Genetic dissection of nonhost resistance of wild lettuce, *Lactuca saligna*, to downy mildew

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CONTENTS

ABSTRACT

Lettuce downy mildew is the most destructive disease in lettuce (*Lactuca* spp.) cultivation and is caused by *Bremia lactucae*. The successful cross between its host *L. sativa* and the nonhost, *L. saligna*, offers a rare chance to study the genetics of the nonhost resistance. From a set of 29 Backcross Inbred Lines (BILs) representing in total 96% of the *L. saligna* genome, 15 introgressions were identified to contribute to this resistance at one to four tested lettuce developmental stages and were provisionally considered as 15 QTLs. QTL pyramiding of four "target QTLs" and the subsequent disease evaluations displayed that the combination of two to three QTLs was enough for almost complete resistance. This shows a redundancy of quantitative genes for nonhost resistance in *L. saligna*. In histological studies, the pathogen development was obviously arrested earlier in *L. saligna* than in the BILs with the "target QTL(s)". The fine mapping of the four "target QTLs" via a Near Isogenic Line (NIL) approach suggested the presence of both single- and multi-QTLs per introgression. In two *L. sativa –L. saligna* crossing combinations, we observed typical 'hybrid necrosis' symptoms, like necrotic lesions on leaves and stems, and retarded growth. This hybrid necrosis is caused by a *RIN4-R* interaction between *RIN4* allele(s) from *L. saligna* and a probable *R* gene in *L. sativa*.

ABBREVIATIONS

ADT_F	Adult plant Disease Test in Field
ADT_G	Adult plant Disease Test in Greenhouse
AFLP®	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
ATTA	*Agrobacterium*-mediated Transient Assay
BIL	Backcross Inbred Line
CAPS	Cleaved Amplified Polymorphic Sequence
dCAPS	Derived CAPS
EHR	Epidermal Hypersensitive Response
EST	Expressed Sequence Tag
HA	Haustorium
HR	Hypersensitive Response
HY	Hypha
IU	Infection Unit
LOD	Limit of Detection
Mal-HY	Mal-formed Hypha-like structure
MAS	Marker Assisted Selection
NIL	Near Isogenic Line
PV	Primary Vesicle
QTL	Quantitative Trait Locus
RIN4	RPM1 Interacting protein 4
RIS	Relative Infection Severity
SDT	Seedling Disease Test
SEHR	Subepidermal HR
SSR	Simple Sequence Repeat (or Microsatellite)
SV	Secondary Vesicle
VIGS	Virus Induced Gene Silencing
YDT	Young plant Disease Test

CHAPTER 1

General Introduction

Lettuce

Lettuce, *Lactuca sativa* is a temperate annual or biennial plant species that belongs to the genus *Lactuca* L. and the family Asteraceae or Compositae. The genus *Lactuca* L. consists of about 100 species (de Vries 1997). Lettuce probably started out as a weed around the Mediterranean basin and has been used as a salad crop for more than 4500 years (Davis et al. 1997). The evidence for this is the depiction of many different lettuce varieties in ancient Greek relics found in tomb paintings in Egypt 2500 B.C. (Davis et al. 1997; de Vries 1997). Probably, lettuce was introduced to northern and western Europe around the Middle Ages by the Romans (Davis et al. 1997; de Vries 1997; Lindqvist 1960). Nowadays, both leafy and stalk lettuce have been included in people's daily diet. Seven commonly recognized edible lettuce cultivar groups appear: Butterhead, Latin, Crisphead (Iceberg types), Cos (romaine type), Cutting (lollo and oakleaf types), Stalk (Davis et al. 1997; de Vries 1997) and Oilseed lettuce (Rulkens 1987). There are types more suitable for field production and others for glasshouse cultivation (de Vries 1997).

The direct ancestor of *L. sativa* (domesticated lettuce) was *L. serriola* (de Vries 1997; Kesseli et al. 1991; Zohary 1991). *L. serriola* is a weed that belongs to the primary gene pool of lettuce that further consists of *L. sativa*, *L. dregeana*, *L. altaica*, and *L. aculeata* (Koopman et al. 1998). *L. saligna* and *L. virosa* branch off this clade and belong to the secondary gene pool. The primary and secondary gene pool species are classified in section *Lactuca* subsection *Lactuca* (Koopman et al. 1998; Lindqvist 1960). *L. sativa* is fully cross-compatible and inter-fertile with wild *L. serriola* and others from the primary gene pool; partly cross-fertile with *L. saligna*; and almost cross-sterile with *L. virosa* (Lindqvist 1960; Zohary 1991). These wild species are used as resources for lettuce improvement.

Modern breeding programs for cultivated lettuce have focused on the improvement of the cultivars with regard to: resistance to disease and insects, improved horticultural type

(color, size, taste, head shape, uniformity, earliness of head formation and yield) and adaptation to environment (temperature, day length, water quality, soil type and low energy need) (Davis et al. 1997; de Vries 1997; Eenink and Smeets 1978).

A major focus has been the introduction of resistance genes to diseases and insects in cultivated lettuce. Resistances to diseases and to insects were found in wild species: *L. serriola* to downy mildew (*Bremia lactucae*) and lettuce mosaic virus (LMV), *L. virosa* to downy mildew and leaf aphid (*Nusonoviu ribis-nigri*) and *L. saligna* to downy mildew and cabbage looper (*Trichoplusia ni*) (Bonnier et al. 1992; de Vries 1997; Eenink and Dieleman 1983; Gustafsson 1989; Whitaker et al. 1974).

L. sativa is autogamous, diploid (2n=18) and its genome size (1 C) is about 2.6 Gb. This is 2.6 and 18 times larger than the tomato and *Arabidopsis* genome, respectively. Such a relatively large genome is common for Compositae species (Doležalová et al. 2002; Kesseli and Michelmore 1996; Michaelson et al. 1991).

Several genetic linkage maps for lettuce have been made. The first one was based on the F_2 of a *L. sativa* x *L. sativa* cross (cv. Calmar x cv. Kordaat), consisting of >13 linkage groups and mainly RFLP and RAPD markers (Kesseli et al. 1994). In 2001, the first genetic map with 9 linkage groups and 476 markers, mainly AFLP, was made on a F_2 of *L. saligna* x *L. sativa* cv. Olof cross (Jeuken et al. 2001). Another genetic map from a recombinant inbred line (RIL) population of *L. sativa* cv. Salinas x *L. serriola* cross was made and had been publicly available on the CGPDB database with 9 linkage groups and 1600 markers (mainly AFLP and EST) (http://compgenomics.ucdavis.edu/).

Recently, Truco et al. (2007) published a consensus map in lettuce comprising over 2700 markers that integrated in total seven individual lettuce maps, including the three genetic maps mentioned above. This integrated lettuce map provides utilities of markers across different genetic backgrounds (Truco et al. 2007).

At the public The Compositae Genome Project website (CGPDB; (http://compgenomics.ucdavis.edu/), a resource for lettuce and sunflower genetic and genomic data is available. To date, this database includes >225,555 expressed sequence tags (ESTs), providing on average >20,000 unigenes per tested *Lactuca* species.

Lettuce downy mildew

Lettuce downy mildew, caused by *Bremia lactucae* Regel, is a devastating disease for lettuce cultivation worldwide. Lettuce downy mildew disease was reported in Europe as early as 1843 (Raid and Datnoff 1992). This foliar disease can directly cause lettuce yield and

postharvest loss. During the past several years, costs to control downy mildew have risen dramatically and resistance to this disease has become the first breeding priority in lettuce.

B. lactucae is an obligate biotrophic pathogen that belongs to the Oomycetes, order Peronosporales and family of Peronosporaceae. Oomycete physically resembles fungus, but shows distinct phylogeny and physiology from fungus. Other well-known plant- pathogenic members of this group are *Pythium* and *Phytophthora*. To date, genomes of several related oomycete plant pathogens have been sequenced: *Phytophthora sojae* (soybean blight), *P. ramorum* (sudden oak death), *P. infestans* (potato late blight), *P. capsici* (root-rot and foliar blight disease in pepper) and *Hyaloperonospora parasitica* (*Arabidopsis* downy mildew) (Lamour et al. 2007; Tyler 2001; Tyler et al. 2006). Important research focus in oomycetes is on the molecular biology of effector genes (Birch et al. 2006; Kamoun 2003; Kamoun 2006)

B. lactucae can infect lettuce and several other *Lactuca* species, for instance *L. serriola* and *L. virosa*, at any developmental stage from seedling to mature plant (Lebeda and Schwinn 1994; Lebeda and Syrovatko 1988; Petrzelova and Lebeda 2004) Symptoms of downy mildew first appear as pale yellow areas on the upper side of lettuce leaves. Under cool, moist conditions favorable for growth of the pathogen, a white cotton-like sporulation generally appears on the lower leaf surface and infected areas are enlarged with time and eventually turn brown. The infected areas may also serve as portals for secondary invaders, such as the fungus *Botrytis cinerea*.

The life cycle of *B. lactucae* starts when a spore (conidium) produces a germ tube and forms an appressorium to directly penetrate leaf epidermal cells (Lebeda et al. 2001). Subsequently formation of a primary vesicle (PV), a secondary vesicle (SV), hyphae (HY) and haustoria (HA) take place in turn within 24 hours post inoculation (hpi) (Ingram et al. 1973; Lebeda and Reinink 1994) (Figure. 1). Colonization occurs when intercellular hyphae grow and penetrate neighbouring cells and conidiophores bearing conidia emerge from the stomata. Wind disseminates the conidia to repeat the infection process. Conidia may also form into zoospores that either directly infect leaf tissue or become encysted for later infection (Figure. 2).

The population structure of *B. lactucae* is complex and consists of multiple races (pathotypes) and two mating types (B1 and B2). Many races of *B. lactucae* are identified from lettuce cultivars and wild relatives. In Europe, at least 25 races of *B. lactucae* were found and described, wherein at least Bl:17, 18, 20-25 were newly discovered between 1998 and 2006. These new races of *B. lactucae* together can overcome all the *Dm/R* genes that were exploited and effective before 1998 (Van Ettekoven 2006; Van Ettekoven and Van der Arend 1999). *B. lactucae* reproduces mainly asexually and occasionally sexually (occurs when the compatible types B1 and B2 are in close proximity) (Crute 1992a).

B. lactucae is known to infect more than 200 species of Compositae from about 40 genera of the tribes Lactuceae, Cynareae and Arctotideae (Crute and Dixon 1981; Lebeda et al. 2002). However, only a limited number of *Lactuca* spp. and closely related genera show infection on naturally growing plants (Lebeda and Syrovatko 1988). The cross-inoculation experiments indicated that *B. lactucae* is highly specific and mostly limited to the same genus of plants. So far, at least 12 *formae speciales* (f.sp.) of *B. lactucae* have been proposed (Koike and Ochoa 2007; Lebeda et al. 2002)

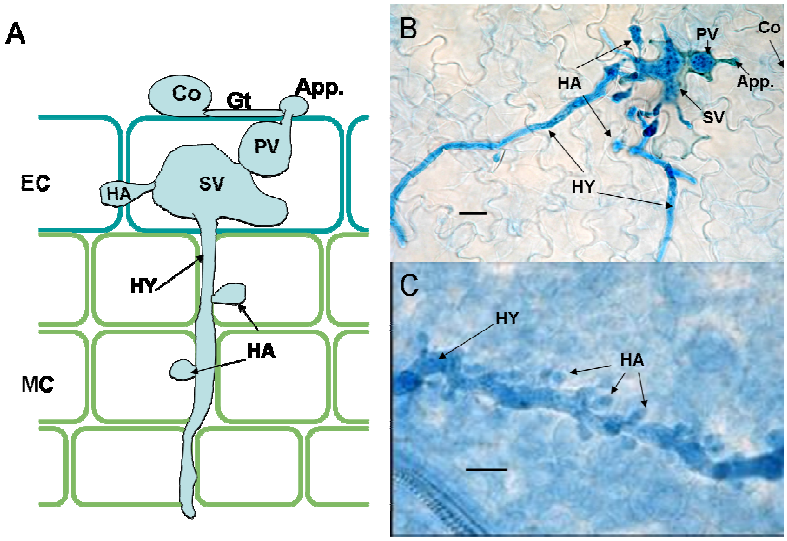

Figure 1. A representation of a compatible interaction between *L. sativa* cv. Olof and *B. lactucae* race Bl:14. **A:** a schematic drawing of the infection process until haustoria are formed. **B:** a trypan blue stained infected *L. sativa* cv. Olof tissue under white light microscope at 48 hours post inoculation (hpi). **C:** infection at 72hpi. The scale bar at the left lower corner represents 20µm. EC=epidermal cell; MC= mesophyll cell; Co=conidium; Gt=germtube; App.=appressorium; PV=primary vesicle; SV=secondary vesicle; HY=hyphae; HA=haustoria.

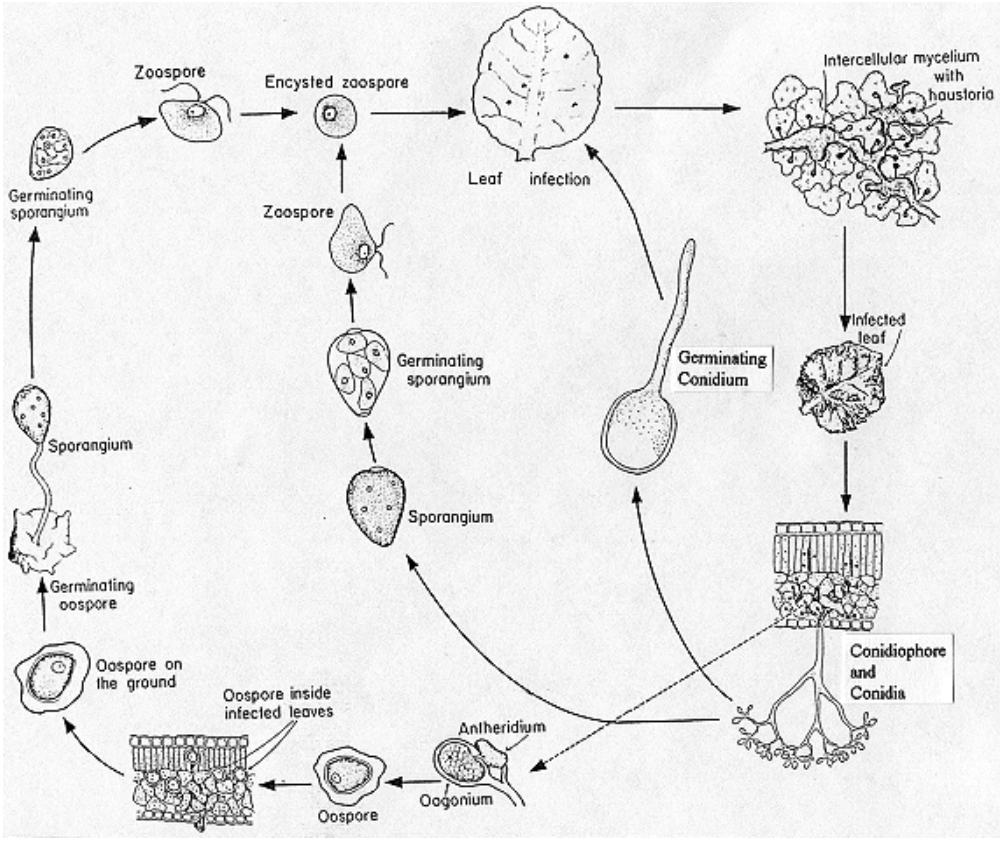

Figure 2. Disease cycle of lettuce downy mildew caused by *Bremia lactucae*. Modified from (Agrios 1988).
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Resistance mechanisms of lettuce to downy mildew

BOX I ***Plant host resistance in general***

Qualitative resistance

Qualitative resistance refers to complete and often race-specific resistance. Its expression is normally independent of environmental conditions and this type of resistance inherits often monogenically. So far, two types of mechanisms have been described for such resistance. One is the "specific toxin" system that involves encoding the toxin reductase and inactivates the pathogenic toxin production (Johal and Briggs 1992). Another mechanism is based on the famous gene-for-gene interaction that is triggered upon direct or indirect recognition of avirulence protein (Avr) of a pathogen by race-specific resistance (R) proteins of the host plant (Flor 1942; Mackey et al. 2002; Reignault and Sancholle 2005; Santangelo et al. 2003). The interaction between Avr-R proteins often leads to a localized necrotic response occurring at the infection site, the hypersensitive response (HR). The largest known family of plant *R*-genes encodes proteins with nucleotide-binding site (NBS) and C-terminal leucine-rich repeat (LRR) domains (Dangl and Jones 2001).

Quantitative resistance

Quantitative resistance refers to all types of resistance that behave in a quantitative way. It associates with many terms and concepts, such as horizontal, partial, intermediate, incomplete, multigenic and field resistance. Quantitative resistance is controlled by several genes whose individual effects are small and together contribute to the resistance. The expression of quantitative resistance is often influenced by environmental conditions and plant developmental stages (Mackay 2001). The commonly used strategy of studying inheritance of quantitative resistance is mapping resistance quantitative traits loci (QTLs) to particular genomic regions (Young 1996). Because no resistance QTL has been cloned yet, knowledge on its molecular features is lacking.

Host resistance in lettuce to downy mildew

Monogenic race-specific resistance

Seeking for genetic resources of resistance to downy mildew has been a major activity in lettuce breeding. The lettuce species *L. sativa, L. serriola* and *L. virosa* show a large variation of resistances to downy mildew (Bonnier et al. 1992). The most common and exploited resistance is qualitative, race-specific and based on single dominant genes, *Dm* genes (downy mildew) or *R* genes (BOX I). To date, many *Dm* genes are known and at least 20 race-specific *Dm* genes have been introgressed into lettuce cultivars (Crute 1992b; Lebeda and Zinkernagel 2003; Meyers et al. 1998). The known *Dm* genes are mainly located in clusters on four different chromosomes (Bonnier et al. 1994; Kesseli et al. 1994). In 1998, the *Dm3* gene was cloned and it was characterized to a class of resistance genes with a nucleotide binding site (NBS) and leucine-rich repeats (LRR) region (Meyers et al. 1998).

Unfortunately, resistance based on *Dm* genes is not durable since the resistance is usually overcome by rapid adaptation of *B. lactucae* races (Lebeda and Zinkernagel 1999). In the last eight years, at least eight new *B. lactucae* races appeared in Europe (Van Ettekoven 2006; Van Ettekoven and Van der Arend 1999). This rapid adaptation is determined by several factors: quick asexual reproduction (ca. 1 week); rapid windblown spread of conidium (migration and gene-flow) and a heterothallic sexual mating system (mutation and new recombination) (McDonald Bruce and Linde 2002). Even the combination of different *Dm* genes in lettuce has been unsuccessful to keep the resistance effective against newly appeared *B. lactucae* isolates (Crute 1992a; Crute and Johnson 1976; Gustafsson 1986). Therefore, breeders are seeking for a good alternative approach to give lettuce durable protection against *B. lactucae*.

Quantitative resistance

Quantitative resistance in lettuce to downy mildew has been reported in *L. serriola* accessions (Gustafsson 1989; Petrzelova and Lebeda 2004), *L. sativa* cultivars Iceberg and Grand Rapids (Grube and Ochoa 2005; Gustafsson 1992) and probably appears in other wild lettuce species. There is not much information about quantitative resistance in *L. serriola*. The quantitative resistance of cv. Iceberg and cv. Grand Rapids is race-non-specific and active in adult plants but not in seedlings. This indicated cv. Iceberg and cv. Grand Rapids have potential to be sources of *B. lactucae* resistance alleles in breeding programs (Grube and Ochoa 2005; Gustafsson 1992). However, the mechanisms and the inheritance of the quantitative resistance in lettuce to downy mildew are not clearly understood and no resistance QTL has been mapped so far.

BOX II ***Plant nonhost resistance in general***

Molecular basis of nonhost resistance

The classical definition of nonhost resistance is: all accessions of a given plant species providing resistance against all races of a given pathogen. This is the most common form of plant resistance against potential pathogens in nature (Heath 1981; Niks 1988).

To date, nonhost resistance is considered to be associated with multiple protective mechanisms, which can be categorized into constitutive barriers and inducible reactions. Constitutive barriers include preformed physical barriers such as cell wall thickness and composition, the cuticle (Nurnberger and Lipka 2005; Thordal Christensen 2003), and preformed chemical barriers such as phenols and alkaloids (Heath 2000; Kamoun 2001; Nurnberger and Lipka 2005). Inducible reactions include cell wall deposition, hypersensitive response (HR), transcriptional activation of defence related genes, phyloalexin production and polarized secretion triggered by the pathogen (Ellis 2006; Nurnberger and Lipka 2005; Trujillo et al. 2004; Wolter et al. 1993).

Recognition of pathogen-associated molecular patterns (PAMPs) in both compatible and incompatible pathogens through specific receptors at the plant cell surface is thought to activate inducible defence responses. These responses are subsequently suppressed by compatible pathogens, but cannot be suppressed by effectors from incompatible pathogens (Li et al. 2005; Zipfel and Felix 2005). The compounds such as ethylene and salicylic acid, heat-shock proteins (Hsps), SGT1, and many more proteins are involved in the signaling pathways of plant defenses (Mysore and Ryu 2004). Two models of plant nonhost resistance are postulated: one model proposes that inducible nonhost resistance depends on PAMP-induced basal resistance in the absence of defence suppression (absence of effective effectors); another model postulates that nonhost resistance depends on stacks of "classical" *R*-genes of the NBS-LRR type. These two models do not necessarily exclude each other which means a combination of the two mechanisms might also occur (Schweizer 2007).

Inheritance of nonhost resistance

Investigating the inheritance of nonhost resistance might allow the identification of the essential regulators that underly nonhost resistance. However, study the inheritance of nonhost resistance requires interspecific crosses between host and nonhost species and such species are often sexually incompatible or produce aberrant or sterile progeny (Atienza et al. 2004; Jeuken and Lindhout 2002; Niks 1988). Therefore, fruitful examples of studies on the inheritance of nonhost resistance are scarce. Only a few studies on wheat and barley to the nonpathogenic leaf and stripe rust fungal species have been reported and suggest that resistance QTLs as well as single *R*-genes contribute to the corresponding nonhost resistance (Rodrigues et al. 2004).

Nonhost resistance in *L. saligna* to downy mildew

Wild species *L. saligna* is considered as nonhost to *B. lactucae* because no *L. saligna* accession (N=52) has been found to be susceptible to any of the 20 tested *B. lactucae* races (Bonnier et al. 1992). Therefore it may be a good resource for the durable protection of *Lactuca* spp. species against *B. lactucae* (Bonnier et al. 1992; Gustafsson 1989; Jeuken and Lindhout 2002; Lebeda and Boukema 1991; Lebeda and Schwinn 1994). The mechanism(s) underlying this nonhost resistance are not clear. Only some histological studies suggested that the resistance mechanisms of *L. saligna* seem to be very different from both race-specific resistance in lettuce cultivars and the partial (field) resistance of lettuce cv. Iceberg (Lebeda and Reinink 1994). Resistance of *L. saligna* seems to be based on restriction of the *B. lactucae* after the formation of secondary vesicle, however, differing between *L. saligna* accessions for both the rate of pathogen development and the proportion of infection sites with HR (Lebeda and Reinink 1994; Lebeda et al. 2006; Sedláová et al. 2001).

Fortunately, the nonhost species *L. saligna* is sexually compatible with the host species *L. sativa*. This creates the possibility to study the inheritance of the nonhost resistance. In order to analyze this resistance, an F_2 population based on a *L. saligna* (nonhost) x *L. sativa* (host) cross has been developed (Jeuken et al. 2001; Jeuken and Lindhout 2004). QTL mapping at adult plant stage (grow in greenhouse) revealed three QTLs, *Rbq1*, *Rbq2* and *rbq3*, involved in the quantitative resistance to both *B. lactucae* races Bl:14 and Bl:16 and a *R*-gene-like resistance (*R39*) to Bl:16 but not to Bl:14. The sterility of many F_2 plants prevented the development of a Recombinant Inbred Line population (RIL) (Jeuken and Lindhout 2002). Later on, a set of 29 Backcross Inbred Lines (BILs) covering 96% of the *L. saligna* CGN05271 genome was generated from the BC_4S_{1-2} and BC_5S_{1-2} of the original *L. saligna* x *L. sativa* cross by Marker Assisted Selection (MAS). Each BIL contains one homozygous *L. saligna* introgression of about 30 cM in a *L. sativa* background. BIL6.3, BIL7.1, BIL7.3 and BIL 8.3 contain one or two additional introgressions in each line. A few BILs contain the introgression in heterozygous state and were designated as “preBILs”.

All 29 BILs were tested against *B. lactucae* Bl:14 and Bl:16 at adult plant stage in the greenhouse using detached leaf discs and 6 BILs showed quantitative resistance. Introgression regions that are associated with quantitative resistance are considered to harbor a QTL. Comparing this result with the results from an already existing F_2 population derived from the same cross, two QTLs are in common and four newly detected recessive QTLs in the set of BILs were designated *rbq4*, *rbq5*, *rbq6* and *rbq7* (Jeuken et al. 2008).

Genetic incompatibility in interspecific crosses

In our study, the plant materials were derived from two interspecific crosses between two distantly related species *L. saligna* and *L. sativa*. Although they are sexually compatible, the crosses are difficult to make and the F_2 progeny are mostly sterile (Jeuken et al. 2001). In the set of BILs, we detected four preBILs that remain heterozygous for introgressions in *L. sativa* background even after several selfing generations (Jeuken and Lindhout 2004), probably because homozygous introgression are lethal for the carrier. This suggests genetic incompatibilities in such interspecific cross between *L. saligna* and *L. sativa*.

One of these preBILs, preBIL9.1 shows symptoms on leaves and stems that resemble a phenomenon described as hybrid necrosis. Hybrid necrosis is a post-zygotic incompatibility leading to necrotic spots on leaves, retarded growth, gradual death of leaves and leaf sheaths in certain hybrid plants. Its phenotypic characteristics are similar to responses induced by environmental stress, including pathogen attack (Bomblies and Weigel 2007; Ren and Lelley 1988). The earliest recognized examples of hybrid necrosis and its association with immunity was in the early 20th century in bean and tobacco (Burkholder and Muller 1926; Kostoff 1930). Consequently hybrid necrosis has been observed in many and studied in few intra- and interspecific plant crosses, such as in wheat, tomato, potato, bean and *Arabidopsis* (Bomblies et al. 2007).

In addition to its role in hybrid necrosis, the heterozygous introgression of preBIL9.1 also carries our previously mapped *R*-gene-like resistance (*R39*) on the top of Chromosome 9 in the F_2 population and preBIL9.1. The mechanism and responsible genes behind hybrid resistance and its related resistance remained unclear, although some studies assumed the involvement of *R* genes in hybrid necrosis (Bomblies et al. 2007; Krüger et al. 2002; Wulff et al. 2004). The observed hybrid necrosis and resistance of preBIL9.1 offer us an opportunity to identify the associated loci and their underlying mechanisms.

Scope of this thesis

Our study aims at (1) studying the inheritance of the nonhost resistance of *L. saligna* to *B. lactucae* at various developmental stages of lettuce; (2) identifying how many introgressed QTLs from *L. saligna* are sufficient to give complete broad-spectrum resistance in *L. sativa*; (3) understanding what are the mechanisms underlying this nonhost resistance; (4) determining more accurate positions of the identified QTLs and (4) finding out the relation between the hybrid necrosis of preBIL9.1 and its resistance to *B. lactucae*.

In Chapter 2, we present the identification and characterization of the QTLs that are responsible for the resistance of *L. saligna* to *B. lactucae* at various plant developmental stages through four types of bioassays.

In Chapter 3, we studied how many QTLs are sufficient to give *L. sativa* a complete protection against *B. lactucae* and determined the resistance mechanisms by histological observations.

In Chapter 4, we fine mapped four target QTLs involved in this nonhost resistance to determine their positions and to determine whether one introgression might contain several QTLs for resistance.

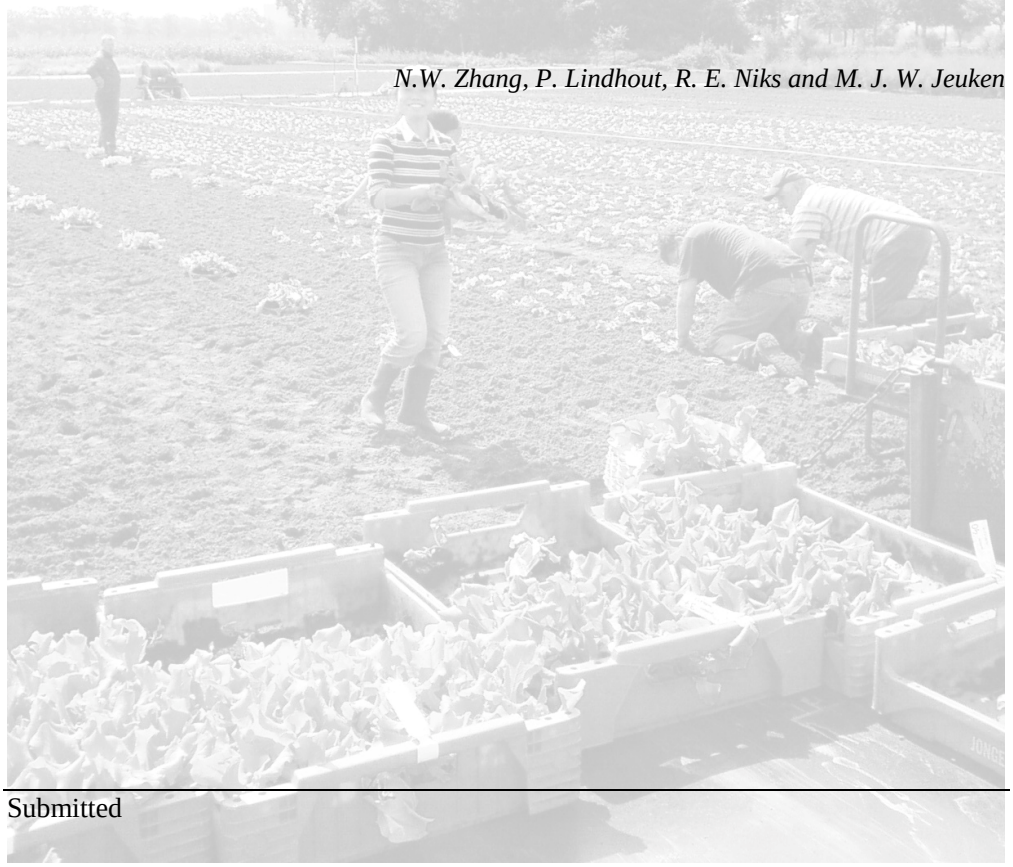
In Chapter 5, we studied genetic and molecular aspects of the hybrid necrosis and the resistance observed from preBIL9.1.

In Chapter 6, the results obtained in the previous chapters are discussed in the light of roles of QTLs for nonhost resistance, the resistance mechanisms and the perspectives for cloning of the identified QTLs, and the genetic and biological model of genetic incompatibilities in particular hybrid necrosis.

CHAPTER 2

Lactuca saligna nonhost resistance to downy mildew is polygenic, and resistance QTL effects are dependent on developmental stage

N.W. Zhang, P. Lindhout, R. E. Niks and M. J. W. Jeuken



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Field Test in July, 2006, Nergena, The Netherlands

Lactuca saligna nonhost resistance to downy mildew is polygenic, and resistance QTL effects are dependent on developmental stage

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Abstract

Nonhost resistance is the most common form of plant immunity to potential pathogens. We used the *Lactuca saligna – Bremia lactucae* pathosystem as a model to investigate the inheritance of nonhost resistance. We focused on the contribution of Quantitative Trait Loci (QTLs) to nonhost resistance at various developmental stages in the lettuce life cycle. A set of 29 Backcross Inbred Lines (BILs) of *L. saligna* CGN05271 (nonhost) introgressions in a *L. sativa* cv. Olof (host) background identified 16 introgressions that contributed to resistance at four plant developmental stages: seedlings, young plants, adult plants in the greenhouse and adult plants in the field. We provisionally considered these introgressions to be 16 QTLs. Of these 16 QTLs, 7 were identified previously and 9 were new QTLs. For 15 QTLs (*Rbq1, Rbq2, rbq3 to 7* and *Rbq8 to 15*), the resistance alleles were derived from the nonhost *L. saligna;* the resistance allele of the other QTL (*Rbq16*) was from the susceptible *L. sativa* cv. Olof. Of the 15 QTLs of *L. saligna*, only 2 QTLs, *rbq5* and *rbq7*, were effective at every plant developmental stage; the other 13 QTLs were only effective at certain developmental stages. Experiments with seven *B. lactucae* races did not provide evidence that any QTL was race-specific. We suggest that nonhost resistance of *L. saligna* might be due to the cumulative effects of many resistance QTLs operating at various developmental stages.

Keywords: Quantitative resistance

Introduction

Nonhost resistance is a phenomenon wherein an entire plant species is strongly resistant to all isolates of a pathogenic species. This is the most prevalent form of disease resistance exhibited by plants, and it is the most durable and complete source of plant immunity to potential pathogens (Heath 1981; Niks 1988). Understanding genes that are responsible for nonhost resistance in plants could potentially result in new strategies for introducing durable resistance into cultivated crops. Therefore, knowledge of both the molecular basis and inheritance of nonhost resistance is extremely valuable.

The molecular basis of nonhost resistance has been investigated intensively, and there is evidence that it relies on multiple protective mechanisms, which can be categorized into constitutive barriers and inducible reactions. Constitutive barriers include physical defences such as cell wall thickness and composition, the cuticle (Thordal Christensen 2003), and chemical compounds such as phenols and alkaloids (Heath 2000; Kamoun 2001; Nurnberger and Lipka 2005). Inducible reactions include cell wall deposition, papilla formation and the Hypersensitive Response (HR) (Ellis 2006; Trujillo et al. 2004; Wolter et al. 1993). Recognition of pathogen-associated molecular patterns (PAMPs) in both compatible and incompatible pathogens by plants is thought to activate inducible defence responses, but these responses are subsequently suppressed by compatible pathogens (Li et al. 2005; Zipfel and Felix 2005). Plant signalling compounds such as ethylene and salicylic acid, heat-shock proteins (Hsps), SGT1 and many more proteins are involved in the induction of plant defence (Mysore and Ryu 2004). Inducible nonhost resistance has been proposed to depend either on PAMP-induced basal resistance in the absence of defence suppression or on stacks of "classical" *R*-genes of the NBS-LRR type, although a combination of the two mechanisms might also occur (Schweizer 2007).

Studies of nonhost resistance have mainly identified the downstream components and transcription factors that are involved in defence signal transduction pathways. These include the *PEN* genes identified in studies of *Arabidopsis* nonhost resistance against incompatible powdery mildew fungi (Ellis 2006) and the *Ror* genes, which are associated with the nonhost resistance of barley to *Blumeria graminis* f. sp. *tritici (Bgt)* (Schweizer 2007; Trujillo et al. 2004). Such genes are unlikely to be the central and most upstream regulators that determine the host/nonhost status of a plant species. Investigating the inheritance of nonhost resistance might allow the identification of the essential regulators that underly nonhost resistance. However, examples of fruitful studies of the inheritance of nonhost resistance are scarce, since they require crosses between host and nonhost species

and such species are often sexually incompatible or produce aberrant or sterile progeny (Atienza et al. 2004; Jeuken and Lindhout 2002; Niks 1988).

Research on the resistance of the progeny of the wheat intra-species cross 'Lemhi' x 'Chinese 166' to the barley yellow rust *P. striiformis* f. sp. *hordei* has identified two QTLs with major effects and two QTLs with minor effects that confer resistance to this pathogen in wheat. The two QTLs with major effects account for 43.5% and 33.2% of the phenotypic variance in resistance, and the two QTLs with minor effects contribute 5.1% and 10.9% of the variance. Three out of the four nonhost QTLs conferred hypersensitive cell death-associated resistance to barley yellow rust. One of the two major QTLs, *QPsh.jic-2B*, was mapped on the long arm of chromosome 2B, where a number of host *R*-genes also reside. These QTLs may therefore serve a classic host-type resistance function by recognizing avirulence factors present in *P. striiformis* f. sp. *hordei* isolates (Rodrigues et al. 2004).

In a series of crosses between "normal" nonhost resistant barley lines and a nonhost susceptible line (SusPtrit), the authors identified a number of resistance QTLs and one *R*-gene responsible for barley nonhost resistance to incompatible rust fungi. They speculated that in barley-rust interactions, nonhost resistance is due to non-suppressed basal resistance, which depends on a group of defence-related genes that associate with nonhost resistance QTLs (Jafary et al. 2006). On the other hand, studies of the resistance of inter-*formae speciales* crosses to powdery mildew fungi support the idea that single or stacked *R*-genes, such as *Pm10*, are the basis of nonhost resistance in wheat (Schweizer 2007).

Studies of the resistance of lettuce species (*Lactuca* spp.) against downy mildew (*Bremia lactucae*) provide an example of the polygenic inheritance of nonhost resistance. The wild lettuce *L. saligna* is considered to be a nonhost to *B. lactucae*, since no *L. saligna* accession has been found to be susceptible to any of the 20 tested *B. lactucae* races (Bonnier et al. 1992; Gustafsson 1989; Lebeda and Boukema 1991; Lebeda and Zinkernagel 2003; Norwood et al. 1981). This wild lettuce is sexually compatible with the cultivated host species *L. sativa*, of which most accessions are susceptible unless they harbour a classical race-specific *R*-gene (Jeuken and Lindhout 2002). To investigate which genes determine host/nonhost status in *L. sativa* (host) and *L. saligna* (nonhost), we initiated the genetic dissection of nonhost resistance in *L. saligna*. Using Marker Assisted Selection (MAS), a set of 29 Backcross Inbred Lines (BILs) were developed from the BC_4S_{1-2} and BC_5S_{1-2} generations of crosses between *L. sativa* cv. Olof and *L. saligna* CGN05271. Each BIL consists of one introgression of *L. saligna* (average of 33cM and ~4% of the genome), in the *L. sativa* cv. Olof background. Together, the 29 BILs cover 96% of the *L. saligna* genome (Jeuken and Lindhout 2004). The identification of seven QTLs for resistance to *B. lactucae*

in adult lettuce plants from the F_2 and the BIL approach together suggest that nonhost resistance is polygenic in *L. saligna* (Jeuken et al. 2008).

