Genetic investigation of the nonhost resistance of wild lettuce, *Lactuca saligna*, to lettuce downy mildew, *Bremia lactucae*

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Thesis

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Contents

Abbreviations

ADT_F	Adult plant disease test in the field
ADT_G	Adult plant disease test in the greenhouse
AFLP®	Amplified fragment length polymorphism
ATTA	Agrobacterium-mediated transient assay
BIL	Backcross inbred line (This thesis: BC_4S_{1-2} plants with *L. saligna* introgression, 20-80 cM long, in a lettuce, *L. sativa*, genetic background)
EST	Expressed sequence tag
ETI	Effector triggered immunity
HI	Hybrid incompatibility
ISL	Infection severity level
MAMPs	Microorganism-associated molecular patterns
PAMPs	Pathogen-associated molecular patterns
Pre-BIL	Line with the same introgression segment as the BIL but in a heterozygous state instead of homozygous *L. saligna* state.
PRRs	Transmembrane pattern recognition receptors
PTI	PAMP-triggered immunity
QTL	Quantitative trait locus
RIS	Relative infection severity level
RRIS	Relative infection severity level in percentage of the susceptible parent (*L. sativa* cv. 'Olof')
SDT	Seedling disease test
Sub-BIL	Line with a smaller introgression segment than the BIL of which it is derived
TRD	Transmission ratio distortion
TRDL	Transmission ratio distortion loci
YDT	Young plant disease test

Schematic and code explanation for generation of offsprings (as used in this thesis)

F_1	An F_1 hybrid (or Filial 1 hybrid) is the first filial generation of offspring of distinctly different parental types**.**
F_2	F_2 hybrids are the result of self-pollination of F_1
F_3	F_3 hybrids are the result of self-pollination of F_2
BC_1	These hybrids are the result of a cross pollination between F_1 and one of the parents of the F_1
BC_1S_1	These hybrids are the result of self-pollination of BC_1 plants
BC_1saligna	These hybrids are the result of a cross pollination between *L. saligna* × *L. sativa* F_1 and *L. saligna*
BC_1sativa	These hybrids are the result of a cross pollination between *L. saligna* × *L. sativa* F_1 and *L. sativa*

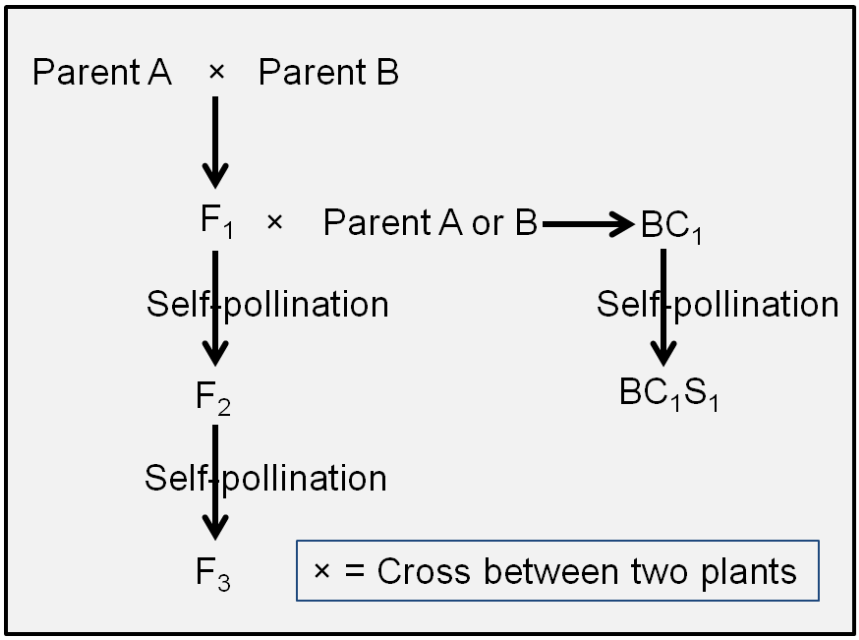


Figure 1. Schematic explanation for generation of offsprings

Code explanation for specific populations used (as used in this thesis)

