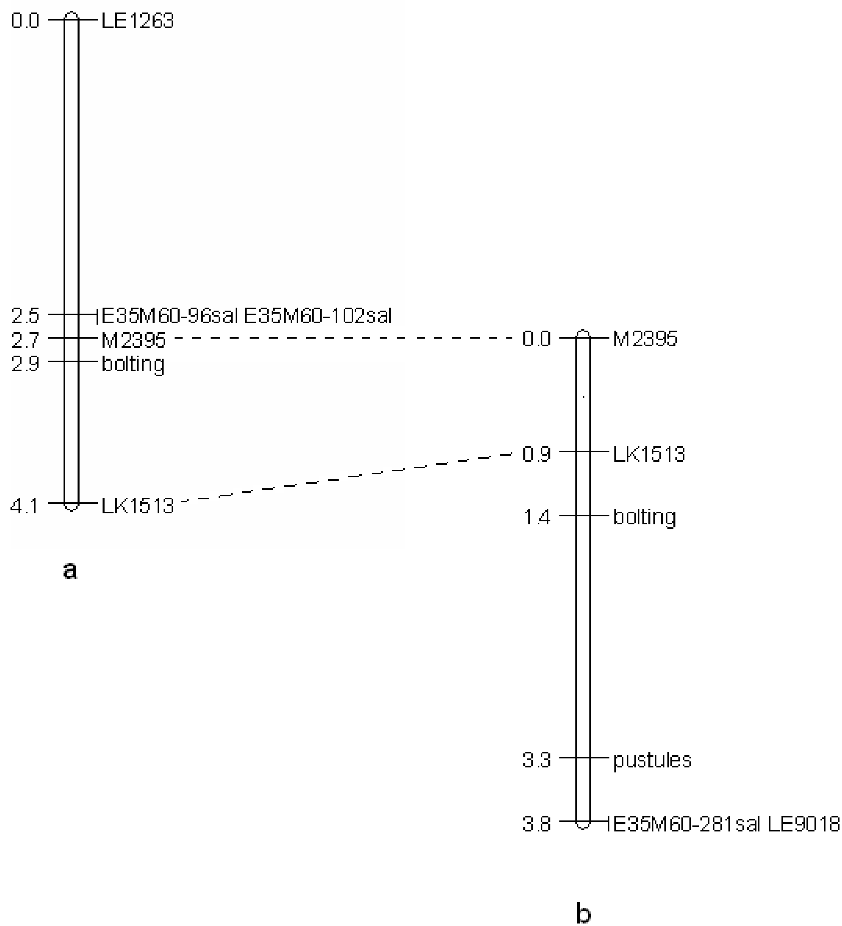

Figure 7. Two genetic linkage maps that were developed based on the segregation analysis on the lines pv02268 (a) and pv02255 (b). These linkage maps consist of AFLP- and EST-markers and morphological markers. The morphological marker early bolting was mapped in line pv02268 (a). The morphological traits early bolting and pustules were mapped in line pv02255 (b).

Figure 8. The plants of line PV02255 were segregating for pustules at the abaxial site of the leaves.

3.2 CombiBILs

3.2.1 *The development of combiBIL 2.2 + 8.2*

For the development of combiBIL 2.2 + 8.2 twenty plants of line pv05115 were sown and genotyped. Five of these plants were homozygous *L. saligna* for introgression 2.2 (Figure 9) and were regarded to as combiBIL 2.2 + 8.2. Among these five plants plant pv05115-16 was regarded as the plant with the most desired genotype, since this plant is homozygous *L. sativa* on the top of Chromosome 4 (Figure 9). These five plants were tested in the disease test, representing plants harboring *rbq4* and *rbq5*, since it was assumed that no resistance QTL is present at the top of Chromosome 4 (Marieke Jeuken, personnel communication).
No distorted segregation was observed among the plants that were tested for the development of combiBIL 2.2 + 8.2.

3.2.2 *The development of combiBIL 6.3 + 8.2*

For the development of combiBIL 6.3 + 8.2 eighty plants of line pv05109 were sown and genotyped. Among these 80 plants no plant with the desired genotype was detected. Distorted segregations were found for the introgressions 6.3 (*L. sativa* 32, *L. saligna* 8, heterozygous 37; χ^2-test P<0.001) and 8.2 (*L. sativa* 36, *L. saligna* 3, heterozygous 40; χ^2-test P<0.001) with preference for L. sativa alleles.
Among the 80 plants of line pv05109 two plants were detected with a recombination event in one of the L. saligna introgressions. These two plants can be used for the fine mapping of *rbq4* and *rbq6*. The candidate for the finemapping of *rbq4* was homozygous *L. sativa* for introgression 6.3 and was homozygous *L. sativa* for the upper part of introgression 8.2 and heterozygous for the lower part of this introgression. The candidate plant for the finemapping of *rbq6* was homozygous *L. sativa* for introgression 8.2 and was heterozygous for the upper part of introgression 6.3 and homozygous *L. sativa* for the lower part of this introgression.

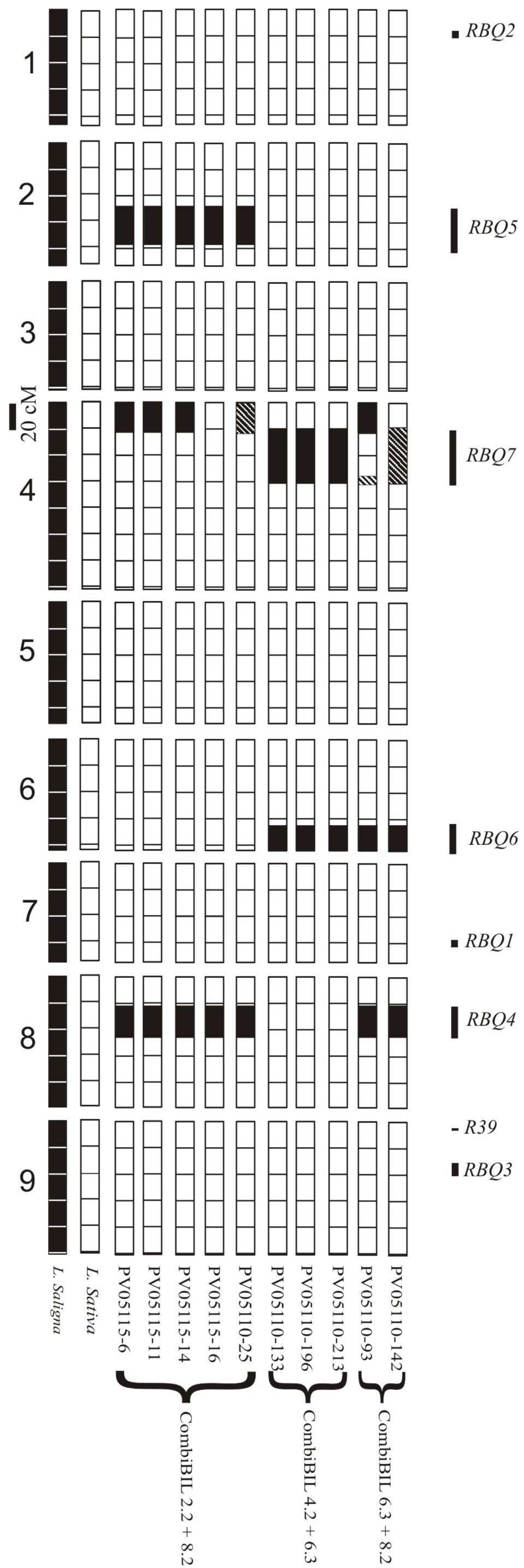
1
2
3
4
5
6
7
8
9
20 cM
RBQ2
RBQ5
RBQ7
RBQ6
RBQ1
RBQ4
R39
RBQ3
L. Saligna
L. Sativa
PV05115-6
PV05115-11
PV05115-14
PV05115-16
PV05110-25
PV05110-133
PV05110-196
PV05110-213
PV05110-93
PV05110-142
CombiBIL 2.2 + 8.2
CombiBIL 4.2 + 6.3
CombiBIL 6.3 + 8.2

Figure 9. The genotypes of the plants that were selected as combiBIL 2.2 + 8.2, combiBIL 4.2 + 6.3 and combiBIL 6.3 + 8.2. *Vertical bars* represent the nine chromosomes of lettuce. The chromosomes are segmented in 20 cM intervals that are delimited by horizontal lines. The genomes of the lines are indicated in *black*, *white* and *diagonal stripes*: *white* homozygous *L.sativa*, *black* homozygous *L.saligna*, *diagonal stripes* heterozygous.