Studies of host resistance QTLs in other pathosystems suggest that QTLs for host resistance vary from being developmental stage-dependent to developmental stage-independent (Castro et al. 2002; Miedaner et al. 2002; Prioul et al. 2004). It is interesting to know which QTLs act in stage-dependent or stage-independent manners. In the case of plant protection in nature or under cultivation, resistance at the young plant or adult stage is more important than at the seedling stage, since these stages cover the largest part of the plant life cycle. An ideal lettuce phenotype would be resistant to *B. lactucae* during the entire lettuce life cycle.

We used the *L. saligna – B. lactucae* pathosystem as a model to investigate the contribution of QTLs to nonhost resistance at various stages in the lettuce life cycle. Results from the following four stages were compared: seedlings, young plants, adult plants under greenhouse conditions (Jeuken et al. 2008) and adult plants under field conditions. Our research questions were fourfold: first, how many QTLs are involved in nonhost resistance over the entire lettuce life cycle? Second, are these QTLs stage-dependent or stage-independent? Third, are the QTLs race-specific? Fourth, what can we conclude about the contribution of any identified QTLs to nonhost resistance in *L. saligna*?

Materials and methods

Plant and pathogen materials

A set of 27 Backcross Inbred Lines (BILs) from a cross between *L. saligna* CGN05271 (resistant donor parent) and *L. sativa* cv. Olof (susceptible recurrent parent) (Jeuken and Lindhout 2004) was used in the present study. The total set of 29 BILs covers 96% of the *L. saligna* CGN05271 genome and consists of 21 lines with a single homozygous introgression, three lines with more than one homozygous introgression and five lines with one or more heterozygous introgressions (preBILs). We excluded preBIL7.2 and preBIL9.1 because they segregate, and their aberrant phenotypes could hamper measurements of infection levels. The nomenclature of the BILs refers to the location of the introgression: for example, BIL1.2 stands for the second introgression on Chromosome 1 (Jeuken and Lindhout 2004).

The following controls were used: (i) the parental lines *L. saligna* CGN05271 (resistant) and *L. sativa* cv. Olof (susceptible); (ii) *L. sativa* cv. Iceberg and *L. sativa* cv. Grand Rapids as control lines for quantitative field resistance (Crute and Norwood 1981; Grube and Ochoa 2005; Norwood et al. 1983); (iii) lines harbouring Downy Mildew (*Dm*)

resistance genes. *L. serriola* LSE/18 with *Dm16* and *L. sativa* cv. Mariska with *Dm18* are resistant to Bl:14 but are susceptible to the most commonly appearing races in Europe, Bl:17-25. Natural infections by *B. lactucae* races from Bl:17-25 can be monitored by these two cultivars. By testing *B. lactucae* races from these two lines with the differential set, we can characterize the putative *B. lactucae* race composition (other than race Bl:14) in field tests (Van Ettekoven 2006; Van Ettekoven and Van der Arend 1999).

We applied *B. lactucae* races Bl:14 and Bl:16 for the Seedling Disease Test (SDT) and Young plant Disease Test (YDT) (Figure 1), and race Bl:14 in the Adult plant Disease Test in Field (ADT_F) in Wageningen. The virulence spectrum of these races, pathogen maintenance, purification, and monitoring, inoculum preparation and inoculation methods were described previously (Jeuken and Lindhout 2002). Inoculum concentrations were 2-4 x 10^5 conidia /ml for SDT and YDT analysis, and 1-4 x 10^4 conidia /ml for ADT_F analysis.

Disease test types

The SDT (Figure 1) used 12 day old seedlings grown in a climate chamber with a photoperiod of 16 h, light intensity of ~250 µmol m^{-2} s^{-1}, relative humidity (RH) of 70% and a constant temperature of 15°C. Two cotyledons of a seedling were detached and submerged in distilled water for approximately 20 min. Cotyledons were then placed abaxial side up on moist filter paper in boxes covered with transparent lids to reach 100% relative humidity (RH) (Eenink and De Jong 1982). The severity of infection for each cotyledon was evaluated as the percentage of sporulating area per total cotyledon area 7 or 8 days post inoculation (dpi).

We performed four independent SDT experiments by inoculation with *B. lactucae*. The first two experiments included all 27 BILs and the control lines. Five seedlings per line per experiment were inoculated by *B. lactucae* races Bl:14 or Bl:16. The seedlings of each genotype were randomly distributed over two inoculation boxes that contained 260 positions in each box. Two cotyledons per seedling were placed next to each other. Afterwards, two additional experiments were performed that included the controls and the 13 BILs selected as likely to be more resistant or more susceptible than the parental line cv. Olof. In the latter two experiments, 16 seedlings per line per test were used to allow for more accurate quantification of infection severity. Two cotyledons per seedling were again placed next to each other; the 16 seedlings of each genotype were divided into two inoculation boxes according to a complete randomized block design (eight seedlings per genotype in each box).

The YDT (Figure 1) used 3- to 4-week old young plants with three to eight true leaves (number of leaves is genotype-dependent). Six plants per genotype were grown randomly in a climate chamber under the same conditions as for the SDT. After inoculation

with a *B. lactucae* conidium suspension, plants were placed in a transparent plastic box to maintain 100% RH. The severity of infection for each plant was evaluated at 9 or 10 dpi as a percentage of sporulating area per total area of a representative leaf. Representative leaves were the two youngest well-expanded leaves at the time of inoculation. Five independent experiments were performed on all 27 BILs plus the control lines: four with Bl:14 and one with Bl:16.

One Adult plant Disease Test in Field (ADT_F) (Figure 1) was carried out in the autumn of 2005 in sandy soil at Wageningen, The Netherlands. We sowed seeds for all BILs and the control lines in moist 5 cm^3 peat blocks with a thin layer of moist silver sand covering. Sowed seeds were first grown at 12°C in the dark for two days to stimulate germination, and then grown in the greenhouse until transplanting. Young plants with three or four true leaves were transplanted to the field in 1.5 m^2 plots. Each plot contained 25 plants from one line that were planted in five rows and five columns with 30 cm between plants. Plots were isolated by oat border rows of at least 30 cm to reduce inter-plot interference. The experimental set up was according to a complete randomized block design with two replicates. One month after transplanting, plants were artificially inoculated by spraying with an inoculum of Bl:14 twice with a one-week interval.

Disease evaluations were performed 20 days after the first inoculation. We first performed a semi-quantitative assessment based on observations in each plot with a scale from 0 (completely resistant) to 10 (most susceptible). Based on the results of this semi-quantitative assessment, the controls, all the BILs that showed resistance and the most susceptible BILs were selected for more accurate quantitative evaluation. We randomly sampled four non-border plants per line for quantitative evaluation of infection severity. This evaluation was performed by estimating the percentage of infected area for each leaf of a plant. An average percentage of infected leaf area was calculated from all the infected leaves of that plant to represent its infection severity.

In addition, breeding companies performed eleven field experiments with natural infection by different *B. lactucae* races on all 27 BILs. These eleven field experiments consisted of eight experiments with at least two replicates and three experiments had a single observation per genotype. Experiments were performed in three consecutive years in several locations with sandy or clay soils in The Netherlands and France. Plants were scored on a 0-10 scale as above.

Data analysis

To improve data normality, we performed an arcsine square root transformation on all percentage data (infection severity) (Jeuken et al. 2008). A two-way ANOVA was used to

analyze data for the ADT_F and a linear mixed model was employed to analyze data for SDTs and YDTs ("experiment", "genotype" and "inoculation box" were fixed factors; "plant" was a random factor in the model). Because different environments might influence plant conditions and resistance levels, experiment x genotype interactions were measured to determine the similarity between the repeated experiments.

A Tukey Honestly Significant Differences (HSD) test was applied for multiple comparisons among all of the BILs and their parental lines (α=0.05). The criterion for detecting an introgression with an effect on the infection level was defined as a significant difference from the susceptible *L. sativa* cv. Olof parent. All statistical analyses were performed with the SPSS12.0.1 for Windows (SPSS Inc., Chicago IL).

Based on statistical analysis, the following categories of resistance were defined: (i) "completely resistant" refers to less than 1% infection severity. (ii) "quantitatively resistant" means significantly less infected than cv. Olof but showing more than 1% of infection severity; (iii) "susceptible" means showing similar infection severity to cv. Olof; (iv) "super susceptible" means significantly higher infection severity than cv. Olof. We considered introgressions that associated with infection levels that were quantitatively lower or higher than cv. Olof to harbour a QTL.

To quantify the resistance level of each line and make comparisons between the disease test types, we calculated the Relative Infection Severity (RIS) for each line using the formula RIS = (b/a)*100%, where the absolute infection severity of *L. sativa* cv. Olof and of the examined line were a and b, respectively. Using such a data transformation, the infection severity of cv. Olof is always 100% in different disease tests, and the resistance effect for different lines is indicated by changes in RIS.

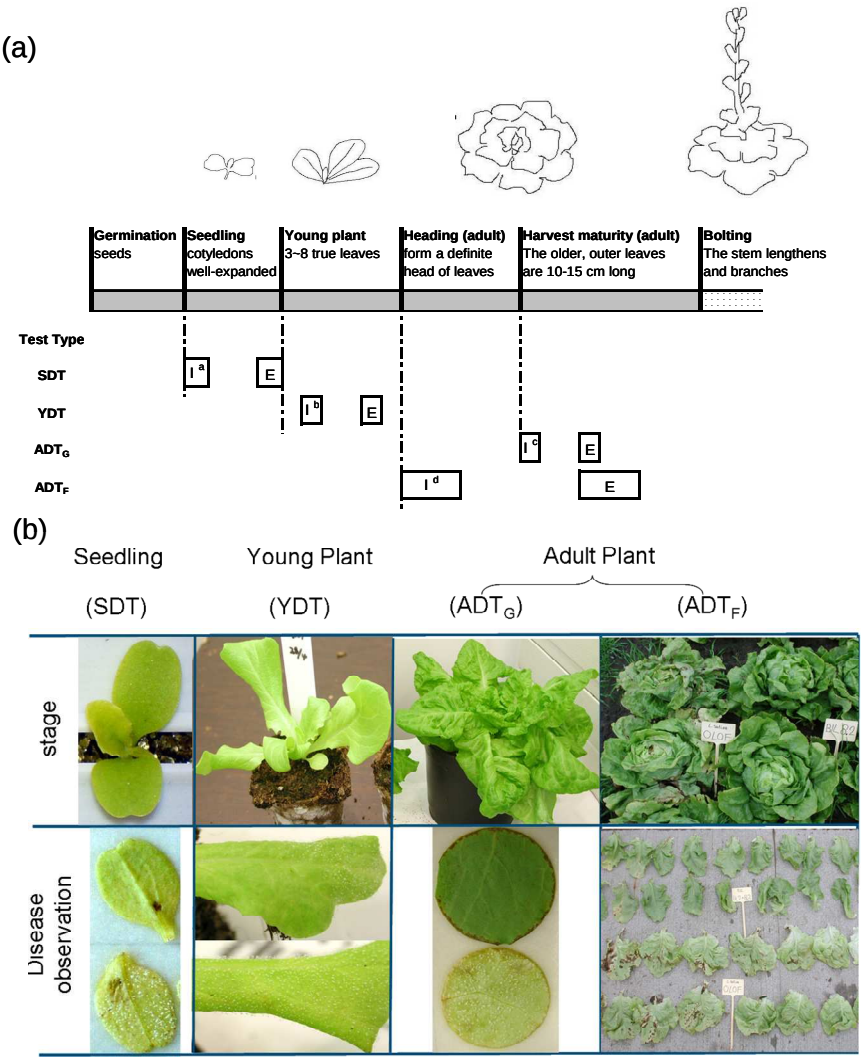

Figure 1. Overview of disease tests performed at four developmental stages in the lettuce life cycle

(a): Grey bar indicates the vegetative growing stages of the lettuce plant. The pictures above the grey bar schematically show the lettuce plant at different growing stages (plants are not drawn to scale). Plants at heading and harvest maturity stages are considered to be adult plants. For each disease test, boxes (I) and (E) indicate the inoculation and evaluation periods, respectively. [a]12-day old plants; [b]3- to 4-week old plants; [c]7- to 9-week old plants; [d]7- to 8-week old plants.

(b): Photo presentation of four disease tests to assess the infection severity of lettuce against *B. lactucae* at different developmental stages. The upper row contains four pictures indicating the four stages tested; the bottom row shows examples of resistant and susceptible observations from the four disease tests.

Results

We developed four different disease tests and evaluated the quantitative resistance of individual BILs to *B. lactucae* (Figure 1). The results of the tests of all BILs grown in greenhouses at the adult stage (ADT_G) have recently been reported (Jeuken et al. 2008). In total, seven resistance QTLs (*Rbq1, 2*; *rbq3* to *7*) have previously been identified using both F_2 and BIL approaches (Jeuken et al. 2008).

Ideally, the same disease tests should be used to compare resistance to *B. lactucae* in the same line at various plant developmental stages. Therefore, we performed leaf disc disease tests from seedlings, young plants and field adult plants. For seedlings, it was effective to detach cotyledons and evaluate resistance levels. However, experiments with leaf discs from young and adult plants from the field failed since the detached leaf tissues could not be kept long enough to assess *B. lactucae* infection. Therefore, we performed disease tests on whole plants at these two stages.

No experiment x genotype interactions within each type of disease test was detected by statistical analysis, indicating that the data could be pooled from multiple experiments to produce general conclusions about each line at a specific developmental stage. None of the quantitative resistance identified showed evidence of *B. lactucae* strain specificity.

Five BILs display quantitative resistance at the seedling stage

At the seedling stage, the resistant parent *L. saligna* CGN05271 displayed 0.3% average infection severity using the SDT and was therefore scored as being completely resistant (data not shown). The susceptible parent *L. sativa* cv. Olof showed an infection severity of 82% (Figure 2A). The control lines for field quantitative resistance, *L. sativa* cv. Iceberg (85%) and cv. Grand Rapids (86%), showed susceptibility that was similar to that of the susceptible parent. Five BILs displayed resistance to *B. lactucae* that the infection severity ranged from 11% to 58% lower than that of the susceptible parent (Figure 2A). The twenty-two other BILs were susceptible to infection (Figure 2A).

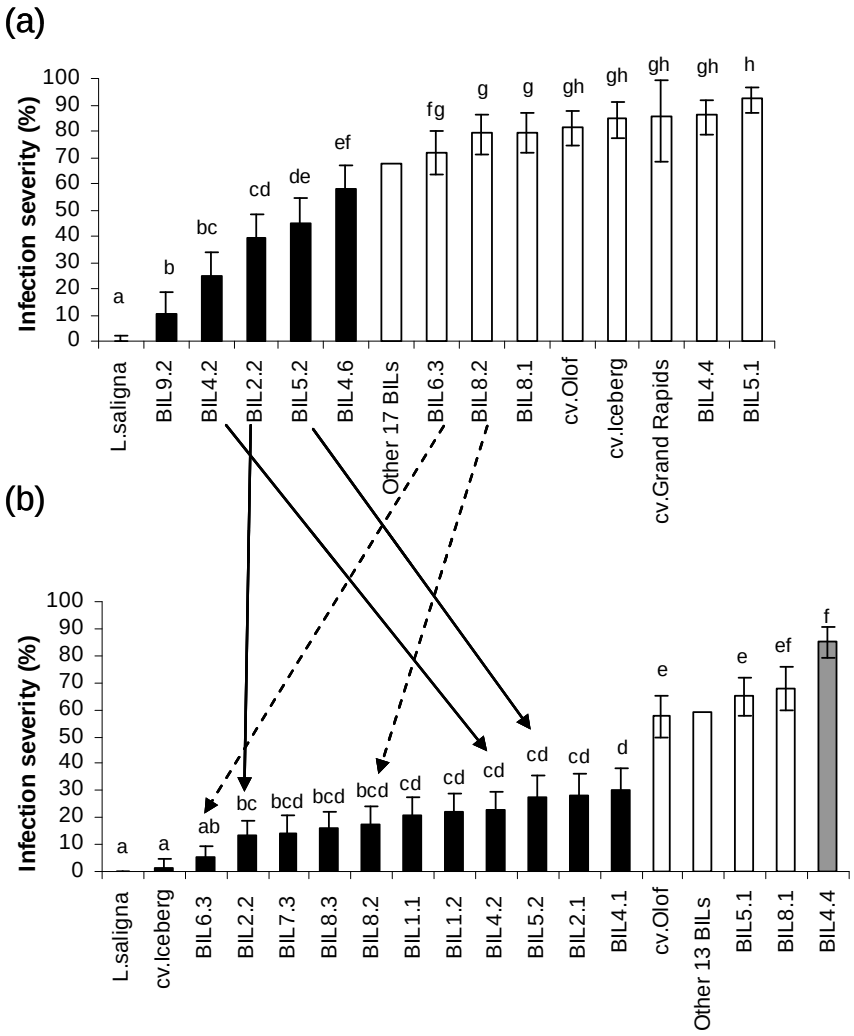

Figure 2. *Bremia lactucae* infection severity in 27 BILs and control lines at seedling and young plant stages

(a) and (b) show infection severities at the seedling stage and at the young plant stage, respectively. Error bars represent 95% confidence intervals. Letters in common indicate no significant difference (α=0.05, Tukey HSD test). Solid bars indicate resistant lines; open bars indicate susceptible lines; grey bar indicates the super susceptible line. In panel A and B, "other 17 BILs" and "other 13 BILs" represent data for 17 and 13 susceptible BILs, respectively, from two experiments. Solid arrows highlight three examples of BILs showing quantitative resistance at both plant stages. Dashed arrows highlight two examples of BILs showing quantitative resistance at only one of the two stages.

Eleven BILs display quantitative resistance and one BIL is super-susceptible at the young plant stage

At the young plant stage, the resistant parent *L. Saligna* CGN05271 remained completely resistant as determined by YDT. The susceptible parent *L. sativa* cv. Olof showed an average infection severity of 57%. *L. sativa* cv. Iceberg showed the highest resistance, with an infection severity of 2%. Eleven BILs displayed resistance, with infection severities ranging from 6% to 28% (Figure 2B). Three of these BILs, 2.2, 4.2 and 5.2, showed resistance at the seedling stage (Figure 2A). BIL4.4 was super-susceptible (84% infection severity). The fifteen other BILs tested were as susceptible as *L. sativa* cv. Olof (Figure 2B).

Eight BILs display quantitative resistance at the adult plant stage in the field

Applying the ADT_F in Wageningen after artificial inoculation with Bl:14, we also detected *B. lactucae* races other than Bl:14 on cv. Mariska and *L. serriola* LSE/18 plants. This natural infection was identified as being due to *B. lactucae* strain Bl:24. Using semi-quantitative assessment, seven BILs were scored at 5 or lower and selected to be quantitatively assessed (Figure 3). The control lines and twelve BILs with scores of ≥7 were also included in the quantitative assessment (Figure 3).

We observed a high correlation (r = 0.79) between the quantitative assessment (percentage of infected leaf area) and the semi-quantitative assessment with a 0-10 scale (Figure 3). *L. saligna* CGN05271 did not show any disease symptoms even two months after inoculation, and the susceptible parent *L. sativa* cv. Olof showed 25% infection severity (semi-quantitative score of 7). The control lines cv. Iceberg and cv. Grand Rapids showed the highest quantitative resistance levels with 2% infection severity and were significantly more resistant than most BILs with quantitative resistance. Nine BILs displayed 7-12% infection severity based on quantitative assessment of infected leaf area (Figure 3). However, BIL8.1 and BIL9.2 were scored 8 and 7, respectively, using the semi-quantitative scoring method (Figure 3). This discrepancy might be due to the dwarf phenotype of BIL9.2, which led to overestimation of the severity of infected plants, and BIL8.1 had an infection severity of 12%, which was at the border between the susceptible group and the resistant group.

To study disease resistance over different seasons and environments, we also performed eleven additional field trials including all of the BILs under normal culture conditions at various locations. The additional field test plants were infected naturally by *B. lactucae* races Bl:16, 18, 22, 24 and 25. In these field trials, the same BILs displayed resistance as in the Wageningen trial (data not shown), except that BIL9.2 had an average score of 4, and BIL8.1 and BIL8.3 had average scores of 8. Therefore, BIL9.2 was scored as

resistant, and BIL8.1 and BIL8.3 were scored as susceptible to *B. lactucae* in these tests (Figure 3). In all of the field tests, BIL4.4 was always the first line to show *B. lactucae* infection symptoms. BIL7.3 plants were very small and bolted earlier than the other lines; hence, the resistance/susceptibility of this line remained unclear after the ADT_F.

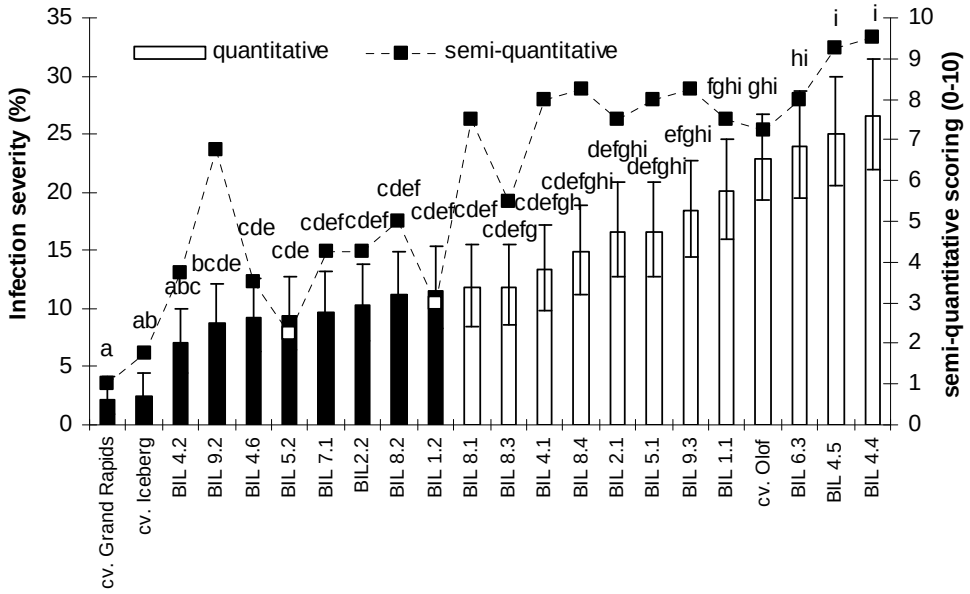

Figure 3. *Bremia lactucae* infection severity in the nineteen BILs and control lines in the field disease test (ADT_F)
L. saligna always showed 0% infection severity and was therefore excluded from the data analysis and figure. Bars and dots indicate results from the quantitative and the semi-quantitative assessments, respectively. Error bars represent 95% confidence intervals. Letters in common indicate no significant difference (α=0.05, Tukey HSD test) from the quantitative assessment. Solid bars indicate resistant lines and open bars indicate susceptible lines.

Discussion

Disease resistance in some BILs indicated that the introgression regions of these BILs were responsible for resistance. We considered the introgression regions in the BILs to be the locations of resistance QTLs. Such large introgression regions (between 20 and 40 cM on average) could each contain several QTLs for resistance, interacting in different ways over the life cycle of the plant. For the present discussion, we assume that only one QTL resides in each BIL introgression region. Future study of plants with smaller introgressions, such as

Near Isogenic Lines (NILs), might identify more precisely the location of one or more QTLs contributing to resistance.

Not all resistance QTLs are effective at each plant developmental stage

Using four types of disease tests, we identified 16 QTLs for resistance to *B. lactucae*: 15 from *L. saligna* and one from *L. sativa* (Figures 2 and 3). We compared the effective developmental stages and the size of the resistance effect for each QTL (Table 1). To describe the developmental stage in which each QTL is effective, we used the terms "stage-independent QTL" for resistance QTLs that were effective at all stages and "stage-dependent QTL" for resistance QTLs that did not act at all stages. Stage-dependent QTLs consisted of three types: "early", in which the QTL was only effective until the young plant stage; "intermediate", in which the QTL was effective from the young plant stage until the adult plant stage; and "late", in which the QTL was only effective at the adult stage (Table 1).

The majority of the 15 QTLs from *L. saligna* caused a 50-90% reduction in RIS as compared to the susceptible cv. Olof parent (Table 1). *Rbq1* to *7* have been detected and assigned previously by ADT_G (Jeuken et al. 2008). *Rbq8* to *15* were assigned to the BILs with newly detected QTLs. We do not know whether these new QTLs are dominant or recessive. Remarkably, only two of the 16 QTLs, *rbq5* and *rbq7*, were effective during the entire lettuce life cycle. The other QTLs showed quantitative resistance at some but not all stages of plant development (Table 1).

BIL4.1, BIL6.3 and BIL8.3 have an introgression in common, from 0cM to 16cM of Chromosome 4 (Jeuken et al. 2004) and they all displayed quantitative resistance at young plant stage (Table 1 and Figure 2). Therefore, *Rbq11* is very likely to locate on the top of Chromosome 4 from 0 cM to 16 cM. Based on this hypothesis, the presence of *Rbq10* from another introgression of BIL8.3 (bottom of Chromosome 8) is doubtful. The quantitative resistance of BIL8.3 is explained either by only *Rbq11* or by the combination of *Rbq10* and *Rbq11*.

The QTL detected in BIL4.4 was named *Rbq16*. The increased susceptibility of BIL4.4 might be caused by the lack of a resistance allele from *L. sativa* cv. Olof or by the presence of an infection-promoting allele from *L. saligna*. It is likely that BIL4.4 lost a resistance allele from cv. Olof, since cv. Olof always required one or two additional days to reach 100% infection severity as compared to BIL4.4.

Many host resistance studies have described the developmental stage-dependence of QTL effects. The correlation between resistance QTLs at the seedling stage and resistance QTLs at subsequent developmental stages is generally low; only a few QTLs are effective at

both seedling and adult stages (Castro et al. 2002; Eenink and De Jong 1982; Mallard et al. 2005; Monteiro et al. 2005; Prioul et al. 2004; Qi et al. 1998).

A few studies of nonhost resistance have shown evidence of polygenic inheritance of nonhost resistance status (Jafary et al. 2006; Jeuken and Lindhout 2002; Jeuken et al. 2008). Our study not only confirmed the involvement of QTL effects in nonhost resistance, but also identified new QTLs and monitored the contribution of different QTLs at multiple developmental stages. Stage-dependent and stage-independent QTLs were found to contribute to nonhost resistance. One factor that might explain the stage-dependence of QTL effects is that the expression of these genes might be limited to a certain plant developmental stage (Prioul et al. 2004).

In the present study, the fewest number of QTLs (five) were effective at the seedling stage; the other three stages had more than six effective QTLs (Table 1). This finding is in an agreement with host resistance studies, where fewer resistance QTLs were effective at the seedling stage as compared to the number of QTLs effective at advanced developmental stages (Castro et al. 2002; Eenink and De Jong 1982; Mallard et al. 2005; Monteiro et al. 2005; Prioul et al. 2004; Qi et al. 1998). This might have evolutionary significance since the seedling stage is relatively short, survival depends on growth and the chances of being confronted with *B. lactucae* spores are low. In contrast, established plants might have a competitive advantage if they can better withstand biotic attacks at later developmental stages. However, when plants reach the adult stage, investment in reproduction might compete with investment in defence against biotic stress. Such a trade-off might result in resistance QTLs that are effective at the young plant stage and not during reproduction. This might also explain why a group of QTLs was young plant-specific in the present study.

Two lettuce cultivars, *L. sativa* cv. Iceberg and cv. Grand Rapids, showed very high levels of quantitative resistance in YDT and ADT_F to all tested *B. lactucae* races, but were susceptible at the seedling stage. Similar stage-dependent resistance was observed for *rbq4* and *rbq6* (Figures 2 and 3 and Table 1). It has been suggested that resistance in cv. Iceberg and cv. Grand Rapids has complex inheritance patterns. However, the resistance loci and mechanisms of cv. Iceberg and cv. Grand Rapids are unknown and are therefore not comparable with our detected QTLs from *L. saligna* (Grube and Ochoa 2005).

The resistance QTLs detected in the BILs are not race-specific

Resistance that is regulated by QTLs is mostly considered to be race-non-specific and durable. However, some studies have described QTL effects that are specific to certain races of virulent pathogens in both host/pathogen and nonhost/non-pathogen reactions (Jafary et al.

2006; Marcel et al. 2007b; Parlevliet 1978; Qi et al. 1999). In the present study of the resistance of BILs against different *B. lactucae* races, all 16 QTLs were most likely race-non-specific. We drew this conclusion based on the following factors: (i) we observed no significant genotype x race interactions with races Bl:14 and Bl:16 at any of the four developmental stages studies, and (ii) all additional field tests confirmed that the same QTLs were also effective against five additional *B. lactucae* races (Bl:18, 22, 23, 24 and 25), which together can overcome 17 of the 20 identified *Dm* genes (Downy mildew resistance genes) (Van Ettekoven 2006; Van Ettekoven and Van der Arend 1999). Therefore, the BILs that harbour resistance QTLs might be a good source for breeding durable resistance to *B. lactucae*. Such QTLs would ideally be applied combinatorially to achieve a high level resistance with broad-spectrum effectiveness.

Table 1. Overview of lines harbouring QTLs for resistance and controls at different developmental stages.
ADT_G results refer to Jeuken *et al*. 2008 (Jeuken et al., 2008). SDT=Seedling Disease Test; YDT=Young plant Disease Test; ADT_G=Adult plant Disease Test in Greenhouse; ADT_F= Adult plant Disease Test in Field. Black grids indicate resistant and quantitatively resistant BILs/lines (significantly lower infection severity than susceptible parent *L. sativa* cv. Olof with 95% confidence) in the corresponding test types; white grids indicate the susceptibility of the BIL/line (not significantly different from the susceptible parent cv. Olof) in the corresponding test types; grey grid represents super susceptibility (significantly higher infection severity than susceptible parent cv. Olof with 95% confidence); "N.D.", not determined. The numbers in the grid indicate the Relative Infection Severity (RIS) of each line compared to cv. Olof. The absolute infection severity of cv. Olof in SDT, YDT, ADT_G and ADT_F were 82%, 57%, 87% and 25%, respectively. "R"= Resistance; "S"= Susceptibility; "?"= likely to be.

BIL / Line	SDT	YDT	ADT_G	ADT_F	Level of R / S	Effects
L.saligna CGN5271	0	0	0	0	Complete R	stage-independent
L. sativa cv.Olof	100	100	100	100	S	stage-independent
BIL4.4 *(Rbq16)*	105	148	110	116	Super S	Early
BIL2.2 *(rbq5)*	48	23	42	45	Quantitative R	stage-independent
BIL4.2 *(rbq7)*	31	39	54	31	Quantitative R	stage-independent
BIL8.2 *(rbq4)*	97	31	51	49	Quantitative R	Intermediate
BIL6.3 *(rbq6)*	88	10	38	104	Quantitative R	Intermediate
BIL9.2 *(rbq3)*	13	123	24	38	Quantitative R	stage-independent?
BIL5.2 *(Rbq8)*	55	48	113	41	Quantitative R	stage-independent?
BIL1.2 *(Rbq9)*	115	38	87	51	Quantitative R	Intermediate
BIL1.1 *(Rbq2)*	117	36	94	88	Quantitative R	Early
BIL8.3 *(Rbq10)*	127	28	106	53	Quantitative R	Early
BIL4.1 *(Rbq11)*	105	52	94	58	Quantitative R	Early
BIL7.3 *(Rbq12)*	107	25	82	N.D.	Quantitative R	Early
BIL2.1 *(Rbq13)*	98	49	77	72	Quantitative R	Early
BIL7.1 *(Rbq14)*	102	120	113	42	Quantitative R	Late
BIL4.6 *(Rbq15)*	71	130	98	40	Quantitative R	Late?
PreBIL7.2 *(Rbq1)*	N.D.	N.D.	72	N.D.	Quantitative R	Late?
L. sativa cv. Iceberg	104	3	N.D.	11	Quantitative R	Intermediate
L.sativa cv.Grand Rapids	105	N.D.	N.D.	10	Quantitative R	Late?

The contribution of QTLs to nonhost resistance

In the present study, 15 QTLs from *L. saligna* were effective in a range of one to four developmental stages, and at least five QTLs were effective at each stage (Table 1). No QTL displayed a particularly high level of resistance. Only *rbq6* (BIL6.3) and *rbq3* (BIL9.2) were more effective than the other QTLs at one developmental stage (Table 1). The major difference between QTLs was the stage at which they were effective during the lettuce life cycle. Thus, we speculate that combining a number of QTLs that are effective at various developmental stages might lead to high or even complete resistance over the lettuce life cycle.

The majority of the 15 QTLs did not coincide with the four known *R*-gene (*Dm* gene) clusters; exceptions might be BIL1.1 (*Rbq2*), BIL2.1 (*Rbq13*) and BIL4.6 (*Rbq15*) (Jeuken et al. 2008). However, the introgressions in these three BILs are around 40 cM long, and it is conceivable that the QTLs in these three BILs lie outside of the *Dm* loci. In addition, the five QTLs that were previously shown to be recessive were also unlikely to be alleles of known *R*-genes or newly detected *R*-genes, since *R*-genes have hitherto displayed dominant inheritance (Jeuken et al. 2008). Hence, nonhost resistance status in *L. saligna* is more likely to be explained by the combined effects of numerous race-non-specific resistance QTL alleles rather than the combined effects of race-specific *R*-genes.

Ideally, all combinations of the 15 QTLs will be made to determine which QTL combinations give complete resistance as for the resistant parent *L. saligna*. Because each effective QTL caused more than a 50% reduction in RIS (Table 1), theoretically the accumulated effects of two or three QTLs might lead to complete resistance (100% reduction in RIS). If two or three QTLs are sufficient to produce complete resistance, why does *L. saligna* have so many QTLs for resistance? One possible reason is that different sets of QTLs (minimally five QTLs/developmental stage) contribute to resistance at various developmental stages.

Two molecular models of nonhost resistance in plants have recently been proposed: one model postulates the absence of compatible fungal effectors that would lead to a PAMP-triggered defence response to suppress further infection (known as the basal defence in host system); the second model postulates the presence of stacks of multiple “gene-for-gene” interactions in which dominant resistance (*R*) genes are responsible for the recognition of pathogen-derived avirulence (*Avr*) genes (Schweizer 2007; Zipfel and Felix 2005). In the nonhost lettuce species *L. saligna*, *B. lactucae* might be stopped by a PAMP-triggered defence by stacks of effective pattern recognition receptors (PRRs) (the QTLs), in combination with ineffective protein effectors from *B. lactucae* that cannot suppress this

defence or otherwise improve pathogenicity. We speculate that during evolution, *L. saligna*, which is relatively distantly related to *L. serriola*, the progenitor of cultivated lettuce (Koopman et al. 1998), escaped from the pathogenicity of *B. lactucae* because *B. lactucae* has not adapted its protein effectors to target proteins from *L. saligna*. One current challenge for science is to identify and recognize these plant targets and to elucidate their biological functions.

CHAPTER 3

Redundancy of QTLs for nonhost resistance in *Lactuca saligna* to *Bremia lactucae*

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Submitted

Hypha and hausoria of *Bremia lactucae* Bl:14 at 72 hours after inoculation

Redundancy of QTLs for nonhost resistance in *Lactuca saligna* to *Bremia lactucae*

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Abstract

The nonhost resistance of wild lettuce (*Lactuca saligna)* to downy mildew (*Bremia lactucae)* is based on at least 15 Quantitative Trait Loci (QTLs), each effective at one or more plant developmental stages. We used QTL pyramiding (stacking) to determine how many of these QTLs from *L. saligna* are sufficient to impart complete resistance towards *B. lactucae* to cultivated lettuce, *L. sativa*. The four most promising QTLs, *rbq4*, *rbq5*, *rbq6+11* and *rbq7* are effective at both the young and adult plant stages. Lines with these four QTLs in all possible combinations were generated by crossing the respective Backcross Inbred Lines (BILs). Using the eleven resulting lines (combiBILs), we determined that combinations of the three QTLs, *rbq4*, *rbq5* and *rbq6+11*, led to increased levels of resistance; however, one QTL, *rbq7*, did not add to the resistance level when combined with the other QTLs. One line, tripleBIL268, which contains the three QTLs *rbq4*, *rbq5* and *rbq6+11*, was completely resistant to *B. lactucae* at the young plant stage. This suggests that these three QTLs are sufficient to explain the complete resistance of the nonhost *L. saligna* and any extra QTLs in *L. saligna* are redundant. Histological analysis of *B. lactucae* infection in *L. saligna*, the BILs, and the combiBILs 48 hours after inoculation revealed that different resistance mechanisms were employed. The interference with early and later phases of the infection process depended on the QTLs.

Keywords: epistatic effects; hypersensitive response (HR); oomycete

Introduction

Nonhost resistance is the most common form of resistance in a given plant species to provide both strong and broad protection against potential pathogens (Heath 1981; Niks 1988). In contrast to host resistance, the mechanisms underlying nonhost resistance are not as well understood (Mysore and Ryu 2004; Schweizer 2007). The investigation of the genetic inheritance of nonhost resistance will lead to the identification of genes that determine whether a plant is a host or nonhost to a particular pathogen. However, it is difficult to study the genetics of nonhost resistance as host and nonhost plant species are usually either not sexually compatible or give aberrant or sterile progeny when crossed (Atienza et al. 2004; Jeuken and Lindhout 2002; Niks 1988). In the lettuce (*Lactuca* spp.) - downy mildew (*Bremia lactucae*) plant-pathosystem, the nonhost species *L. saligna* is crossable with the host species *L. sativa*. This thus makes it possible to study the inheritance of nonhost resistance.

We were able to identify 15 BILs displaying race-non-specific quantitative resistance at one or more plant developmental stages (Chapter 2) by infecting a set of 27 Backcross Inbred Lines (BILs) covering 91% of the *L. saligna* genome; each BIL contains one introgression of *L. saligna* in *L. sativa* background (Jeuken and Lindhout 2004). The 15 BIL introgressions are considered to be 15 QTLs, but it is possible that an introgression can contain more than one gene that contributes to quantitative resistance. These QTLs displayed Relative Infection Severities (RIS) of 10-71% compared with the susceptible parental line. These findings suggest that the nonhost resistance of *L. saligna* is based on at least 15 QTLs, each with moderate to large effect (Chapter 2).