CO	Plant material derived from *L. saligna* accession 275-5, that have been collected from the island Corsica in France
FR	Plant material derived from *L. saligna* accession CGN05271, that have been collected from mainland France
GEO	Plant material derived from *L. saligna* accession CGN15705, that have been collected from Georgia

CHAPTER 1

General introduction

Plant pathogen resistance

Plants are able to defend themselves to most potential pathogens of other plant species although their immunity is lacking the circulatory and mobile immune cells which are present in the vertebrate defence system. Plants have developed many different passive and active strategies and barriers to defend themselves (Spoel and Dong, 2012). Some defence mechanisms are functional against many pathogens and others are very specific against a single pathogen species or even against a particular isolate of the pathogen. The level of resistance to pathogens varies from absolute resistance to a slight reduction in pathogenicity or to tolerance in which the pathogen is able to infect the host but does not cause visible or severe symptoms and might incur hardly any damage to its host.

First layer of defence

The first layer of defence is based on avoidance in which the plant's morphology prevents establishment of intimate contact between the host and the pathogen. Plant morphology like leaf shape or size, and stoma morphology, density, arrangement in the epidermis and closure influence the level of disease resistance (Bradley et al., 2003; Melotto et al., 2006; Niks et al., 2011). Trichomes can prevent pathogenic microorganisms to reach the leaf surface or they can produce secondary antimicrobial metabolites (Nonomura et al., 2009; Harada et al., 2010). To penetrate the cell wall, pathogens require to develop specialised structures and / or to form enzymes to digest plant cell wall components (Carapito et al., 2008; Choi et al., 2011; Łaźniewska et al., 2012).

Second layer of defence

When a potential pathogen passes the first layer of defence the plant has to activate other resistance mechanisms to defend itself against the pathogen. First the plant can prevent the pathogen entering the cell by fortifying the cell wall by papillae, which is a common active plant response against penetration attempts by pathogens (Schmelzer, 2002). Plants also contain or produce secondary metabolites and anti-microbial enzymes which can inhibit or slow down the infection attempt or process (Bennett and Wallsgrove, 1994). For resistance often recognition of the pathogen is required. Plant pathogens can be detected extracellularly as well as intercellularly.

On the external face of the plant cell, pattern recognition receptors (PRRs) detect conserved microbial elicitors, called pathogen or microorganism-associated molecular patterns (PAMPs or MAMPs) and endogenous molecules released by pathogen invasion (damage-associated molecular patterns, DAMPs) (Jones and Dangl, 2006; Dodds and Rathjen, 2010; Spoel and Dong, 2012). The activation of PRRs leads to a defence reaction called PAMP, MAMP-triggered immunity (PTI, MTI) or basal immunity. Examples of PAMPs/MAMPs are chitin from fungi (Kaku et al., 2006), flagellin or lipopolysaccharides from bacteria (Zeidler et al., 2004;

Zipfel et al., 2004) and elicitins, trans glutaminase and cellulose-binding proteins from oomycetes (Kamoun, 2006; Stassen and Van den Ackerveken, 2011). Examples of DAMPs are cell wall fragments released by microbial enzymes, cutin monomers released by fungal cutinases and systemin which is expected to be released after cell injury (Darvill and Albersheim, 1984; Schweizer et al., 1996; Ryan and Pearce, 2003; Boller and Felix, 2009).

If the pathogen is able to overcome or to avoid the basal immunity, resistance can be triggered by recognition of pathogen virulence molecules which are called effectors. This recognition is based on highly specific, direct or indirect, interactions of proteins from plant resistance genes (*R*-genes) with these effector proteins. Recognition of the effector leads to a hypersensitive cell death response, which is known as effector triggered immunity (ETI), *R*-gene based, qualitative resistance or vertical resistance (Boller and Felix, 2009). Such ETI has been demonstrated or hypothesised to occur in many plant-pathosystems. For an overview see (Sanseverino et al., 2010).