3.2.3 The development of combiBIL 4.2 + 6.3

Among the 240 plants of line pv05110 that was segregating for the introgressions 4.1, 4.2, 6.3 and 8.2 three plants were selected as combiBIL 4.2 + 6.3 (Figure 9). These plants had all the desired genotype, since they were all homozygous *L. sativa* for introgression 4.1. These three plants were all tested for resistance in the leaf disc test.

Since no target plant for combiBIL 6.3 + 8.2 was detected in the 80 plants from line pv05109 the plants of line pv05110 were screened for combiBIL 6.3 + 8.2. Two plants were found that were homozygous *L. saligna* for the introgressions 6.3 and 8.2 (Figure 10). Plant pv05110-27 (Figure 10) is heterozygous for introgression 4.2, however since *rbq7* is recessive this plant is tested as combiBIL 6.3 + 8.2. Both these plants were used as combiBIL 6.3 + 8.2 in the leaf disc test.

Introgression 6.3 showed a distorted segregation in favour of *L. sativa* alleles (*L. sativa* 60*, L. saligna* 45*,* heterozygous 68; χ^2-test $P<0.01$) among the 240 plants of line pv05110. The segregation pattern of the other introgressions was not analyzed, since only the plants which were homozygous *L saligna* for introgression 6.3 were genotyped for these introgressions.

3.2.4 Disease test on the combiBILs

In this disease test five plants per line were tested, using four leaf discs per plant. For BIL4.2, combiBIL 4.2 + 6.3 and combiBIL 6.3 + 8.2 more leaf discs per plant were taken in order to test 20 leaf discs per plant since less than five plants were available of these lines.

The infection severity for the leaf disc test in general was lower than the previously performed leaf disc tests (see appendix 4 for an overview of these tests). The lines Olof, BIL 2.2, BIL, 4.2, BIL 4.4 and BIL 8.4 showed the highest infection severity (Figure 10). This was according to our expectations for the susceptible controls Olof, BIL 4.4 and BIL 8.4, however based on previously performed leaf disc tests BIL 2.2 and BIL 4.2 were not expected to have an infection severity equal to the susceptible controls.

A lot of leaf discs showed brown spots, which were probably caused by too high moisture content in the boxes. These brown rotten spots were found on leaf discs of all lines.

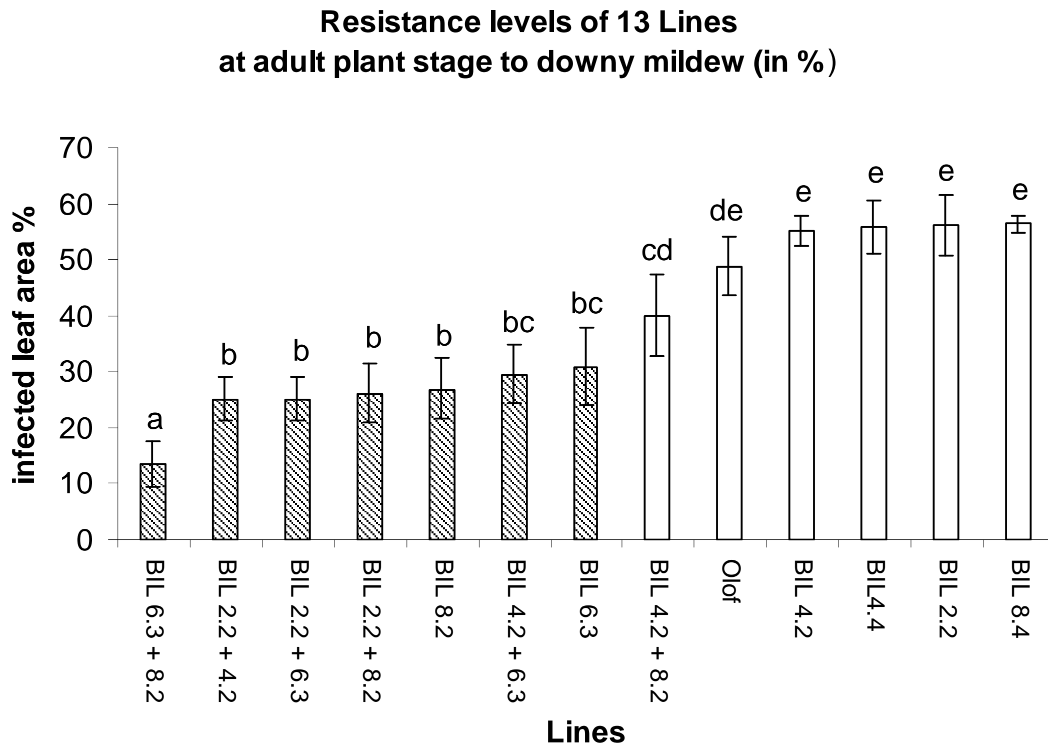

Figure 10. Infection severities to lettuce downy mildew of the lines quantitatively assessed in a leaf disc test. The error bars stand for the 95% confidence interval. The letters above the error bars represent the results of Tukey test with α= 0.05. The box-plots with diagonal stripes indicate the lines which were significantly more resistant than the susceptible control Olof and the white box-plots indicate the lines that were as susceptible as cv Olof. Five plants were tested except for BIL 4.2, combiBIL 4.2 + 6.3 and combiBIL 6.3 + 8.2 from which4, 3 and 2 plants were tested respectively. The resistant control, *L. saligna* CGN 5271 had an infection severity of 0.3% and is therefore excluded from this figure.

The non-host *L. saligna* was resistant and some leaf discs showed an HR. On some leaf discs of *L. saligna* a very small infection was detected on the edge. The average infection severity of *L. saligna* was 0.3%.

CombiBIL 6.3 + 8.2 was the most resistant combiBIL in this test with a 36% decrease of infection severity compared to Olof and it was more resistant than the single BILs 6.3 and 8.2. The other combiBILs except combiBIL 4.2 + 8.2 were significantly not more resistant than the corresponding BILs although they were more resistant than Olof.

The leaf discs were also scored for infection severity eight days post inoculation. Compared to the observations of seven days post inoculation the differences between the lines were smaller eight days post inoculation (results not shown). The quality of the leaf discs was deceased eight days post inoculation compared to seven days post inoculation, since more and larger brown rotten spots were observed on the leaf discs scored eight days post inoculation.

3.3 The fine mapping of *RBQ1*

3.3.1 Recombinant screening

For the recombinant screening for *RBQ1* 320 plants of line pv02268 and 250 plants of line pv02255 were sown. The germination percentage of the seeds from line pv02268 was higher than line pv02255. The growth rate at early stage of both lines was low, however the growth rate of line pv02268 was higher than line pv02255.

Based on the first genotyping round 31 plants of line pv02268 were selected and a second round of genotyping was performed. Twenty-two plants confirmed their recombinant genotype for line pv02268 (Figure 11). Five groups of different recombinant genotypes were discriminated in this line.

Based on the new information on the marker positions (Figure 6) suppression of recombination was observed between the EST-markers LE1263 and M2395 (chi-square $P<0.001$) and between the EST-markers M2395 and LK1513 (chi-square $P<0.05$). Between LE1263 and M2395 the recombination was three times suppressed and between M2395 and LK1513 recombination was suppressed two times, which was in both cases lower than the recombination suppression found in preBIL 2.2 and preBIL 4.2 (Jeuken et al 2005).