Exploiting this nonhost resistance in cultivated lettuce might be beneficial, since the resistance might prove more durable than the hitherto extensively deployed *Dm* genes (Lebeda and Schwinn 1994). In order to exploit this nonhost resistance in cultivated lettuce (*L. sativa*), we need to know how many of the about 15 QTLs are sufficient to give *L. sativa* complete protection to *B. lactucae*. One way to address this question is to pyramid (stack) different QTLs in the *L. sativa* background and evaluate their effects on infection severity. However, it is almost impossible to develop all thousands of different combinations of 2 to 15 QTLs. Therefore, we selected four recessive QTLs, *rbq4*, *rbq5*, *rbq6* and *rbq7* that are effective at both the young and adult plant stages, and combined them into *L. sativa* cv. Olof background (Chapter 2). These QTLs were mapped in the *L. saligna* introgressions of BIL8.2, BIL2.2, BIL6.3 and BIL4.2, respectively. In BIL6.3, the presence of *rbq6* is always combined with another QTL, *Rbq11* that is only effective at the young plant stage and is very likely to be located at 0-16 cM of Chromosome 4 (Chapter 2). We refer to this situation as

rbq6+11, and count them as one inseparable QTL. To study the interactions between these selected QTLs, eleven lines that contain homozygous *L. saligna* introgressions with more than one resistance allele of the respective QTL in the *L. sativa* background were developed and designated as combiBILs.

In most biotrophic plant-pathosystems, the mechanism of resistance to incompatible (nonhost) pathogens differs from qualitative resistance in a host to its compatible pathogen species. The latter case is usually based on post-haustorial Hypersensitive Response (HR) (Niks and Dekens 1991; Niks and Rubiales 2002), while nonhost resistance is often characterized by pre-penetration defence. Most examples of such mechanisms involve fungal biotrophic pathogens, especially powdery mildews and rusts (Ferreira et al. 2006; Heath 2000; Heath 2001; Niks and Rubiales 2002). In oomycetes, HR seems to play a comparatively larger role as it is associated with both *R*-gene-mediated and nonhost resistance (Kamoun et al. 1999; Vleeshouwers et al. 2000).

We carried out histological observations to characterize different resistance mechanisms of the target QTLs to *B. lactucae*, an obligate biotrophic oomycete pathogen. Different mechanisms of resistance in accessions of *Lactuca* spp. to *B. lactucae* have been described, including differences in the timing and rate of pathogen infection structure development, the occurrence of HR (Lebeda et al. 2002; Lebeda et al. 2001; Maclean and Tommerup 1979), accumulation of phenolics (Bennett et al. 1996) and occurrence of oxidative stress (Bestwick et al. 2001).

The nonhost resistance mechanisms of *L. saligna* accessions seem to be very different from race-specific resistance conferred by *Dm* genes in lettuce cultivars and from the partial (field) resistance of lettuce cv. Iceberg (Lebeda and Reinink 1994). At 48 hours post inoculation (hpi), *L. saligna* CGN05271 had a much lower frequency of hypha and haustorium formation, but a higher frequency of epidermal necrosis at the infection sites compared to susceptible *L. sativa* cultivars (Lebeda and Reinink 1994). Some studies have reported variation between *L. saligna* accessions in their predominant mechanisms of resistance to *B. lactucae* (Lebeda and Reinink 1994; Lebeda et al. 2006; Sedláová et al. 2001).

The present study reports on: (1) the development of eleven combiBILs; (2) the comparison of resistance levels between the eleven combiBILs and the BILs carrying individual resistance QTLs, primarily at the young plant stage; (3) determination of QTLs that lead to additive or epistatic effects when combined with other QTLs; and (4) the resistance mechanisms of *L. saligna* and the QTLs based on histological studies.

Materials and Methods

Plant materials

For QTL pyramiding, four BILs harbouring quantitative resistance alleles of QTL to *B. lactucae* were used, namely, BIL2.2 (*rbq5*), BIL4.2 (*rbq7*), BIL6.3 (*rbq6+11*) and BIL8.2 (*rbq4*). The nomenclature of the BILs refers to the location of the introgression. For example, BIL2.2 stands for the second introgression in Chromosome 2 (Jeuken and Lindhout 2004). Each BIL contains one homozygous *L. saligna* CGN05271 (non-host) introgression in *L. sativa* cv. Olof (host) background, except BIL6.3, which contains an additional introgression on top of Chromosome 4 from 0 to 32 cM harbouring *Rbq11*. The resistance alleles of QTLs on introgressions 2.2, 4.2, 6.3 and 8.2 are effective at both the young and adult plant (greenhouse) stages, and for BIL2.2 and 4.2, also at the seedling stage (Chapter 2).

The following genotypes were included in the disease tests to evaluate the resistance level of each combiBIL (lines containing more than one target QTL): (1) the eleven combiBILs that were developed in the present study, including six doubleBILs, four tripleBILs and one quatroBIL. (2) the individual BILs with QTLs, BIL2.2, 4.2, 6.3 and 8.2 ; (3) the parental lines: *L. saligna* CGN5271 (nonhost), *L. sativa* cv. Olof (host), and (4) three extra-susceptible reference lines: BIL4.4, BIL5.1 and BIL8.1 (Chapter 2).

Pyramiding QTL procedure

According to the formula C(n,m)=n!/(m!*(n-m)! (c=number of combinations; m=number of QTLs in one line; n=total number of QTLs), we could develop eleven combiBILs from four target BILs to obtain all the possible QTL combinations. We first crossed BIL2.2 with BIL6.3 and BIL4.2 with BIL8.2. The F_1 progeny from these two crosses were selfed to obtain F_2 plants. Among these F_2 plants, we selected doubleBIL2.2+6.3 and doubleBIL4.2+8.2, respectively, by Marker Assisted Selection (MAS). We then crossed doubleBIL2.2+6.3 with doubleBIL4.2+8.2, followed by one round of selfing to obtain a second F_2 population. This second F_2 population and subsequent generations from selfing (F_3 and F_4) were genotyped and selection was carried out for the other combiBILs (Figure 1). The combiBILs are designated by the chromosome numbers in which the introgression is located, For example, we refer to the doubleBIL2.2+6.3 as “doubleBIL26” and tripleBIL2.2+6.3+8.2 was designated “tripleBIL268” (Figure 2).

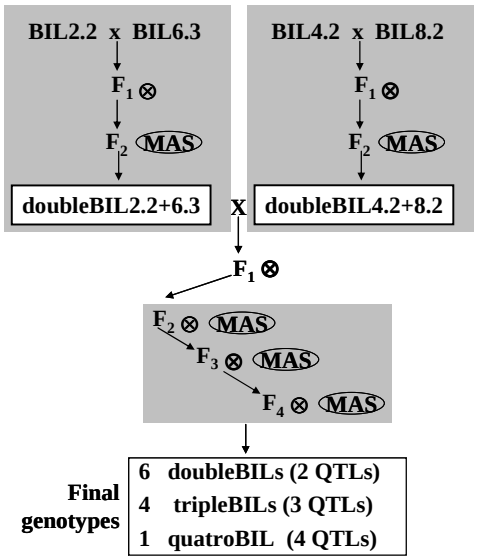

Figure 1. Development of the lettuce downy mildew resistance QTL pyramided population. MAS stands for Marker Assisted Selection. ⊗=selfing

Genotyping

First, the F_2 plants were genotyped by introgression-specific-EST markers (EST: expressed sequence tags) that are mapped on BIL2.2, 4.2, 6.3 and 8.2 introgressions. The positions and primers of these markers were described in Jeuken et al. (2008). To confirm that the complete introgression was present, selected plants were again genotyped using genome-wide Amplified Fragment Length Polymorphism (AFLP®) analyses (Jeuken et al. 2001). DNA isolation for the first screening using EST-markers was done by the NaOH method (Wang et al. 1993). DNA isolation for confirmation and genome wide genotyping by AFLP® was performed according to the modified CTAB method (Jeuken et al. 2001; Stewart and Via 1993). The amplification of EST-markers, Cleaved Amplified Polymorphic Sequence (CAPS) digestion and AFLP® analysis were described previously (Jeuken et al. 2008). Each final candidate combiBIL plant was genotyped independently at least three times.

Disease evaluation

To evaluate the infection severities of combiBILs and reference lines, we performed disease tests at the seedling, young plant and adult plant stages. The experimental procedures of the SDT (Seedling Disease Test), YDT (Young plant Disease Test), ADT_G (Adult plant Diseases Test in Greenhouse) and ADT_F (Adult plant Disease Test in Field) were described previously (Jeuken et al. 2008) (Chapter 2).

We first tested six doubleBILs, together with the individual BILs, the parental lines and the reference lines using four different types of bioassays. For SDT, YDT and ADT_G, two independent tests were performed for each bioassay type, one with *B. lactucae* race Bl:14 and another with race Bl:16. The concentrations of the inoculum used in these tests were 2~4 x 10^5 conidia/ml. For ADT_F, we performed artificial inoculation by spraying with Bl:14 at a concentration of 1 x 10^4 conidia/ml. The virulence spectra of race Bl:14 and Bl:16

are stated in Jeuken and Lindhout (2002). Pathogen maintenance, inoculum preparation and the method of inoculation were done as described previously (Jeuken and Lindhout 2002).

For the SDT, we tested 14 seedlings per line per test in a climate chamber. For the YDT, we tested six young plants (three to four weeks old) per genotype per test, under the same conditions as those used for the SDT. For the ADT_G, seven plants were used per genotype per experiment with four detached leaf discs per plant. In all three bioassay types, we evaluated infection severities from 8 to 10 dpi by estimating the percentage of sporulating area per cotyledon, representative leaf, and leaf disc. The representative leaves are two of the youngest, well expanded leaves at the moment of inoculation of each plant.

The ADT_F was carried out in sandy soil at Wageningen in The Netherlands in the autumn of 2006 according to a randomized, complete block design with five replicates. Each plot contained 5x5 plants from one genotype and inter-plant spacing of 30 cm. One month after transplantation, the plants were artificially inoculated with *B. lactucae* race Bl:14. The disease evaluations were done at 20 days after inoculation. We performed a semi-quantitative assessment based on observations per plot with a scale ranging from 0 (completely resistant) to 10 (the most susceptible) as described in Chapter 2.

In subsequent experiments, we evaluated all the eleven combiBILs, four individual BILs, the parental lines and the reference lines using three additional YDTs by inoculation with race Bl:14 at a similar inoculum concentration as for previous YDTs on doubleBILs. The YDT is the most efficient test in terms of time and space, and is relevant since the level of resistance is positively correlated with the level of resistance in adult plants (Chapter 2).

All the severity percentage data were arcsine square root transformed to improve the homogeneity of the residual variance. A two-way ANOVA was performed on the ADT_F data and the linear mixed model was employed to analyze the SDT, YDT and ADT_G data. We set “genotype” and “inoculation box” as fixed factors and “plant” as the random factor. The results are presented as the relative infection severity (RIS) for each examined line, which was calculated using the formula RIS = (b/a)*100%, where the absolute infection severity of *L. sativa* cv. Olof and the examined line were a% and b%, respectively. Hence, the severity of *L. sativa* cv. Olof was always 100% in different bioassay types.

The Tukey Honestly Significant Differences (HSD) test (α=0.05) was used for multiple comparisons between all the genotypes within one type of disease test. The criterion for detecting an introgression with an effect on the infection level was set using a significant difference from the susceptible parental line *L. sativa* cv. Olof. We considered introgressions that were associated with significantly higher or lower infection levels than *L. sativa* cv. Olof, to harbour a QTL allele for resistance or susceptibility. To detect epistatic effects of the QTLs, we set a significance level of 0.05 for the multiple comparisons between the

individual BILs and the combiBILs in a pairwise manner. All statistical analyses were performed with SPSS12.0.1 (SPSS Inc., Chicago IL).

We used the terms “additive effect” and “epistatic effect” to describe the results of QTL combinations. Additive effects refer to situations where the combined effect of the QTLs is (almost) equal to the sum of the individual QTL effects, i.e. the QTL combination showing significantly lower RIS than the individual QTL lines and the decrease in RIS should be similar to the sum of the reductions in RIS from the individual QTLs. An epistatic effect refers to a situation where there is interaction between QTLs such that the combined effect of the QTLs is lower or higher than the sum of the individual QTLs effects.

Histology

An additional YDT was performed for a histological study on variants, including *L. sativa* cv. Olof, *L. saligna* CGN05271, BIL4.4, BIL2.2, BIL4.2, BIL6.3, BIL8.2, BIL4.1, one doubleBIL that showed an epistatic effect on resistance (later called doubleBIL68) and one tripleBIL that showed complete resistance to *B. lactucae* at the young plant stage (later called tripleBIL268). The other combiBILs were not included since they did not display more outstanding results from the disease evaluation than doubleBIL68 and tripleBIL268. Six four-week-old plants of each genotype were inoculated with *B. lactucae* race Bl:14 conidia suspension ($3x10^5$ conidia/ml). For each genotype, three plants were used for microscopic observations, and the remaining three for macroscopic observations (the infection severity evaluation and data analysis were as described for YDT).

For microscopic observations, two leaf segments, 1 x 2 cm^2 in size, per plant were sampled at 48 hpi. The sampled leaf segments were first fixed in acetic acid-ethanol (1:3, v:v) solution and stained with lacto-phenol and trypan blue as described by Van Damme et al. (2005). The leaf segments were examined with a phase contrast (Zeiss Axiophot) microscope equipped with a digital camera at 400 x magnification. Photographs were taken by the digital camera and transferred to a computer using an AxioVison LE Rel.4.6 (Carl Zeiss).

The typical compatible infection process of *B. lactucae* starts from conidium germination, followed by formation of an appressorium and penetration of the epidermal cell wall, then by formation of a primary vesicle (PV), a secondary vesicle (SV), hyphae (HY) and haustoria (HA) within 24 hours post inoculation (hpi) (Ingram et al. 1973; Lebeda and Reinink 1994).

On each leaf segment, we counted the total number of infection units (IUs) and recorded the presence of the various infection structures of *B. lactucae*. We defined an IU as a germinated conidium with a PV. Except for the PV, the frequency of subsequent infection structures was calculated as a proportion (percentages) of the structures from which they

were derived. To identify the HR, autofluorescence of trypan blue-stained tissue was observed as described by Vleeshouwers et al, (2000). The number of IUs that were associated with only epidermal HR (EHR) and also subepidermal HR (SEHR) cells was recorded, respectively. HR frequency (HR%) was calculated using the number of IUs that showed HR (EHR and SEHR) at infection sites divided by the total number of IUs.

For data analysis, we combined the number of IUs from two leaf segments of the same plant to obtain a better representation of pathogen development in that plant. The frequency of each infection structure was compared to that on *L. saligna* CGN05271 and *L. sativa* cv. Olof, respectively, using the Mann-Whitney U test at the 95% level. The statistical analyses were performed with SPSS12.0.1.

For *L. sativa* cv. Olof, *L. saligna* CGN05271, BIL4.4, BIL2.2, BIL4.2, histological observations were also carried out in a similar way, but in a separate experiment. The results were similar between the previous and the present histological test. Here, we only show the results from the present and the most extensive study.

Results

Development of the combiBILs

DoubleBIL26 and doubleBIL48 were selected from the F_2 populations from crosses between the target BILs by Marker Assisted Selection (MAS) using the EST-markers at the introgression regions (Figure 1). Next, 48 plants from the F_2 population of doubleBIL26 x doubleBIL48 were screened using EST-markers. This resulted in the selection of doubleBIL24 and six plants with potential genotypes that could lead to the other eight types of combiBILs. These six plants were selfed to obtain the F_3 and the F_4 generation (Figure 1). After genotyping the F_3 and the F_4 plants, the other eight combiBILs were selected by MAS, and genotypes of all eleven combiBILs were confirmed using 94 molecular markers scattered across the introgression segments.

Due to the extra introgression on top of Chromosome 4 (0-32 cM) in BIL6.3, all the combiBILs that were derived from BIL6.3 also had this introgression. Severe preference of *L. saligna* alleles on the top of Chromosome 4 prevented the selection of genotypes without this introgression. Therefore, *rbq6* and *Rbq11* were always combined in each combiBIL derived from BIL6.3. DoubleBIL28 contained a small introgression from 29-43 cM on Chromosome 4, but this doubleBIL may not contain either *Rbq11* (located on segment 4.1) or *rbq7* (located on segment 4.2) for two reasons: (1) *Rbq11* is likely to locate on the top of

Chromosome 4 from 0-16 cM (Chapter 2); (2) BIL4.2 introgression is nearly 60 cM (29-83 cM) long and the chance for *rbq7* residing in 29-43 cM is low.

Introgression segments in BIL2.2, BIL6.3 and BIL8.2 where *rbq5*, *rbq6+11* and *rbq4* reside did not cause abnormal *L. sativa* morphology; however, the introgression in BIL4.2 (*rbq7*) caused dark-green, long-narrow leaf and non-heading (Jeuken and Lindhout 2004). The same morphological features were logically pyramided in the subsequent combiBILs.

CombiBILs containing introgressions of BIL6.3 (*rbq6+11*) and BIL8.2 (*rbq4*) were expected to be difficult to retrieve due to severely distorted segregations with a deficit of homozygous *L. saligna* alleles in the F_2 population (Jeuken et al. 2001; Jeuken et al. 2008). The segregation ratios of introgression 6.3 and 8.2 during combiBIL development are listed in Table 1. Surprisingly, the segregation of introgression 6.3 was not as distorted as observed in the original F_2 population from *L. saligna* CGN05271 x *L. sativa* cv. Olof (n=126). The allele frequency of *L. saligna* was 0.46 and the genotype frequency of homozygous *L. saligna* was 0.26, close to the respective normal values of 0.5 and 0.25. The segregation of introgression 8.2 was severely distorted compared to a hypothetical ratio of 1:2:1 (Chi-square test, $P<0.001$) and with a clear deficit against homozygous *L. saligna* genotype and excess of heterozygous genotype, while the allele frequency stayed normal for the *L. saligna* allele (Table 1).

Table 1 The segregation ratio, genotype frequency and allele frequency of BIL6.3 (*rbq6+11*) and BIL8.2 (*rbq4*) introgression segments.

Introgression	Segregation ratio (a : h : b)		Genotype frequency of homo. *L.saligna*		Allele frequency of *L. saligna*	
	F_2	BIL	F_2	BIL	F_2	BIL
BIL6.3	36:46:9 (P <0.001)	60:68:45 *(P <0.005)	0.10	0.26	0.35	0.46
BIL8.2	30:90:6 (P <0.001)	192:228:8 **(P <0.001)	0.05	0.02	0.40	0.55

The segregation ratios were examined by Chi-square test against hypothetic ratio that is a:h:b=1:2:1. a=homozygous *L. sativa*; h=heterozygous; b=homozygous *L. saligna*
* data from the progeny of one line
** data from the progeny of four lines with similar segregation ratio

Disease evaluation for combiBILs

We first evaluated the infection severities of the six doubleBILs compared to the four individual BILs using the four different types of bioassays. In subsequent experiments, all

eleven combiBILs (quatro-, triple- and doubleBILs) were evaluated only at the young plant stage due to time and space limitations (Figure 2).

	Line	QTL presence	Graphical genotype				Relative infection severity (%)			
			C2	C4	C6	C8	SDT	YDT	ADT_G	ADT_F
Reference Lines	*L. saligna*	≥15 QTL †					0.0 *a*	0.0 *a*	0.0 *a*	0.0 *a*
	L. sativa	No QTL					100 *gh*	100 *g*	100 *f*	100 *ef*
Target BILs	BIL2.2	*rbq5*					28 *bc*	12 *bc*	47 *bc*	32 *abcd*
	BIL4.2	*rbq7*					40 *bcd*	40 *ef*	64 *de*	51 *bcd*
	BIL6.3	*rbq6+11* ††					73 *defgh*	16 *cd*	38 *bc*	65 *de*
	BIL8.2	*rbq4*					86 *efgh*	57 *f*	28 *bc*	41 *abcd*
	BIL4.1	*Rbq11* †††					108 *h*	44 *ef*	91 *f*	*N.D.*
doubleBILs	doubleBIL68	*rbq6+11+rbq4*					89 *fgh*	0.2 *a***	10 *a***	22 *ab**
	doubleBIL26	*rbq5+rbq6+11*					51 *cdef*	0.3 *a***	46 *bc*	35 *abcd*
	doubleBIL28 ‡	*rbq5+rbq*					14 *b**	3 *ab**	29 *bc*	10 *a**
	doubleBIL24	*rbq5+rbq7*					33 *bcd*	31 *de*	50 *cd*	46 *bcd*
	doubleBIL46	*rbq7+rbq6/11*					90 *fgh*	17 *cd*	29 *bc*	55 *bcd*
	doubleBIL48	*rbq7+rbq4*					45 *bcde*	38 *ef*	27 *b*	41 *abcd*
tripleBILs	tripleBIL246	*rbq5+rbq7+rbq6/11*					*N.D.*	0.1 *a*	*N.D.*	*N.D.*
	tripleBIL248	*rbq5+rbq7+rbq4*					*N.D.*	6 *bc*	*N.D.*	*N.D.*
	tripleBIL468	*rbq7+rbq6+11+rbq4*					*N.D.*	0.4 *a*	*N.D.*	*N.D.*
	tripleBIL268	*rbq5+rbq6+11+rbq4*					*N.D.*	0.0 *a***	*N.D.*	*N.D.*
quartoBIL	quartoBIL2468	*rbq5+rbq7+rbq6+11+rbq4*					*N.D.*	0.0 *a*	*N.D.*	*N.D.*

Figure 2. The genotypes of the lettuce downy mildew resistance QTL pyramid population and their Relative Infection Severities (RIS) to *B. lactucae* at various plant development stages.

In the genotype presentation, Cx stands for Chromosome x. A solid bar indicates the homozygous *L. saligna* introgression; an open bar indicates the homozygous *L. sativa* background. For RIS compared to *L. sativa* cv. Olof, the absolute infection severity of *L. sativa* cv. Olof in SDT, YDT, ADT_G and ADT_F were 82%, 57%, 87% and 25%, respectively. SDT=Seedling Disease Test; YDT=Young plant Disease Test; ADT_G=Adult plant Disease Test in Greenhouse and ADT_F= Adult plant Disease Test in Greenhouse. Letters next to the RIS numbers indicate the significance between different lines within one type of disease test. Letters in common means no significant difference (α=0.05, Tukey HSD test). Black grids indicate the completely resistant lines in the corresponding bioassay types; grey grids indicate the quantitatively resistant lines (significantly lower

infection severity than *L. sativa* cv. Olof with 95% confidence); white grids indicate the susceptible lines (not significantly different from cv. Olof). N.D. = not determined.
† fifteen QTLs are identified in *L. saligna*;
†† The presence of both *rbq6* and *Rbq11*is designated *rbq6+11*
††† *Rbq11* is only effective at young plant stage (Chapter 2)
‡doubleBIL28 contains an introgression on Chromosome 4 that has a small overlap with BIL4.1 and BIL4.2, but the precise locations of *Rbq11* and *rbq7* are unknown.
*no significant difference from the individual BILs, but infection severity decreased suggestively than the individual BILs.
**lines showed epistatic effect with significantly decreased infection severity compared to the individual BILs.

The results are presented as relative infection severity (RIS) compared to the susceptible parental line *L. sativa* cv. Olof as described in Materials and Methods. Therefore, *L. sativa* cv. Olof was always set as 100% RIS, although it showed 59%, 83% and 60% absolute infection severity at the seedling (SDT), young plant (YDT) and adult plant (greenhouse) stages (ADT_G), respectively, and scored 7 on a 0-10 scale (resistant to susceptible) in the field test (ADT_F). *L. saligna* showed 0% infection severity in all the experiments. The extra-susceptible reference lines, BIL4.4, BIL5.1 and BIL8.1 all showed either a similar or higher infection severity (90~153%) compared to *L. sativa* cv. Olof. All RIS values for the target BILs were as expected and in accordance with earlier results at all developmental stages (Figure 2) (Chapter 2). In summary, BIL2.2 and BIL4.2 showed quantitative resistance at all four developmental stages, BIL8.2 was resistant in the YDT, ADT_G and ADT_F and BIL6.3 in the YDT and ADT_G. BIL4.1 showed quantitative resistance only in the YDT.

At none of the tested developmental stages did we observe additive effects for any of the QTL combinations, as the increased resistance level of each combiBIL was not equal to the sum of the effects of the individual BILs (Figure 2).

At the seedling stage (SDT), doubleBIL28 had a RIS of 14%, which is lower than BIL2.2 and BIL8.2, but not significantly so. We assumed that doubleBIL28 is likely to show an epistatic effect for the resistance (Figure 2). The RIS of doubleBIL24 and doubleBIL26 were similar to their corresponding individual BILs. As expected, doubleBIL68 showed susceptibility with similar RIS to *L. sativa* cv. Olof because both BIL6.3 and BIL8.2 are susceptible at the seedling stage (Chapter 2) (Figure 2). Surprisingly, doubleBIL46 was susceptible, although we expected it would have a similar or lower RIS to BIL4.2 (*rbq7*) (Figure 2). This suggested a negative epistatic effect at the seedling stage, when *rbq6+11* and *rbq7* are combined.

At the adult plant stage in the greenhouse (ADT_G), all doubleBILs had a significantly lower RIS (10~50%) than *L. sativa* cv. Olof. Only doubleBIL68 showed a significantly lower RIS (10%) than its corresponding individual BILs (BIL6.3, 38% and BIL8.2, 28%), which suggests epistatic effects of QTLs *rbq6+11* and *rbq4*, leading to an increased level of resistance. The RIS of the other five doubleBILs were close to the levels of their respective individual BILs (Figure 2). In the field disease test (ADT_F), similar results were obtained as in the greenhouse, except that doubleBIL68 and doubleBIL28 had a lower (but not significantly so) RIS than their corresponding individual BILs (Figure 2).

At the young plant stage (YDT), the correlation between the two experiments with only doubleBILs and the two with all combiBILs was high (r=0.9). Therefore, we only present the results from the latter of these (Figure 2). All combiBILs showed a significantly lower RIS than the susceptible *L. sativa* cv. Olof. For doubleBILs, doubleBIL68 (0.2%) and doubleBIL26 (0.3%) stood out and showed very little sporulation. Moreover, the RIS was significantly lower for the combiBILs than for the corresponding individual BILs (Figure 2). DoubleBIL28 also had a low RIS (3%), but it was not significantly lower than that of BIL2.2 (12%).

TripleBIL268 showed complete resistance (0% RIS) to *B. lactucae*, similar to the nonhost *L. saligna*. The corresponding doubleBILs, doubleBIL68, doubleBIL28 and doubleBIL26 all showed very little infection severity, but were not completely resistant. This result confirms the epistatic effects of *rbq4*, *rbq5*, and *rbq6+11* alleles for the resistance. The other three tripleBILs, tripleBIL246, tripleBIL248 and tripleBIL468 showed very low RIS (0.1% to 6%) that were significantly lower than the corresponding individual BILs, but not significantly lower than the corresponding doubleBILs (Figure 2). For instance, tripleBIL468 (0.4%) showed a similar severity level as doubleBIL68 (0.2%). These three tripleBILs have, in common, introgression 4.2. Furthermore, the results confirmed that the resistance alleles of *rbq7* do not contribute to a higher resistance level. QuatroBIL2468 also showed complete resistance like tripleBIL268 (Figure 2).

Histological observation

Macroscopic infection severity observations of the examined genotypes confirmed the results (Table 2) presented in Figure 2. In this set of histological observations, non-penetrating appressoria were very rarely observed in all tested lines. Therefore, we excluded the frequency of appressoria formation from this report.

At 48 hours after inoculation with *B. lactucae* race Bl:14, the susceptible genotypes *L. sativa* cv. Olof and BIL4.4 showed a very high frequency (>90%) of SV formation (SV / PV), hyphae formation (HY / SV) and haustoria formation (HY / HA) and an HR frequency

of 56% and 43%, respectively. The HR was mostly restricted to the epidermal cell where the penetration took place (Table 2 and Figures 3 and 4).

Table 2. Development of *B. lactucae* race Bl:14 in the tested *Lactuca* genotypes at 48hpi.

Genotype	RIS %	# PV (IU) /plant	Freq. SV/PV	Freq. mal-HY/SV	Freq. HY/SV	Freq. HA/HY	HR%	EHR%	SEHR%
L.sativa	100^{S}	81	97^{S}	0^{S}	94^{S}	94^{S}	56^{S}	98	2
L. saligna	0.0^{O}	167	86^{O}	23^{O}	0^{O}	0^{O}	88^{O}	97	3
BIL4.4	$119^{O,S}$	43	94^{S}	0^{S}	94^{S}	96^{S}	43^{S}	100	0
BIL4.2	$43^{O,S}$	28	87^{O}	9	$77^{O,S}$	79^{S}	65	93	7
BIL2.2	$15^{O,S}$	30	$75^{O,S}$	24^{O}	$15^{O,S}$	83^{S}	88^{O}	$33^{O,S}$	$67^{O,S}$
BIL8.2	$56^{O,S}$	32	86^{O}	35^{O}	$29^{O,S}$	$60^{O,S}$	68	$47^{O,S}$	$53^{O,S}$
BIL4.1	$37^{O,S}$	39	$69^{O,S}$	49^{O}	7^{O}	$60^{O,S}$	56^{S}	$32^{O,S}$	$68^{O,S}$
BIL6.3	$19^{O,S}$	66	83^{O}	$88^{O,S}$	0^{O}	0^{O}	64	$19^{O,S}$	$81^{O,S}$
doubleBIL68	0.3^{O}	77	$73^{O,S}$	$82^{O,S}$	0^{O}	0^{O}	95^{O}	$19^{O,S}$	$81^{O,S}$
tripleBIL268	0.0^{O}	129	83^{O}	$74^{O,S}$	0^{O}	0^{O}	84^{O}	$15^{O,S}$	$85^{O,S}$

^{O,S} Significantly different from *L. sativa* (cv. Olof) and *L. saligna* (CGN05271), respectively.

RIS=Relative Infection Severity compared to *L. sativa* cv. Olof at 10dpi. The absolute infection severity of *L. sativa* cv. Olof was 84%.
PV=primary vesicle; IU=infection unit; SV=secondary vesicle; mal-HY=malformed hyphae like structure; HY=hyphae; HA=haustorium; Freq.= frequency / proportion in percentage;
HR%=percentage of IUs showed hypersensitive response; EHR%=percentage of IUs that showed HR were only epidermal-HR; SEHR%=percentage of IUs that showed also subepidermal-HR.

The most striking observations for the nonhost *L. saligna* compared to *L. sativa* cv. Olof were that (1) no real healthy hyphae were observed (consequently the frequency of haustorium was 0%) and (2) 23% of SVs formed sort of malformed hypha-like structures (mal-HY) (Figure 3). The mal-HY and 0% real hyphae formation suggested that all the IUs in *L. saligna* were arrested after their SV were formed. (3) The higher frequency of HR appeared in *L. saligna* (88%) than in *L. sativa* cv. Olof (56%) (Table 2). The resistance mechanism of *L. saligna* seemed to be based mainly on pre-hyphal resistance and a high frequency of HR (Table 2 and Figures 3 and 4).

The *B. lactucae* development process in BIL2.2, BIL8.2 and BIL4.1 showed similar patterns to each other in the frequency of formation of each pathogen structure, in which the responses were intermediate between the susceptible and resistant parental lines. In these BILs, the infection was largely stopped before HY formation since the HY/SV ratios in these lines were significantly lower than in the susceptible parent *L. sativa* cv. Olof (Table 2 and

Figure 3). All three BILs had a higher proportion of Subepidermal-HR (SEHR) occurrence, in contrast to *L. sativa* cv. Olof and *L. saligna* which were mainly associated with epidermal-HR (EHR) (Table 2 and Figure 4).

BIL6.3, doubleBIL68 and tripleBIL268 plants all had a high proportion of IUs that formed SV, but no IUs that formed healthy hyphae and haustoria. Large proportions (~80%) of the mal-HY were observed in these three lines and often, the mal-HY was accompanied by SEHR (Table 2 and Figures 3 and 4). Such a pre-hyphal resistance mechanism was similar to that of *L. saligna*; however, the pathogen development was obviously arrested earlier in *L. saligna* than in tripleBIL268, doubleBIL68 and BIL6.3 because of the different frequencies of mal-HY formation and the occurrence of SEHR. For BIL4.2, we observed only minor differences in pathogen development from that of *L. sativa* cv. Olof at 48 hpi (Table 2 and Figure 3).

Discussion

The present study aims to determine how many *L. saligna* resistance QTLs are sufficient to impart complete resistance to *B. lactucae* and to identify the resistance mechanisms underlying each target QTL. To achieve the first objective, we selected four "target BILs" with "target QTLs" and developed eleven combiBILs, in which *L. saligna* introgressions carrying QTLs for resistance were combined in the *L. sativa* background. In parallel, histological studies were carried out to achieve the second objective of understanding the resistance mechanisms.

Redundancy of QTLs in the nonhost resistance of *L. saligna*

We observed only epistatic effects of the eleven combiBILs at different developmental stages, as the increased resistance level of each combiBIL was not equal to the sum of the effects of the individual BILs (Figure 2). At both young and adult plant stages, the epistatic effects of QTLs *rbq4* (introgression 8.2), *rbq5* (introgression 2.2) and *rbq6+11* (introgression 6.3+4.1) in combiBILs led to an increased level of resistance (decrease RIS) compared to their respective individual BILs. This decrease in RIS was 97~99% at the young plant stage and 54~90% at adult plant stage (ADT_G and ADT_F), compared to *L.sativa* cv. Olof (Figure 2).

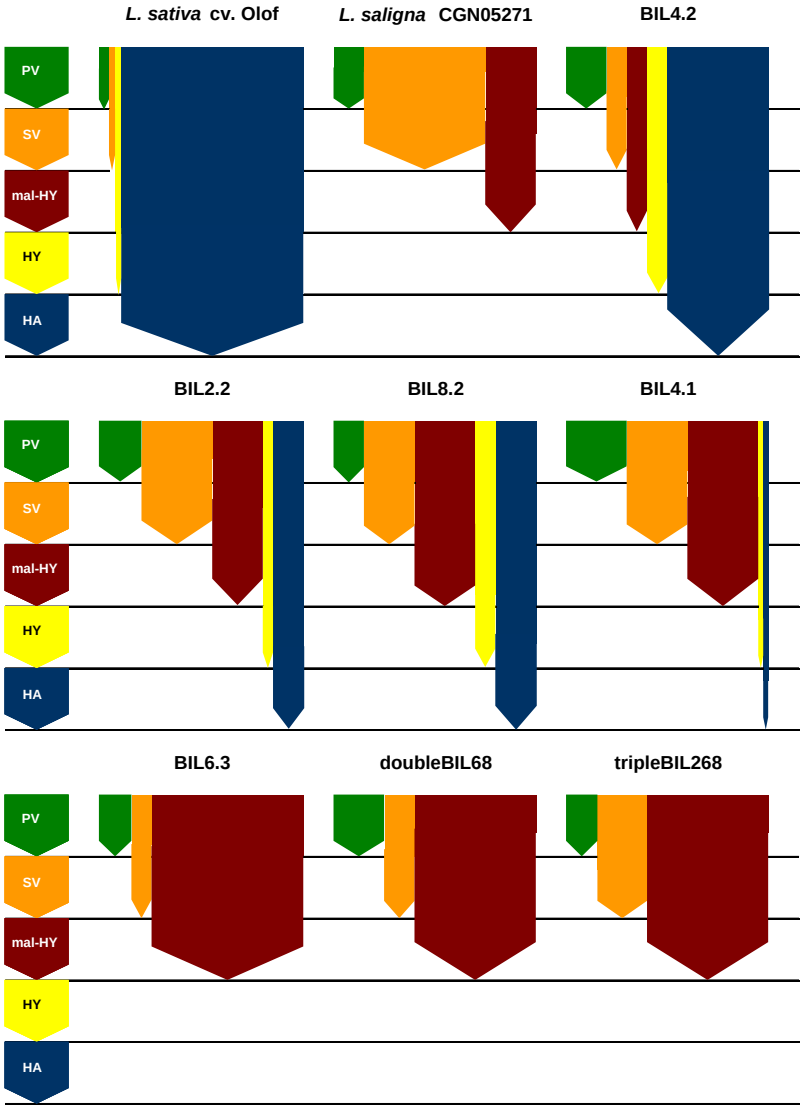

Figure 3. Diagrammatic representation of the infection process of *B. lactucae* race Bl:14 in tested *Lactuca* genotypes. The barriers which *B. lactucae* may encounter while invading lettuce plants are represented on the left side with different colored arrows. PV=primary vesicle, SV=secondary vesicle; mal-HY=malformed hyphae like structure; HY=hyphae formation and HA=haustorium formation. The widths of the arrows are proportional to the fractions of the infection units encountering the respective barriers.

Such effects even resulted in complete resistance of tripleBIL268 towards *B. lactucae* at young plant stage. In contrast, the resistance alleles of *rbq7* (introgression 4.2) in combiBILs did not contribute to increased resistance levels and only showed similar resistance levels as the individual BILs (Figure 1). At the seedling stage, *rbq6+11* and *rbq7*, when combined, showed a negative epistatic effect because *rbq7* lost its resistance effect after being combined with *rbq6+11*. Similar situations of epistatic effects were reported previously in other QTL pyramiding studies. For example, in barley to stripe rust (Castro et al. 2003a; Castro et al. 2003b; Richardson et al. 2006) and in wheat (*Triticum aestivum*) to leaf rust caused by *Puccinia recondita* f.sp.*tritici* (Kloppers and Pretorius 1997).

The complete and almost complete resistance of, tripleBIL268, quatroBIL2468 and doubleBIL68, respectively, suggests a redundancy of QTLs in the nonhost resistance of *L. saligna*, where 15 QTLs for resistance have been identified. So far, we only have evidence for the almost complete to complete resistance of doubleBIL68, tripleBIL268 and quatroBIL2468 at the young plant stage, but we expect that these three lines will also show very high levels of resistance at the adult plant stage. At the seedling stage, tripleBIL268 might not be more resistant than doubleBIL28 since BIL6.3, BIL8.2 and doubleBIL68 were all susceptible (Chapter 2). But the resistance level of quatroBIL2468 is more difficult to predict as more interactions between the QTL may occur. TripleBIL268 may need one or more QTLs to be resistant at other developmental stages. We hypothesize that the redundant QTLs might be a good backup in case some QTLs are overcome by *B. lactucae* races.