The current view of the plant immune system is a zigzag model in which both the plant and the host are adapting to each other by natural selection in four different phases, described by Jones and Dangl (2006). In phase one, pathogen PAMPs or MAMPs are recognised by PRRs, resulting in PAMP or MAMP-triggered immunity. In phase two, the pathogen develops effectors to overcome this resistance and in phase three the plant develops *R*-genes to detect these effectors. And in phase four the pathogen avoids recognition by not expressing recognised effectors, changing the structure of these effectors or developing additional effectors that suppress somehow the ETI.

Nonhost resistance

"A species is considered as a nonhost when the complete species is resistant to all different variants of a pathogen" (Heath, 1981; Niks, 1988). This strict definition of "nonhost resistance" is a theoretical definition which implies that nonhost status cannot be ultimately proven, as it is impossible to test all genotypes of a plant species to all different isolates of a pathogen that have appeared in the past, present and future and under all different conditions (Niks et al., 2011). However in practice, testing a reasonable number of genotypes from a plant species against a reasonable number of isolates of a pathogen can already show a clear difference in the general trend of a plant species being compatible with the pathogen (host) or being incompatible (nonhost). Apart from obvious host and nonhost status, different intermediate levels of host status can be found (Niks, 1987). Examples of extensive plant-pathosystem host status studies are described for: barley (*Hordeum vulgare*)-rust (*Puccinia*) with 110 barley genotypes and 13 heterologous and one homologous rust species collected from various cereal and grass species(Atienza et al., 2004; Niks and Marcel, 2009), and lettuce-downy mildew with four lettuce (*Lactuca)* species and 20 downy mildew (*B. lactucae*) isolates (Bonnier et al.,1991). In the barley host status study, barley was a marginal host to nine heterologous rust species and a nonhost to

four heterologous rust species. Within the lettuce study, 1789 plant genotypes (cultivated lettuce *Lactuca sativa* n= 1288, wild lettuce species: *L. serriola* n= 399, and *L. saligna* n= 52 and *L. virosa* n= 50) were tested with two isolates or 20 isolates when the line was resistant for the first 2 tested isolates (Bonnier et al., 1991). *L. sativa* and *L. serriola* showed a compatible response (host) and *L. saligna* a consistently incompatible response (nonhost). *L. virosa* took an intermediate position, as several accessions of *L. virosa* were resistant to all races used, while other accessions gave a race-specific interaction with *B. lactucae*.

Nonhost resistance is the most common form of resistance as plant species are resistant to almost all the pathogens that specialised to other plant species. Besides possible explanations by the first layers of defence, the genetic mechanism behind nonhost resistance is believed to be explained by a combination of the pathways of PAMP and ETI which are also involved in host resistance (Schweizer, 2007; Schulze-Lefert and Panstruga, 2011). Fan and Doerner (2012) imply that there is no single mechanism that explains the nonhost resistance in all crops to different pathogens because a multitude of mechanisms seem to play a role in nonhost resistance, including recognition-based defences and recessive susceptibility factors within the primary plant metabolism. Studying nonhost resistance is often difficult due to crossing and fertilisation barriers between host and nonhost species. Nonhost resistance is scientifically very interesting because the molecular mechanisms behind this resistance can give an answer to the more conserved resistance mechanisms. It is of interest for crop breeding because related nonhost species might be an alternative source of durable resistance.