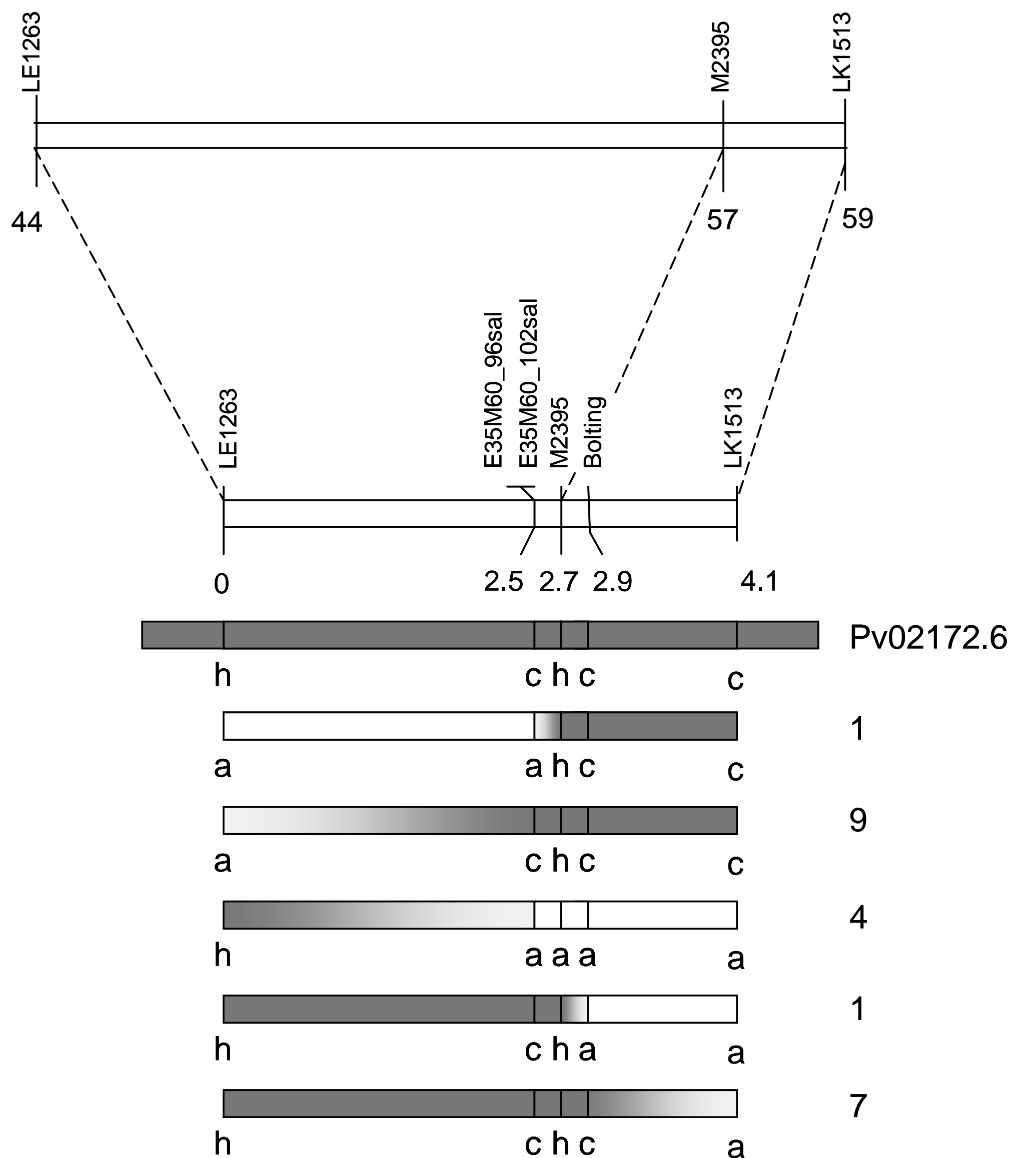

Figure 11. The recombinant genotypes for the *RBQ1* interval for which the plants of line pv02268 were segregating. The upper bar represents the marker positions based on the original F2 population (Jeuken at al 2001). The second Bar represents the marker positions based on the progeny of line PV02268 (selfed from pv02172.6). The third bar represents the introgression of the parental plant for line pv02268, based on 28 AFLP markers. The genomes for the *RBQ1* introgressions are indicated in *grey, white* and *shading*: *white* homozygous *L. sativa, grey* heterozygous and within the shading interval a recombination event took place. The scores of the markers are presented as *a, c* and *h*.: *a* homozygous *L. sativa, c* heterozygous or homozygous *L. saligna* and *h* as heterozygous. The number of plants that were found for a certain genotype is presented to the right of the lower five bars. Since only the genotype between the markers is known, only this part is presented for the recombinants in this figure.

For line pv02255 twenty-eight plants with a recombinant genotype were identified in the first genotyping. The genotypes of these plants were checked again in a second genotyping and twenty-three recombinant plants were confirmed (Figure 12). Six groups of different recombinant genotypes were discriminated in this line with the six used markers. The plants of line pv02255

segregated for EST-marker LE1263, which was erroneously assumed to be homozygous *L. sativa*. The introgression was longer than assumed based on AFLP analysis.

Based on the new information on the marker positions (Figure 6) suppression of recombination was observed between the EST-markers M2395 and LE9018 (chi-square P<0.05). The recombination between these EST-markers was suppressed 1.6 times, which is lower than the suppression of recombination found in BIL 2.2 and BIL 4.2 (Jeuken et al 2005).

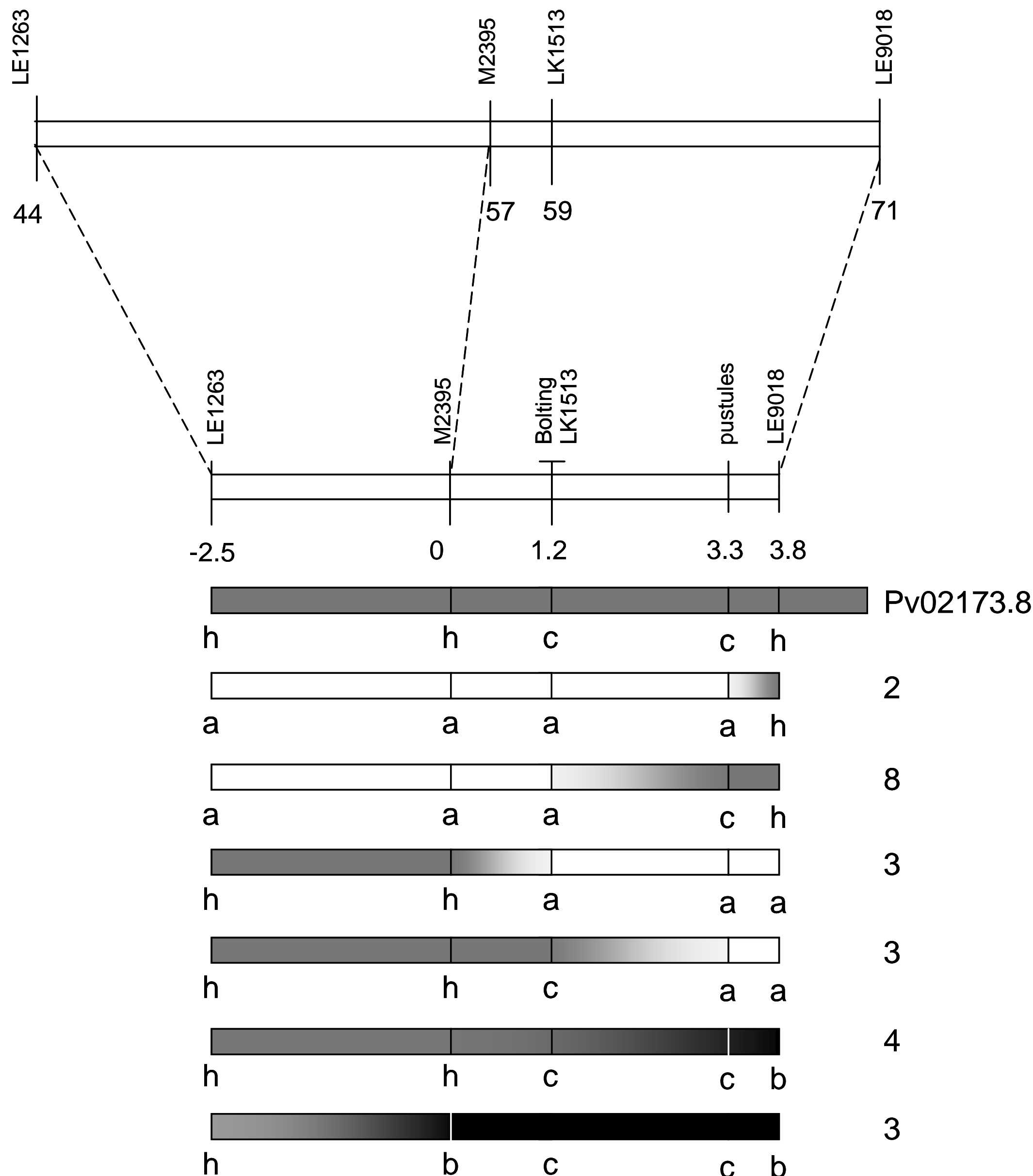

Figure 12. The recombinant genotypes for the *RBQ1* interval for which the plants of line pv02255 were segregating. The upper bar represents the marker positions based on the original F2 population (Jeuken at al 2001). The second Bar represents the marker positions based on the progeny of line PV02268 (selfed from pv02172.6). The third bar represents the introgression of the parental plant for line pv02268, based on 28 AFLP markers. The genomes of the *RBQ1* introgressions from the recombinant plants are indicated in *black, grey, white* and *shading*: *black* homozygous *L. saligna, white* homozygous *L. sativa, grey* heterozygous and within the shading area a recombination event took place. The scores of the markers are