Resistance mechanisms underlying the QTLs

We observed that the mechanism of the nonhost resistance of *L. saligna* CGN05271 mainly involves pre-hyphal resistance, which is similar to the previous findings based on the inoculation of *L. saligna* accessions with *B. lactucae* races Bl:5 and Bl:16 on detached tissues (Lebeda and Reinink 1994; Sedláová et al. 2001). Compared to previously reported results, our samples showed generally higher HR frequencies (HR%) in all tested lines, even in the susceptible reference lines. This is probably due to differences in growth conditions or tissue types used between studies.

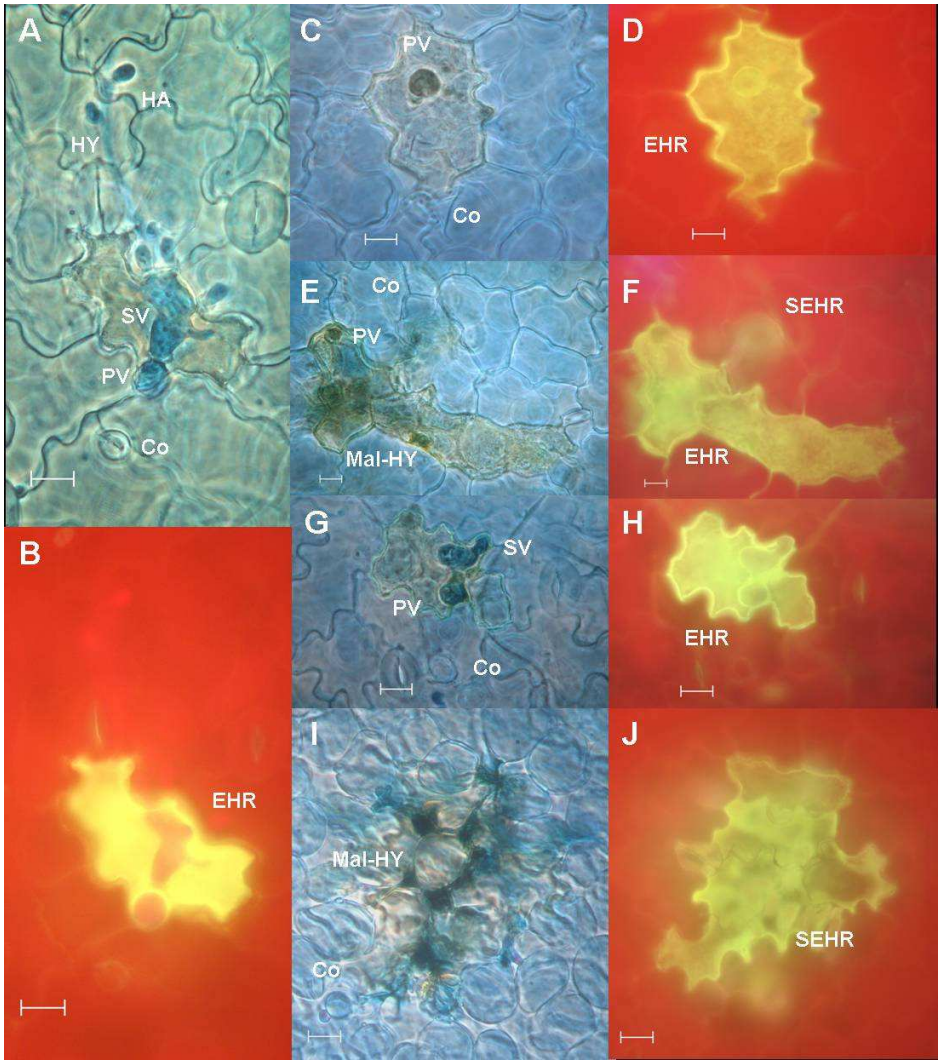

Figure 4. Microscopic illustration of *B. lactucae* race Bl:14 development stages observed in tested *Lactuca* genotypes. The scale bar at the left lower corner equals to 20µm. Co=conidium; PV=primary vesicle; SV=secondary vesicle; HY=hyphae; HA=haustorium; EHR=epidermal HR cell; SEHR=subepidermal HR cell.
A, B IU in *L. sativa* cv. Olof with hypha and haustoria and HR epidermal cell
C, D IU in *L. saligna* CGN05271 with primary vesicle (PV) and HR epidermal cell
E, F IU in *L. saligna* CGN05271 with deformed hypha-like structure and HR epidermal cell
G, H IU in BIL2.2 with secondary vesicle (SV) and HR epidermal cell
I, J IU in doubleBIL68 with deformed hypha-like structure and HR epidermal and subepidermal cells

The resistance mechanisms in BIL2.2 BIL8.2 and BIL4.1 might be characterized as incomplete pre-hyphal resistance associated with subepidermal HR (Table 2 and Figures 3 and 4). For BIL6.3 (*rbq6+11*), doubleBIL68 (*rbq4+rbq6+11*) and tripleBIL268 (*rbq4+rbq5+rbq6+11*), the resistance mechanism most resembled that in *L. saligna.* However, the pathogen could form haustoria during the later phase of infection in BIL6.3 and doubleBIL68, else we would not have observed 16% and 0.3% RIS in BIL6.3 and doubleBIL68, respectively (Table 2). The resistance mechanism of doubleBIL68 and tripleBIL268 might be based on the respective combination of BIL2.2, BIL6.3 and BIL8.2, but the effect of *rbq6+11* in BIL6.3 seemed to mask most of the effect from *rbq4* in BIL8.2 and *rbq5* in BIL2.2 (Table 2 and Figure 3). The epistatic effects of these QTLs might be due to complementary effects from their different resistance mechanisms. BIL4.2 (*rbq7*) might show a delayed (after 48 hpi) defensive action compared to other QTL lines. Such a delayed defensive action might be redundant with the earlier acting resistance mechanisms of *rbq6+11*, *rbq4* and *rbq5* and therefore explain why the resistance level did not increase when it was combined with other QTLs.

Interestingly, the resistance mechanisms of tripbleBIL268 do not completely resemble that of *L. saligna* CGN05271, although this line showed the complete resistance. The subepidermal HR-based resistance in doubleBIL68 and tripleBIL268 were derived from BIL2.2, BIL6.3 and BIL8.2, which was not observed in *L. saligna* CGN05271 and *L. sativa* cv. Olof. The reasons for such deviation between mechanisms of dissected resistance and of the intact nonhost resistance remain unknown. One possible explanation may be that in the nonhost *L. saligna*, the resistance level is very high and the pathogen is inhibited early, before it can induce SEHR. In the BILs and combiBILs, the resistance is less strong, hence the inhibition might be slightly later and/or weaker, allowing the pathogen to more greatly affect the tissue and hence, induce HR at a later phase like in subepidermal cells. In the compatible interaction with either lower doses of QTLs or none at all, the pathogen organizes its infection well and suppresses HR more than in the nonhost parent.

Observations at time points later than 48 hpi may be useful for further characterizing the resistance mechanisms of these QTLs. In the future, histological studies may assist determination of the QTL effect and facilitate both the fine mapping of QTLs and ongoing QTL cloning.

Perspectives for breeding

Pyramiding or stacking QTLs for resistance by MAS has been reported as an effective strategy for increasing the resistance level and the probability of durable resistance towards different pathogens or pests (Richardson et al. 2006; Varshney et al. 2006; Yang et al. 2005).

Our four target resistance QTLs are recessive and most probably not specific to individual *B. lactucae* races (Jeuken et al. 2008 and Chapter 2). We pyramided these four target QTLs in eleven combiBILs. The best combiBILs were doubleBIL68 and tripleBIL268 as they showed very low or no infection severity to *B. lactucae* (Figure 2).

QTLs in BIL2.2 (*rbq5*), BIL6.3 (*rbq6+11*) and BIL8.2 (*rbq4*) are more interesting for introgression in lettuce cultivars than the QTL in BIL4.2 (*rbq7*) for three reasons: (1) the former three QTLs showed epistatic effects with increased resistance levels while *rbq7* did not; (2) the resistance mechanism of these three QTLs acted at early phase of pathogen development compared to *rbq7*; and (3) the former three BILs have a normal lettuce cultivar morphology while BIL4.2 showed undesired traits (Jeuken and Lindhout 2004). All the above features of the QTLs, *rbq4*, *rbq5* and *rbq6+11*, suggest that introducing them into a lettuce cultivar might lead to more durable resistance to *B. lactucae*.

CHAPTER 4

Fine mapping of four QTLs for nonhost resistance to lettuce downy mildew, reveals both single- and multi-QTL per introgression segment

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To be submitted

A portrait of lettuce leaf discs from experiment LDTW06

Fine mapping of four QTLs for nonhost resistance to lettuce downy mildew, reveals both single- and multi-QTL per introgression segment

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Abstract:

An earlier study identified four *Lactuca saligna* introgressions with recessive Quantitative Trait Loci (QTLs), *rbq4* (BIL8.2), *rbq5* (BIL2.2), *rbq6* (BIL6.3) and *rbq7* (BIL4.2) contributing to the nonhost resistance of this donor to *Bremia lactucae* (downy mildew) in a backcross population derived from a cross of *L. saligna* CGN05271 (nonhost) x *L. sativa* cv. Olof (host). These QTLs are effective at both the young and adult plant stages and are considered to play a major role in this nonhost resistance. The QTLs were located in varying sized introgressions, between 20 cM and 60 cM. To fine map these QTLs and to separate them from undesired agronomic traits, we developed four sets of Near Isogenic Lines (NILs) with smaller *L. saligna* introgressions by marker assisted selection (MAS). The disease evaluation revealed that the introgression 8.2 and 6.3 likely each contained one single QTL for the resistance to *B. lactucae*. These QTLs (*rbq4* and *rbq6*) were fine mapped in 11 cM and 14 cM intervals, respectively. Unlike BIL8.2 and BIL6.3, results on BIL4.2 suggested multi-QTL in its introgression and the putative locations of sub-QTLs *rbq7a*, *rbq7b* and *rbq7c* were proposed. The location of *rbq5* was assumed in a 0.7 cM interval, although BIL2.2 introgression seemed also to contain multi-QTL. This is the first report where four QTLs contributing to the nonhost resistance have been fine mapped and introduced into cultivated lettuce species. The results also offer the unique possibility to clone these nonhost resistance QTLs.

Keywords: Backcross Inbred Lines (BILs), *Lactuca* spp., Substitution mapping

Introduction

Resistance to *Bremia lactucae* is an important trait in lettuce (*Lactuca* spp.) breeding and cultivation. To date, many downy mildew resistance genes (*Dm* genes) are known (Crute 1992b; Kesseli et al. 1994; Lebeda et al. 2001; Lebeda and Zinkernagel 2003) and at least 20 race-specific *Dm* genes have been introgressed into lettuce cultivars (Crute 1992b; Lebeda and Zinkernagel 2003). However, this type of qualitative resistance is not durable since the resistance is usually overcome by rapid adaptation of *B. lactucae* races. The nonhostresistance of *L. saligna* is an alternative option for the durable protection of *Lactuca* spp. species against *B. lactucae* (Bonnier et al. 1992; Jeuken and Lindhout 2002; Lebeda and Boukema 1991). We are interested in identifying the genes contributing to this nonhost resistance.

Lactuca species are diploid inbreeding/self fertilizing plant species with nine chromosome pairs. In the lettuce (*Lactuca* spp.) - downy mildew (*Bremia lactucae*) pathosystem, we studied the inheritance of nonhost resistance in *L. saligna*. Originally, we made a cross of *L. saligna* CGN05271 x *L. sativa* cv. Olof resulting in an F_2 population of 126 plants. A set of 29 Backcross Inbred Lines (BILs) from the BC_4S_{1-2} and BC_5S_{1-2} were developed by Marker Assisted Selection (MAS). The nomenclature of the BILs refers to the location of the introgression. For example, BIL2.2 stands for the second introgression in Chromosome 2 (Jeuken and Lindhout 2004).

We identified 15 introgressions that contribute to the nonhost resistance of *L. saligna* and provisionally assigned them as 15 QTLs, *Rbq1* to *15*, by evaluating infection severity levels of the F_2 population at adult plant stage and each BIL at seedling, young plant and adult plant stages (Jeuken et al. 2008 and Chapter 2). Such polygenic inheritance implies that the nonhost resistance of *L. saligna* is due to the combined effects of the resistance alleles at these QTLs. The effects of the 15 QTLs appear to be mainly plant developmental stage dependent. However, four recessive QTLs, *rbq4* (BIL8.2), *rbq5* (BIL2.2), *rbq6* (BIL6.3) and *rbq7* (BIL4.2), are effective at both the young and adult plant stages and each reduces the Relative Infection Severity (RIS) by 46%~90% compared to the susceptible parental line, *L. sativa* cv. Olof. We consider these four QTLs to play a major role in the nonhost resistance of *L. saligna* (Jeuken et al. 2008) (Chapter 2). They are the four target QTLs in the present study. The size of the introgression segments of BIL8.2 (*rbq4*), BIL2.2 (*rbq5*), BIL6.3 (*rbq6*) and BIL4.2 (*rbq7*) are between 20 cM and 60 cM (Jeuken and Lindhout 2004).

Fine mapping of the QTL effects on these four introgressions will lead to identification of the genes contributing to the nonhost resistance in *L. saligna* and the

underlying resistance mechanisms. An effective method to dissect QTLs is substitution mapping. With this strategy, donor introgression segments that affect the quantitative trait are broken-up in smaller and overlapping segments in order to identify more precisely the position of the genetic factor(s) responsible for the QTL effect (Paterson et al. 1990; Thomson et al. 2006).

In this study, we developed sets of Near Isogenic Lines (NILs) with smaller, overlapping introgressions across the introgression segments of BIL8.2 (*rbq4*), BIL2.2 (*rbq5*), BIL6.3 (*rbq6*) and BIL4.2 (*rbq7*) in *L. sativa* background to: (1) identify the number of loci that contribute to the resistance on each segment (single- or multi-QTL per introgression); (2) find flanking markers as closely linked as possible to the genetic factors affecting resistance; (3) separate the resistance QTLs from genes on the same introgression that affect plant morphology.

Materials and Methods

Map saturation

To saturate and improve the genetic linkage map of the *L. saligna* CGN05271 x *L. sativa* cv. Olof cross, additional marker analyses were performed on the F_2 population (n=126, Jeuken et al. 2001). New Amplified Fragment Length Polymorphism (AFLP) analyses with two new primer combinations E48M59 (selective nucleotides CAC and CTA) and E33M59 (selective nucleotides AAG and CTA) were performed. More than 100 EST markers from lettuce EST sequences of the *Compositae* Genome Project Database were mapped in the F_2 population (CGPDB, compgenomics.ucdavis.edu) and 12 SSR markers were developed and mapped by Syngenta Seeds B. V., The Netherlands.

Linkage analyses

Linkage analyses were performed using JoinMap 4.0 software (Van Ooijen 2006) on this F_2 population with the following mapping conditions: for grouping Regression Mapping was used with weak linkages recombination and LOD thresholds of 0.45 and 0.5. Markers were assigned to nine linkage groups at a LOD threshold of 8. Calculations of the linkage maps were done by using all pair-wise recombination estimates smaller than 0.40, LOD scores higher than 1, jump threshold of 5, and Haldane's mapping function. For this research, new linkage maps, marker information and data are shown for the introgression regions of BIL2.2, BIL4.2, BIL6.3 and BIL8.2 only.

We also used additional AFLP markers that can be easier scored in BILs and NILs than in F_2 plants due to a less busy banding pattern in BILs and NILs. The map positions of

these AFLP markers were defined on segregations of several backcross populations. We have tagged these AFLP-markers with "_b", like E44M49-128_b. Markers labeled with the extension "sal", such as E38M54-140sal, gave amplification products for *L. saligna* alleles. AFLP-markers without "sal" gave amplification products for *L. sativa* alleles.

NIL development procedure

We developed plant genotypes with smaller, overlapping *L. saligna* introgressions than in the four BILs harbouring the selected QTLs, BIL2.2 (*rbq5*), BIL4.2 (*rbq7*), BIL6.3 (*rbq6*) and BIL8.2 (*rbq4*). To select the recombinant plants that have crossover sites within each introgression, we used selfed segregating populations from the preBILs. PreBILs are heterozygous parental lines of the corresponding BILs. PreBIL2.2, preBIL4.2 and preBIL8.2 have no other *L. saligna* introgressions, but preBIL6.3 contains one additional heterozygous *L. saligna* introgression, i.e. on top of Chromosome 4 from 0 to 32 cM, called here introgression 4.1.

We used two codominant PCR-markers (Polymerase Chain Reaction, PCR) that are near the extremities of the introgression to screen on average 400 plants per introgression for recombinant plants. The selected recombinant plants were transplanted and their DNA was isolated for the second round of genotyping. In the second round of genotyping, we used five PCR-markers including the two markers near the extremities of the introgression to confirm the genotype of each selected plant. Afterwards, we performed more extensive genotyping by AFLP markers to validate genotypes of the recombinant plants that were confirmed by the PCR-markers.

The next step was to develop homozygous lines from the recombinant plants. To save labour, we selected only one or two plants from the ones that had the same cross-over point according to the current map resolution. If later the QTL is mapped close to this cross-over point, we will use the selfing progeny of the other recombinant plants to increase the map resolution. After selfing the recombinant plant, a progeny of about 20 plants was screened with PCR-markers. The plants that were homozygous for the *L. saligna* introgression were selected and we confirmed their genotypes with more markers. These homozygous recombinant lines were designated NILs as they contain smaller introgressions than the BILs in uniform *L. sativa* background. The NILs that were obtained are presented in Figures 1 to 4.

Genotyping

For recombinant screening in the preBIL progeny (>250), we used PCR-markers based on EST or SSR. The primer sequences for each marker and the restriction enzymes that were used for Cleaved Amplified Polymorphic Sequence (CAPS) digestion are listed in Table 1.

The PCR and CAPS procedure were as described by Jeuken et al, (2008). DNA extractions were performed by two methods: for the first round of recombinant screening, we used the simple NaOH method (Wang et al. 1993); for the confirmation of the selected recombinants and AFLP genotyping, we used the CTAB method (Jeuken et al. 2001). The two PCR-markers near the extremities of most introgression were less than 10 cM away from the ends of the introgression. Only BIL8.2 had markers further away from the extremities of the introgression at 11cM and 14cM; BIL4.2 had a distal marker away from the top of the introgression at 19 cM (Figures 1 to 4). AFLP analyses were run as described previously (Jeuken et al. 2001; Vos et al. 1995).

Disease evaluation

Plant materials used in the disease evaluation are listed in Table 2. Experimental procedures of Young plant Disease Test (YDT) and Adult plant Diseases Test in Greenhouse (ADT_G) were as described previously (Jeuken et al. 2008) (Chapter 2). For fine mapping of each QTL, two independent YDT were performed. Additionally, two independent ADT_G were carried out to fine map QTL *rbq6*.

For YDT, we used four-week-old young plants with six to eight true leaves (number of leaves is genotype dependent). Six young plants per genotype per experiment were grown in a randomized complete block design with six blocks. The infection severity of each plant was evaluated at nine or ten days post inoculation (dpi) as percentage of sporulating area per representative leaf (the two youngest, well expanded leaves at the moment of inoculation of each plant). For ADT_G, seven plants per genotype per experiment were grown randomly in the greenhouse. Four detached leaf discs from each plant were placed on moist filter paper according to randomized complete block design (two blocks per experiment and two discs from one plant in each block). Infection severity was evaluated by scoring the percentage of sporulating area per leaf disc.

We applied artificial inoculation using *B. lactucae* race Bl:14 for all the disease tests as the QTL effects were previously shown to be race-non-specific (Chapter 2). The concentrations of the inoculum used in these experiments were 2~4 x 10^5 conidia/ml. The virulence spectrum of race Bl:14, the pathogen maintenance, inoculum preparation and the way of inoculation were as described previously (Jeuken and Lindhout 2002).

Table 1. PCR-markers used for genotyping recombinants. The markers that used for the first round of recombinants selection are highlighted in bold and underlined.

Marker Name	Chr.	Marker Type	Restriction Enzyme	Primer sequences (5' - 3')
KLK1473_850	2	dCAPS	HhaI	Fw: AATCGGAACTCCACCACAA
				Rv: GTGGTTTACAAATAGGGTGATTACAGCG
NL0212	2	SSR		Fw: CCAGTGAAGAAACCAAAGG
				Rv: CTTCTCCTTCATCGTCACC
LE4034	2	SCAR		Fw: AATCTCTGACATGAAATCGGC
				Rv: TGCCCTCTTCCAAGATTATCA
LE0350	2	CAPS	HinfI	Fw: CGGTTGCTCAAGACCTCTCA
				Rv: AGCGAACGACCCTCTAACG
LE1276	2	SCAR		Fw: TTTGGGTTCCTTCAGTTTGC
				Rv: CACAGTTTGGGATGAACACG
LE7003	2	SCAR	DdeI	Fw: GGTCTACTGGTTCGCAGAGC
				Rv: AAGCCTCACATGTTCTTCCC
LK1336	2	CAPS	Eco88I	Fw: TGAGGAGTCCATGGATACGG
				Rv: CGATGCAACAGCATGGATAC
LE1244	2	SCAR		Fw: CATCCGCTTCCTCTTCAGTC
				Rv: ACGAGCACCTGCATCTACAA
LE0142	2	CAPS	HinfI	Fw: AGCAGTGGTGGATCGATTTC
				Rv: TTGGTTCTGCAAGTTGCTTC
LK1475	2	SCAR		Fw: GGAGTTCAGGGCCTCTGTC
				Rv: CCGATTCTGCGGTTATCTTC
LE1114	2	CAPS	MseI	Fw: CAAGAGGTGAATGGGAAGGA
				Rv: TACCACACAAACAAGCGGAA
NL0157	2	SSR		Fw: ATTGATCCATGGCTACGAC
				Rv: GAGCCTATTTCATCCATGC
LK1525	4	CAPS	HaeIII	Fw: CAGACGTCCACCTGGAATTG
				Rv: ATTCAGTGCGTCTGTTGCAG
NL1186	4	SSR		Fw: AGGGGCTGATGATGATATG
				Rv: AGTACATACTTGTGTCTTGTGG
LE0351	4	CAPS	NlaIV	Fw: GAATATGCGGCGGAGATAAG
				Rv: AATCACATGAATGGATGCAAA
NL1088	4	SSR		Fw: ATTTGAAAGCCATGGAAAC
				Rv: TTGCTTCAAATTTTCCACC
LE0333	4	SCAR		Fw: GGACCGGGTTTTTAAGTCGT
				Rv: TTTCTCTGTATATATGCAATCTCCATT
LE0337	4	CAPS	ScrFI	Fw: CCATGGCTAAAAAGCAAACC
				Rv: ACATTAGCCAAGCGACAACA
LK1406	4	CAPS	RsaI	Fw: CACCACCCTCACCTTAGCTC
				Rv: ACCGTTGAATATCGGACACG
LE1106	4	SCAR		Fw: TGATTATGGAGGCGAAGAGG
				Rv: CACAAAGATTCATTACTTGCCATC
KLE0053	4	CAPS	AvaI	Fw: TACCCCTAAAGCCCACCTCT
				Rv: CGGTGGTGAAGATTCGTTTT
LsB104 *	4	SSR		Fw:
				Rv:
NL0589	6	SSR		Fw: AACGAATGTATACCGCAGC
				Rv: ACGATTGGTCAAGGAAGTG
LE1126	6	CAPS	AluI	Fw: CTTTGCTCCAATTCCTCTCG
				Rv: AATGCCATAGTGAAGCTGGG
NL1114	6	SSR		Fw: AAGGCCATTGTAGGTGATG
				Rv: GCTTCACTTGCTCTTGGAC
NL0653	6	SSR		Fw: TCTCAATCCTGTGGCTTTC
				Rv: GCGAATGATCGAGAAGAAG
NL1084	6	SSR		Fw: CAACAGCAACAATCTGCAC
				Rv: AGCACTTCCAAATTTCAGC
M7120	6	CAPS	SSiI	Fw: ACAGCAACAGCCGACCG
				Rv: CGCACATTATTCGGCTCAAA
NL0833	6	SSR		Fw: ATGTCTAGAGGCGCAACAG
				Rv: CTTGTTCCTCCCATGACTC
LE1211	6	CAPS	HinfI	Fw: CGGGTGATTACATCGGCTAT
				Rv: CGCAACCAACCAAATTTACC
NL0418	6	SSR		Fw: AAGCCCAAAGAAGAAGAGG
				Rv: ATGCATTTGGATTCTCGTC
LE0232_dCAPS	8	dCAPS	HinfI	Fw: CCATCGCGTAAACATGCCCGGGAGT
				Rv: TAAAGGTCGATTAGGGCACG
NL0935	8	SSR		Fw: GTGAACCAATGAGTGGAGG
				Rv: GAACATCCACTTGGTCCAG
KLE0263	8	CAPS	MnlI	Fw: CAACCTCACCGGAGTTTTGT
				Rv: GCCGGAAAGTTTGTTGTTGT
NL1187	8	SSR		Fw: ACCTTCATCCTATGAAACCC
				Rv: TCTCCCTCCAAAACCAAC
KLK1366	8	CAPS	MnlI	Fw: GAATCGCTCAGGCAAACAAT
				Rv: TGGCCTCTCAAGCAGATTTT
LK1225	8	SCAR		Fw: CGCAGTGAACATTACGAACG
				Rv: CCACGTATGAACACGTCAGC
LE1111	8	SCAR		Fw: AATTCACTCCACCACCGAAG
				Rv: CTACGTCAGTGCCTATGCCA
LE0026	8	CAPS	MseI	Fw: AGGTATTTTCCGGCGAACTT
				Rv: CTTTGTGCCTCAAACCCAAT
LE3019	8	CAPS	ClaI	Fw: ATTGCTGGAGTCGTGGTTTC
				Rv: CTTTGTGCCTCAAACCCAAT

* This SSR refers to (Van de Wiel et al. 1999)

Table 2. Plant materials used for the disease evaluation. YDT=young plant disease test and ADT_G=adult plant disease test in greenhouse. x=lines that were used

	Lines	Description	Disease Evaluation	
			YDT	ADT_G
Controls	*L. sativa* cv. Olof	susceptible parent	x	x
	L. saligna CGN05271	non-host resistant parent	x	x
	BIL4.4	*Rbq16,* super susceptible control	x	x
Target BILs	BIL2.2	*rbq5*	x	
	BIL4.2	*rbq7*	x	
	BIL6.3 [a]	*rbq6+Rbq11* [b]	x	x
	BIL8.2	*rbq4*	x	
Neighboring BILs [c]	BIL4.1	*Rbq11* [b]	x	x
	BIL2.1	*Rbq13* [b]	x	
	BIL8.3	*Rbq10* [b]	x	
	BIL2.3	susceptible	x	
	BIL4.3	susceptible	x	
	BIL8.1	susceptible	x	
NILs	2.2-NILs	developed from the present study	11 NILs	
	4.2-NILs	developed from the present study	11 NILs	
	6.3-NILs	developed from the present study	5 NILs	5 NILs
	8.2-NILs	developed from the present study	12 NILs	

[a] BIL6.3 contains an additional introgression on the top of Chromosome 4 where *Rbq11* was located.
[b] QTLs that are effective only at young plant stage (Chapter 2)
[c] Neighbouring BILs are the lines with overlapping introgression to the target BILs (Figures 1 to 4)

Data analysis

The infection severity recorded in percentage was transformed (arcsine square root) to improve the normality of the distribution. Differences in mean infection severity values of each tested line were analysed by univariate analysis of variance, using the general linear model module of the statistical package SPSS version 12.0.1. For each analysis, infection severity values were used as a dependent variable and genotypes were used as a fixed factor. Tukey's Honestly Significant Differences (HSD) test (α=0.05) was applied for multiple comparisons between all the genotype pairs in one type of disease test. The pair-wise multiple comparisons allowed us to compare infection severity levels between NILs and BILs. If all the NILs that shared one common introgression region showed lower infection severity than the susceptible reference line and that was similar to the infection severity of the target BIL, we concluded the QTL to be residing in the overlap introgression region of these NILs.

QTL-mapping was executed using MapQTL®5.0 (Van Ooijen 2004). Interval Mapping (IM) was run and markers at the LOD peaks were used as cofactors for running the

Multiple-QTL Model (MQM) mapping. The explained phenotypic variance was estimated with the MQM results. The threshold values of detecting a QTL were determined by a permutation test and they were 2.2, 1.1, 1.9 and 2.3 in the introgression 8.2, 2.2, 6.3 and 4.2, respectively.

Results

Map saturation and development of NILs

The target introgression segments 2.2, 4.2, 6.3 and 8.2 were saturated with 45 additional markers. The updated linkage maps for the four target BIL introgression regions are shown in Figures 1 to 4.

To develop multiple NILs, which contain smaller *L. saligna* introgression in *L. sativa* background than the target BIL introgression region, we started with a recombinant screening of a selfed preBIL progeny. Table 3 presents the number of plants that were screened per preBIL, the number of recombinant plants detected and the number of homozygous NILs per BIL/QTL region developed. The recombination frequency of the selfed progeny from preBIL8.2, preBIL2.2, preBIL6.3 and preBIL4.2 between the two markers near the extremities of the introgression that were used for the recombinant screening was 4.0%, 1.2%, 1.6% and 1.6%, respectively. Compared to the original F_2 population, recombination in the introgression regions for BIL8.2 (*rbq4*), BIL2.2 (*rbq5*), BIL6.3 (*rbq6*) and BIL4.2 (*rbq7*) were suppressed 2 to 15 times, respectively (Table 3).

Table 3. Summary of development of the substitution lines
= number of, Rec. = Recombinant; Rec. freq. = Recombination frequency;
Rec. suppre.= times of recombination suppression compared to the F_2 population

Material	QTL	# Plants	# Rec	# NILs	Rec freq. in BIL	Rec freq. in F_2	Rec suppre.
Progeny of preBIL8.2	*rbq4*	361	29	12[a]	4.0%	9.1%	2
Progeny of preBIL2.2	*rbq5*	442	11	11	1.2%	15.1%	12
Progeny of preBIL6.3	*rbq6*	448	14	5[b]	1.6%	10.3%	7
Progeny of preBIL4.2	*rbq7*	258	8	11[c]	1.6%	22.6%	15

[a] selected 11 out of 29 recombinant plants including one double-recombinant to make 12 homozygous NILs
[b] selected 9 out of 14 recombinant plants to make homozygous NILs but four died before seeds harvesting
[c] three additional NILs developed from other experiment

For the BIL8.2 introgression region, we found 29 recombinant plants and selected 11 of them as described in Material and Methods, with different cross-over points to further

develop homozygous NILs (Table 2 and Figure 1). For BIL2.2 region, we identified 11 recombinant plants and selfed them to obtain 11 homozygous NILs (Table 2 and Figure 2). For the BIL6.3 introgression region, a hot spot of recombination was discovered between marker E45M48-300 and E44M49-352sal (a 0.4 cM interval). Five out of the fourteen recombinant plants discovered from preBIL6.3 progeny had cross-over at this marker interval. In addition, five recombinant plants died before seed harvesting. Finally, five 6.3-NILs were developed and they all contained the extra homozygous *L. saligna* 4.1 introgression. Due to the distorted segregation of BIL4.1 introgression with a preference for *L. saligna* alleles as reported previously (Jeuken et al. 2008 and Chapter 2), we were not able to select against the 4.1 introgression as this segment was never homozygous *L. sativa*. NIL6.3-2 contains a smaller introgression on the top of Chromosome 4 from 0 to 16 cM, and all other four 6.3-NILs had an entire homozygous 4.1 introgression from 0 to 32 cM. For the BIL4.2 introgression region, we detected eight recombinant plants in this study and three additional recombinant plants from previous experiments (unpublished data) and have developed eleven NILs. The final genotypes and the corresponding marker positions of each set of NILs are presented in Figures 1 to 4.

Disease evaluation in general

In all disease tests (both YDT and ADT_G), the nonhost parent *L. saligna* and the susceptible parent *L. sativa* cv. Olof, BIL4.4, the four target BILs and the neighbouring BILs (Table 1) all had a similar infection severity as observed in previous experiments (Chapter 2). We did not observe experiment x genotype interaction, therefore, data from different experiments for the same lines were pooled to generate following results.

Fine mapping of *rbq4*

We performed YDTs on all 8.2-NILs (Table 1) to fine map *rbq4* at both young plant and adult plant stages. At young plant stage, five 8.2-NILs, (NIL8.2-2 to –6) all showed quantitative resistance with similar low severities (12% to 28%) as BIL8.2 (18%), and significantly lower than the susceptible reference line *L. sativa* cv. Olof (63%). The other six NILs, NIL8.2-7 to 12 and neighboring BIL8.1 showed high infection severity (39%~70%) similar as *L. sativa* cv. Olof which suggested that the common introgression region of NIL8.2-2 to NIL8.2-6 was the most likely location of *rbq4*, which was a 7cM interval between marker E38M54-268_b and E44M49-97sal. However, NIL8.2-1 showed 39% infection severity which was significantly lower than the susceptible reference line, although it was significantly higher than BIL8.2. Therefore, *rbq4* might also be flanked by marker NL0935 and E35M59-299 in a 5 cM region (Figure 1).

MapQTL® calculation revealed one single peak with two markers E35M59-299 and KLE0263, which showed the highest LOD value of 4.3. The results also showed that this QTL explained 71% of the phenotypic variance. Furthermore, this indicated that *rbq4* region was more likely located between markers E38M54-268 and E44M49-97sal than between markers NL0935 and E35M59-299.

In addition, neighboring BIL8.3 showed significantly lower infection severity (21%) than *L. sativa* cv. Olof confirming the previous detection of *Rbq10* (Chapter 2). *Rbq10* resides from marker E48M59-197 towards the distal end of BIL8.3 introgression as NIL8.2-7 to 12 showed a similar infection severity level as *L. sativa* cv. Olof (Figure 1).

Fine mapping of *rbq5*

We evaluated the infection severity of all 2.2-NILs by YDT. BIL2.2 showed significantly lower infection severity (20%) than *L. sativa* cv. Olof (64%) (Figure 2). The 11 NILs showed a gradient level of infection severity and there was no obvious delimitation between the susceptible and resistant group among the NILs. Only NIL2.2-7 (31%) and NIL2.2-10 (30%) stood out and showed significantly lower infection severity than *L. sativa* cv. Olof. However, NIL2.2-7 and NIL2.2-10 did not share a unique introgression segment. The rest of 2.2-NILs and neighboring BIL2.3 showed an infection severity with a range from 37% to 76% and were not significantly different from the level of *L. sativa* cv. Olof (Figure 2). The above mentioned results suggest that the BIL2.2 introgression region contains more than one resistance QTL which contributed to the quantitative resistance of BIL2.2. This seems to preclude determination of an exact location of *rbq5* or of possible sub-QTLs in this region. The resistance shown by BIL2.2 introgression is likely explained by a multiple interacting loci.

Nevertheless, based on the multiple comparisons between each pair of lines, NIL2.2-2, -4, -5, -6 and -8 had intermediate infection severity of 37% to 48%, which were higher but not significantly different from that of BIL2.2 and lower but not significantly different from that of *L. sativa* cv. Olof. The tendency of infection severity of this group of NILs leads to a hypothesis that the infection severity levels of these five NILs might be in one category that was similar to BIL2.2. Interestingly, these five NILs shared a common marker, E35M59-107sal_b with NIL2.2-7 and NIL2.2-10. Therefore, the effective gene(s) contributing to the quantitative resistance of BIL2.2 are likely to be located in the 0.7cM interval flanked by markers E38M54-63_b and E35M48-153_b (Figure 2). This hypothesis was supported by a peak in the LOD profile (LOD 1.4) at markers E38M54-63_b and E35M59-107sal_b. The effect of this QTL was estimated to explain only 34% of the phenotypic variation.

In addition, neighboring BIL2.1 showed significantly lower infection severity (18%) than on *L. sativa* cv. Olof, which confirmed the presence of *Rbq13* (Chapter 2). NIL2.2-1 and NIL2.2-3 showed similar infection severity as on *L. sativa* cv. Olof (Figure 2) which suggested the location of *Rbq13* should be above marker E35M59-396sal towards the top of chromosome 2.

Fine mapping of *rbq6*

In YDT, the severity of infection on BIL 6.3 (12%), all five 6.3-derived NILs (13% to 37%) and BIL4.1 (18%) was significantly lower than on *L. sativa* cv. Olof (63%). As the BIL6.3 and the five 6.3-NILs all contain the 4.1 introgression, which carries *Rbq11*, the low infection severity are most likely due to *Rbq11* or sometimes in combination with *rbq6* (Figure 3). Therefore, it is difficult to fine map *rbq6* through disease tests at young plant stage.

As expected at adult plant stage, *Rbq11* on introgression 4.1 was not effective against *B. lactucae* . NIL6.3-2 and -3 showed infection severity as low as BIL6.3, while NIL6.3-1, and -5 showed similar infection severity compared to the susceptible reference line *L. sativa* cv. Olof. NIL6.3-4 showed an infection severity lower than *L. sativa* cv. Olof but higher than BIL6.3. Therefore, the location of *rbq6* was probably between markers E45M48-102sal_b and E44M49-352sal. Most likely *rbq6* located in the interval between markers E45M48-102sal_b and NL0653 because NIL6.3-4 showed significantly higher level of infection severity than BIL6.3 (Figure 3).

The marker loci located between markers E45M48-102sal_b and E44M49-352sal gave highest LOD value (2.51) and explained 76% of the phenotypic variance. This suggested that a single-QTL, *rbq6*, contributes to the resistance of 6.3 introgression segment.

Fine mapping of *rbq7*

In YDT, BIL4.2 had 15% infection severity which was significantly lower than for the reference line *L. sativa* cv. Olof. All 4.2-NILs showed a gradient level of infection severities similar as for 2.2-NILs, which suggest the presence of multiple sub-QTLs in this segment.