presented as *a, b, c* and *h.*: *a* homozygous *L. sativa, b* homozygous *L. saligna, c* heterozygous or homozygous *L. saligna* and *h* as heterozygous. The number of plants that were found for a certain genotype is presented to the right of the lower five bars. Since only the genotype between the markers is known, only this part is presented for the recombinants in this figure.

In the progeny of line pv02172.6 and pv02173.8 distorted segregations favouring *L. sativa* alleles were observed for the introgressions on Chromosome 7 (Figure 4). The progeny of line pv02172.6 segregated 119:102:0 (homozygous *L. sativa*: heterozygous: homozygous *L. saligna*) for the introgression between EST-markers LE1263 and LK1513, and the progeny of line pv02173.8 segregated 119:86:2 (homozygous *L. sativa*: heterozygous: homozygous *L. saligna*) for the introgression between EST-markers M2395 and LE9018.

The plants of preBIL7.2 were segregating into three groups based on their genotype. The first group which contained 4 plants were homozygous *L. sativa* for the introgression for which this line was segregating (Figure 4). Three plants were heterozygous for this introgression and one plant was detected with a recombinant genotype. This plant was heterozygous for the introgression for EST-marker M2395 and homozygous *L. sativa* for the rest of Chrom 7.

3.3.2 Disease test on the recombinants of RBQ1

A leaf disc disease test was performed on the recombinant plants. The plants that were tested in this disease test were heavily infected by aphids just before the leaf disc test was performed. Serious damage was observed on the leaves of these plants as a result of this infection.

The leaf disc test was performed on the plants that were selected as recombinant during the first genotyping and on the control plants. The plants which were not confirmed as recombinant in the second genotyping were also tested. Per plant four leaf discs were tested with *Bremia* race Bl:14 and Bl:16.

The infection severity level of the leaf disc test (appendix 5) was for Bl:14 and Bl:16 in general lower than in previously performed leaf disc tests (appendix 4). The density of the sporangiophores on the leaf discs of the recombinant plants was in general lower than for Olof, but the sporangiophores appeared in general all over the leaf. In the leaf discs of Olof the sporangiophores appeared in high density in groups on the leaf discs.

Two groups of recombinants from line pv02268 were more resistant than the susceptible control Olof (appendix 5a). Also the non recombinant plants from this line, which were still heterozygous for the introgression on Chromosome 7, and BIL 7.3 were more resistant than Olof. The result of

BIL 7.3 was remarkable, since BIL 7.3 proved susceptible in all previously performed disease tests (Marieke Jeuken, personnel communication).

In the disease test with Bl:16 a group of recombinant plants of line pv02268 and a group with non recombinant plants, which still were heterozygous for the introgression on Chromosome 7 were more resistant than Olof according to the Dunnett test (appendix 5a). These two groups proved also more resistant than Olof in the disease test with Bl:14. BIL 7.3 was in the disease test using Bl:16 not more resistant to Olof.

The progeny of preBIL 7.2 was segregating into two groups. One group became homozygous *L. sativa* for the heterozygous introgression of preBIL 7.2 and one group was still heterozygous for this introgression (Figure 5). Both these groups in which the progeny of preBIL 7.2 segregated, were in both disease tests with Bl:14 and Bl:16 significantly not more resistant than Olof according to the Dunnett test (appendix 5a).

The infection severities of the groups of recombinant plants of line pv02255 did not significantly differ from Olof in the leaf disc test performed with Bl:14 according to the Dunnett test (appendix 5b).

Based on the results on the leaf disc test on the recombinants of line pv02255 four groups of recombinants were more resistant than Olof according to the Dunnett test (appendix 5b). Three of these groups were homozygous *L. saligna* or heterozygous between LE1263 and LE9018. The other group that was more resistant than Olof was heterozygous for at the bottom site of Chrom 7, where *RBQ1* was not expected. Based on these results no relations could be detected with regard to the location of *RBQ1.*

BIL 7.3 was not significantly different from Olof in this disease test, which was according to our expectations.

Discussion

4.1 The development of molecular and morphological markers

4.1.1 Molecular markers

Eight new EST-markers were developed on the Chromosomes 2, 7, 8 and 9 (Table 2). Most of the EST-markers that were developed were mapped in regions on which already EST-markers had been developed. LK1513 was mapped within the interval where *RBQ1* was estimated (Jeuken and Lindhout 2002) and used for the recombinant screening of *RBQ1*. LK1330 can be used for the finemapping of *RBQ3,* since this marker is mapped in the interval where *RBQ3* is expected (Jeuken et al 2002) and no other EST-markers are mapped in this region. Remarkable was EST-marker KLK1473_850 which was mapped on Chromosome 8 by University of California at Davis and on Chromosome 2 by me based on the map developed by Jeuken et al (2001).
For Chromosome 7 a new linkage map was calculated (Figure 6), since new EST-markers were developed on this chromosome and more data from some previously developed EST-markers were generated. The distance between the markers in this new linkage map changed slightly compared to the previous linkage map of Chromosome 7 developed in 2005 (appendix 6), although the order of the markers did not change. The positions of the EST-markers showed a little difference compared to the estimated positions based on the manual ordering of these markers on the F2 population (Figure 4).

4.1.2 Morphological markers

During the screening for recombinants for the *RBQ1* interval the morphological characteristics early bolting and pustules became obvious in the line pv02255. In line pv02268 only the morphological marker early bolting was observed. The morphological trait early bolting has also been observed during the development of BIL 7.3 (Jeuken and Lindhout 2004). *L. saligna* also shows early bolting, whereas this morphological characteristic is not observed in *L. sativa* cv Olof. Pustules were not found in *L. saligna* or in *L. sativa* cv Olof, which makes it likely that the gene or genes responsible for this traits has an interaction with alleles which are located on an other chromosomal regions.

Early bolting was mapped on different positions on lines pv02268 and pv02255 (Fig 7a and 7b). It is assumed that early bolting is associated with *L. saligna* alleles between the EST-markers M2395 and LK1513 (Figure 7a), since more informative observations were done in this line pv02268 with regard to early bolting than in line pv02255. Based on the new linkage map of Chrom 7 (Figure 6) this morphological trait is mapped between 57 and 59 cM. However early bolting was mapped in BIL 7.3 (Figure 5) by Jeuken and Lindhout (2004), which supports the mapping of early bolting based on the data of line pv02255 (Figure 7b). Probably there are two different genes involved in early bolting. Another likely explanation for this result is that the data of EST-marker LK1513 were not reliable in line pv02268. Based on the progeny of the recombinant plants it can be investigated whether there are two genes responsible for early bolting or whether the mapping of early bolting in line pv02268 was incorrect.
The morphological trait pustules was mapped on line pv02255 and was associated with *L. saligna* alleles between the EST-markers LK1513 and LE9018 in close proximity with EST-marker LE9018 (Figure 7b).
Both traits are considered dominant, since both traits were associated with genotypes that are homozygous *L. saligna* and heterozygous for the introgression on Chrom 7. These traits could be used as dominant phenotypic markers in the future, but they are not very useful because they do not appear in the young plant stage.

4.2 CombiBIL development and assessment of their resistance level

In this experiment three new combiBILs were successfully developed. Pv05115-16 is the best target plant for combiBIL 2.2 + 8.2. For combiBIL 4.2 + 6.3 three plants were selected, which all had the desired genotype. The progeny from plant pv05110-142 should be used to select combiBIL 6.3 + 8.2. The progeny of this plant segregates for introgression 4.2 and therefore both combiBIL 6.3 + 8.2 and combiBIL 4.2 + 6.3 + 8.2 can be developed from this plant.
The progeny of these lines that were used for the development of combiBIL 6.3 + 8.2 and combiBIL 4.2 + 6.3 (+ 8.2) provided information about segregation ratios, which could be compared to skewed segregation ratios observed in the F_2 population and during the BIL development (Jeuken et al 2001, Jeuken and Lindhout 2004). During the development of combiBIL 6.3 and 8.2 and combiBIL 4.2 + 6.3 (+ 8.2) distorted segregations favouring *L. sativa* alleles were observed for introgression 6.3. This distorted segregation was also observed for introgression 6.3 favouring *L. sativa* alleles in the F_2 population (Jeuken et al 2001), but not during

the development of the BILs (Jeuken and Lindhout 2004). This distorted segregation can be explained by genetic effects on pollen fitness or zygote viability (Jeuken et al 2004).
The distorted segregation favouring *L. sativa* alleles which was observed in line pv05109 for introgression 6.3 (χ^2-test $P<0.001$) was more severe than in line pv05110 (χ^2-test $P<0.01$). During the development of combiBIL 6.3 + 8.2 from line pv05109 I selected by accident for larger seedlings during the transplanting of seedlings. These results suggest that a lower germination rate for plants which are homozygous *L. saligna* for introgression 6.3 could also be a cause for the distorted segregations that were found.
A distorted segregation (favouring *L. sativa* alleles) was also observed for introgression 8.2 during the development of combiBIL 6.3 + 8.2. The plants of line pv05110 (combiBIL 4.2 + 6.3 (+8.2)) were not analyzed for distorted segregations on introgression 8.2, since only the selected plants which were homozygous *L. saligna* for introgression 6.3 were tested for introgression 8.2. For introgression 8.2 a distorted segregation was also found in the F_2 population, but in the favour of heterozygous alleles (Jeuken et al 2001). This was the most likely explanation that *RBQ4,* which is located on introgression 8.2, was not detected in the F_2 population (Jeuken 2002, chapter 5), however no distorted segregation was observed for this introgression during the BIL development (Jeuken and Lindhout 2004). The distorted segregation which was observed might be explained by genetic effects of *L. saligna* alleles in this introgression on pollen fitness or zygote viability (Jeuken et al 2001).

The infection severity in the leaf disc test was in general lower than in previously performed leaf disc tests. The leaf discs were also scored eight days post inoculation but also at this time point the infection severity was in general lower than expected (results not shown). One of the explanations of the lower infection severity might be that brown rotten spots appeared on a lot of leaf discs. *Bremia* is not able to grow and reproduce on death material, since it is an obligate parasite (Lebeda and Syrovatko 1988). These brown rotten spots were possibly caused by too high moisture content in the boxes. Controlling the humidity in a leaf disc test is important, because the quality of the leaf discs decreases when the humidity in the box is too high. This seems especially be the case when droplets of water are formed on the leaf discs. However it is also important that the humidity is not too low, since the sporulation of *Bremia* is affected by the relative humidity, with sporulation increasing at RH ≥ 90% (Su et al 2004). In conclusion it is important that a balance should be found with regard to the humidity in the boxes.
BIL 6.3 and 8.2 and the combiBILs, except combiBIL 4.2 + 8.2, were more resistant than the susceptible controls Olof, BIL 4.4 and BIL 8.4 in this disease test. BIL 2.2 and BIL 4.2 were not

more resistant in this leaf disc test than the susceptible controls (Figure 10). These results were observed incidentally in previously performed leaf disc tests. In six leaf disc tests performed in the past, in which BIL 2.2 and BIL 4.2 were included, one time BIL 2.2 and two times BIL 4.2 proved as susceptible as Olof based on a Tukey test (Marieke Jeuken, personnel communication). A likely explanation for these aberrant results is that resistance QTLs can be influenced by the environmental conditions (Reignault and Sancholle 2005). These results makes it is hard to draw conclusions about interactions between *rbq 5* and *rbq 7* with the other resistance QTLs. However combiBIL 2.2 + 4.2 was more resistant than both single BILs, which gives a slight indication that *rbq 5* and *rbq 7* have an additive effect on resistance.

The result of combiBIL 6.3 + 8.2 suggests that *rbq6* and *rbq 4* might have an additional effect on the resistance. However the result of combiBIL 6.3 + 8.2 should be handled with care, since only two plants of this combiBIL were tested. If *rbq 4* and *rbq6* have an additive effect, it is likely that *rbq 4* and *rbq6* are involved in different pathways of different responses leading to resistance, like for instance *rbq4* is involved in the pathway leading to papilla formation and *rbq6* is involved in the pathway resulting in the expression of antimicrobial proteins. Histological observations and gene expression studies might help in the future to investigate which defense a response is regulated by a QTL.

Further research is required for to research whether there is an interaction between the resistance QTLs and if there is an interaction to investigate the type of interaction. In the leaf disc test performed for this thesis maximal five plants were tested per combiBIL. In the near future new leaf disc tests could be performed using more plants per combiBIL, since more seeds are available per combiBIL. It is also important that different types of disease tests will be performed on these combiBILs like seedling tests and field tests. With these experiments it can be investigated whether the results from the leaf disc test correlate with the results of the plants in different developmental stages. For plant breeders it is important that their plants are resistant against *Bremia* in all developmental stages.

In *L. saligna* sometimes a hypersensitive response (HR) was observed macroscopically. An HR was also observed on *L. saligna* in some previously performed leaf disc tests and disease tests on young plants (Ningwen Zhang, personnel communication).

4.3 The finemapping of *RBQ1*

For the screening of recombinants for the *RBQ1* interval two lines were used. For both lines a low growth rate was observed. This was also observed in preBIL 7.2 (Marieke Jeuken, personnel

communication). The lower germination percentage and growth rate of line pv02255 might be associated with *L. saligna* alleles located on the lowest part of Chromosome 7 or on the introgression on Chromosome 5 (Figure 4).

In lines pv02255 and pv02268 twenty-eight and thirty-one plants were found respectively which had a recombinant genotype. These recombinants were divided in several groups, based the recombinant genotype (figure 11 and 12).

In both lines that were screened for recombinants a suppression of recombination was observed. However the suppression of recombination was for both lines lower than observed in the recombinant screening of preBIL 2.2 and preBIL 4.2 (Jeuken et al 2005). A likely explanation for the suppression of recombination could be a lack of homology between *L. sativa* and *L. saligna* in this genomic region. This results in inferior binding of the nonsister chromatids in this region during meiosis, which eventually leads to the formation of less chiasmata and a lower recombination frequency. Recombination suppression due to a lack of homology was also observed in hybrids between the cultivated tomato (*Lycopersicon esculentum*) and the wild nightshade *Solanum lycopersiscoides* (Chetelat et al 2000) and in wide hybrids of *Petunia hybrida* (Robbins et al 1995).

In the progeny of line pv02172.6 and pv02173.8 distorted segregations favouring *L. sativa* alleles were observed for the introgressions on Chromosome 7. These distorted segregations favouring *L. sativa* alleles were also observed in the F_2 from 0 to 75 cM (Jeuken et al 2001) and in the BIL population from 45 to 72 cM (Jeuken and Lindhout 2004). Based on the results of the progeny of the lines pv02172.6 and pv02173.8 the distorted segregations favouring *L. sativa* alleles were associated with *L. saligna* alleles between 44 and 65 cM, which is the region for which the introgressions in both lines overlap. This distorted segregation can be explained by assuming that the *L. saligna* introgression contains genetic information which has a negative effect on the pollen or egg fitness (Jeuken et al 2004).

The amplification products of *L. sativa* and *L. saligna* of EST-marker LE1263 were hard to discriminate, but when these scores were correct the introgression between the EST-markers LE1263 and M2395 was not observed homozygous *L. saligna* among the plants of both lines that were tested (figure 11 and 12). This makes it likely that homozygosity of *L. saligna* alleles in this region is lethal. In the F_2 population this introgression was found homozygous *L. saligna* for some F_2 plants. This lethality seems to be compensated in the F_2 plants by the presence of *L. saligna* alleles on other chromosomes, which are absent in these lines. Based on a superficial screening on the F_2 population we suggest that these *L. saligna* alleles might be located on the middle part

or at the bottom site of Chrom 8. To investigate this hypothesis a cross could be made between plants of line pv02268 or pv02255 with BIL 8.2 and BIL 8.4, followed by a round of selfing.