Infection severity levels of four 4.2-NILs (-4, -5, -6 and -8) were in a range of 8% to 37% and similar as on BIL4.2. The other 4.2-NILs (-1,-2,-3,-7,-9,-10 and -11) and the neighboring BIL4.3 all showed similar infection severity as on cv. Olof in a range of 41% to 70%. Strikingly, the infection severity level of NIL4.2-7 (43%) did not show a significant difference from *L. sativa* cv. Olof while that of NIL4.2-8 (37%) did, although these two lines had an identical genotype based on the current map resolution. However, the small difference between their severity levels allowed us to place NIL4.2-7 in the group of NIL4.2-4, -5, -6

and -8. Therefore, *rbq7* was possibly located on the overlapping region of these five NILs for an 8.3 cM interval between marker NL1088 and E35M60-84. This region was designated as *rbq7a*.

Interestingly, NIL4.2-11 without introgression on *rbq7a* region showed a relatively lower infection severity (43%) than on *L. sativa* cv. Olof (64%), although not significantly so. This suggested possible sub-QTL presence in the region outside *rbq7a* location. Because NIL4.2-6 had a significant lower severity level than NIL4.2-7 and NIL4.2-8, we assume another sub-QTL, *rbq7b*, to be located from 28cM to 48cM on top of introgression 4.2, which was absent in NIL4.2-7 and NIL4.2-8. Analogously, to explain the severity difference between NIL4.2-6 and NIL4.2-4, NIL4.2-5, we assume the presence of sub-QTL, *rbq7c*, on the bottom of the BIL4.2 introgression between marker LE1106 and E45M48-156_b. The high infection severity level of NIL4.2-1, -2, -3, -9 and NIL4.2-10 suggested that the sole presence of either *rbq7b* or *rbq7c* was not sufficient to lead to quantitative resistance. However, when any two of these sub-QTLs from *rbq7a*, *rbq7b* and *rbq7c* were combined, the subsequent NILs showed a decreased infection severity compared to *L. sativa* cv. Olof.

MapQTL® showed two peaks of LOD value of 3.7 and 2.5 for markers E35M60-85 and E35M49-298sal, respectively. The position of these two peaks exactly coincided with the former proposed region of *rbq7a* and *rbq7b*, respectively (Figure 4). Therefore, we propose that at least two sub-QTLs were present in BIL4.2 introgression. The phenotypic variations explained by these two sub-QTLs were 44% and 27%, respectively.

Additionally, NIL4.2-1, NIL4.2-2 and NIL4.2-3 showed similar severity levels as on *L. sativa* cv. Olof suggested that *Rbq11* (in BIL4.1) was mapped above NL1186, the last common marker between BIL4.1 and BIL4.2.

Fine mapping of morphological traits

Some of the BILs studied in this work showed deviating morphology, i.e. long dark green and twisted leaf and non-heading phenotype in BIL 4.2 (Jeuken and Lindhout 2004) and a lobed leaf shape in BIL8.2. We fine mapped these traits within the BIL introgressions by scoring presence or absence of the traits in the NILs. The morphological traits “dark green leaf” from *L. saligna* and “twisted leaf” were both mapped between marker LE1106 and E44M48-474 on Chromosome 4, designated as “*Darkgreen_sal*” and “*Twisted*”, respectively (Figure 4). It was not obvious whether the trait “twisted leaf” was from *L. sativa* or from *L. saligna* as both parental lines did not show such a phenotype. The traits “long narrow leaf” from *L. saligna* and “heading” from *L. sativa* were fine mapped in a 2.9 cM interval between marker LE0351 and LE2211 on Chromosome 4 and designated “*Longnarrow_sal*” and “*Heading_sat*”, respectively (Figure 4). Morphological trait “lobed leaf” was mapped to a

3.6 cM interval flanked by markers KLK1366 and E44M49-385 on Chromosome 8 (Figure 1). Traits “Darkgreen leaf”, “Twisted leaf” and “Lobed leaf” were dominant because they were also observed in plants heterozygous for the mapping region.

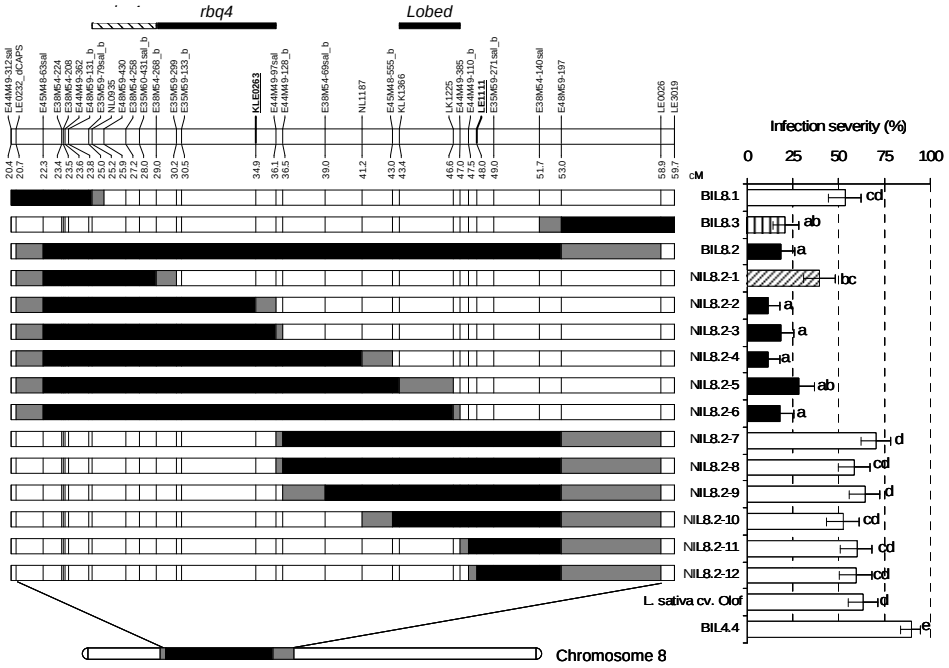


Figure 1 Genotypes and disease evaluation at young plant stage of lettuce NILs covering BIL8.2 (*rbq4*) introgression, the neighbouring BILs and the reference lines. In the genotype graph, the marker positions are presented in cM. Markers in bold and underlined are the distal markers used for recombinant screening. Open bars represent homozygous *L.sativa*, solid bars represent homozygous *L. saligna* and grey bars represent intervals containing a recombination event. The putative positions of *rbq4* and morphological trait gene *Lobed* are indicated by the bars above the markers. It is more likely that *rbq4* resides in the region indicated by the black bar rather than the hatched bar region as the infection severity of NIL8.2-1 was significantly higher than of BIL8.2. In the *Bremia* disease evaluation graph, infection severities are presented. Error bars stand for the 95% confidence interval. Letters in common, right of the error bars, indicate no significant difference (α=0.05, Tukey HSD test). Black columns stand for the lines that showed significantly lower infection severity than *L. sativa* cv. Olof and similar infection severity as BIL8.2; white columns indicate the lines that showed similar or higher infection severity than *L. sativa* cv. Olof; hatched column stands for the line showing infection severity that was significantly lower than cv. Olof, but significantly higher than BIL8.2; vertically striped column stands for the neighbouring BIL contains overlapping introgression with BIL8.2 and showed significantly lower infection severity than cv. Olof.

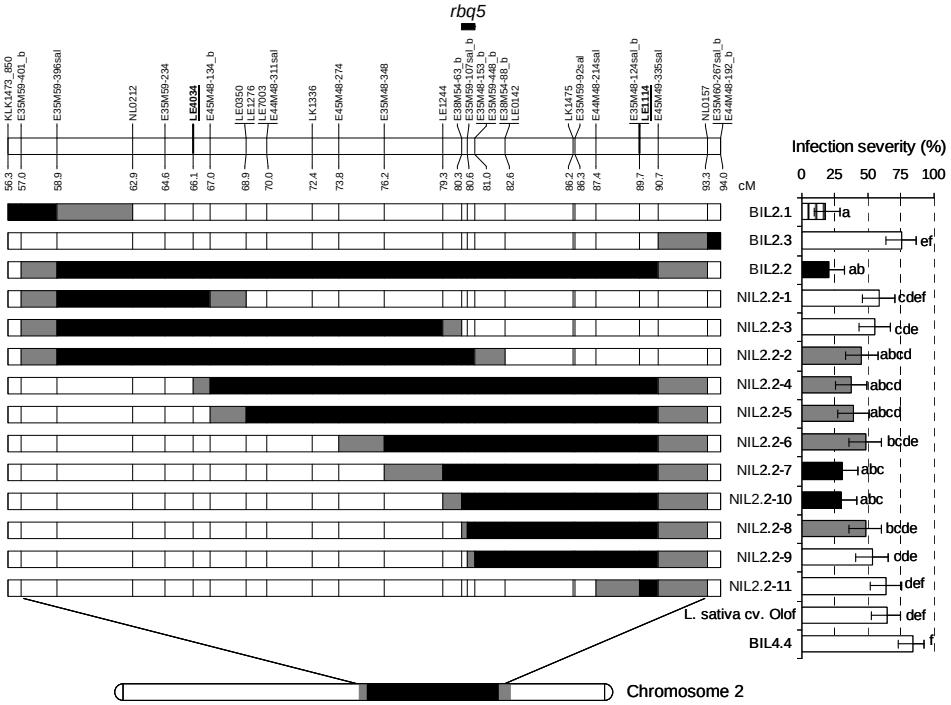


Figure 2 Genotypes and disease evaluation at young plant stage of lettuce NILs covering BIL2.2 (*rbq5*) introgression, the neighbouring BILs and the reference lines. In the genotype graph: for explanations refer to Figure1. The putative position of *rbq5* is indicated by the black bar above the markers. In the *Bremia* disease evaluation graph, error bars stand for the 95% confidence interval. Letters in common, right of the error bars, indicate no significant difference (α=0.05, Tukey HSD test). Black columns stand for the lines that showed significantly lower infection severity than *L. sativa* cv. Olof and similar infection severity as BIL2.2; white columns indicate the lines that showed similar or higher infection severity than *L. sativa* cv. Olof; grey bars stand for the lines that showed infection severity levels higher than BIL2.2 and lower than *L. sativa* cv. Olof, but not significantly different from both reference lines; vertically striped column stands for the neighbouring BIL that has overlapping introgression with BIL2.2 and showed significantly lower infection severity than cv. Olof.

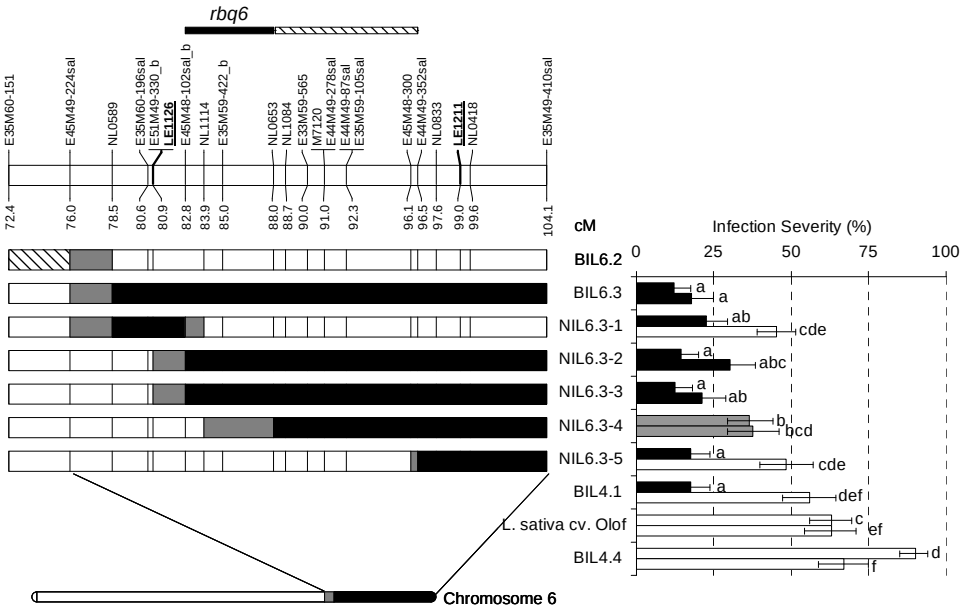


Figure 3 Genotypes and disease evaluation at both young and adult plant stages of lettuce NILs covering BIL6.3 (*rbq6*) introgression, the neighbouring BILs and the reference lines. In the genotype graph, the explanations refer to Figure 1. Grey bars represent intervals containing a recombination event. The putative position of *rbq6* is indicated by the bar above the markers. It is more likely that *rbq6* resides in the region indicated by the black bar rather than the hatched bar region as the infection severity of NIL6.3-4 was significantly higher than of BIL6.3. In the *Bremia* disease evaluation graph, the upper and lower columns of each line stand for the corresponding results from Young plant Disease Tests (YDT) and from Adult plant Disease Tests in Greenhouse (ADT_G), respectively. Error bars stand for the 95% confidence interval. Letters in common, right of the error bars, indicate no significant difference (α=0.05, Tukey HSD test). Black columns stand for the lines that showed significantly lower infection severity than *L. sativa* cv. Olof and similar infection severity as BIL6.3; white columns indicate the lines that showed similar or higher infection severity than *L. sativa* cv. Olof; grey columns stand for the line showing the infection severity that was significantly lower than cv. Olof, but was significantly higher than BIL6.3. BIL6.2 contains heterozygous introgression (hatched bar) which does not overlap with BIL6.3 based on current map resolution and this BIL showed similar infection severity as cv. Olof according to Chapter 2.

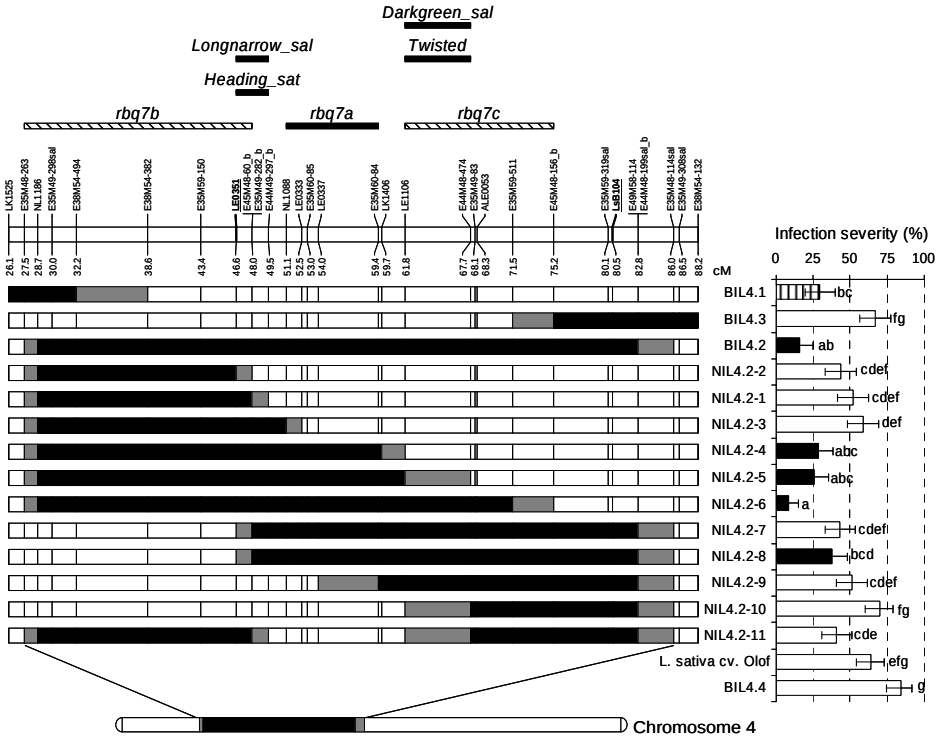


Figure 4 Genotypes and disease evaluation of lettuce NILs covering BIL4.2 (*rbq7*) introgression, the neighbouring BILs and the reference lines. In the genotype graph, the explanations refer to Figure 1. The putative positions of *rbq7a* and traits *Darkgreen_sal*, *Twisted*, *Longnarrow_sal* and *Heading_sat*, are indicated by the black bars above the markers. The proposed positions of *rbq7b* and *rbq7c* are indicated by the hatched bars. In the *Bremia* disease evaluation graph, error bars stand for the 95% confidence interval. Letters in common, right of the error bars, indicate no significant difference (α=0.05, Tukey HSD test). Black columns stand for the lines that showed significantly lower infection severity than *L. sativa* cv. Olof and similar infection severity as BIL4.2; white columns indicate the lines that showed similar or higher infection severity than *L. sativa* cv. Olof; grey column stands for the line that the lower but not significantly lower infection severity than cv. Olof and the infection severity was also not significantly higher than BIL4.2; vertically striped column stands for the BIL that has overlapping introgression with BIL4.2 and showed lower infection severity than cv. Olof.

Discussion

Single- and multi-QTL detection within one target introgression

In the present study, the quantitative resistances of the target BILs can be explained two times by a single-QTL and two times by multi-QTL. More experiments for phenotyping the NILs for resistance level are probably helpful to corroborate the hypothesis of several QTLs within the same introgression.

We successfully fine mapped *rbq4* from 39cM into an 11cM interval (Figure 1) and fine mapped *rbq6* from 28cM into a 14cM interval (Figure 3). In contrast to this, fine mapping of *rbq5* and *rbq7* suggested multi-QTL presence in one introgression. Sub-QTLs *rbq7b* and *rbq7c* hardly gave any contribution to the resistance towards *B. lactucae* when they were present solely in the NILs. Only when one of these two sub-QTLs was combined with *rbq7a*, the NIL showed a reduction in severity as strong as observed in BIL4.2 (Figure 4). Such multi-QTL presence in one single chromosomal segment has been reported previously in rice for flowering-time QTLs *dth1.1a* and *dth1.1b*, and for heading date QTLs *Hd3a* and *Hd3b*, and in *Arabidopsis* for two tightly linked growth-rate QTLs (Thomson et al. 2006). A common feature of above mentioned traits controlled by the QTLs is the genetic complexity.

Our fine mapped QTL locations showed no coincidence with known *Dm*-gene (downy mildew resistance gene) clusters in lettuce genome. The introgression regions of the QTLs, *rbq5*, *rbq6* and *rbq7* do not overlap with any of the known *Dm*-gene clusters. Only BIL8.2 (*rbq4*) introgression region could possibly overlap with *Dm13* locus (Jeuken et al. 2008). However, we have mapped *RGC1*, a candidate of *Dm13* (Shen et al. 1998), to the position of 9.5cM away from the top of Chromosome 8 based on our saturated F_2 map. This result indicated that *rbq4* region did not overlap with the *Dm13* locus because our putative position of *rbq4* was between 25.2 cM to 36.1 cM.

We detected the QTLs, *rbq4, rbq5, rbq6 and rbq7* in disease tests on the set of BILs, but not in the original F_2 population (*L. saligna* x *L. sativa*). Earlier, it was reported that the failure of detection of *rbq4* and *rbq6* was probably due to the combination of recessiveness of the traits with skewed segregation against the recessive wild species alleles (Jeuken et al, 2008). Results from the present study indicate that the failure of detection of *rbq5* and *rbq7* in the F_2 population might be due to the genetic complexity of these introgression segments, i.e. presence of multi-QTL.

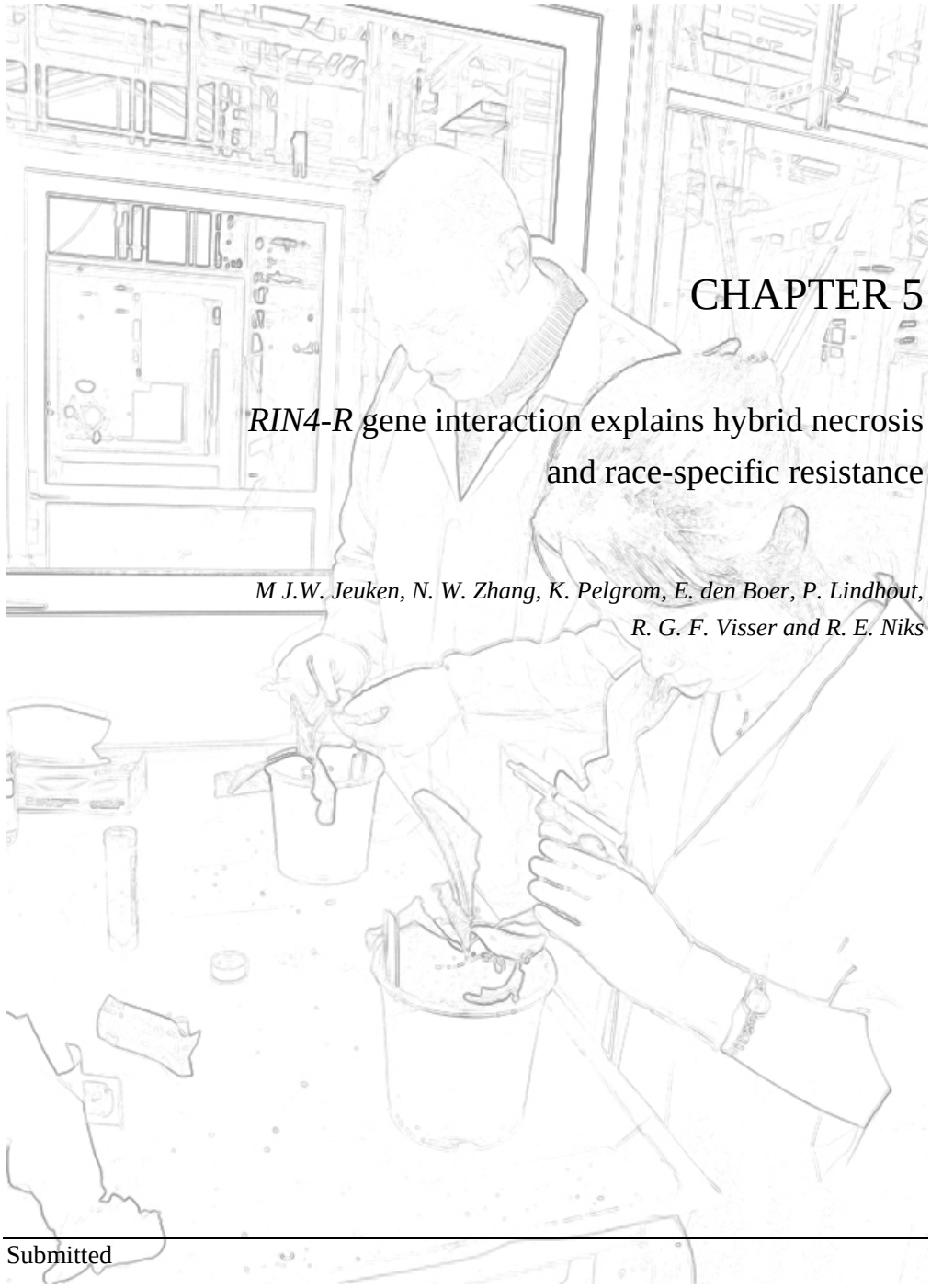
Recombination suppression

The recombination frequencies in the BILs were suppressed at different levels (2 to 15 times) compared to that in the F_2 population for the same chromosomal region (Table 3). Such recombination suppression was discovered in introgressed segments from wild tomato species (Brouwer and St Clair 2004; Paterson et al. 1990), but not in intra-specific NILs of maize (Graham et al. 1997) and rice (Wissuwa and Ae 2001). The reduction of recombination in inter-specific cross offspring may be explained by reduced reciprocal exchange, together with positive interference (Paterson et al. 1990). Recombination suppression was more severe when the introgressed segment was smaller (Brouwer and St Clair 2004).

Our plant materials were derived from an inter-specific cross and the two parental lines, *L. sativa* and *L. saligna*, have great sequence divergence. This resulted in the recombination suppression in the subsequent backcross generations compared to the F_2 population (Jeuken and Lindhout 2004). Recombination suppression increases the difficulties to identify recombinants in the target introgression region. Therefore, we need a large segregating population to identify sufficient recombinants and to further fine map the QTLs by development of sub-NILs. However, our introgression segments with single-QTL only had 2x and 7x recombination suppression compared to the ones with multi-QTL that had 12x and 15x suppression. This offers opportunities for cloning the single-QTLs.

Breeding perspectives

In the present study, the NILs carrying single-QTL, *rbq4* and *rbq6*, are very useful genetic stocks to be exploited in resistance breeding programs. (1) These two recessive QTLs show high level resistance to at least seven *B. lactucae* races at both young and adult plant stages (Jeuken et al, 2008 and Chapter 2). (2) The resistance alleles of *rbq4* and *rbq6* also showed epistatic effects that led to increased resistance level when combined with each other (chapter 3, this thesis). (3) Different resistance mechanisms were postulated for these two QTLs based on the histological studies (chapter 3, this thesis). (4) The fine-mapped intervals containing these two QTLs did not carry genes conferring undesired morphological effects as far as we see (Figures 1 and 3). (5) The simple PCR-markers presented in this study are useful diagnostic markers in practical breeding programs for MAS. Therefore, further characterization of these two loci will provide more information and materials for breeders to incorporate high level and likely durable downy mildew resistance into lettuce cultivars.



CHAPTER 5

RIN4-R gene interaction explains hybrid necrosis and race-specific resistance

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RIN4-R gene interaction explains hybrid necrosis and race-specific resistance

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Abstract:

Among other things evolutionary plant biology studies the mechanisms that underlie the birth and perpetuation of plant species. Speciation requires gene flow barriers that reduce or prevent hybridization between diverging plant populations derived from a common ancestor species. A well-described postzygotic barrier is 'hybrid necrosis'. Earlier studies indicated that hybrid necrosis is mostly due to an interaction between alleles in a two-locus system, in which one of the loci encodes a resistance (R-) (like) gene. Here we show a case in lettuce hybrids where hybrid necrosis involves an interaction between *RIN4* and what an *R* gene is probably, resulting in autoimmunity and race-specific resistance. A molecular and genetic model is proposed. In *Arabidopsis*, RIN4 is known to interact with an *R*-gene product, where their disturbed interaction results in hypersensitive resistance. The present case indicates that RIN4 may play a role in establishing interspecific hybridization barriers between closely related plant taxa.

Introduction

During evolution, ancestral plant species can diverge into several derived species that become genetically isolated from each other due to pre- and postzygotic barriers, reducing the capacity for hybridization (Rieseberg and Willis 2007).

One of the best described postzygotic barriers is 'hybrid necrosis', having been recognized for more than eight decades (Bomblies and Weigel 2007). This type of genetic incompatibility is manifested as necrotic lesions in seedlings or adult plants and is often associated with phenotypes like wilting, chlorosis, stunted growth and lethality. Hybrid necrosis has been reported in several interspecific and some intraspecific plant crosses, and its genetic architecture is explained in a two-locus system in the classical Bateson-Dobzhansky-Muller model reviewed in (Bomblies and Weigel 2007). Studies on interspecific hybrids in tomatoes and intraspecific hybrids in *Arabidopsis* imply that hybrid necrosis may result from changes in resistance genes (*R*-genes) that induce autoimmunity-like responses when combined with a particular allele of a gene elsewhere in the genome (Bomblies et al. 2007; Krüger et al. 2002; Wulff et al. 2004).

We detected an interesting example of hybrid necrosis in the progeny of cultivated lettuce (*Lactuca sativa*) and a wild lettuce species, *L. saligna.* The latter is a nonhost species to *Bremia lactucae*, lettuce downy mildew (Bonnier et al. 1992). Both species are autogamous and therefore homozygous. In order to elucidate the inheritance of this nonhost resistance, we made interspecific crosses between *L. saligna* and *L. sativa*. We developed two F_2 populations (populations A and B) based on different *L. saligna* accessions and *L. sativa* cultivars and a set of 29 Backcross Inbred Lines (BILs) with single *L. saligna* introgressions into a *L. sativa* genetic background (Jeuken et al. 2001; Jeuken and Lindhout 2004; Jeuken et al. 2008).

From nonhost related experiments on these hybrid plant materials we observed three possibly correlated traits - necrotic lesions on leaves, lethality, and resistance to downy mildew - which all mapped to the same locus and led to our assumption of hybrid necrosis.

Of these three traits, the first one that we detected was *R*-gene-like resistance from the wild lettuce species in the two F_2 populations (formerly designated *R39*) that mapped to Chromosome 9 (hereafter referred to as C9, See Figure S1). Introgression of this C9 segment into a BIL (in the cultivated lettuce background) resulted only in plants that were heterozygous, and not homozygous, for the C9 introgression. Lethality was also observed. These plants heterozygous for the introgression (designated preBIL9.1, 'pre'-suffix indicates that the introgression is still heterozygously present) were resistant to both *B. lactucae* race

Bl:16 and Bl:14, while in both F_2 populations plants heterozygous for the C9 introgression were resistant to Bl:16 and susceptible to Bl:14. This result constituted a remarkable discrepancy between F_2 and preBIL9.1 in the specificity of the resistance.

PreBIL9.1 plants showed a remarkable phenotype of necrotic lesions on leaves and stem and retarded growth, especially in winter (Figure 1). This observation suggested a possible temperature-dependent phenotype.

Seedlings homozygous for the *L. saligna* C9 introgression (BIL9.1) did arise but were extremely necrotic and died within a week (Figure 1). The apparent lethality effect for BIL9.1 plants corresponded to the presence of only a few plants that were homozygous for the *L. saligna* C9 introgression in the F_2 populations (severe distorted segregations with preference for *L. sativa* alleles for this C9 segment (Jeuken et al. 2001). Since, of course, the *L. saligna* alleles at the C9 locus are not lethal in a pure *L. saligna* background, the lethality of BIL9.1 plants should be due to one or more *L. sativa* alleles elsewhere on the genome that epistatically interact with the *L. saligna* allele at the C9 locus, causing extreme necrosis that leads to lethality.

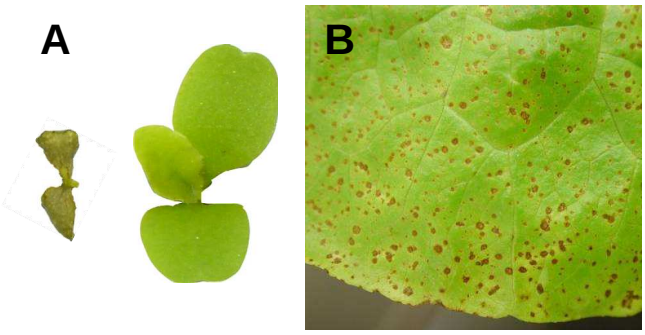

Figure 1. 'Hybrid necrosis' phenotypes in lettuce. (A) Completely necrotic seedling (6A9B) unable to survive compared to normal seedling phenotype (6A9A) grown at 15ºC. (B) Detail of leaf with a high density of necrotic lesions in 6 weeks old plants of preBIL9.1 (6A9H) grown under greenhouse conditions.

All these observations of necrotic lesions, lethality and resistance resemble previously described cases of hybrid necrosis (Bomblies and Weigel 2007) and its association with resistance, and indicate a genetic incompatibility due to the interaction of two or more loci.

Here we provide evidence for the identity of two interacting loci and their candidate genes. We furthermore show that there is a relation between hybrid necrosis and the resistance response, and we propose a genetic and molecular model to explain this relation.

Results

Two epistatic loci are involved in hybrid necrosis

To determine the epistatic loci for hybrid necrosis, we focused on the "lethality" of plants that were homozygous for the *L. saligna* C9 introgression in a *L. sativa* background. We analyzed the genotypes of the F_2 populations *(L. saligna* x *L. sativa* crosses) by sorting for individuals that were homozygous *L. saligna* at the top of C9 (*R39* locus), and we found one such genotype in cross A and seven such genotypes in cross B (See Table S1, panel A). All eight plants were either homozygous *L. saligna* type or heterozygous for a region from about 37 to 42 cM on Chromosome 6 (hereafter referred to as C6, Table S1 panel A). This result suggested an interactive role for the *L. sativa* allele in this C6 segment in inducing necrosis leading to lethality and absence of this interaction when at least one *L. saligna* allele is present at this C6 locus. This C6 locus segregated normally (1:2:1) in the F_2 populations (α=0.05 cross A, α=0.01 cross B).

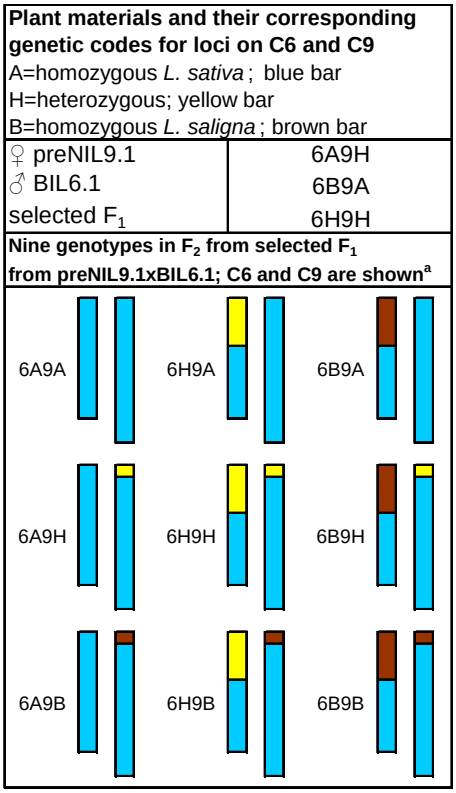

Figure 2. Genetic nomenclature of genotypes
[a] C6 and C9 are Chromosome 6 and 9; all other chromosomes are homozygous *L. sativa* Olof; The C6 and C9 introgressions are 40 and 11 cM long respectively.

To corroborate a role for C6 in hybrid necrosis, we crossed preNIL9.1 and BIL6.1 that cover the putative epistatic loci (BIL6.1 has an introgression from 0-42 cM on C6). We used preNIL9.1 plants instead of preBIL9.1 plants because preNIL9.1 plants have identical phenotypes for hybrid necrosis and show resistance to *B. lactucae*, but they have a smaller heterozygous C9 segment (0-11 cM) than preBIL9.1 (0-48 cM).

The F_2 progeny from a selected F_1 plant (heterozygous at C6 and C9 loci) segregated, and by DNA marker analyses we identified nine genotype classes for which we introduce an

appropriate nomenclature (See Figure 2). We screened for F_2 plants that were homozygous *L. saligna* for the top of C9. Besides detecting seedlings of 6A9B genotype that shriveled, became necrotic and died after a week, we also detected adult plants with the genotypes 6B9B and 6H9B with a normal and a slightly necrotic phenotype, respectively.

Similar phenotypes and a similar segregation occurred in the progeny of a BC_4S_1 line from cross B (backcrosses to *L. sativa*) that had a 6H9B genotype. These results demonstrated that for both *L. saligna* accession/ *L. sativa* cultivar combinations, the lethality due to the homozygous *L. saligna* C9 segment was nullified when at least one *L. saligna* C6 allele was present.

Seeds were collected from the eight different F_2 genotypes resulting from preNIL9.1 x BIL6.1 cross A; the ninth, 6A9B, was lethal after one week (Figure 2). Plants from six genotypes were further analyzed for their phenotypes compared to the original parental lines *L. sativa* cv. Olof and *L. saligna* CGN05271 (6A9B plants until they died). Of the six genotypes, four contained at least one C6-C9 allele pair that should lead to at least some degree of necrosis (6A9B, 6A9H, 6H9B and 6H9H) and two representative lines are expected to lack a necrotic interaction (6B9A and 6B9B). Macroscopic symptoms of hybrid necrosis were extreme for genotype 6A9B, severe for 6A9H, less severe for 6H9B, and nil for 6H9H (*L. sativa* cv. Olof served as reference, Figure 3). The level of necrosis was negatively correlated with the dry weight of the plants. We conclude that macroscopic necrosis only occurred in plants that carry two *L. sativa* alleles at the C6 locus and at least one *L. saligna* allele at the C9 locus, or at least one *L. sativa* allele at C6 and two *L. saligna* alleles at C9. The most extreme hybrid necrosis phenotype of the lethal seedlings of genotype 6A9B was explained by two *L. sativa* alleles at C6 interacting with two *L. saligna* alleles at C9.

For cross B we observed macroscopically-similar necrosis phenotypes and a reduced growth rate for 6A9B and 6H9B. Therefore, in cross B the same epistatic interaction for hybrid necrosis occurred as in cross A.

Hybrid necrosis is temperature sensitive

Our earlier observations of less severe macroscopic necrosis phenotypes in summer than in winter suggested that hybrid necrosis was temperature sensitive. Therefore, we tested the genotypes at 15°C and 30°C. The 6A9B genotype, which died quickly after germination at 15°C, survived at 30°C without any necrosis and grew at the same rate as *L. sativa* Olof (Figure 4). At 30°C the genotypes 6A9H and 6H9B also grew at similar rates as *L. sativa* Olof and without necrosis, while at 15°C they showed reduced growth and hybrid necrosis.

Plant material				Hybrid necrosis			Resistance levels					
		genotype[k]		macrosc.	microsc.	dry	T=17°C [h]		T=19°C [i], 6 hr ↑ 24C			
specific name	genetic code	C 6	C 9	necrosis level	necrosis level[a]	weight[c]	rAUDPC[df] Bl:14	rAUDPC[f] Bl:16	rAUDPC[eg] Bl:14	rAUDPC[eg] Bl:16	IS[j] Bl:14	IS[j] Bl:16
	6A9B			+++ lethal[b]	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
preNIL9.1	6A9H			++	12.28 a	0.4 a	0.03 a	0.00 a	0.24 a	0.00 a	21	0
	6H9B			+	0.72 b	9.2 b	0.32 ab	0.08 a	1.02 b	0.04 a	82	2
	6H9H				0.50 bc	13.3 c	0.81 abc	0.25 a	1.10 bc	0.15 a	82	6
BIL6.1	6B9A				0.39 bc	13.9 c	2.68 e	2.35 c	1.24 bc	1.81 c	89	99
	6B9B				0.39 bc	12.6 c	1.35 cd	1.13 b	1.30 c	1.88 c	96	98
Lactuca sativa					0.01 c	13.2 c	1.00 bc	1.00 b	1.00 b	1.00 b	80	58
Lactuca saligna					0.29 bc	15.6 c	0.00 a	0.00 a	0.00 a	0.00 a	0	0
BIL4.4					N.D.	N.D.	2.11 de	1.49 b	1.21 bc	1.88 c	92	98

Figure 3. Hybrid necrosis and resistance levels

Letters in common within a column indicate that the values are not significantly different (α=0.05, Tukey HSD procedure).