In general the infection severity level in these leaf disc tests was lower than observed in previously performed leaf disc tests. This could be caused by the infection of the aphids in the lettuce plants just before the leaf disc test was performed. The infection of with aphids might have lead to local acquired resistance or to systemic acquired resistance. Also the quality of the leaf discs, especially of the leaf disc test performed with Bl:14, was poor. Brown rotten spot appeared on the leaf discs, and *Bremia* which is an obligate parasite can not grow and reproduce on these spots. These brown rotten spots were probably caused by too high humidity content in the boxes in which the leaf disc test was performed. The consequences of changing the humidity in the leaf disc test are discussed in chapter 4.2. Scoring the infection severity at a later time point, which should in general result in a higher infection severity level, was not possible, due to the poor quality of the leaf discs.

The density of the sporangiophores on the leaf discs of plants which had a recombinant genotype was in general lower than on the leaf discs on Olof. This was also observed in the past by (Marieke Jeuken, personnel communication). In general the sporangiophores appear in high density as groups on the leaf discs. The appearance of the sporangiophores all over the leaf disc, but in lower density, made it difficult to score the infection severity.

Two groups plants with a recombinant genotype from line pv02268 and the group of non recombinant plants from this line, which still were heterozygous for the introgression on Chromosome 7, were more resistant than the susceptible control Olof in the leaf disc test with Bl:14 (appendix 5a). Based on the genotypes of the plants in these three groups, *RBQ1* is expected between the EST-markers LE1263 and LK1513 and is expected dominant. This is in confirmation with the interval where *RBQ1* was expected based on previously performed disease tests (Jeuken et al manuscript in preparation). *RBQ1* was expected dominant based on disease tests performed in the past (Marieke Jeuken, personnel communication).

The group of plants of preBIL 7.2, which were heterozygous for the introgression on Chrom 7 for which this line was segregating (Figure 5), was not more resistant than Olof (appendix 5a). This line was expected to be more resistant than Olof, because this line is partial homozygous *L. saligna* and partial heterozygous for the interval where *RBQ1* is expected. These results might be explained by environmental influences on the resistance QTL, since resistance QTLs can be influenced by the environment (Reignault and Sancholle 2005).

BIL 7.3 was significantly more resistant than Olof based on the disease test with Bl:14. In previously performed leaf disc tests this BIL proved susceptible (Marieke Jeuken, personnel communication). Local or systemic acquired resistance as a result of the infection of the plants with aphids might have caused this result. Also the poor quality of the leaves might have caused this aberrant result.

In the disease test with Bl:16 a group of recombinant plants of line pv02268 and a group with non recombinant plants, which were heterozygous for the introgression on Chromosome 7, were more resistant than Olof according to the Dunnett test (appendix 5a). These lines confirmed the interval where *RBQ1* was expected. However one group of recombinants of this line which was heterozygous for the interval where *BRQ1* was expected proved not more resistant than Olof according to the Dunnett test. Also the group of preBIL 7.2, of which the plants had the same genotype as their parent (Figure 5), proved not more resistant than Olof in this disease test although these plants were homozygous *L. saligna* or heterozygous for the interval where *RBQ1* is expected. When *RBQ1* is located in this interval, a likely explanation for not detecting *RBQ1* in these groups might be that *RBQ1* was influenced by environmental factors in the plants of these groups.

Based on the disease test on the recombinant plants of line pv02255 no conclusions could be drawn with regard to the mapping of *RBQ1.* In the disease test with BL:14 no significant differences were detected with regard to the infection severities between the different groups of recombinant plants and Olof. In the disease test using Bl:16 some groups of plants of this line proved a significantly lower infection severity than Olof, however no relations could be observed with regard to the location of *RBQ1.*

Further research is required to further finemap *RBQ1*. New leaf disc tests and other types of disease tests should be performed on the progeny of the recombinant plants.

Other types of disease tests like seedling tests and field tests should be performed in order to investigate whether the results of the leaf disc test correlate with the influence on the resistance of *RBQ1* in normal growing plants. Disease tests on the progeny of the recombinant plants should be performed on different developmental stages in order to research whether *RBQ1* has an effect on the resistance in a certain developmental stage or during the complete life cycle of the plant. An advantage of this new tests is that more repeats can be included compared to my disease tests. A disadvantage is that most recombinant plants that were detected are still heterozygous for the interval where *RBQ1* is expected. The progeny of these plants have to be genotyped for this introgression, before they can be used in a disease test.

In Figure 13 an overview is presented of the chromosomal intervals on which different characteristics were associated or expected to be associated with *L. saligna* alleles.

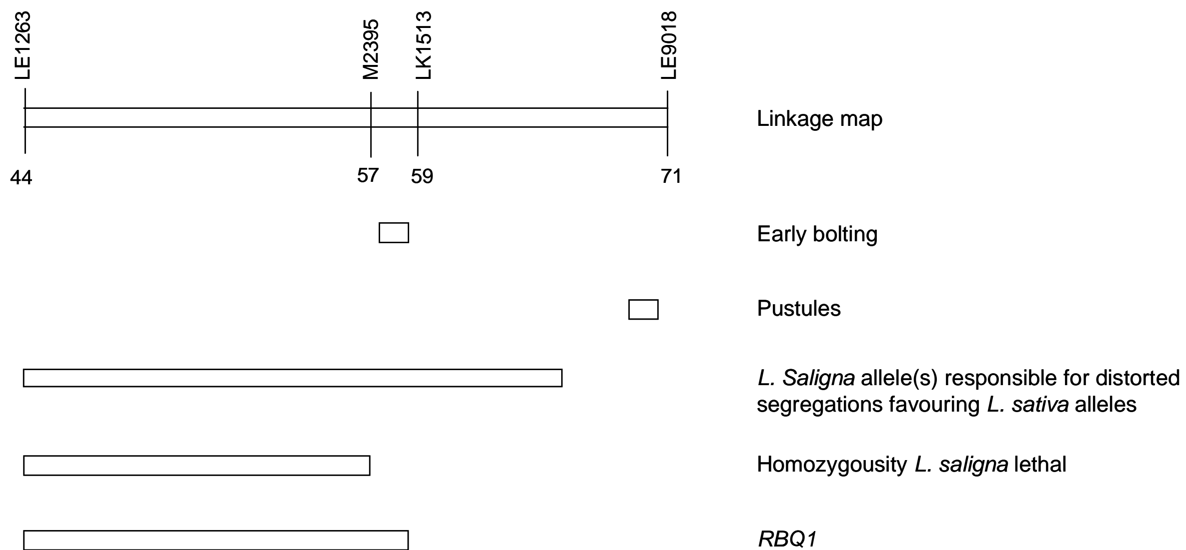

Figure 13. The different chromosomal regions, on which different characteristics were associated or expected to be associated with *L. saligna* alleles. The upper bar represents the position of the four EST-markers that were used for the recombinant screening. The chromosomal region on which the alleles responsible for the morphological traits early bolting and pustules were mapped, are presented in the second and third bar respectively. Within the fourth bar *L. saligna* alleles are expected which might cause distorted segregation in favour of *L. sativa* alleles. The fifth bar presents a chromosomal region which was never observed homozygous *L. saligna.* It is hypothesized that homozygosity for *L. saligna* alleles in this region is lethal. The fifth bar represents the interval where *RBQ1* is expected.

4.4 General

The non-host resistance in *L. saligna* against *Bremia* has a quantitative character, since 7 resistance QTLs have been detected (Jeuken and Lindhout 2002,Jeuken et al 2005). This polygenically character makes it likely that the non-host resistance in *L. saligna* is based on basal resistance. Basal resistance to heterologous pathogens has also been reported to inherit polygenically in barley (*Hordeum vulgare*) which is near non-host to several heterologous rust species (Jafari et al 2006).

In the leaf disc test that I performed an HR was observed macroscopically on some leaf discs of *L. saligna.* An HR was also observed incidentally in previously performed leaf disc tests and young plant tests (Ningwen Zhang, personnel communication). Although non-host resistance can be

involved in non-host resistance (Heath 2001) it is not expected to be an important factor involved in the non-host resistance of *L. saligna*.

4.5 Recommendations:

Molecular Markers

- For the candidate EST-markers which gave no amplification product in the first test on *L. sativa* cv Olof and *L. saligna*, it is possible to develop new primers. Maybe it is also an opportunity to change the annealing temperature of the PCR reaction.
- When a lot of plants have to be genotyped with just a few EST-markers it is better to use the NaOH DNA isolation method (appendix 3) than the CTAB method (appendix 2), since this method is much faster and gives better results. For the CTAB method the concentration of the isolated DNA has to be diluted, which is difficult, because the concentration in general differs much among the samples using this method.
- For the DNA isolation it is in general important that the leaf discs are put carefully on the bottom of the tubes before they are grinded. This will result better and more similar grinding, which results in higher efficiency and less drop-outs, since the leaf can not curl around the pin of the grind machine.
- dCAPs markers offer a nice opportunity when no suitable CAPs restriction enzyme is found on a polymorphism. Using dCAPs markers can avoid the use of strange enzymes in order to develop CAPs markers.

Recommendations with regard to the finemapping of RBQ1

Since LE1263 is a SCAR marker which is very difficult to score, I advice to sequence the amplification products of *L. sativa* cv Olof and *L. saligna* of this marker and develop a better scorable codominant marker on LE1263. Since LE1263 is a SCAR marker it is possible that via the development of new primers an easy scorable codominant SCAR marker can be developed.
It is also important that the development of new EST-marker keeps going for the interval where *RBQ1* is expected, because the distance between LE1263 and M2395 is 11 cM which is very large.
It is important that in the future the introgression on which *RBQ1* is located will be obtained homozygous *L. saligna.* Homozygosity is a very valuable prerequisite for the quality of trait

analyses in research as every phenotypic difference between the introgression lines can immediately be associated with the unique introgressed *L. saligna* segment (Jeuken and Lindhout 2004). This homozygosity can probably be obtained by searching recombinants in which *RBQ1* is uncoupled from the *L. saligna* gene or genes which cause the lethality. Another possibility is to map the *L. saligna* allele(s) which have an interaction with the *L. saligna* gene or genes causing the lethality. When this allele(s) is mapped, it can be combined with the *L. saligna* interval where *RBQ1* is expected, in order to obtain a plant homozygous for the *RBQ1* interval. Also for lettuce breeders it is important that the plants can be homozygous for the introgression where *RBQ1* is located, since the commercial lettuce cultivars are completely homozygous.
When breeders want to exploit *RBQ1* it should be uncoupled from the *L. saligna* allele which is responsible for early bolting, since this is an undesired genotype in commercial lettuce cultivars. Also the *L. saligna* alleles that are responsible for low viability and the different leaf shape have to be uncoupled from *RBQ1*.
If *RBQ1* is finemapped in the future it is interesting to investigate the interaction of this QTL with the other resistance QTLs. Probably this QTL can be involved in the combiBILs with *rqb4, rbq5, rbq6* and *rbq7.*

Leaf disc test

For the leaf disc test it is important to find a balance with regard to the humidity in the box in which the leaf disc test is performed. To keep the humidity at a high level it is important to sometimes spray water in the box. It is best to spray this water against the walls of the box to avoid that water droplets are formed on the leaf discs, because this is the most likely cause of appearance of brown rotten spots on the leaf discs.

General

It is important that during the transplanting of plants no unnoticed selection is made. This implies that in practice every plant, no matter how poor it looks, should be included in the experiments. Via this selection it is possible to lose the plants with the desired genotype.

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At the end of this thesis I have come to the end of my study Plant Sciences at Wageningen University. Doing this thesis I have learned a lot and it has developed me further and extended my knowledge on many aspects. Also the techniques I have learned during my thesis will hopefully be of great use for my future work.
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Used internet sites:

- http://cgpdb.ucdavis.edu/sitemap.html
- http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
- http://helix.wustl.edu/dcaps/dcaps.html

Appendices

Appendix 1

PCR:

The PCR mix:

1 µl primer forward (5mM)
1 µl primer reverse (5mM)
2.5 µl SuperTaq-buffer
1 µl dNTP (5mM)
0.1 µl SuperTaq polymerase
17.4 µl MilliQ water

The PCR reaction:

1. 2 minutes 94.0°C
2. 30 seconds 94.0°C
3. 30 seconds 52.0°C
4. 1 minute 72.0°C
5. 2 minutes 72.0°C
6. undetermined time 10.0°C

The steps 2, 3 and 4 together are repeated 40 times.

Restriction:

For the digestion 1 unit of enzymes and 3 µl of restriction enzyme buffer were added per PCR sample. The incubation took place at 37°C for at least 3 hours.

Appendix 2

DNA isolation protocol (CTAB):

1. Take two leaf discs with a diameter of 1 cm
2. Grind the leaf discs the Tissue striker KS-TSC 100 in 50 µl fresh buffer
3. Add 450 µl fresh buffer and put the solution at 65°C for at least 30 minutes
4. Add 500 µl chloroform and invert 40 times
5. Spin down for 15 minutes at 3500 rpm
6. Take of the supernatant
7. Add 0.8 volume isopropanol and invert 40 times
8. Spin down for 15 minutes at 3500 rpm
9. Poor of the supernatant
10. Wash the pellet in 500 µl 70% ethanol
11. Spin down for 5 minutes at 3500 rpm
12. Dry the pellet
13. Suspend the pellet in 100 µl T0.1E
14. Measure the DNA concentration using a BIOphotometer
15. Adjust the DNA concentration to 5 ng/ µl using MilliQwater
16. 3 µl DNA mix is used for a PCR reaction

Buffers:

Extraction buffer:

0.35 M Sorbitol

0.1 M Tris

5 mM EDTA

Lyses buffer:

0.2 M Tris pH 7.5

0.05 M EDTA

2 M NaCl

2% CTAB

Sarcosyl 5% ($^{w}/_{v}$)

Fresh Buffer:

25 ml extraction buffer

25 ml lyses buffer

10 ml sarcosyl

0.3 g sodium disulfite

T0.1E

100 ml MQ

1 ml 1 M Tris

20 µl 0.5 M EDTA

Appendix 3

DNA isolation protocol (NaOH) according to Wang et all 1993):

1. Take two leaf discs with a diameter of 1 cm
2. Grind the leaf discs in 20 µl 0.5 M NaOH using the Tissue striker KS-TSC 100
3. Add 20 µl 100mM Tris
4. Dilute 5 µl sample in 200 µl 100mM Tris
5. 1 µl DNA mix is used for a PCR reaction

Appendix 4

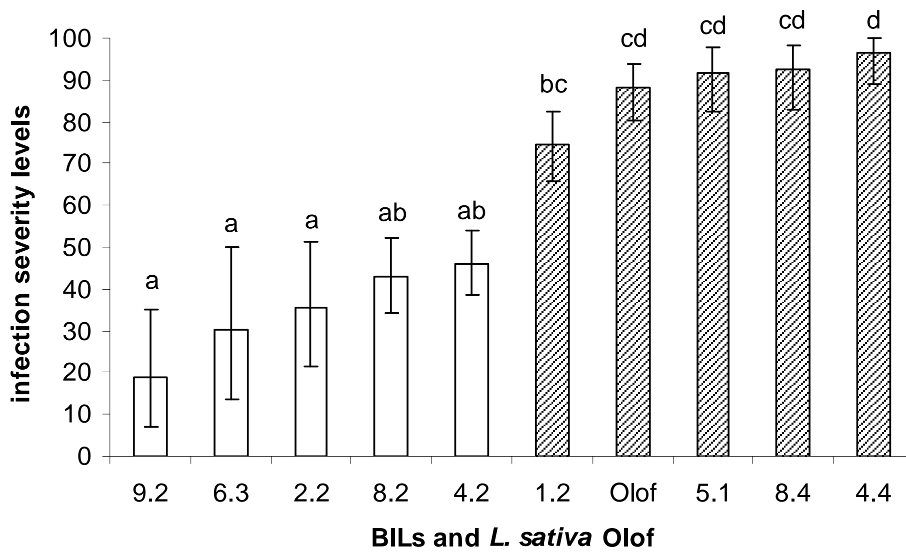

Figure. Corrected infection severity levels and 95% confidence intervals of ten lines are shown based on infection indexes analyses of six leaf disc tests. Letters in common indicate that the values are not significantly different (α =0.05, Tukey HSD procedure) (Jeuken et al manuscript in preparation).