[a] Percentage of necrotic leaf area. Per genotype 2 leaf segments x 3 plants were examined.

[b] Seedling gets completely necrotic and shrivels after several days

[c] Dry weight in grams from 11 week old plants grown in the greenhouse (n=7).

[d] Relative AUDPC from YDT in climate cell calculated from observations for infection severity at 8, 10 and 12 dpi. *L. sativa* Olof was set at 1.00.

[e] Relative AUDPC from YDT in greenhouse calculated from observations for infection severity at 8, 9, 10 and 11 dpi. *L. sativa* Olof was set at 1.00.

[f] Similar results were observed with disease tests with race Bl:14 on cotyledons of these genotypes.

[g] Similar results were observed with four disease tests with race Bl:14 and Bl:16 on detached leaf discs from 9 and 12 week old plants of these genotypes.

[h] In the climate chamber with day/ night cycles of 19°/12°C (block intervals) and an average temperature of 17°C.

[i] In the greenhouse with an average temperature of 19°; minimal night temperature of 15°C; and the day temperature was 6 hours above 24°C and 12 hours above 22°C.

[j] Average Infection Severity level of downy mildew at 11 dpi on young plant (YDT) scored as percentage of leaf area showing sporangiophores.

[k] graphical genotype of C6 and C9; blue means homozygous *L. sativa*, brown means homozygous *L. saligna* and yellow is heterozygous.

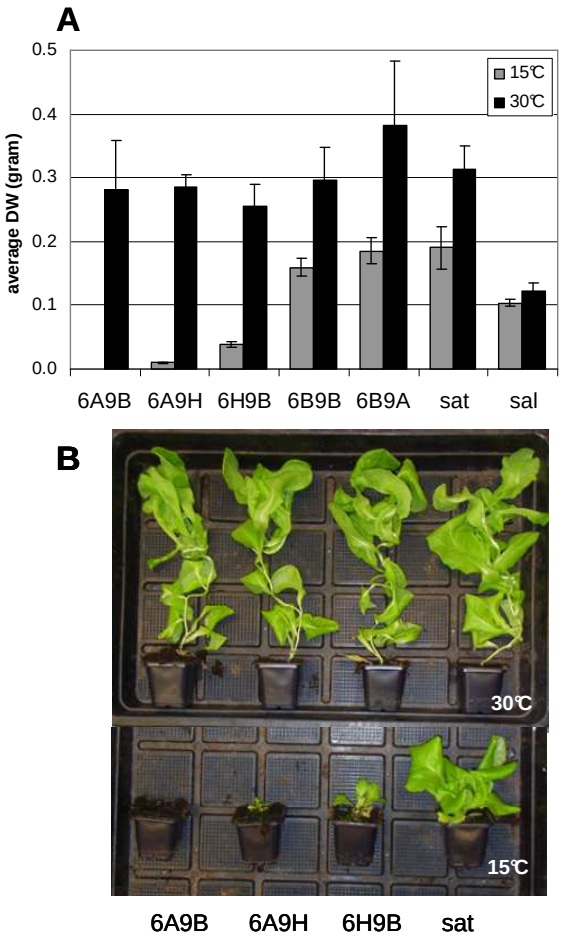

Figure 4. Temperature sensitivity of hybrid necrosis
(A) Average dry weight (n=5, 45 days old); sat= *L. sativa* cv. Olof (6A9A), sal = *L. saligna* (6B9B); 95% confidence intervals are shown. (B) 7 weeks old C6C9-genotypes, grown at 15°C and 30°C

After seven weeks, we transferred a selection of plants (6A9B, 6A9H, 6A9A and 6B9B) grown at 30°C to 15°C. The first symptoms of hybrid necrosis appeared as brown, necrotic lesions in the youngest leaf at 48 hours for 6A9B and at 80 hours for 6A9H, especially close to major veins (Figure S2). All leaves of 6A9B plants started to wilt after five days and shriveled, becoming completely necrotic after eight days. For 6A9H plants similar symptoms appeared but at a slower rate. Most leaves showed necrotic spots after eight days and were completely necrotic and shriveled after twenty days (Figure S3).

Hybrid necrosis and resistance level correlate

Earlier observations in the F_2 population and in preBIL9.1 plants suggested a relation between hybrid necrosis (necrotic lesions and lethality) and resistance (see introduction). Hybrid necrosis is explained by two loci (on C6 and C9) as described above, and this explanation could be expected for the associated resistance as well. However, QTL mapping for resistance to *B. lactucae* with the original F_2 data (Jeuken et al 2001, Figure S1) only resulted in one race specific resistance QTL effective to Bl:16 but not to Bl:14, namely at C9 and no such QTL at C6.

To investigate whether there is a relationship between the resistance response and hybrid necrosis we performed disease tests with two downy mildew races on the same C6C9-

genotypes as used in the experiment for hybrid necrosis phenotyping. The results of the disease tests were very similar between the three plant stages: seedling, young plant and adult plant. The results for young plants are shown in Figure 3.

Most surprisingly, genotype 6B9B was susceptible to both downy mildew races, indicating that the *L. saligna* C9 introgression does not lead to resistance *per se*. A discrepancy in the resistance specificity against races Bl:16 and Bl:14, as observed for the F_2 and preBIL9.1 (6A9H), was observed again, since genotype 6H9H was only resistant to Bl:16 and 6A9H was resistant to both Bl:16 and Bl:14.

A complete race-specific resistance to downy mildew race Bl:16 was only observed in genotypes 6A9H, 6H9B and 6H9H, namely in the presence of at least one *L. sativa* allele on C6 and at least one *L. saligna* allele on C9. One possible explanation for this race-specific resistance to Bl:16 is the combination of an *R*-gene and a gene "required for resistance", one from *L. sativa* and the other from *L. saligna*. However at present we cannot conclude which gene is located on C6 and which on C9.

The three genotypes 6A9H, 6H9B and 6H9H showed infection severity levels to race Bl:14 that ranged from very low to high to almost the same level as the susceptible parent *L. sativa* Olof at 17° C (Figure 3). With a 2ºC higher growth temperature, the Bl:14 infection severity levels of all genotypes rose significantly, which resulted in only 6A9H still being strongly quantitatively resistant, while 6H9B and 6H9H became as susceptible as *L. sativa* Olof (Figure 3). The resistance to Bl:14 depended on the number of interactive alleles at C6 and C9 and also on the temperature, paralleling the level of hybrid necrosis (necrosis, reduced dry weight) observed in uninoculated plants (Figure 3). Consequently, we hypothesized that the interaction between an *R* gene and a gene "required for resistance" triggered a Hypersensitive Response both in the presence and absence of pathogen attack (=an autoimmune like response). This quantitative autoimmune-like resistance response was overshadowed in the tests with race Bl:16 by the complete character of the race-specific resistance. At least one *L. sativa* allele on C6 with at least one *L. saligna* allele on C9 was sufficient to give strong resistance to Bl:16.

The correlation between hybrid necrosis, the autoimmune-like resistance response and the race-specific resistance response to *B. lactucae* is consistent with a hypothesis that all phenomena depend on the same interactive alleles at the C6 and C9 loci (Figure S1).

In the F_2 population of *L. saligna* x *L. sativa*, we detected only one peak LOD value for race-specific resistance at the C9 locus and none at the C6 locus (Jeuken and Lindhout 2002) (Figure S1). In hindsight, this absence of a peak LOD value on C6 in this specific F_2 can be explained by the differences being very small, between the three genotype classes for the C6 locus caused by the distorted segregation of the C9 locus (Table S1, panel B). Normal

segregation at the C9 locus would have resulted in a peak LOD value for resistance to Bl:16 at the C6 and C9 loci (Table S1, panel C).

***RIN4* is the candidate gene on C9**

EST marker LE0478 was most closely associated with the resistance to *B. lactucae* Bl:16 in the F_2 population, and it was also located in the *L. saligna* C9 introgression of preNIL9.1 (Figure S1). Primer design for LE0478 was based on a contig of lettuce EST sequences (QG_CA_Contig7104 in CGP1 database by The Compositae Genome Project, http://compgenomics.ucdavis.edu). This contig showed the highest homology with At3g25070 in a blastx search against the *Arabidopsis* TIGR database (predicted ORFs) of unigene set, and is known as RIN4, RPM1 INTERACTING PROTEIN 4. RIN4 is one of the first and best studied molecules that provide concrete support of the "guard" function of an indirectly working *R* gene. RIN4 is a 211 amino acid, acylated, plasma membrane associated protein in *Arabidopsis*, being a negative regulator of basal defense and a virulence target for three effectors from *Pseudomonas syringae* (AvrRpm1, AvrB and AvrRpt2), which is "guarded" by two independent R proteins (RPM1 and RPS2) (Axtell and Staskawicz 2003; Ingle et al. 2006; Mackey et al. 2003; Mackey et al. 2002).

We obtained the full cDNA sequences of the RIN4 alleles from our parental lines *L. sativa* Olof and *L. saligna* CGN5271 and from the wild lettuce species *L. virosa* CGN05978. From each Lactuca species we detected two RIN4 transcript versions with different lengths of 735 and 732 base pairs long (from start to stop codon, named Transcript1 and Transcript2), due to a CAG indel at base pair position 705; the deduced amino acid sequences are 244 and 243 amino acids long. By sequencing the genomic DNA we detected only one gene version in *L. sativa* and *L. saligna that* with the CAG sequence. An alternative splicing event seems responsible for the two transcript versions (see Figure S4). The deduced RIN4 amino acid sequences showed six amino acid differences between *L. sativa* and *L. saligna* (Figure 5, panel A). *L. virosa* contained at the six mentioned specific polymorphic sites four times the same amino acid as *L. sativa* and two times the same amino acid as *L. saligna* (Figure 5, panel A).

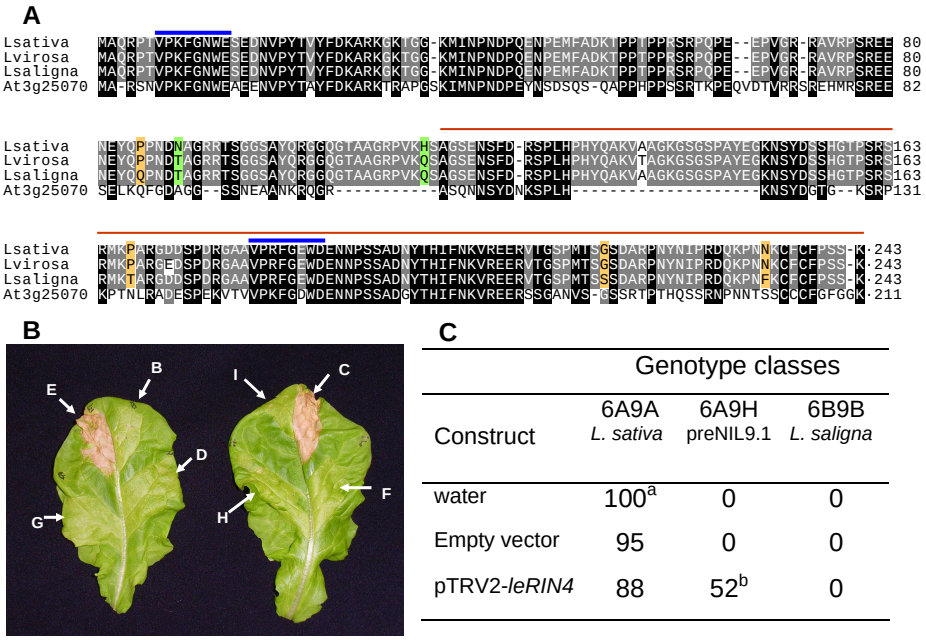

Figure 5. *RIN4* is the candidate gene on C9

(A) RIN4 homologues from *L. sativa*, *L. saligna*, *L. virosa* and *A. thaliana* (At3g25070). The deduced amino acid sequences of lettuce *RIN4* transcript2 were aligned using the Clustal W software. Transcript1 has one extra amino acid, a glutamine, Q, between the lysine, K, and the cysteine, C, at positions 236 and 237 (not shown). Conserved residues between *A. thaliana* and *Lactuca* species are shaded in black, conserved residues within *Lactuca* species are shaded in grey. Orange and green shaded amino acids highlight amino acid differences between *L. saligna* and *L. sativa*, while *L. virosa* has at this position the same amino acid as *L. sativa* or *L. saligna*, respectively.

The blue lines represent AvrRpt2 cleavage sites in *RIN4* of *A. thaliana* (Chisholm et al. 2005). The red line marks the DNA sequence that was engineered into pTRV2 vector for VIGS silencing experiments.

(B) Transient expression with *RIN4* alleles/ transcripts infiltrated in *L. sativa* cv. Olof (8 days post infiltration). Per genotype class, 5 plants x 2 leaves. Alleles: sat= *L. sativa*, sal= *L. saligna* and vir= *L. virosa*. T1 and T2 are *RIN4* transcript versions 1 and 2. Infiltrations: B= empty vector, C= PsojNIP, D= RIN4salT1, E= RIN4salT2, F= RIN4satT2, G= RIN4satT1, H= RIN4virT1 and I= RIN4virT2

(C) Relative Infection Severity (RIS) to *B. lactucae* Bl:16 of *RIN4* silenced lettuce genotypes by VIGS. [a] The absolute infection severity of 6A9A, *L. sativa* cv. Olof, was 82% at 10 dpi in Young plant Disease Test. [b] The infection severity was estimated for the entire leaves, although we could not observe whether the entire leaf was silenced for *RIN4*. Therefore the presented RIS value may underestimate the effect of the silencing.

The *RIN4salT2* allele causes necrosis

To examine whether one or both RIN4 proteins from *L. saligna* were directly involved in the hybrid necrosis, *Agrobacterium*-mediated transient assays were executed to over-express different *RIN4* transcripts. Transcript2, but not Transcript1, of the *L. saligna* allele of *RIN4* (*RIN4salT2*) caused a severe necrotic reaction in *L. sativa* plants harboring the *L. sativa* allele at the C6 locus (Figure 5, panel C and Table S2). Neither of the *RIN4* transcripts of *L. sativa* and *L. virosa* caused necrosis. As the amino acid sequence of the *L. virosa* RIN4 protein is intermediate between *L. sativa* and *L. saligna*, the polymorphic amino acids in the *L. saligna* RIN4 protein are now reduced from six to four (Figure 5, panel A).

Silenced RIN4 impairs resistance

To validate the involvement of *RIN4* in the hypersensitive resistance response of different C6C9 genotypes to *B. lactucae*, we reduced *RIN4* transcript levels by VIGS and challenged these plants with *B. lactucae* Bl:16. The silencing of *RIN4* did not cause abnormal plant phenotypes in any of the tested genotypes. Silencing of *RIN4* rendered resistant preNIL9.1 (6A9H) susceptible (Figure 5, panel C). This result confirms that *RIN4* is involved in the *R* gene mediated resistance reaction.

We propose that the candidate gene on the C9 introgression is the *L. saligna* allele of *RIN4*, which is known to be a virulence target and is guarded by R genes in *Arabidopsis*. Therefore *RIN4* represents the gene that is "required for resistance", and is also a partner in the two-locus interaction leading to hybrid necrosis.

***R* gene may be the candidate gene on C6**

As the candidate gene on the C9 locus is the *RIN4* gene, we presume that the candidate gene at C6 is most likely an *R* gene from *L. sativa*. There are no resistance genes against downy mildew or any other pathogen yet mapped in lettuce on C6 (our Chromosome 6 corresponds with linkage group 8 of the integrated lettuce map (Truco et al. 2007). The smallest introgression region on C6 associated with hybrid necrosis lies between marker E35M48-399_b at 37 cM and marker E54M48-154_b at 43.5 cM (Figure S1). In the search for candidate *R* genes we selected *RGC3* and *RGC4*, which have been reported as resistance gene candidates by PCR with degenerate primers based on the *Dm3* gene against downy mildew (Shen et al. 2002). *RGC3* and *RGC4* do not map to any of the four resistance clusters in lettuce and have been reported to co-segregate with markers that map at a linkage group that corresponds to our Chromosome 6 (Shen et al. 2002). PCR markers, developed for *RGC3* and *RGC4* (GenBank accession numbers AF017753 and AF017754) mapped to C9 32 cM and C6 62 cM, respectively, i.e. not in or near our C6 locus, 37-42 cM.

Genetic and molecular model

The results on hybrid necrosis and the resistance level differences to both *B. lactucae* races between C6C9 genotypes are visualized in our proposed genetic model (Figure 6). Analogous to RIN4 in *Arabidopsis*, we assume that in lettuce the RIN4 protein is also guarded by an R protein (gene on C6) and that RIN4 is a target for pathogen effectors that can modify it (e.g. by phosphorylation or degradation as in *Arabidopsis- P. syringae*). This R-RIN4 protein communication can be influenced by sequence variants of the partner molecules or by the action of a pathogen effector resulting in the Hypersensitive Response.

For our model we introduce the definition “guarding capacity”, which refers to the capacity of an R protein to watch over its target protein, perceive any modifications and trigger a Hypersensitive Response.

In the present case, two aspects of hybrid necrosis are still unsolved and need a molecular explanation: 1) The gene dose of interacting alleles at the C6 and C9 loci parallels the level of autoimmunity (necrosis, reduced dry weight, quantitative resistance to Bl:14), but of the two genotypes with three interacting alleles, 6H9B has a considerably lower autoimmunity level than 6A9H; 2) The genotype with two interacting alleles, 6H9H, shows race-specific resistance to Bl:16, but does not show any level of autoimmunity.

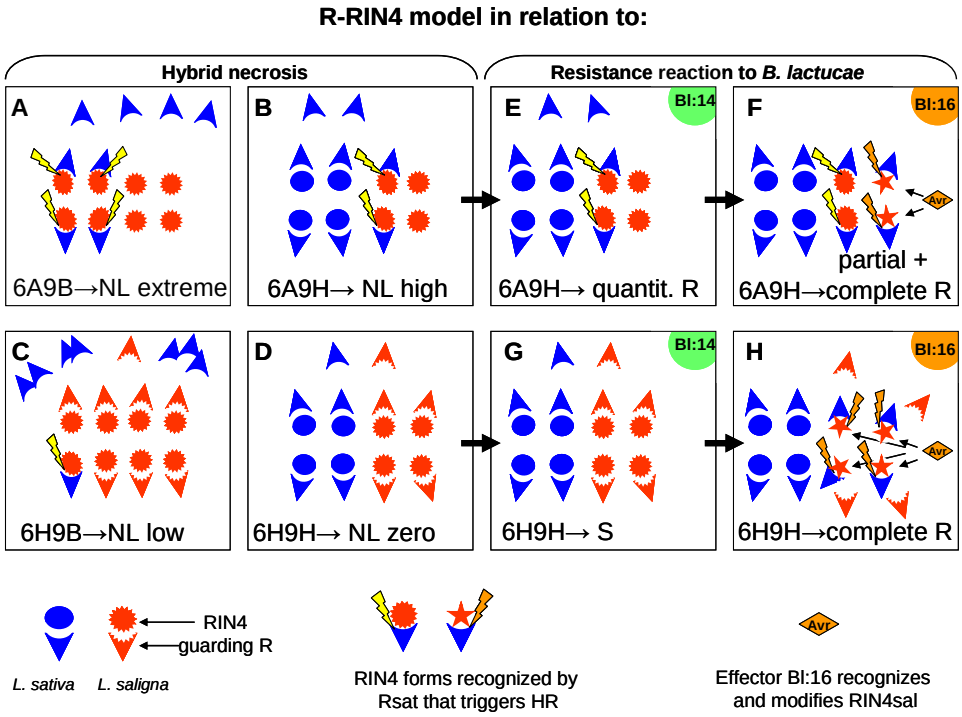

Figure 6. Proposed genetic and molecular model for hybrid necrosis and resistance to *B. lactucae* in lettuce (at lower temperatures < 30°C).
Abbreviations: "sat" and "sal" refer to *L. sativa* and *L. saligna* alleles or protein versions; "NL" means necrosis level; "S" means susceptible, "R" means resistant, "quantit. R" means quantitative resistant.
Genotype codes A= homozygous *L. sativa*, H= heterozygous, B= homozygous *L. saligna.*
Assumptions for the model: 1. There is an abundance of R protein to guard its target RIN4. 2. "guarding capacity"= the capacity of an R protein to watch over its target protein, perceive any modifications and trigger a Hypersensitive Response .To explain the lower hybrid necrosis level of 6H9B (Rsat+Rsal ↔RIN4sal) compared to 6A9H (Rsat ↔RIN4sat+RIN4sal), the guarding capacity of Rsal for RIN4sal must be higher than of Rsat. If the guarding capacities of Rsat for both RIN4 alleles were the same, the same necrosis level would be expected for 6A9H and 6H9B.
Proposed guarding capacities to illustrate this hypothesized model are - RIN4sat is guarded best by its own Rsat and RIN4sal is guarded best by its own Rsal, both a 100% guarding capacity; and - RIN4sal can be guarded by Rsat with a 50% guarding capacity.

R-RIN4 model in relation to hybrid necrosis: In *L. sativa* and *L. saligna* RIN4 proteins are guarded by their native R proteins, no modifications are sensed, and no activation of HR is triggered (not illustrated) **A**: Half of all RIN4 proteins is sensed by Rsat and HR is activated extremely, visible as extreme and lethal hybrid necrosis. **B**. A quarter of all RIN4 proteins are sensed by Rsat and HR is activated visibly as severe hybrid necrosis. **C**. Majority of RIN4sal is guarded by its own R proteins due to the higher binding capacities. Minority of RIN4sal is sensed by Rsat and HR is activated, visible as light hybrid necrosis (16 instead of 8 R proteins were drawn here, to better illustrate the result of guarding capacity differences) **D**. RIN4sat and RINsal are guarded by there own R proteins.

R-RIN4 model in relation to resistance reaction: **E.** The autoimmunity induces constant HR (See B) and quantitative resistance to Bl:14. **F**. An effector of Bl:16 recognizes and modifies RIN4sal (and not RIN4sat), which is sensed by the R gene, leading to activation of local hypersensitive response resulting in complete resistance to Bl:16, masking the quantitative autoimmune-like resistance response illustrated in E. **G**. The plant is susceptible as its infection is not stopped by any form of HR. **H.** An effector of Bl:16 recognizes and modifies RIN4sal (and not RIN4sat), which is sensed by the R gene, leading to activation of local hypersensitive response resulting in complete resistance to Bl:16.

We have developed a hypothetical molecular model to explain these two aspects. We hypothesize that aspects 1 and 2 are only explicable if all three of the following assumptions hold true: 1) there is an abundance of R protein to guard its target RIN4 in all genotypes; 2) the guarding capacity of an R protein for its native RIN4 is higher than for a nonnative RIN4; and 3) only the *L. saligna* RIN4 is modified by a Bl:16 effector, and only the *L. sativa* R perceives this modified RIN4 and triggers a HR.

The higher guarding capacity of *L. saligna* R for *L. saligna* RIN4 than of *L. sativa* R explains the lower autoimmunity level for 6H9B. (Figure 6, panels B and C). If the guarding capacities of *L. sativa* R for both *L. sativa* and *L. saligna* RIN4 proteins were the same, the same necrosis level would have been expected for 6A9H and 6H9B.

The higher guarding capacity of *L. sativa* R for its native RIN4 than for the *L. saligna* RIN4 explains the absence of an autoimmune like response in 6H9H (Figure 6, panel D).

Discussion

Earlier studies showed that hybrid necrosis cases are mostly due to two interacting genes, causing some autoimmune-like responses (Bomblies and Weigel 2007). In at least two cases, one of the interacting genes was demonstrated or suggested to be an *R* gene (Bomblies et al. 2007; Krüger et al. 2002). In the present study on lettuce, the interacting pair of genes is a *L. sativa* allele on C6 interacting with a *L. saligna* allele on C9. The C9 gene was demonstrated to be *RIN4*, most probably interacting with an *R* gene on C6.

In the absence of infection, the *R* gene interacting with the *L. saligna* allele of *RIN4* tends to trigger an autoimmune-like response macroscopically visible as the hybrid necrosis phenotype. This autoimmune-like response causes race-non-specific resistance to *B. lactucae* (demonstrated for Bl:14). The severity of the autoimmune-like response depends on the gene dose of interacting alleles and the presumed higher guarding capacity of an R protein for its native RIN4 than for a nonnative RIN4 (See the genetic and molecular model in Figure 6).

In the presence of infection, this *R*-gene apparently requires the *RIN4* allele of *L. saligna* to confer complete HR resistance to Bl:16. We assume that a pathogen effector recognizes and modifies RIN4sal (and not RIN4sat), which is sensed by the *L. sativa* R protein, leading to the activation of a complete local HR resistance to Bl:16. Such indirect interaction between a pathogen effector and an R-protein via RIN4 was demonstrated for AvrRpm1 and AvrB versus RPM1, and AvrRpt2 versus RPS2, in the *Pseudomonas syringae- Arabidopsis* pathosystem (Axtell and Staskawicz 2003; Mackey et al. 2003; Mackey et al. 2002).

The R protein in lettuce may guard and interact with its endogenous *L. sativa* RIN4 to protect the plant from some pathogen, but not from the pathogen *B. lactucae* (at least not the known and described races Bl:1-Bl:24, Table S3).

A difference of only a few amino acids between *L. saligna* RIN4 and *L. sativa* RIN4 was sufficient to initiate interaction between R and RIN4. We hypothesize that during the speciation of a common *Lactuca* ancestor into *L. sativa* and *L. saligna*, this R-RIN4 complex

has diverged. In *L. saligna*, a Bl:16-specific effector modifies RIN4, but the R protein does not recognize this modification anymore. As *L. saligna* possibly developed into a non-host species, relying on a different resistance mechanism explained by QTL rather than *R* genes, there was no selective advantage for a functional *R* gene (Jeuken and Lindhout 2002; Jeuken et al. 2008) (Chapter 2). In *L. sativa*, a Bl:16-specific effector either does not modify RIN4 or it modifies RIN4 in an unperceivable way; consequently RIN4 is not recognized by its native *R* gene. The divergence of this R-RIN4 complex has resulted in a genetic incompatibility in the form of hybrid necrosis.

This R-RIN4 complex seems conserved in *L. sativa* and *L. saligna* as the same hybrid necrosis was demonstrated for two different parent pairs (cross A and B). More knowledge about the allele frequency of the *R* gene and about polymorphisms in *RIN4* alleles in the Lactuca germplasm will give us more insight into the events leading to this diversification and the molecular evolution of the R-RIN4 complex.

In *Arabidopsis* RIN4 has been demonstrated to be a negative regulator of basal defense and a target for pathogen effectors. Our study demonstrated that RIN4 may also contribute to speciation by its involvement in hybrid necrosis.

Experimental procedures

Material

Lettuce material

L. sativa and *L. saligna*

Two crosses were made between wild lettuce *L. saligna* and cultivated lettuce *L. sativa*- *L. saligna* CGN05271 x *L. sativa* cv. Olof (cross A) and *L. saligna* CGN11341 x *L. sativa* cv. Norden (cross B). From both crosses the F_1 was selfed and backcrossed to its recurrent *L. sativa* parent. The selfed F_1 resulted in an F_2 population of 126 and 54 plants from crosses A and B, respectively. The genetic linkage map and infection severity levels in adult plants to downy mildew in the F_2 populations were described in (Jeuken and Lindhout 2002; Jeuken et al. 2001). Both BC_1 populations were further backcrossed with the respective cultivated *L. sativa* parent until the BC_4 generation. For cross B, we selfed the BC_4 and genotyped the BC_4S_1. For cross A we developed a set of Backcross Inbred Lines (BILs) from the $BC_4S_{1\text{-}2}$ and $BC_5S_{1\text{-}2}$ by Marker Assisted Selection. This resulted in a set of 29 BILs that was genotyped with more than 700 DNA markers (AFLP, ESTs and SSRs) and covers 96% of the *L. saligna* genome (Jeuken and Lindhout 2004). Most BILs contained one homozygous introgression fragment of the wild species with an average genetic length of 33 cM (about

20-40% of a chromosome) in a *L. sativa* Olof background. For some lines it was not possible to obtain the introgression in a homozygous state and the best alternative was a line with the introgression in heterozygous state. We designated these lines "preBILs".

An overview of lettuce material is shown in Table S3.

C6C9 plant materials

PreNIL9.1 showed hybrid necrosis and contained one heterozygous introgression, from 0-11 cM on Chromosome 9. This line was designated Near Isogenic Line (NIL) as the introgression size was smaller than the introgression in preBIL9.1 (introgression from 0-48 cM) from which it was derived. BIL6.1 harbored one homozygous introgression from 0 to 42 cM on Chromosome 6.

From a cross between preNIL9.1 and BIL6.1, an F_1 plant that was heterozygous for both loci was selected and was selfed. The F_2 progeny segregated according to classical genetics in nine different genotypes for the two loci on Chromosome 6 and 9. For facilitating a description of the results we introduce genetic codes for these nine genotype classes of the F_2 progeny (see Figure 2). By genotyping the F_2 of preNIL9.1 × BIL6.1 we identified the nine genotypes and after selfing we harvested seeds, except from one genotype that was lethal. These eight viable genotypes consisted of three homozygous lines and five lines that segregated at one or both chromosome regions (See Figure 2). Six of these nine genotypes were used in all following experiments described.

Pathogen material

The complete virulence spectrum of *B. lactucae* races Bl:14 and Bl:16 is described in (Jeuken and Lindhout 2002) and in the evaluation report by the International Bremia Evaluation Board (IBEB).

B. lactucae races were maintained on lettuce seedlings at 15°C, at 100% relative humidity, and in cycles of 16 h light/8 h darkness after inoculation with *B. lactucae* conidia (Jeuken and Lindhout 2002).

Methods

Linkage analyses, genotyping and QTL mapping

Additional markers, preferably codominant ones, were necessary to saturate and improve the genetic linkage map of the *L. saligna* x *L. sativa* cross in general, especially near the interactive loci. Therefore, marker analyses were performed on the F_2 population of cross A. Markers consist of AFLP markers from two primer combinations E48M59 (primer+CAC, Primer+CTA) and E33M59 (Primer+AAG, Primer+CTA), SSR markers and EST markers

that were developed from lettuce EST sequences by the Compositae Genome Project (Table S4, http://compgenomics.ucdavis.edu/). Polymorphisms of EST and SSR markers between *L. saligna* and *L. sativa* were visualized by the size differences of their PCR products on agarose gels (directly or after enzyme digestion) as described in Jeuken et al, (2008).

Linkage analyses were performed by using JoinMap 4.0 software (Van Ooijen 2006) on the F_2 population of Cross A with the following mapping conditions. For grouping, Regression Mapping was used with weak linkages recombination and LOD thresholds of 0.45 and 0.05. Markers were assigned to nine linkage groups at a LOD threshold of 8. Calculations of the linkage maps were done by using all pair wise recombination estimates smaller than 0.40, LOD scores higher than 1, a jump threshold of 5, and Haldane's mapping function. As the integration of former linkage maps for F_2 populations from Cross A and B showed high co-linearity with respect to marker order and distance, we consider the linkage map for cross B to be identical (Jeuken et al. 2001). Genotypic nomenclature is as follows: A= homozygous *L. sativa*, H=heterozygous, B=homozygous *L. saligna*.

To fine map the race-specific resistance to the new F_2 linkage map we performed QTL mapping procedures like simple Interval Mapping and approximate multiple QTL mapping (MQM) by MapQTL 5.0 (Van Ooijen 2004).

All plant material used in the following described experiments was genotyped for C6 and C9 with a minimum of 8 DNA markers (combination of EST, SSR and AFLP markers) per introgression segment at an early plant stage to select the desired genotypes.

Phenotyping hybrid necrosis

Plants were phenotyped by observing cotyledons or leaves for macroscopically visible necrotic lesions or areas. Plants were categorized according to a general impression of abundance and size of necrotic lesions compared to other plants under the same conditions.

A microscopic evaluation was performed to quantify the level of necrotic leaf area. Three plants per genotype were randomly grown in a greenhouse at 15ºC for five weeks. The 4th true leaf from each plant was sampled by cutting two leaf segments, 1x2 cm in size. Leaf segments were discolored for three days in acetic acid/ethanol solution (v:v=1:3) and then cleared and stored in saturated chloral hydrate solution (5 g / 2 ml). The cleared samples were mounted in 70% glycerol. Slides were observed under the light microscope and the necrotic area was recognized by cytoplasm granulation and darkening with a yellow or brown color. The necrotic area was captured through a digital camera and measured by AxioVison LE. 4.6 (Carl Zeiss) in μm^2. The percentage of necrotic area per leaf segment was calculated. For multiple comparisons of the percentage of necrotic area and the dry weights per genotype, we used two-way ANOVA and the Tukey HSD test (α=0.05).

To quantify the retardation of growth, dry weight was measured from 11-week old plants that were grown in a randomized block design in a greenhouse. Seven plants per genotype were examined. Above-ground parts were harvested and dried for 16 hours at 105ºC. Weight was measured in grams per plant. For multiple comparisons of the dry weights between genotypes, we used one-way ANOVA and the Tukey HSD test (α =0.05).

Resistance levels

To measure the level of infection severity to *Bremia lactucae* race Bl:14 and race Bl:16 on different genotypes at three plant developmental stages, we executed Seedling Disease Tests (SDT) (14 seedlings per genotype), Young plant Disease Tests (YDT) (8 plants per genotype) and Adult plant Disease Tests in the Greenhouse (ADT_G) (7 plants x 8 leaf discs per genotype) (Jeuken and Lindhout 2002) (Chapter 2).

For all three disease tests (SDT, YDT and ADT_G) infection severity levels were scored daily between 8 and 11 dpi as the percentage of sporulating area per cotyledon/representative leaf/leaf disc. For each test the Area Under Disease Progress Curve (AUDPC) was calculated. The relative AUDPC was calculated as relative to the susceptible control parent *L. sativa* cv. Olof (set at 1.00). For multiple comparisons of the AUDPC data between genotypes, we used one-way ANOVA and Tukey HSD tests (α =0.05).

Young plant Disease Tests (YDT)

Attached leaves were tested from young, three to four week-old plants. One test with race Bl:14 and one test with Bl:16 were performed in a climate chamber. In parallel, one test with Bl:14 and one test with Bl:16 were performed in a greenhouse compartment. The same inoculum was used for the tests with Bl:14 and for the tests with Bl:16. The temperature was conditioned during growth and after inoculation at both locations, which is of course more variable in the greenhouse due to a natural day/night cycle (in June: 17 h day/7 h night) and natural light conditions. In the greenhouse the temperature ranged gradually with a lowest point of 15°C during the night to a peak of 29°C at noon during day; the average was 18.6°C. In the climate cell the temperature shifted in blocks from 19.6°C in the artificial day of 16 hours to 12.3°C in the artificial night, with an average temperature of 17.2°C. Representative leaves of young plants were scored at 8, 9, 10 and 11 dpi.

RIN4 sequences

We used Qiagen RNeasy plant mini kit with on column DNase treatment per the manufacturer's directions for RNA isolation and used iScript for cDNA synthesis (Bio-Rad).

Additional exon DNA sequences of RIN4 from three lettuce genotypes, *L. sativa* cv Salinas, *L. serriola* accession UC96US23, and *L. saligna* CGN5322, were provided by Leah McHale (Michelmore lab UC Davis). Additional cDNA sequences of ESTs homologues to RIN4 from *L. virosa* and *L. saligna* were selected from the Compositae Genome Project Database (http://compgenomics.ucdavis.edu/). Based on these sequences, internal oligonucleotide primers and two primers at the start and the end of the cDNA sequence of the *RIN4* gene were designed (Table S5). In subsequent PCR experiments, the cDNA and genomic sequences spanning the complete open reading frames (ORFs) of *RIN4* were obtained for *L. sativa* cv. Olof, *L. saligna* CGN05271 and *L. virosa* CGN05978.

Agrobacterium-mediated transient assays

Transient assays were executed to overexpress different *RIN4* transcripts. Total RNA isolation and cDNA syntheses were described above. Full length cDNAs encoding the *RIN4* alleles (sat, sal, vir) and transcript versions (T1, T2), *RIN4satT1, RIN4satT2, RIN4salT1, RINsalT2, RIN4virT1 and RIN4virT2,* were amplified from lettuce cDNA from leaves of *L. sativa* cv. Olof, *L. saligna* CGN05271 and *L. virosa* CGN05978. PCR products with proofreading enzyme Phusion DNA polymerase (Finnzymes) were cloned into pENTR/D-TOPO entry vector (Invitrogen), followed by recombination into the GATEWAY^{TM} T-DNA binary vector pK7WG2 (Karimi et al. 2002) by LR clonase (Invitrogen). The resulting binary vector pK7WG2 with target genes were electroporated into an *A. tumefaciens* strain C58C1 (pGV2260).

The *A. tumefaciens* strain C58C1 (pGV2260) and the strain containing the *PsojNIP* gene cloned in the binary vector pB7WG2 were provided by G. Van den Ackerveken (Utrecht University). PsojNIP is a necrosis-inducing protein from *Phytophthora sojae* (Qutob et al. 2002). LB medium (bacteriological peptone 10g/L; NaCl 10g/L;yeast extract 5g/L) was used for liquid and solid (15g/L agar) bacterial cultures. Spectomycine (50mg /L) was used to maintain pB7WG2 in *A. tumefaciens*.

Two independent experiments were performed. Plants were grown in a greenhouse at 21ºC in the daytime and at 19ºC in the nighttime until the sixth to seventh leaf stage. The 5th and 6th true leaves were infiltrated. The culture preparation and leaf infiltrations were performed as described by (Wroblewski et al. 2005).

Virus Induced Gene silencing (VIGS)

In the first place, a VIGS protocol was adapted for lettuce. Next, the VIGS approach was used to validate the potential involvement of *RIN4* in the resistance response to *B. lactucae* Bl:16.

Lettuce is a host for tobacco rattle virus (Mojtahedi et al. 2003). We examined the ability of the tobacco rattle virus (TRV)-based VIGS vector of Dinesh Kumar to suppress the expression of the endogenous phytoene desaturase gene (*PDS*) of lettuce in *L. sativa* and *L. saligna*, following the protocol for tomato(Liu et al. 2002a; Liu et al. 2002b). Details on the construction of above clones are given below.

In the initial experiments to set up the TRV-VIGS system for lettuce, two independent sets of infiltrations were performed, with 20 *L. sativa* and 20 *L. saligna* plants infiltrated with a mixture of *Agrobacterium* culture containing the pTRV2-le*PDS* and pTRV1. The same numbers of plants were also infiltrated with the pTRV1 and empty pTRV2 (empty vector control) and mock infiltrated with water. Twenty-three days post *agro*-infiltration the expected photo-bleaching phenotype, caused by inhibition of carotenoid synthesis, and was observed on the 4^{th} to 14^{th} leaf in *L. sativa* and on 3^{rd} to 10^{th} leaf in *L. saligna* on at least 75% of the plants (Figure S5). *PDS* suppression effect was visible uniformly throughout the entire leaf (Figure S5), especially for 5^{th} to 8^{th} leaves of *L. sativa* and 3^{rd} to 5^{th} leaves of *L. saligna*.

We validated the silencing by measuring the transcript levels for lettuce *PDS* using a quantitative RT-PCR. Primers that anneal to the *PDS* gene outside the region targeted for silencing were used (primer pair le-PDS-RT3 Table S5). Experiment was conducted in an iCycler MyiQ detection system (Bio-Rad), using the iQ SYBR Green Super mix (Bio-Rad). Assays were done in duplicate. Relative quantification of *Le-PDS* transcript level was normalized to results from the lettuce ubiquitin control by applying the $2^{-\Delta Ct}$ formula. Three plants for each group and RNA target were analyzed. Tukey Honestly Significant Differences (HSD) test with α=0.05 was applied for pair-wise multiple comparisons between the groups.

After confirming the effectiveness of the VIGS approach (Figure S5 and Figure S6), a new TRV construct pTRV2-*leRIN4*, was made with 285 base pairs fragment of lettuce *RIN4* (Figure 5). In several independent experiments, plants were agro-infiltrated with pTRV2-*leRIN4* and 30 days after agro-infiltration the plants were challenged with *B. lactucae* Bl:16 as in a normal Young Plants Disease Test (YDT, see description disease tests). Infection severities were measured and analyzed as described (see method disease tests). Similar trends were displayed in different experiments. Detailed results of one experiment are presented.

Plasmid construction VIGS

pTRV1 and pTRV2 VIGS vectors have been described in (Liu et al., 2002a). pTRV2-lePDS: a 315-bp fragment of PDS cDNA fragment corresponding to bases 1334–1648 of lettuce

PDS gene (Contig CLS_S3_Contig8919) was PCR amplified from *L. sativa* cv. Olof cDNA using Taq DNA polymerase and the primer pair le-PDS1 (Table S5). The resulting PCR product was cloned into the pGEM-T Easy vector as described by the manufacturer (Promega) and later was ligated into EcoRI-cut pTRV2.

pTRV2-leRIN4: a 285-bp fragment of RIN4satT1 cDNA fragment corresponding to bases 451-735 of lettuce cDNA (Figure 5) was PCR amplified from lettuce cDNA using Taq DNA polymerase and the primer pair RIN4_TOT (Table S4). The resulting PCR product was digested with EcoRI and the 285 fragment was ligated into EcoRI-cut pTRV2.

Agro-infiltration

For *Agrobacterium tumefaciens*-mediated virus infection, cultures of *A. tumefaciens* GV3101 containing pTRV1, empty pTRV2 vector control, and each of the constructs derived from pTRV2 were grown, harvested and subsequently infiltrated as described (Bai et al. 2008; Van der Hoorn et al. 2000). The infiltration was performed on the abaxial side of both cotyledons of each lettuce seedling at nine days after sowing using a needleless syringe. The infiltrated plants were grown under normal greenhouse conditions (20°C daytime and 18°C nighttime), and were checked for virus symptoms at regular intervals. The only symptoms of TRV infection were the restricted plant growth compared to mock infiltrated plants.

Temperature sensitivity test

Per genotype class eight plants were grown in a randomized design at 15ºC and at 30ºC in identical climate chambers with identical conditions except for the temperature. Plants were weekly observed for necrotic lesions or any other aberrant phenotypes. After 45 days the dry weight of five plants per genotype was measured as described above. Forty-nine days after sowing, plants of four genotypes, grown at 30ºC, were transferred to room temperature for 21 hours, and next to 15ºC. The plants were monitored every 12 hours until 14 days for necrotic lesions or any other aberrant phenotypes.

Endnotes:

Author contributions: M. J. W. Jeuken and N. W. Zhang designed the experiments and analyzed the data; M. J. W. Jeuken, N. W. Zhang, K. Pelgrom and E. den Boer performed the research; and M. J. W. Jeuken wrote the paper.

Supplementary Figures and Tables

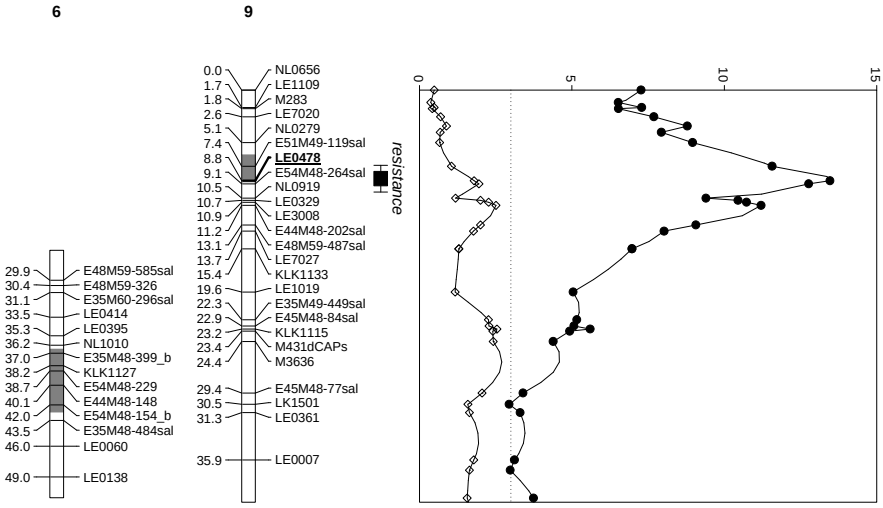

Figure S1. Map positions of hybrid necrosis loci.
Maps with the two interactive loci on chromosome 6 and 9 (C6 and C9) and the LOD profile for racespecific resistance on C9 to *B. lactucae* Bl:16 (formerly designated *R39*). Genetic map is based on F_2 from *L. saligna* x *L. sativa*. The grey bars show the two smallest fine mapped segments that are associated with hybrid necrosis based on F_2 populations and introgression lines from cross A and B.
The LOD profile of race specific resistance to *B. lactucae* Bl:16 (solid bullets) and susceptibility to Bl:14 (open diamants) is shown for C9 with one LOD and two LOD support confidence intervals (approximate multiple QTL mapping, MQM). The highest associated marker is underlined and bold. LE0478 and its derived markers (LE0478INT, LE0478indel not shown) all mapped at the same position.

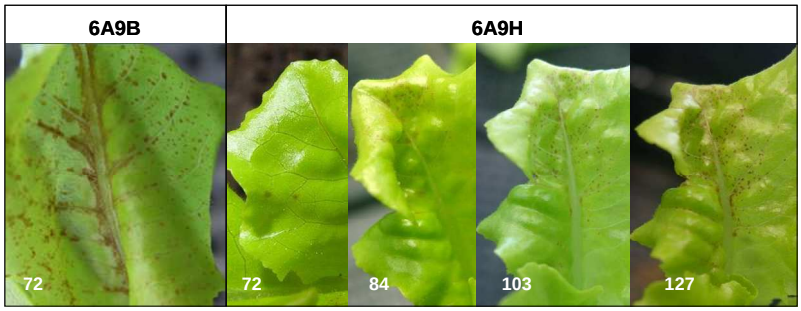

Figure S2. Hybrid necrosis symptoms in youngest leaf after temperature shift. Observations on genotypes classes with extreme to severe hybrid necrosis symptoms at lower temperatures: 6A9B and 6A9H, in hours after shift from 30° to 15°C. Left: detail of necrotic cells in 6A9B appearing near the major veins. Right: time frame of appearance of necrotic lesions in a 6A9H leaf.

Figure S3. Hybrid necrosis symptoms in whole plants after temperature shift. Observations on genotypes classes with extreme to severe hybrid necrosis symptoms at lower temperatures: 6A9B and 6A9H, and genotypes with no hybrid necrosis symptoms 6A9A *L. sativa* Olof and 6B9B doubleBIL6.1+9.1, at 5 and 17 days after a shift from 30° to 15°C.

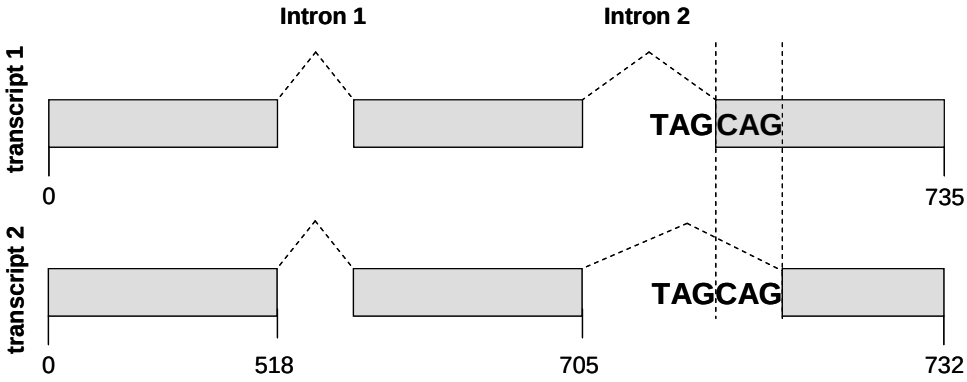

Figure S4. Proposed alternative splicing of RIN4.
The proposed alternative splicing event that utilizes different 3'splice sites within the second intron at a 'TAGCAG' sequence leading to two transcript versions in *L. sativa*, *L. saligna* and *L. virosa*. Transcript1 and transcript2 arose from a splice after the 'TAG' and the 'CAG' motif, respectively. This results in one extra amino acid, namely glutamine at position 237 in transcript1.

Figure S5. Silencing of PDS in lettuce by VIGS
L. sativa Olof and *L. saligna* CGN05271 at 23 days after infiltration. Infection with TRV-le*PDS* silences endogenous *PDS* and causes inhibition of carotenoid biosynthesis resulting in photo-bleaching phenotype.

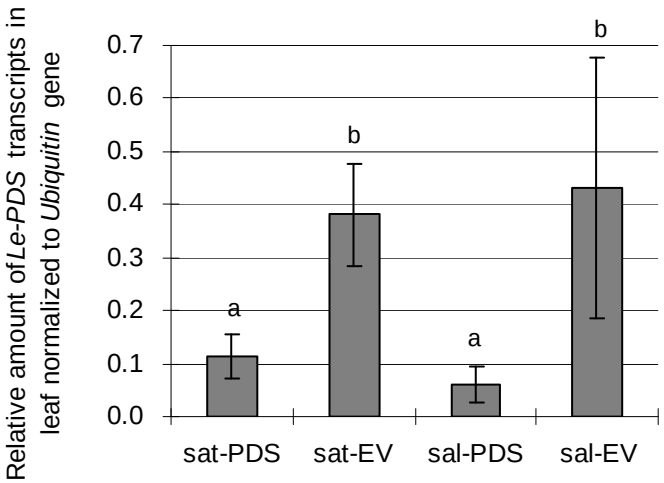

Figure S6. Real-time PCR expression data of *Le-PDS* in lettuce leaves.

mean ± s.d., sat= *L. sativa* cv. Olof and sal= *L. saligna* CGN05271. PDS means leaves showing photo bleaching for 75%-100% leaf area; EV means infiltrated with empty vector (leafs are green). Letters in common, above the error bars, indicate no significant difference between groups (α=0.05, Tukey HSD test).

Table S1. F_2 Segregation ratios (panel **A**) and real infection severities to *B. lactucae* race Bl:16 per genotype class at epistatic loci C6 and C9 (panel **B**). Predicted infection severities per locus in case of normal segregation ratios are shown in Panel **C**. White bold numbers indicate low infection severities (resistance specific to Bl:16). Italic numbers indicate predicted values.

A. Segregation ratios

F_2 cross A

	C9[a]			
C6[a]	*A*	*H*	*B*	tot
A	20	12	0	32
H	27	25	0	52
B	18	10	1	29
tot	65	47	1	113[b]

F_2 cross B

	C9[a]			
C6[a]	*A*	*H*	*B*	tot
A	5	0	0	5
H	5	24	2	31
B	2	10	5	17
tot	12	34	7	53[c]

B. Infection severity[d]

	C9[a]			
C6[a]	*A*	*H*	*B*	tot
A	2.19	**0.00**		**1.37**
H	1.96	**0.53**		**1.27**
B	1.94	1.50	1.13	**1.76**
tot	**2.03**	**0.60**	**1.13**	

	C9[a]			
C6[a]	*A*	*H*	*B*	tot
A	2.53			**2.53**
H	2.33	**0.56**	**0.00**	**0.81**
B	3.33	1.23	1.00	**1.41**
tot	**2.58**	**0.76**	**0.71**	

C. Predicted infection severity per locus in case of normal segregation ratios (1:2:1 and n=112)[e]

	C9[a]			
C6[a]	*A*	*H*	*B*	tot
A	2.19	**0.00**	***0.00***	***0.55***[f]
H	1.96	**0.53**	***0.00***	***0.76***[f]
B	1.94	1.50	1.13	***1.52***[f]
tot	***2.01***	***0.64***	***0.28***	

[a] Epistatic loci at Chromosome 9 (C9) around 8 cM and Chromosome 6 (C6) around 40 cM. A=homozygous *L. sativa*, H=heterozygous, B=homozygous *L. saligna*.

[b] From 126 F_2 plants 13 plants could not be classified as the genotype was unknown for one or both loci.

[c] From 54 F_2 plants 1 plant could not be classified as the genotype was unknown for both loci.

[d] Average infection severity per genotype class, based on one (cross B) and two (cross A) disease tests with *B. lactucae* Bl:16. Scoring classes ranged from 0-4, resistant to susceptible (class 0: no sporulation, class 1: 1-25% of leaf disc area sporulates; class 2: 26-50%; class 3: 50-75% and class 4: 75-100%; Jeuken and Lindhout 2002).

[e] Predicted average infection severities per genotype class per locus. The values of the seven of the nine genotype classes are used from the real data (see above). The values for genotype 6H9B was predicted to be 0, based on F_2 cross B. The value of genotype 6A9B was predicted to be 0, as 6A9H and 6B9H were both 0. A normal segregation ratio of 1:2:1 for both loci and 112 F_2 individuals were used for calculations.
[f] In panel C , the predicted infection severities for the genotypes at the C6 locus show a larger difference between B genotypes and the A or H genotypes in case of normal segregation ratios than in case of the real segregation ratios in panel B. This implies that a higher LOD value at the C6 locus would have been detected in case of normal segregation.

Table S2. Transient expression of *RIN4* alleles and transcripts. Macroscopic observations at leaf area at 8 days after infiltration. '+'= necrosis phenotype, '−'= no symptoms . sat=*L. sativa* allele, sal= *L. saligna* allele, vir=*L. virosa* allele, T1 and T2 are transcript versions 1 and 2.

		RIN4 alleles/transcripts [a]						controls		
line	genotype	sat T1	sat T2	sal T1	sal T2 [b]	vir T1	vir T2	water	ev[c]	PsojNIP[d]
L. sativa [e]	6A9A	−	−	−	+	−	−	−	−	+
L. saligna [f]	6B9B	−	−	−	−	−	−	−	−	+
BIL6.1+9.1 [g]	6B9B	−	−	−	−	−	−	−	−	+
BIL6.1[h]	6B9A	−	−	−	−	−	−	−	−	+

[a] Alleles: sat=*L. sativa*, sal= *L. saligna*, vir=*L. viros.* T1 and T2 are RIN4 transcript versions 1 and 2.
[b] first symptom visible 6 dpi
[c] empty vector
[d] first symptom visible 4 dpi, by *Phytophthora sojae* necrosis-inducing protein
[e] cv. Olof and cv. Norden
[f] accessions CGN05271 and CGN11341
[g] like *L. sativa* Olof but with *L. saligna* introgression at C6 and C9 locus respectively
[h] like *L. sativa* Olof but with *L. saligna* introgression at C6 locus

Table S3 Lettuce material

genotype	accessions	features
L. sativa	cv. Olof cv. Norden	Cultivated lettuce species, host for downy mildew, Olof harbors no known *R* genes and is susceptible to race Bl:1-Bl:24, Norden harbors *Dm3* and *Dm11*
L. saligna	CGN05271 CGN11341	Wild lettuce species, nonhost to downy mildew
L. virosa	CGN05978	Wild lettuce species
preNIL9.1	Background cv. Olof	Hybrid necrosis phenotype, Heterozygous *L. saligna* introgression on C9
BIL6.1	Background cv. Olof	Homozygous *L. saligna* introgression on C6
BIL4.4	Background cv. Olof	Homozygous *L. saligna* introgression on C4, super susceptible to downy mildew

Table S4. DNA markers

Marker name [a]	Restrict enzyme	Forward primer	Reverse primer
LE0395	DdeI	GCGTGATGTCGCTTTGTTAAT	ACAGTGAGTGTGTCGCAAGG
LE0414		CGATTGGGAACACATGTCAG	TCCTAAAGAACCACGCAACG
NL1010		CTTCCCAATCTGAAAGCTG	CAAATGCATAAGGGAGCAC
KLK1127		GCAACACCACTTCGGATTCT	CCATGAAAATTGCAAGAAAACA
LE0060	RsaI	GGCACATCTGCAAGAAACAA	ATTGTGCCCCAAATCTGAAG
LE0138	HinfI	GGGCTGTAGCTGGTGTTTGT	CCAGCTAAAGATCTGCGCTC
NL0656		GCAATGGAGATGAAAGAGC	TTTTTGGTTTCACTTTCGG
LE1109		GGACGTTCAAATCCAGCAAT	GCAAATCAGCCGATAAATCC
M283		ATGTGTCTGGGGTGGCTTTA	TTGCCCCATAATCATAAAGAATG
LE7020	HinfI	CGCTGTCATCGGAGTTGTAA	CCAGTGGAATTTGGGAAGAA
NL0279		AGCTTGACCAGTTCCACAG	GTCCTTTCTGACTCCTCCC
LE0478 [b]	HincII	GCATATGGCCCATGAATTCTCGG	GCCCTTTAGAGCTATTCACAACA
LE0478indel		ATAGACCAAATTGCCGTCCA	CCCCTTTCAATTTTGATCGT
LE0478INT		GGTAAAACCGGTGGGAAGAT	TTGGTCACGTGGAATGTTGT
NL0919		CTGAGGGTTGCTCTTTCTG	TGTTCATTTCAAAGTTAACCAC
LE0329	ApoI	GGATATCAACGATCGGAGGA	GTTATCGCTGGCCTCAAGTC
LE3008	MseI	CATGCTGAACTCCACGTAACA	CAAATTCCCTGCAGCAATAGA
LE7027	MnlI	CTATCGCCGGACTATGGAAA	GCATTAGGACGGATGATGCT
KLK1133		AGGCAGAACACCAACTCCAG	CTCCTTGTTGTTGGGGAAGA
LE1019		TTTTTCCCGATCTTTGCATC	AGCGAATCTTTGCTTTTTCG
KLK1115dCAPs	HindIII	GAAGATGGATATTGAAGTTCTGGACAAGCT	ACCCATCACCAAAGAATCCA
M431dCAPs	DdeI	GATCGATCGTTCATCGTTCTCTCA	TTGTTGAAACAAGTTCACTATTTGG
M3636		CCAGAGACATTCCACAAGCA	CAACAACACGATAATGGGACA
LK1501	NlaIII	GTTGAACATGCGGTGTCTTG	TAAATCCTCCCATGCAGGTC
RGC3	DdeI	CACTCAAGCACCCAACAAGA	CTTTCGAAAAGAAGCCATGC
RGC4	AluI	TCCGGGAGGAATCAAGTAAG	CCACGTCTTTTGGAAGAAGC

[a]For markers that start their name with M, LE or LK primers were originally designed on EST contigs in the Compositae Genome Project Database (CGPDB). For markers that start with

KLK we designed new primers on EST contigs from the Compositae Genome Project Database. Marker names starting with NL are SSR markers.
[b]LE0478 is the original primer pair derived from CGPDB, LE0478INT and LE0478indel were developed on additional sequence information of lettuce RIN4.

Table S5 Primer pairs for RIN4 sequencing, cloning and RT-PCR

Primer pairs for sequencing RIN4 cDNA and gDNA			
	Forward primer	Reverse primer	begin-end bp[a]
RIN4_TOT	ATGGCGCAGCGTCCAACTGTA	TCACTTCGACIATGGGAAG	0-732/735
RIN4_INT	GGTAAAACCGGTGGGAAGAT	TTGGTCACGTGGAATGTTGT	91-667
RIN4_END	GTTTGGTGAGTGGGATGAAAACAA	TCACTTCGACIATGGGAAG	546-732/735
RIN4_STA	ATGGCGCAGCGTCCAACTGTA	GACAGGTTCTTCCGGTTGAG	0-208
Primer pairs for Agro-assay fragment cloning			
RIN4-topoall	CACCATGGCGCAGCGTCCAACT	TCACTTCGACIATGGGAAG	
VIGS and RT-PCR			
le-PDS1[b]	TACCCGAAGAATGGAAACCA	CAGCTGCAATTTCATCAGGA	
le-PDS-RT3[c]	CCACCCACCATAACATCCATTCAG	TGGCAGAAACATTTCCAAACAGAG	
le-Ubiquitinc	GAAGAAGACCTACACCAAGCCAAAG	ACTCAGCATTAGGGCACTCTTTCC	

[a] begin and end base pair number of PCR product of cDNA sequence RIN4
[b] primer pair used for fragment cloning in pTRV2 for VIGS
[c] primer pair used in RT-PCR on VIGS silenced plants

CHAPTER 6

General Discussion

The genetic architecture of *L. saligna* nonhost resistance to *B. lactucae*

L. saligna nonhost resistance is polygenic

The inheritance of nonhost resistance has not been intensively studied due to the difficulties such as sexually incompatibility between most host and nonhost species. In this research we provide one of the few fruitful examples in which the genetic architecture of nonhost resistance can be unraveled, viz. in the *L. saligna – B. lactucae* pathosystem. Based on a successful cross between *L. saligna* (nonhost) and *L. sativa* (host), we developed an F_2 population and a set of BILs to dissect the *L. saligna* genome into the *L. sativa* background. QTL mapping in the F_2 population at adult plant stage (ADT_G) detected three resistance QTLs, *Rbq1*, *Rbq2* and *rbq3* that locate on Chromosome 7, 1 and 9, respectively (Jeuken and Lindhout 2002). Later on, Jeuken et al. (2008) detected six QTLs in BILs compared to only three in the F_2 population, of which two QTLs were in common. Each of the BIL introgressions contributing to the quantitative resistance was provisionally considered to be due to one QTL.

The four newly detected QTLs *rbq4*, *rbq5*, *rbq6* and *rbq7* were proven to inherit recessively. They remained unnoticed in the F_2 population. The reasons for unnoticing *rbq4* and *rbq6* in the F_2 are probably due to a combination of recessiveness of the trait and skewed segregation causing a deficit of the wild species alleles (Jeuken et al. 2008). The reason for missing *rbq5* and *rbq7* in F_2 mapping were understood only after fine mapping these two QTLs by NILs (see below).

In this thesis, we report that in total 15 BIL introgressions from *L. saligna* contributed to the quantitative resistance against downy mildew at four developmental stages: seedling stage, young plant stage, adult plant stage in the greenhouse and in the field. The 15 BIL introgressions are provisionally considered QTLs, including 9 new QTLs and 6 QTLs that

were identified before (Jeuken and Lindhout 2002; Jeuken et al. 2008). Each QTL was effective at one to four developmental stages. Interestingly, none of these 15 QTLs showed race specificity against 7 different *B. lactucae* races (Chapter 2). Moreover, the majority of these 15 QTLs did not coincide with the four known *R*-gene (*Dm* gene) clusters according to the updated genetic map of lettuce (Figure 1 and Chapters 1 and 4). This indicates that nonhost resistance is due to different genes from those that are responsible for monogenic resistance based on *Dm*/*R*-genes, and are unlikely to represent weak alleles of *Dm* genes (Chapter 2). The above mentioned results strongly suggest that the nonhost resistance of *L. saligna* to *B. lactucae* is polygenic and the effectiveness of these QTLs is mainly plant developmental stage dependent (Chapter 2). We focused on four "target BILs" in the subsequent studies, BIL2.2 (*rbq5*), BIL4.2 (*rbq7*), BIL6.3 (*rbq6*) and BIL8.2 (*rbq4*). They contributed to a high level of quantitative resistance at both the young and adult plant stages (Chapters 2, 3 and 4).

The polygenic inheritance of the nonhost resistance has not been reported comprehensively elsewhere, except by Jafary (2006), who reported that several QTLs are involved in the nonhost resistance of barley to heterologous rust fungi, and by Shafiei et al. (2007) who identified three QTLs in the nonhost resistance of *Arabidopsis thaliana* against wheat leaf rust (*Puccinia triticina*).

We did not only introgress resistance alleles into a *L. sativa* background, but also seemed to introduce a susceptibility allele from *L. saligna* into *L. sativa.* This was concluded from the increased infection severity in BIL4.4 (*Rbq16*) compared to the susceptible parental line *L. sativa* cv. Olof at all stages but particularly at young plant stage. Such super susceptibility is due to an introgression of a gene for a susceptibility factor from *L. saligna* or replacement of a resistance allele in *L. sativa* by a neutral *L. saligna* introgression (Chapter 2).

Redundancy of QTLs in the nonhost resistance

The results of the experiments on pyramiding the four QTLs of the "target BILs" suggested a great redundancy of QTLs in the nonhost species *L. saligna* CGN05271. Introducing three QTLs from *L. saligna* into the *L. sativa* background was sufficient to give *L. sativa* complete protection to downy mildew. Combining the three QTLs, *rbq4*, *rbq5* and *rbq6*, led to complete resistance at young plant stage and likely will also lead to a high level or complete resistance at adult plant stage. Significantly increased resistance was observed even when only two out of these three QTLs were combined. The QTL, *rbq7* did not lead to increased resistance when we combined it with any other target QTLs (Chapter 3).

We determined the resistance mechanisms underlying *L. saligna* CGN05271 and these four QTLs and their (dis)similarities through histological studies. At 48 hpi, *L. saligna* (nonhost) showed pre-hyphal resistance that arrested most of the *B. lactucae* infection units before hypha formation, while >90% of the infection units on *L. sativa* cv. Olof (host) formed hyphae and haustoria. The BILs with QTLs displayed several grades of incomplete pre-hyphal resistance, allowing only <30% of the *B. lactucae* infection units to form hyphae and haustoria. The extent of incomplete pre-hyphal resistance varied for different QTLs from similar to *L. saligna* CGN05271 (BIL6.3 and doubleBIL68) to almost none (BIL4.2). Particularly, BIL2.2, BIL6.3 and BIL8.2, showed sub-epidermal HR, while *L. saligna* and *L. sativa* cv. Olof did not.

Redundancy of resistance QTLs from *L. saligna* is shown by the many resistance QTLs of *L. saligna* CGN05271 operating at various plant developmental stages (Chapter 2) and the sufficiency of three QTLs to lead to the complete resistance to downy mildew (Chapter 3). With such abundance of QTLs governing resistance to downy mildew, one may wonder whether different *L. saligna* accessions share a common set of genes to keep their nonhost status or mainly different sets of genes. Related to this question, Jafary and his colleagues (2006) have used three different barley mapping populations to identify the QTLs contributing to nonhost resistance to heterologous rusts. Their results suggested that different populations only had very few QTLs (genes) in common and indicated a high diversity of genes for nonhost resistance to heterologous rusts among the populations (Jafary et al. 2008). Therefore, we do not rule out the possibility that different *L. saligna* accessions might harbor different sets of QTLs for resistance, while a certain QTL combination may occur in a high frequency in most of *L. saligna* accessions. During evolution, *L. saligna* may have developed a strategy, whereby high frequency of a certain QTL combination occurs to prevent the rapid adaptation of *B. lactucae* races. One way to prove this assumption is to cross *L. saligna* accessions and perform disease tests on many large F_2 populations. Our hypothesis about different *L. saligna* containing different sets of loci for resistance will be supported if susceptible *L. saligna* F_2 individuals appear.

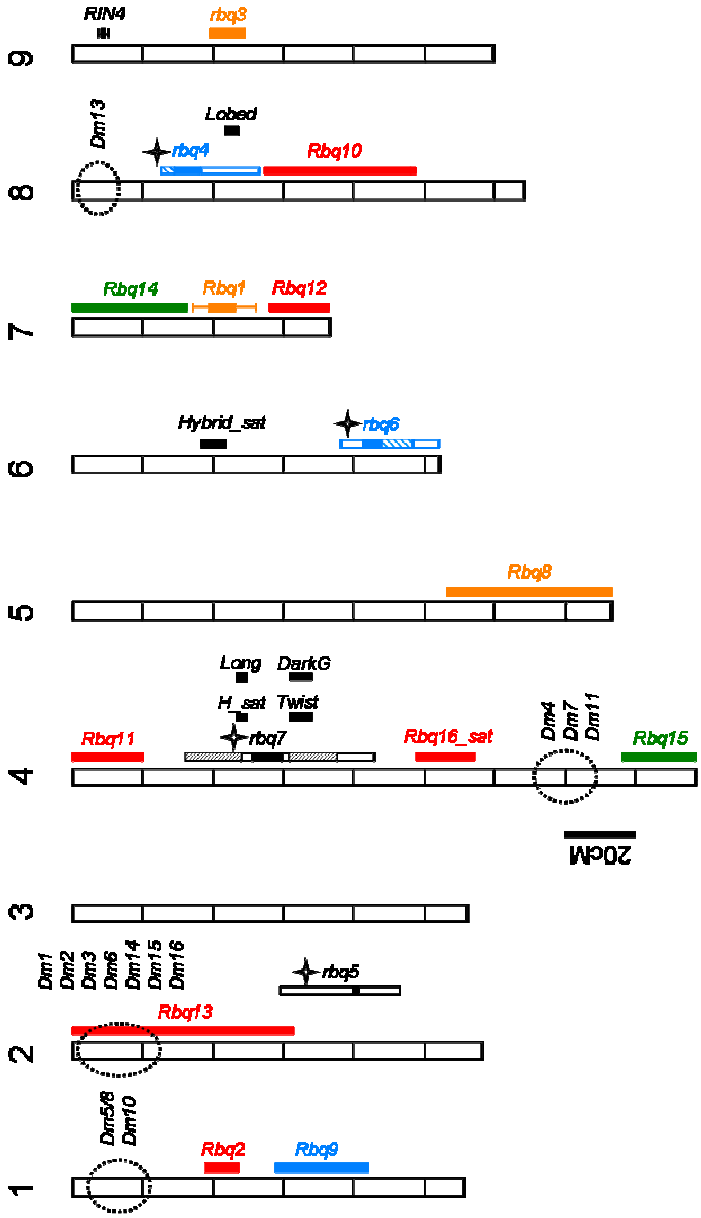

Figure 1. Position of resistance QTLs to downy mildew, the hybrid necrosis related genes, some morphological traits, and formerly mapped positions of four known resistance gene (*Dm*) clusters in lettuce linkage maps, according to Kesseli et al., (1994), Jeuken et al, (2002), Truco et al, (2007) and Chapters 4 and 5.

In the F_2 population of the cross between *L. saligna* CGN05271 and *L. sativa* cv. Olof, QTLs *Rbq1*, *Rbq2* and *rbq3* were mapped with LOD value >3.5 (Jeuken and Lindhout 2002). QTL bar of *Rbq1* indicates the QTL interval in which the inner bar shows a one LOD and the outer bar shows a two LOD support confidence interval based on the results of Multiple-QTL Mapping (MQM). *Rbq2* and *rbq3* with LOD value of only 1.1 and 2.2, respectively in the latest updated genetic map with 784 markers became doubtful QTLs (M. Jeuken, personal communication). Therefore, the QTL bars for *Rbq2* and *rbq3* indicate the intervals with LOD >1.0.

In the sets of BILs and NILs, the following traits were mapped: resistance QTLs, *rbq4* to *7*, *Rbq8* to *16* were mapped according to the introgression regions of each BIL and NIL that showed quantitative resistance to downy mildew at various developmental stages (Jeuken et al. 2008) (Chapter 2). The colors of the QTL bars stand for the different categories of each QTL according the developmental stage(s) at which they are effective. Black: the QTL was effective at all four plant developmental stages, seedling, young plant, adult plant in greenhouse and adult plant in field ("stage-independent"); Red: only effective until the young plant stage ("early"); Blue: effective from young plant stage until adult stage ("intermediate"); Orange: effective at three non-continuous developmental stages ("likely stage-independent"); Green: only effective at adult plant stage ("late"). Four QTLs tagged with ✧ are our target QTLs described in Chapters 3 and 4 that were used for QTL pyramiding and fine mapping. The entire QTL bar for each target QTL indicates the original BIL introgression region. The solid region within the QTL bar indicates the most likely positions of this QTL fine mapped by the NILs with smaller introgressions; hatched region indicates the possible extension of the QTL interval.

Morphological traits: *DarkG=dark green leaf*, *Twist=twisted leaf*, *Long=long narrow leaf* and *H_sat=heading*, *Lobed=lobed leaf*

Genes related to hybrid necrosis were mapped and indicated by *Hybrid_sat* and *RIN4*. The loci with a "*_sat*" tag means this QTL is from *L. sativa*, otherwise from *L. saligna.*

The hypothetic molecular basis of *L. saligna* nonhost resistance

Two models have been postulated to explain the molecular basis of nonhost resistance: one is based on the discovery of PAMP-triggered defense and another one is based on effector-triggered defense which is associated with stacks of multiple "gene-for-gene" interactions between *R*-genes and corresponding *Avr* genes from the pathogen (Nurnberger and Lipka 2005; Schweizer 2007). As we did not detect any gene for resistance of *L. saligna* CGN05271 to be localized in an *R*-gene cluster and the resistance mechanisms of the QTLs are not mainly based on HR (Chapter 3), downy mildew infection is likely to be arrested by a PAMP-triggered defense in this wild lettuce species, wherein the effectors from *B. lactucae* cannot suppress this defense. We presume that the QTLs we identified in this thesis (or their transcripts) may act as the plant targets for pathogen effectors and determine the success or failure of suppression of basal defense in plant cells. Future fine mapping and cloning of the

QTLs, and genome sequencing will facilitate the identification and understanding of these plant targets and their contribution to nonhost resistance.

Fine mapping QTLs and the feasibilities of QTL cloning

In Chapter 4, we used sets of NILs to fine map the four target QTLs residing in each BIL introgression. The mapping intervals for the two QTLs, *rbq4* and *rbq6* were narrowed down from 38 cM to 11 cM (BIL8.2, *rbq4*) and 28 cM to 14 cM (BIL6.3, *rbq6*), respectively. In contrast to those genes, *rbq5* and *rbq7* were more likely to be explained by several QTLs in their respective introgressions and with the present resolution capacity of these NILs it was not possible to clearly fine map all the sub-QTLs. The presence of several quantitative resistance genes (sub-QTLs) in one *L. saligna* introgression suggests a genetic complexity of these chromosomal regions. This complexity might also explain why these QTLs went unnoticed in the F_2 population (Chapter 4).

The single QTLs *rbq4* and *rbq6* provide better possibilities for future map-based cloning of the underlying genes. Particularly, since *rbq4* is also effective in the adult plant stage in the field while *rbq6* is not, we will focus on *rbq4* to discuss the feasibilities for cloning.

In general, cloning of lettuce QTLs is a great challenge due to the limited sequence information for the lettuce genome and the lack of linearity between lettuce and any other model plants' chromosomes that have been fully sequenced. No genome of a Compositae species has been sequenced so far, although plans are made to sequence lettuce, sunflower and *Leontodon taraxacoides* when sequence costs are further reduced (Compositae White Draft, http://compgenomics.ucdavis.edu/cwp/draft). Nevertheless, we list the current feasibilities of cloning *rbq4* by map-based cloning in combination with a candidate gene approach.

For the map-based cloning, the availability of many additional mapped EST markers would enhance the genetic map resolution and increase the chance to find candidate genes at the same time. The number of markers can be increased by ESTs that come available from the lettuce genome chip (http://chiplett.ucdavis.edu). Our plant material permits us to further backcross NIL8.2-2 with *L. sativa* cv. Olof to identify more recombinants within the fine mapped *rbq4* region. Fortunately, the recombination was not as much suppressed in the BIL8.2 introgression as it was for other introgressions (Chapter 4, Table 3). A similar approach has been utilized in the fine mapping of a QTL for basal resistance of barley to rust fungi, in which the authors precisely pinned down the QTL *Rphq2* to a 0.1cM interval flanked by two markers. The cloning of this QTL is now ongoing (Marcel et al. 2007a). Further on, construction of Bacterial Artificial Chromosomal (BAC) Libraries of the nonhost

species *L. saligna* and host species *L. sativa* might be essential to land on the genes closely associated with the resistance.

So far, the only EST marker mapped in the 11cM interval of *rbq4* is KLE0263 and it showed the highest LOD value for resistance (Chapter 4). This EST is homologous to a leucine-rich repeat transmembrane protein kinase in *Arabidopsis* (At3g02130) with E-value of 5e-22 (tBlastx against NCBI). This protein is required for the establishment of *Arabidopsis* cotyledon primordia (Nodine and Tax 2008). It is too early to consider this gene a serious candidate for *rbq4*.

Hybrid Necrosis

Hybrid necrosis is one of the phenomena that is found in intra- and inter-specific plant hybrids to prevent gene exchange. Such a hybridization barrier contributes to genetic isolation and delimitation of related plant species. Hybrids in which hybrid necrosis occurs show clear symptoms such as necrotic spots on leaves and retarded growth (Chapters 1 and 5). We found that the necrosis of preBIL9.1 is associated with race-non-specific and race-specific resistance against *B. lactucae* races. In addition, we found that also a locus on Chromosome 6 is involved and two interactive loci on Chromosome 6 and 9 respectively were implicated in this hybrid necrosis and the resistance. Over-expression and silencing the *RIN4* alleles of *L. saligna* proved the involvement of one of the two translation products of this gene in the hybrid necrosis and the resistance (Chapter 5).