Appendix 5

Table A. The average infection severity of the leaf disc tests performed with Bl:14 and Bl:16 on the recombinant plants found in line pv02268. As positive controls Olof and BIL 7.3 were used in this test. Also preBIL 7.2, which is segregating for a certain introgression on Chromosome 7, was tested. The recombinants were divided into groups based on their recombinant genotypes. These infection severities of these different groups were compared to the susceptible control Olof using a Dunnet test. The scores of the markers are presented as *a, b, c* and *h*: *a* homozygous *L. sativa, b* homozygous *L. saligna, c* heterozygous or homozygous *L. saligna* and *h* as heterozygous. A "y" in the column of the Dunnett test indicates that this group is significantly different from Olof (α = 0.05), whereas an "n" indicates not significantly different from Olof.

	Markers										Bl:14		Bl:16	
Line	LE1263	E35M60-102sal_b	E35M60-96sal_b	M2395	Bolting	LK1513	Pustules	E35M60-281sal	LE9018	# plants	Average infection severity %	DUNNETT	Average infection severity %	DUNNETT
pv02268	a	c	c	h	c	c	a	a	a	9	18,0	y	43,9	n
pv02268	h	c	c	h	c	a	a	a	a	7	13,0	y	33,4	y
pv02268	h	a	a	a	a	a	a	a	a	4	36,3	n	42,5	n
pv02268*	h	c	c	h	c	c	a	a	a	9	11,6	y	34,0	y
Olof	a	a	a	a	a	a	a	a	a	8	50,2	n	56,6	n
preBIL7.2	b	c	c	a	u	a	a	a	a	4	46,4	n	49,7	n
preBIL7.2	b	c	c	h	u	h	h	h	h	3	54,1	n	34,7	n
BIL7.3	a	a	a	a		a	c	b	b	6	29,4	y	53,0	n

* Non recombinant

Table B. The average infection severity of the leaf disc tests performed with Bl:14 and Bl:16 on the recombinant plants found in line pv02255. As positive controls Olof and BIL 7.3 were used in this test. Also preBIL 7.2, which is segregating for a certain introgression on Chromosome 7, was tested. The recombinants were divided into groups based on their recombinant genotypes. These infection severities of these different groups were compared to the susceptible control Olof using a Dunnet test. The scores of the markers are presented as *a, b, c* and *h*: *a* homozygous *L. sativa*, *b* homozygous *L. saligna*, *c* heterozygous or homozygous *L. saligna* and *h* as heterozygous. A "y" in the column of the Dunnett test indicates that this group is significantly different from Olof (α = 0.05), whereas an "n" indicates not significantly different from Olof.

	Markers										Bl:14		Bl:16	
Line	LE1263	E35M60-102sal_b	E35M60-96sal_b	M2395	Bolting	LK1513	Pustules	E35M60-281sal	LE9018	# plants	Average infection severity %	DUNNETT	Average infection severity %	DUNNETT
pv02255	a	a	a	a	a	a	c	h	h	8	32,1	n	32,8	y
pv02255	h	c	c	b	c	c	c	b	b	3	32,2	n	30,4	y
pv02255	h	c	c	h	a	a	a	a	a	3	31,7	n	40,1	n
pv02255	h	c	c	h	c	c	a	a	a	3	39,7	n	51,3	n
pv02255	h	c	c	h	c	c	c	b	b	4	38,7	n	36,8	y
pv02255*	a	a	a	a	a	a	a	a	a	4	44,3	n	48,4	n
Olof	a	a	a	a	a	a	a	a	a	8	50,2	n	56,6	n
preBIL 7.2	b	c	c	a	u	a	a	a	a	4	46,4	n	49,7	n
preBIL 7.2	b	c	c	h	u	h	h	c	h	3	54,1	n	34,7	y
BIL 7.3	a	a	a	a	a	a	c	b	b	6	29,4	n	53,0	n

* Non recombinant

Appendix 6

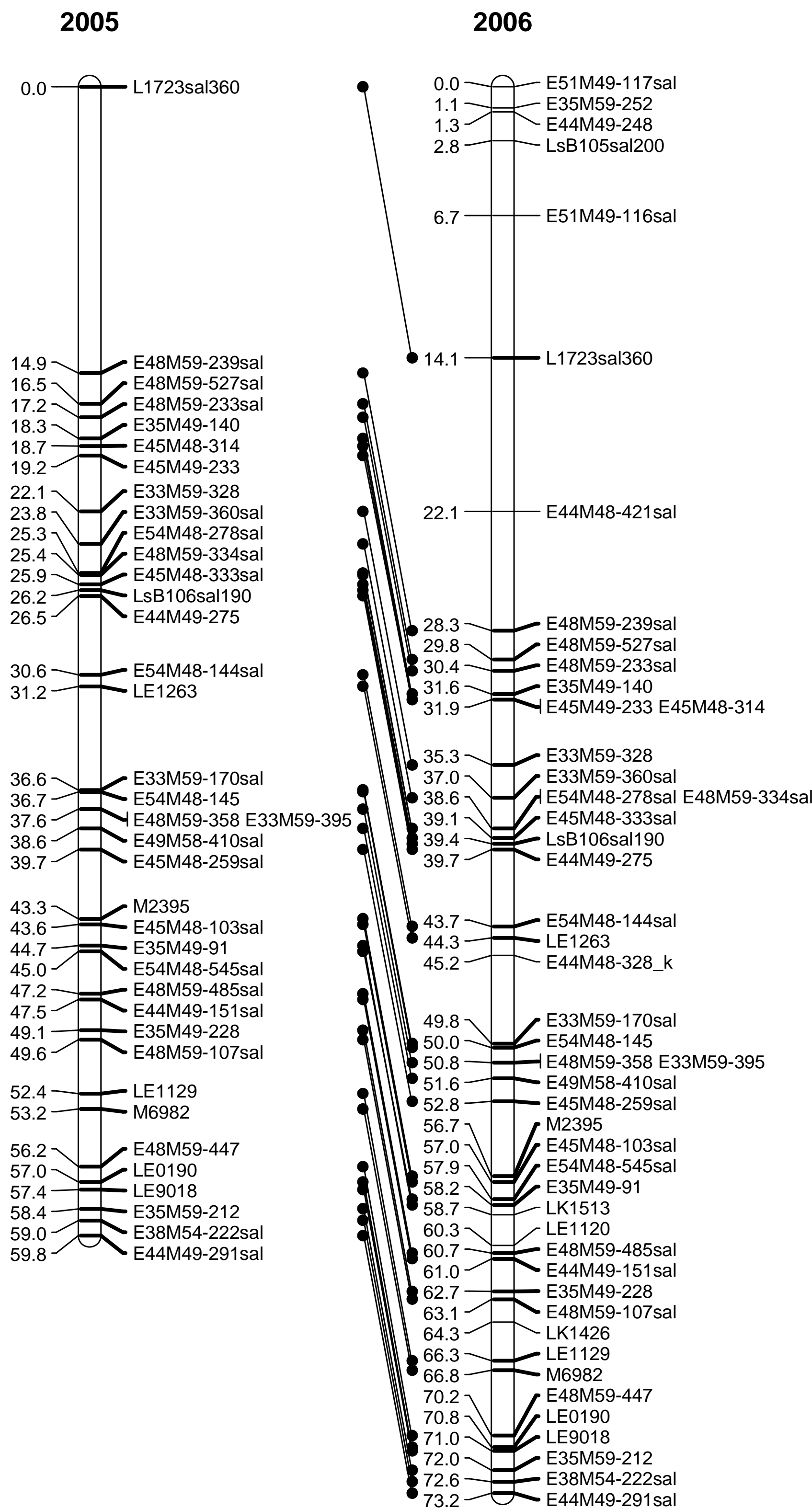

Figure. A comparison of the linkage maps of Chromosome 7 based on data of 2005 and 2006. A few AFLP-markers were lacking in the linkage map of 2005. In 2006 new EST-markers were developed and extra data were generated from existing EST-markers on the F2 population (Jeuken et al 2001).