To our knowledge, we are the first to show concrete evidence that *RIN4* is involved in hybrid necrosis (Chapter 5). In *Arabidopsis thaliana* to *Pseudomonas syringae* studies, RIN4 is already known to interact with an *R*-gene product and leads to resistance accompanied by hypersensitive response (HR) after pathogen attack (Mackey et al. 2002; Shang et al. 2006). Bomblies et al. (2007) reported evidence that resistance gene is involved in hybrid necrosis in *Arabidopsis*. In our case, the locus on Chromosome 6 that interacts with *L. saligna RIN4* on Chromosome 9 is very likely an *R*-gene from *L. sativa*. In the light of rapidly increasing lettuce genome information and modern technologies (454 sequencing^{TM}, microarray, Virus Induced Gene Silencing (VIGS) and *Agrobacterium*-mediated Transient Assay (ATTA), the identification of the other interactive locus, on Chromosome 6 is practically “just around the corner”.

As we detected this hybrid necrosis from two hybrids between different *L. sativa* cultivars and *L. saligna* accessions (Chapter 5), one may wonder whether all the *L. sativa* cultivars harbor the same gene that interacts with the *L. saligna RIN4* or not and whether all *L. saligna* accessions carry a *RIN4* gene that interacts with *L. sativa* to elicit an auto-necrosis and resistance response. By ATTA, it is possible to determine the induction of auto-necrosis

in cv. Olof and cv. Norden (Chapter 5). This ATTA test should be extended to more cultivated lettuce accessions, in order to find the frequency of the *RIN4*-interacting allele in *L. sativa*. Inheritance studies should demonstrate whether such an interacting gene is also in those accessions located on Chromosome 6 or not. In addition, different disease tests can be performed to find out whether this hybrid necrosis leads to a general defense of plants to various diseases or only to a specific resistance to downy mildew.

Another chromosomal region with symptoms of a genetic incompatibility and resistance by a QTL is on Chromosome 7 around *Rbq1*. Firstly, *Rbq1* was detected in the F_2 of a cross between *L. saligna* x *L. sativa* by both *B. lactucae* race Bl:14 and Bl:16 (Jeuken and Lindhout 2002). Later on, in the BILs, the introgression region of preBIL7.2 coincided with *Rbq1*. So far it has been impossible to obtain BIL7.2 with a homozygous *L. saligna* introgression from 38 to 73cM on Chromosome 7 (Jeuken and Lindhout 2004). Moreover, preBIL7.2 also shows retarded growth but also fast bolting and the plants are much smaller than the cultivated lettuce cv. Olof (Jeuken and Lindhout 2004). We may assume that this incompatibility is also due to an epistatic interaction between an allele on Chromosome 7 and another allele from another locus in the genome.

Additionally, BIL6.3 has two homozygous introgressions from *L. saligna* on Chromosome 4 and 6. A severe distorted segregation with preference for *L. saligna* alleles at the location of the Chromosome 4 segment prevented the selection of genotypes without this segment in BIL6.3 and subsequent combiBILs and NILs that were derived from BIL6.3 (Chapters 3 and 4). This phenomenon may indicate a two-locus incompatibility between *L. saligna* and *L. sativa* similar to the *RIN4-R*-gene case.

***L. saligna* - *B. lactucae* pathosystem is a good model to study nonhost resistance**

A good model system brings light to a particular research. There are different types of resistance involved in the lettuce-downy mildew pathosystem, consisting of basal defense, major (*Dm*/*R*) gene-mediated resistance and nonhost resistance (Chapter 1). In particular, the successful cross between wild lettuce *L. saligna* (nonhost) and cultivated lettuce *L. sativa* (host) offers a unique chance to study the inheritance of this nonhost resistance.

Nonhost resistance has been a hot topic for plant pathologists for a long time. Recent studies on plant nonhost resistance used both forward and reverse genetic approaches. Especially in *Arabidopsis thaliana*, two types of systems are discovered to explain the nonhost resistance, including PAMP triggered immunity (PTI) and effector triggered immunity (ETI). Many genes and transcription factors have been identified to be involved in nonhost resistance and the suppression of this resistance (Nurnberger and Lipka 2005; Ryan et al. 2007; Schweizer 2007; Thordal Christensen 2003). However, most of these findings are

unlikely to be the central and most upstream regulators that determine the host or nonhost status of a plant species. Studying inheritance of the nonhost resistance in *L. saligna x L. sativa* allows identification of genes responsible for the natural variation in (non)host status of the two species to *B. lactucae*, especially with the following materials and genomic tools.

Materials:

In the recent 10 years, we have developed an F_2 population, a set of 29 BILs, a set of combiBILs with QTL combinations (Chapter 3), four sets of NILs derived from four target BILs (Chapter 4) and also a set of hybrids for studying hybrid necrosis (Chapter 5). These plant materials with uniform genetic background can be used in breeding programs and also provide an excellent basis to set up future applied and fundamental researches.

Genomic tools:

Although the genome of lettuce has not been fully sequenced, the current tools available are nevertheless impressive. High density genetic maps with thousands of molecular markers, an EST database for Compositae genomes (compgenomics.ucdavis.edu), accurate bioassays for quantitative disease evaluation at various plant developmental stages (Chapter 2), practical functional assays such as ATTA and VIGS (Chapter 5), and protocols for histological studies (Chapter 3). Furthermore, an Affymetrix chip will become available and is currently being tested with the cv. Salinas x *L. serriola* Recombinant Inbred Line (RIL) population for massively parallel genetic mapping (Michelmore, personal communication). This chip has a total of 6.6 million features representing >35,000 unigenes, each with ~200 oligonucleotides with a 2 bp stagger (http://chiplett.ucdavis.edu).

Future research focus

Based on above mentioned results, information and speculations, our future research may focus on (1) further fine mapping of QTLs, especially *rbq4* and *rbq6*; (2) cloning the QTL by map-based cloning approach and functionally validate the candidate genes; (3) further characterization of the mechanisms underlying the resistance QTLs; (4) identifying the assumed *R*-gene in *L. sativa* that interacts with *RIN4* from *L. saligna* and test this resistance to other *B. lactucae* races and even other diseases; (5) screening *L. sativa* cultivars to look for genes interacting with *L. saligna RIN4*.

The information delivered by this PhD thesis provides a solid basis for both applied and fundamental studies on *L. saligna* nonhost resistance to downy mildew. We are confident that the materials and tools developed in this study will facilitate the future research on plant defense and its related aspects.

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SUMMARY

Lettuce (*Lactuca sativa*) is a popular leafy vegetable worldwide. Lettuce downy mildew is the most destructive disease in lettuce cultivation and is caused by *Bremia lactucae*, an obligate biotrophic oomycete pathogen. To date, downy mildew control heavily relies on the use of chemicals and on breeding of host resistance by introgressing disease resistance (*Dm/R*) genes into cultivars. So far, many *Dm/R* genes are known and more than 20 of them have been deployed in lettuce breeding programs. However, the application of pestcides raises environmental concerns and develops the tolerance of *B. lactucae* to some pesticides, and use of host resistance mediated by *Dm/R* genes offers no durable solution due to rapid development of new *B. lactucae* races.

Introduction of nonhost resistance of the wild lettuce species *L. saligna* to *B. lactucae* is a potential alternative strategy of introducing more durable resistance into lettuce cultivars. The successful cross between the nonhost *L. saligna* CGN05271 and the host *L. sativa* cv. Olof offers a rare chance to study the genetics of this nonhost resistance. From a selfed F_1 of this cross, we developed an F_2 population of 126 plants and a set of 29 Backcross Inbred Lines (BILs) representing in total 96% of the *L. saligna* genome.

In **Chapter 2**, the set of BILs were evaluated against *B. lactucae* infections at three plant developmental stages, seedling (SDT), young plant (YDT) and adult plant in field (ADT_F). The QTLs that were identified in these tests were compared with seven previously determined QTLs at adult plant stage in greenhouse (ADT_G). In total, 16 introgressions were identified to contribute to the resistance at one or more of the four above mentioned developmental stages and were provisionally considered as 16 QTLs (*Rbq1* to *16*). For 15 of these QTLs, the resistance allele is contributed by the nonhost *L. saligna*; for *Rbq16* the resistance allele is contributed by the host *L. sativa*. All the 15 QTLs from *L. saligna* seemed to be race-non-specific against *B. lactucae*. Interestingly, only 2 out of the 15 QTLs from *L. saligna* were effective at all tested stages, the effects of the other 13 QTLs depended on the plant developmental stage. We focused on four BILs, viz. BIL2.2, BIL4.2, BIL6.3 and BIL8.2, which contained QTLs that were effective at both young and adult plant stages, *rbq5*, *rbq7*, *rbq6+11* and *rbq4*, respectively. They were selected as “target BILs” (and “target QTLs”, respectively) for the subsequent studies.

To find out how many of these four target QTLs from *L. saligna* are sufficient for complete protection against *B. lactucae* when introgressed into *L. sativa* background, **Chapter 3** describes the development, disease evaluation and histological studies on 11 combiBILs that each contains two to four target QTLs in *L. sativa* background (doubleBIL, tripleBIL and quartoBIL). The results showed that the three QTLs, *rbq4*, *rbq5* and *rbq6+11* led to increased levels of resistance when they were combined; but *rbq7* did not add to the resistance level when combined with the other QTLs. Most interestingly, one tripleBIL, with QTL combination of *rbq4*, *rbq5* and *rbq6+11* displayed complete resistance at young plant stage. This suggests a redundancy of quantitative genes for nonhost resistance in *L. saligna*.

In histological studies, we determined the resistance mechanisms underlying *L. saligna*, four target BILs, doubleBIL6.3+8.2 and tripleBIL2.2+6.3+8.2. The results suggested that *L. saligna* arrested all the *B. lactucae* infection units (IUs) before normal hypha formation; whereas in the susceptible *L. sativa*, >90% of IUs formed hyphae and haustoria. Most BILs with resistance QTLs allowed a few IUs to form hyphae and haustoria and displayed incomplete pre-hyphal resistance. The extent of incomplete pre-hyphal resistance varied for different QTLs:

- In BIL6.3, doubleBIL6.3+8.2 and tripleBIL2.2+6.3+8.2, we did not observe normal hyphae and haustoria, only malformed hypha-like structures. The resistance mechanisms in these lines resembled most those of *L. saligna*, but in addition they showed a subepidermal hypersensitive response.
- BIL2.2 and BIL8.2 showed mechanisms similar to those of *L. saligna* but in addition allowed a few hyphae and haustoria to form.

The pathogen in BIL4.2 displayed a similar infection development as in *L. sativa*. The quantitative resistance observed in BIL4.2 is therefore probably due to defense mechanisms activated later during the infection process (**Chapter 3**).

In **Chapter 4,** we describe the fine mapping of these four target QTLs via a Near Isogenic Line (NIL) approach. The QTLs were mapped originally in various-sized introgressions, between 20 cM and 60 cM. We first developed four sets of NILs with smaller *L. saligna* introgressions. Disease evaluations on these sets of NILs indicated that the intervals in which the resistance QTLs were likely to be located were narrowed down from 38 to 11 cM (BIL8.2, *rbq4*) and from 24 to 11 cM (BIL 6.3, *rbq6*). Unlike BIL8.2 and BIL6.3, results on BIL2.2 (*rbq5*) and BIL4.2 (*rbq7*) suggested multi-QTL in each introgression and the putative locations of three sub-QTLs, *rbq7a*, *rbq7b* and *rbq7c*, were determined. More experiments for phenotyping resistance levels in these sets of NILs are required to corroborate the hypothesis of several QTLs within the same introgression. In **Chapter 6**, the feasibility to clone *rbq4* is further discussed.

We observed necrotic lesions on leaves and stems, and retarded growth in hybrids from two interspecific crossing combinations, *L. saligna* CGN05271 x *L. sativa* cv. Olof (cross A) and *L. saligna* CGN11341 x *L.sativa* cv. Norden (cross B) (**Chapter 5**). Such phenomena are symptoms of "hybrid necrosis": a type of genetic incompatibility that occurs in intra- or interspecific crosses. **Chapter 5** describes that this hybrid necrosis is caused by a *RIN4-R* interaction between *RIN4* allele(s) from *L. saligna* (located on top of Chromosome 9) and a probable *R* gene in *L. sativa* (located on Chromosome 6). This hybrid necrosis correlated positively with resistance levels to *B. lactucae*, and was a temperature sensitive trait, since it did not occur at 30°C. Definitive proof of involvement of *RIN4* transcripts of *L. saligna* in hybrid necrosis and the resistance to *B. lactucae* race Bl:16 was obtained from over expression experiments (*Agrobacterium*-mediated Transient Assay, ATTA) and silencing experiments (Virus Induced Gene Silencing, VIGS).

In **Chapter 6**, the main results reported in this thesis are discussed in a broader perspective. The contributions of the QTLs to *L. saligna* nonhost resistance, the redundancy of QTLs in this nonhost resistance and the more precise mapping positions of the target QTLs are discussed. The possibilities to use the hybrid necrosis in lettuce as an example to study other genetic incompatibilities caused by interactive loci in hybrids are also addressed. At last, we also discuss the perspective and future use of this *L. saligna – B. lactucae* pathosystem to investigate the nonhost resistance.

SAMENVATTING

Sla (*Lactuca sativa*) is wereldwijd een populaire bladgroente. Valse meeldauw in sla (ook wel “het wit”genoemd) is de meest schadelijke ziekte in slateelten en wordt veroorzaakt door *Bremia lactucae*, een obligate biotrofe oomyceet. Op dit moment is de beheersing van valse meeldauw sterk aangewezen op het gebruik van chemische pesticiden en op de toepassing van waard-resistentie door introgressie van resistentie genen (*Dm/R*) in slarassen. Tot op heden zijn er veel *Dm/R* genen bekend en meer dan 20 ervan zijn toegepast in sla-veredelingsprogramma’s. Echter het gebruik van pesticiden is potentieel schadelijk voor het milieu, valse meeldauw kan tolerantie voor pesticiden ontwikkelen, en het gebruik van waard-resistentie (*Dm/R* genen) is niet duurzaam door de snelle ontwikkeling van nieuwe *B. lactucae* fysio’s.

Niet-waard-resistentie in de wilde sla-soort *Lactuca saligna* tegen *B. lactucae* zou benut kunnen worden als een meer duurzame resistentie in commerciële slarassen. De succesvolle kruising tussen de niet-waard *L. saligna* CGN05271 en de waard *L. sativa* Olof biedt een zeldzame kans om de genetica van niet-waard resistentie te bestuderen. Uit de zelfbevruchte F_1 van deze kruising ontwikkelden we een F_2 populatie van 126 planten en een set van 29 terugkruisingslijnen, zogenaamde “Backcross Inbred Lines (BILs). Het totale aantal introgressies binnen deze 29 BILs vertegenwoordigde 96% van het genoom van *L. saligna*.

In **Hoofdstuk 2** werd de set van BILs geëvalueerd voor *B. lactucae* aantasting in drie plantontwikkelingsstadia: zaailing, jonge plant en volwassen plant (veldtoets). De geïdentificeerde QTLs (=loci voor kwantitatieve eigenschappen) in deze tests werden vergeleken met zeven QTLs die eerder geïdentificeerd waren in volwassen planten in een kastoets. In totaal werden 16 introgressies geïdentificeerd die bijdragen aan de resistentie in één of meer van de vier genoemde ontwikkelingstadia en deze worden voorlopig beschouwd als 16 QTLs (*Rbq1* tot *16*). Voor 15 van deze QTLs is het resistentie-allel afkomstig van de niet-waard *L. saligna*; voor één QTL (*Rbq16*) is het resistentieallel afkomstig van de waard *L. sativa*. Alle 15 QTLs van *L. saligna* leken fysio-niet-speciek te zijn.

Interessant was dat slechts 2 van de 15 QTLs van *L. saligna* effectief waren in alle geteste plantontwikkelingsstadia; de effecten van de andere 13 QTLs waren

ontwikkelingsstadiumafhankelijk. Vier BILs, BIL2.2, BIL4.2, BIL6.3 en BIL8.2 bevatten QTLs die effectief waren in zowel jong als in volwassen plant stadium, *rbq5*, *rbq7*, *rbq6+11* en *rbq4*. We concentreerden ons voor vervolgstudies op deze BILs en hun QTLs voor *B. lactucae*-resistentie ("target-BILs" en "target-QTLs").

Om te bepalen hoeveel van deze vier introgressies van *L. saligna* voldoende zijn voor volledige bescherming tegen *B. lactucae* na inkruising in *L. sativa*, worden in **Hoofdstuk 3** de ontwikkeling, de ziekte-evaluatie en de histologische studies van 11 combinatie-BILs (combiBIL) beschreven. Deze combiBILs combineren ieder twee tot vier target-QTLs in een *L. sativa* achtergrond (doubleBIL, tripleBIL en quartoBIL). De resultaten lieten zien dat combinaties van de drie QTLs, *rbq4*, *rbq5* en *rbq6+11* tot verhoogde resistentie niveaus leidden, terwijl *rbq7* het effect van de andere QTLs niet versterkte. Wetenswaardig was dat één tripleBIL met *rbq4*, *rbq5* en *rbq6+11* volledig resistent was in het jonge plant-stadium. Dit suggereert dat er in *L. saligna* een overschot aan kwantitatieve genen voor niet-waard resistentie is, meer dan strikt nodig voor volledige bescherming.

In histologische studies bepaalden we de resistentiemechanismen in *L. saligna*, de vier target-BILs, de doubleBIL6.3+8.2 en de tripleBIL2.2+6.3+8.2. De resultaten weren uit dat *L. saligna* het alles *B. lactucae* infectie eenheden (IE) stopt vóór vorming van hyfen, terwijl in de vatbare *L. sativa* >90% van de IE hyfen en haustoria vormden. De meeste BILs met resistentie QTLs lieten enkele IE tot hyfen en haustoria uitgroeien en vertoonden dus een onvolledige pre-hyfen resistentie. De mate van incomplete pre-hyfen resistentie varieerde voor verschillende QTLs:

- In BIL6.3, doubleBIL6.3+8.2 en tripleBIL2.2+6.3+8.2 zagen we geen normale, maar alleen abnormale (verschrompelde) hyfen en haustoria. Hun resistentiemechanismen leken het meest op die van *L. saligna* met daarbovenop een subepidermale overgevoeligheidsreactie.
- BIL2.2 en BIL8.2 lieten soortgelijke mechanismen zien als *L. saligna*, maar deze lijnen stonden daarnaast de vorming van enkele hyfen en haustoria toe.

In BIL4.2 vertoonde *B. lactucae* eenzelfde infectieontwikkeling als in *L. sativa*. De kwantitatieve resistentie, waargenomen in BIL4.2, wordt daarom waarschijnlijk veroorzaakt door defensiemechanismen die later in het infectie proces geactiveerd worden (**Hoofdstuk 3**).

In **Hoofdstuk 4** beschrijven we de fijnkartering van de vier target-QTLs volgens een Near Isogenic Line (NIL) benadering. De QTLs werden oorspronkelijk gekarteerd in introgressies van verschillende grootten, tussen de 20 en 60 centiMorgan. Eerst ontwikkelden we vier sets van NILs met kleinere *L. saligna* introgressies. Vervolgens bleek na analyse van de ziektetoetsen dat de intervallen waarin de resistentie QTLs waarschijnlijk gelegen zijn verkleind zijn van 38 tot 11 cM (BIL8.2, *rbq4*) en van 24 tot 11 cM (BIL 6.3, *rbq6*). In

tegenstelling tot deze resultaten voor BIL8.2 en BIL6.3, suggereerden de fijnkarteringsresultaten van BIL2.2 (*rbq5*) en BIL4.2 (*rbq7*) meerdere QTLs in elke introgressie en de mogelijke locaties van de drie sub-QTLs, *rbq7a*, *rbq7b* en *rbq7c*, werden bepaald. Meer fenotyperings-experimenten voor resistentieniveau zijn in deze sets NILs nodig om de hypothese van meerdere QTLs in dezelfde introgressie te bevestigen. De mogelijkheden tot het cloneren van *rbq4* worden besproken in **Hoofdstuk 6**.

Wij observeerden necrotische vlekjes op bladeren en stengels, en achtergebleven groei in hybriden van twee interspecifieke kruisingscombinaties, *L. saligna* CGN05271 x *L. sativa* cv. Olof (cross A) en *L. saligna* CGN11341 x *L. sativa* cv. Norden (cross B) (**Hoofdstuk 5**). Dergelijke verschijnselen zijn symptomen van "hybride necrose", dat een type van genetische incompatibiliteit is die voorkomt in intra- of interspecifieke kruisingen. **Hoofdstuk 5** beschrijft dat deze hybride necrosis wordt veroorzaakt door een *RIN4-R* interactie tussen *RIN4* allele(n) van *L. saligna* (locus bovenaan Chromosoom 9) en waarschijnlijk een *R* gen in *L. sativa* (locus op Chromosoom 6). Deze hybride necrose is positief gecorreleerd met resistentieniveaus tegen *B. lactucae*, en was temperatuurgevoelig, want trad niet op bij 30°C. Definitief bewijs van de betrokkenheid van *RIN4* transcripten van *L. saligna* in hybride necrose en resistentie tegen *B. lactucae* Bl:16 werden verkregen uit overexpressie-(*Agrobacterium*-mediated Transient Assay, ATTA), en uitschakelings-experimenten (Virus Induced Gene Silencing, VIGS).

In **Hoofdstuk 6** worden de belangrijkste resultaten van dit proefschrift besproken en in een breder perspectief geplaatst. De bijdragen van de QTLs voor niet-waard resistentie van *L. saligna*, de overtolligheid van het aantal QTLs dat bijdraagt aan deze niet-waard resistentie en de preciezere kaartposities van de target-QTLs worden bediscussieerd. De mogelijkheden om deze hybride necrose in sla te gebruiken als een voorbeeld om genetische incompatibiliteiten tengevolge van wisselwerking tussen loci te bestuderen worden besproken. Tot slot, behandelen we de perspectieven en het toekomstige gebruik van dit *L. saligna – B. lactucae* pathosysteem voor het onderzoeken van niet-waard resistentie.

中文摘要

博士论文

英文题目

Genetic dissection of nonhost resistance of wild lettuce, *Lactuca saligna*, to downy mildew

中文题目

野生种生菜(*Lactuca saligna*)抗霜霉病非寄主抗性的遗传剖析

张凝文

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生菜（*Lactuca sativa*）是一种重要的叶用类蔬菜。生菜霜霉病是由活体寄生卵菌 *Bremia lactucae* 引起的，是生菜最严重的病害。该病害的防治工作主要依赖于杀菌剂的使用和在栽培种中渗入主效抗性基因（*Dm/R*）使其获得抗病性。至今，至少有 20 个 *Dm/R* 基因被成功应用于生菜育种项目中。然而，杀菌剂的使用会造成严重的环境问题， 也会促使病原菌对杀菌剂产生一定程度的耐药性，而且主效抗性基因的抗病性由于病原菌的快速变异又通常不能持久。所以，一直以来人们都在寻找一种更安全更持久的病害防治措施。

利用非寄主抗性的抗性机制来使作物自身产生持久抗病性是一种很有潜力的既安全又可持续的病害防治策略。本课题主要对非寄主抗性进行遗传剖析并研究其抗性机制。野生种生菜 *Lactuca saligna*（CGN05271）是霜霉病的非寄主，同时也能够和栽培种 *Lactuca sativa* (cv. Olof) 杂交，从而为进行该非寄主抗性遗传剖析提供了难得的实验材料。Jeuken 博士通过将该杂交系的杂种一代自交得到 126 株杂种二代， 同时将杂种二代植株与轮回亲本回交数代，再将回交后代自交后最终得到一套由 29 个品系组成的回交重组自交系（Backcross Inbred Line, BIL）。整套回交重组自交系共将野生种生菜 *L. saligna* 基因组的 96%导入了栽培种 *L. sativa* 的遗传背景中。

本论文的第二章通过三种生物鉴定方式，分别在苗期、幼株期和田间成株期三个生菜不同生长发育阶段，对该回交重组自交系的全部品系进行抗病性鉴定。我们将鉴定结果与已经报导的在温室成株阶段对该自交系各品系鉴定后确定的七个数量性状位点（Quantitative Trait Locus, QTL）进行了比较。 结果显示，共计 16 个 品系被鉴定为在一个或多个生长发育阶段表现出抗病性。 这 16 个品系的渗入片段的位置被暂定为 16 个 QTL，命名为 *Rbq1* 到 *Rbq16*。 其中，有 15 个位点的抗病等位基因是来自野生种（非寄主），而 *Rbq16* 则是来自栽培种（寄主）。 所有 15 个来自野生种的 QTL 都表现出非小种专化抗病性。 有趣的是这 15 个 QTL 中，只有两个位点在所有鉴定的生长发育阶段都表现出了抗病性，而其他的 13 个位点抗病性只在特定的一个或几个生长发育阶段才表现出来。本研究将其中四个品系，即分别含有数量性状位点 *rbq5*, *rbq7*, *rbq6+11* and *rbq4* 的 BIL2.2, BIL4.2, BIL6.3 和 BIL8.2，选取为“目标品系”，它们所含的 QTL 为“目标 QTL”，因为这四个品系在幼株期和成株期都表现出了不同程度的抗病性，从而具有较高的生产实用性。

为了研究同时导入多少个“目标 QTL”才能够使栽培种具有完全的抗病性，论文第三章报导了构建 11 个 QTL 聚合系的过程和对其进行抗病性鉴定和组织学方面研究

的成果。 这些 QTL 聚合系分别在栽培种的背景上渐渗有两个到四个目标 QTL（doubleBIL, tripleBIL 和 quartoBIL）。 结论指出，QTL 聚合系的抗病性由于其中三个 QTL，*rbq4*, *rbq5* 和 *rbq6+11* 的聚合而增强，相反，*rbq7* 与其他 QTL 聚合后并不能带给品系比原来更强的抗病性。最特别的是其中一个 tripleBIL, 含有 *rbq4*, *rbq5* 和 *rbq6+11*，在幼株阶段表现出了完全抗病性。 该结果表明在非寄主 *L. saligna* 的基因组中，有大量冗余的数量性状位点存在，而他们在非寄主抗性中所起的作用有待进一步讨论。

本研究通过显微镜分析，对非寄主 *L. saligna*, 四个目标品系, doubleBIL6.3+8.2 和 tripleBIL2.2+6.3+8.2 的抗性机制进行了注释。结果显示 *L. saligna* 能抑制所有接种的病原菌产生正常的菌丝和吸器， 而在感病亲本中，大于 90% 的病原菌能够形成正常的菌丝和吸器。大多数经过鉴定的含有 QTL 的渐渗系中，我们观测到只有很少一部分病原菌形成了正常的菌丝和吸器，并且把这一现象叫做菌丝前抗性（pre-hyphal resistance）。 菌丝前抗性的程度因 QTL 的不同而异：

- 我们没有在渐渗系 BIL6.3, doubleBIL6.3+8.2 和 tripleBIL2.2+6.3+8.2 中观测到正常的菌丝和吸器，只观测到类似变态菌丝的结构。其中的抗性机制与非寄主亲本所表现出的十分相似。但是，在这几个渐渗系中还额外观测到了表皮下快速过敏反应（Subepidermal Hypersensitive Response, SEHR）
- 渐渗系 BIL2.2 和 BIL8.2 的抗性机制也与非寄主亲本类似，所不同的是，接种在这两个品系上的病原菌有一小部分形成了正常的菌丝和吸器。

由于我们没有观测到渐渗系 BIL4.2 与感病亲本间的显著差异，所以推测该系所含 QTL 的抗性机制属于迟缓型，可能会在接种后期才表现出来（第三章）。

论文的第四章通过构建近等基因系（NIL, Near Isogenic Line），对四个目标 QTL 进行了精细定位。这四个目标 QTL 最初被定位在 20 cM 到 60cM 不等的渗入片段上。我们首先在四个目标 BIL 的基础上构建了四套近等基因系。其中每一套近等基因系中的各品系含有比原有 BIL 的渗入片段更小的渗入片段，在一起互相重叠覆盖整个 BIL 渗入片段。 通过对每一套近等基因系进行抗病性鉴定，从而将各 QTL 精细定位。 最终， QTL *rbq4* 的定位从原来的 38 cM 精确到 11 cM 的渗入片段上，*rbq6* 的定位从原来的 24 cM 精确到 11 cM。 同时，鉴定结果显示含有另外两个 QTL， *rbq5* 和 *rbq7* 的渗入片段极有可能分别含有多个细分数量性状位点。 本研究还进一步估计了细分数量性状位点，*rbq7a*, *rbq7b* and *rbq7c* 的大致定位。 当然，更加精确的 QTL 定位

有待于对同一近等基因系进行更多次重复鉴定。 论文还在第六章讨论了克隆 *rbq4* 的可行性方案。

在研究过程中，我们还在两组种间杂交组合的后代的叶片及茎秆上发现了坏死斑，并伴随有植株迟缓发育现象（第五章）。这一现象是典型的“杂交坏死斑”，通常由于种间或种内杂交的遗传不亲和性而导致。第五章的研究表明这种杂交坏死斑是由分别位于 9 号染色体端部的 *RIN4* 基因和位于 6 号染色体的 *R* 基因互作而产生的。该坏死斑的程度还与植株抗病性成正相关，而与环境温度成负相关（因为在 30°C.的条件下该杂交种不呈现坏死斑）。 我们通过农杆菌介导的瞬态检测（*Agrobacterium*-mediated Transient Assay, ATTA）和病毒诱导的基因沉默（Virus Induced Gene Silencing, VIGS）进行 *RIN4* 基因转录物的过度表达实验和基因沉默实验，从而证明了该 *RIN4* 基因是杂交坏死斑的成因之一，而且该基因的存在也导致了杂交种对于霜霉病小种 Bl:16 的完全抗病性。

论文第六章总结并更加深入地讨论了本研究的各项结果，包括多个微效性状位点在野生种所具备的非寄主抗性中的贡献，冗余 QTL 的发现以及对于目标 QTL 的精细定位。 同时我们也提出了利用所发现的生菜杂交坏死斑现象及目前的研究成果作为模式，进一步研究其它由基因互作而引起的遗传不亲和性现象的构想。最后，我们还讨论了野生种生菜——霜霉病抗病系统作为今后研究非寄主抗性的模式系统的应用前景 。

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I had also an unforgettable experience as being a representative of Chinese students at Wageningen and became a member of Association of Chinese Students and Scholors in The Netherlands (ACSSNL). During the work over there, I met **Prof. Tong Guangwu, Prof. Dong Huiqing** and **Prof. Zhang Xiaodong** from Chinese Embassy in The Netherlands and **the board members of ACSSNL**. I would like to express my gratitude to all of you for supporting me and sharing this wonderful experience with me.

I am a lucky person. Besides all above, the love from **my grandparents, parents, parents in law, sister in law and brother in law and all the relatives** is the key reason of building-up my positive attitude. **My parents** used to say that home is my haven, I can return whenever I want. Yes, Mum and Dad, I am a kite flying on the sky. Whenever you want, just pull the line and I will return…….

我是一个幸运的人。我的幸运除了上述的所有际遇外，我的家人们（我的祖父、祖母、爸爸妈妈、公公婆婆、姐姐，姐夫和亲戚们）对我无限的关爱是让我拥有乐观个性的关键。爸爸妈妈常说，无论何时家都是我永远的避风港，在外面漂泊的倦了、累了，随时回家。是的，爸妈，我就像一个风筝，一直在天上飘呀飘，但只要你们轻轻扯动手中的绳，我就会回家。

There are many regrets and failures in my thesis and also in my personal life, but I would never regret to marry **Lin Ke**. Ke, you are such a sunny guy and I even think it is hopeless to see you becoming a mature man. You told me that you will keep this childlike innocence for reminding me to be happy and carefree. I admit that your confidence and passion have brought perspective and depth in my life. My only hope is to hold your hand and grow old.

Wageningen, September 2, 2008

About the author

Ningwen Zhang was born on September 21st, 1980 in Kunming City, Yunnan Province, China. In Nanjing Agricultural University (NAU), she obtained her BSc as Ornamental Horticulture and Landscape Design major in 2002.

In September 2002, she came to Wageningen University and Research Centre (WUR) and obtained her MSc as Genetic Plant Breeding and Resources major in April 2004.

From 2004 to 2008, she works as a research assistant (AIO/ PhD) in the Laboratory of Plant Breeding, Graduate School of Experimental Plant Sciences (EPS) of WUR, on a project of "Functional genomic characterization of nonhost resistance to downy mildew species in lettuce and related plant species". This thesis is the product of her four years PhD research, which was financed by the Dutch Technology Foundation STW, the applied science division of NWO and the Technology Program of the Ministry of Economic Affairs and the six Dutch breeding companies.

List of Publications

- Huang S, van der Vossen E.A., Kuang H, Vleeshouwers V.G., **Zhang N,** Borm T.J., van Eck H.J., Baker B, Jacobsen E, Visser R.G. F. Comparative genomics enabled the isolation of the R3a late blight resistance gene in potato. *Plant J. 2005 Apr;42 (2):251-61.*

- **Zhang, N.W.,** Lindout, P, Niks, R.E. and Jeuken M.J.W. *Lactuca saligna* nonhost resistance to downy mildew is polygenic, and resistance QTL effects are dependent on developmental stage. Submitted

- **Zhang, N. W.,** Pelgrom, K., Niks, R. E., Visser, R.G.F. and Jeuken, M. J. W. Redundancy of QTLs for nonhost resistance in *Lactuca saligna* to *Bremia lactucae*. Submitted

- Jeuken, M. J. W., **Zhang, N. W.,** Pelgrom, K., Den Boer, E., Lindhout, P., Visser, R.G.F. and Niks, R. E. Hybrid necrosis reveals autoimmunity and hidden downy mildew resistance by *RIN4-R* gene interaction. Submitted

- **Zhang, N. W.,** Pelgrom, K., Niks, R. E., Visser, R.G.F. and Jeuken, M. J. W. Fine mapping of four QTLs for nonhost resistance to lettuce downy mildew, reveals both single- and multi-QTL per introgression region. To be submitted

Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Ningwen Zhang
Date: September 02, 2008
Group: Laboratory of Plant Breeding, Wageningen University

1) Start-up phase	date
► **First presentation of your project**	
Functional genomic characterisation of nonhost resistance to downy mildew species in lettuce and related plant species	May 18, 2004
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
Plant-Microbe interaction (PHP30306)	Feb-Apr 2005
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student day 2004 (Free University of Amsterdam)	Jun 03, 2004
EPS PhD student day 2005 (Radboud University Nijmegen)	Jun 02, 2005
EPS PhD student day 2006 (Wageningen University)	Sep 19, 2006
► **EPS theme symposia**	
EPS symposium: topic Intracellular Trafficking (University of Amsterdam)	Feb 02, 2006
EPS theme 2 symposium in combination with Willy Commelin Scholten day	Feb 02, 2007
► **NWO Lunteren days and other National Platforms**	
NWO-ALW Lunteren meeting	Apr 05-06, 2004
NWO-ALW Lunteren meeting	Apr 04-05, 2005
NWO-ALW Lunteren meeting	Apr 03-04, 2006
NWO-ALW Lunteren meeting	Apr 02-03, 2007
NWO-ALW Lunteren meeting	Apr 07-08, 2008
► **Seminars (series), workshops and symposia**	
Flying seminars	2004-2008
Weekly seminars at Plant Breeding	2004-2008
► **Seminar plus**	
► **International symposia and congresses**	
Plant Genomics European Meetings (GEMs), Lyon, FR	Sep 21-25, 2004
EUCARPIA Leafy Vegetables 2007 Conference, Warwick, England	Apr 18-20, 2007
► **Presentations**	
Oral presentation at summerschool 'Environmental signaling: Arabidopsis as a model'	Aug 23, 2005
Poster presentation at EUCARPIA Leafy Vegetables 2007, Warwick, England	Apr 18-20, 2007
Oral presentation at EUCARPIA Leafy Vegetables 2007, Warwick, England	Apr 19, 2007
Oral presentation at NWO-ALW Lunteren meeting	Apr 08, 2008
► **IAB interview**	Sep 08, 2006
► **Excursions**	
Subtotal Scientific Exposure	*11.8 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
Springschool 'Bioinformatics: Triple I'	Mar 31-Apr 02, 2004
Summerschool 'Environmental signaling: Arabidopsis as a model'	Aug 22-24, 2005
PhD course 'Systems Biology: Principles of ¬omics data analysis'	Nov 07-10, 2005
PhD course 'Basic statistics'	Jun 12-16, 2006
Summerschool 'Signaling in Plant Development and defence: towards Systems Biology'	Jun 19-21, 2006
► **Journal club**	
Member of a literature discussion group at Plant Breeding, WUR	2004-2008
► **Individual research training**	
Subtotal In-Depth Studies	*8.4 credits**

4) Personal development	date
► **Skill training courses**	
Project and Time Management	Nov-Dec, 2004
Teaching and Supervising Thesis Students	Jun 01-02, 2005
Academic Writing II (CENTA, Wageningen)	May 03-Jul 05, 2007
Scientific Writing (CENTA, Wageningen)	Sept 27-Nov 15, 2007
Career Perspectives	Mar 26-May 07, 2008
► **Organisation of PhD students day, course or conference**	
Organisation of weekly seminars at Plant Breeding, WUR	2005-2006
Organisation of PhD Meeting at Plant Breeding, WUR	Jun 2007
► **Membership of Board, Committee or PhD council**	
Member of the EPS PhD student counil	Oct 2004-Nov 2007
Subtotal Personal Development	*11.9 credits**

TOTAL NUMBER OF CREDIT POINTS*	**39.6**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*

The research described in this thesis was performed at the Laboratory of Plant Breeding of Wageningen University and Research Center and was financially supported by the Dutch Technology Foundation STW, the applied science division of NWO and the Technology Program of the Ministry of Economic Affairs (Project no. WGC06212).

Lay-out: Ningwen Zhang

Cover: Ningwen Zhang, Ke Lin and Nicolas Champouret

Fighting Saligna vs Lettuce Downy Mildew
Our wild lettuce *Lactuca saligna* is fully equipped and ready to fight with the pathogen (background). The four weapons stand for our four "target QTLs".

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