Lettuce- downy mildew

A well-studied crop pathogen interaction is lettuce, *Lactuca sativa*, with its downy mildew pathogen *Bremia lactucae* (Figure 1). This interaction has been studied well for a few decades, because lettuce is one of the most valuable leafy vegetable crops and *B. lactucae* is one of the major pathogens (Michelmore et al., 2009). Susceptibility to this pathogen is not only observed in cultivated lettuce *L. sativa* but also in wild relatives from the primary gene pool like *L. serriola*, *L. altaica* and *L. aculeata* and from the secondary gene pool like *L. virosa* (Lebeda and Boukema, 1991; Petrželová and Lebeda, 2004; Beharav et al., 2006). Both quantitative (Crute and Norwood, 1981; Norwood et al., 1983; Jeuken and Lindhout, 2002; Grube and Ochoa, 2005; Zhang et al., 2009a) and qualitative (reviewed by Michelmore and Wong, (2008) resistance against *Bremia lactucae* are present in lettuce and in related *Lactuca* species. The qualitative resistance to this pathogen is studied and applied in breeding more extensively than the quantitative resistance.

Figure 1. Downy mildew infection on a young plant with sporulation (indicated by arrow, left), leaf disc (middle), and on an adult plant (right).

Cultivated lettuce *Lactuca sativa*

Cultivated lettuce *Lactuca sativa* belongs to the genus *Lactuca* L. and to the family of Asteraceae or Compositae. *L. sativa* is mostly grown as an annual crop for its leaves, or for its stem (stem lettuce) or seeds (oilseed lettuce). *L. sativa* is a diploid self-pollinator with 18 chromosomes (2n = 2x =18). Probably lettuce is cultivated already for 4500 years. Cultivation started in the middle east where wall paintings of lettuce in Egyptian Tombs are found that are as old as 2500 years old (Lindqvist, 1960; de Vries, 1997). *L. serriola* is probably one of or the only direct ancestor of cultivated lettuce *L. sativa* (Kesseli, 1991; Vries and Raamsdonk, 1994).

Most of the lettuce is grown in the open field but production also takes place in the greenhouse. Cultivation on substrate or water is used rarely. Lettuce is consumed as a salad crop mainly in the western world. The seven most commonly grown lettuce cultivar groups are: crisp head (iceberg) with a large spherical head, romaine (cos) with an elongated head, butter head with a small spherical head and pliable leaves with oily texture, cutting lettuce with non-heading loose leaves, stalk lettuce with thick stem and small leaves and oilseed lettuce, a plant that forms no head or rosette of leaves but 50% larger seeds (de Vries, 1997). Cultivation and consumption of the lettuce cultivar groups is region dependent. Preferences are: north-western Europe: butter head, USA: crisp head, southern Europe: Romaine and China: cutting and stalk lettuce (personal communication J. Schut, lettuce breeder Rijk Zwaan).

Breeding and research in lettuce is done for many traits like: yield (as plant size and total weight per hectare), plant morphology, shelf-life, heat, salt and drought tolerance, nitrate level, colour, late bolting, resistance to physiological deficiencies like tip burning and disease and pest resistance (Ryder, 2010; Simko, 2013). The most important breeding objective is the improvement for resistance to *B. lactucae*, which causes serious yield losses worldwide. Most problematic for *Bremia lactucae* resistance breeding is that the pathogen is rapidly adapting to

the latest introduced *R*-genes. New R-genes are stacked and added to existing gene combinations of desired traits. The different lettuce cultivar groups complicate the breeding for resistance to *Bremia lactucae* as the new resistance genes need to be introgressed in all different lettuce groups without changing the plant morphology of that group. This requires time and labour intensive backcrossing.

The oomycete *Bremia lactucae*

The oomycete *B. lactucae* is an obligate biotrophic pathogen belonging to the Peronosporales. Although the oomycete is a fungal-like pathogen it is evolutionary related to brown algae and not to fungi. Oomycetes are destructive pathogens in many other cultivated plant species besides lettuce, for instance: *Phytophthora infestans* causing late blight in potato, *Phytium sojae* causing root rot in soybean, *Plasmopara viticola* causing downy mildew in grapes, *Peronospora farinosa* causing downy mildew in spinach, and *Plasmopara obducens* causing downy mildew on the garden flower *Impatiens walleriana* (Wegulo et al., 2004). Oomycetes can be necrotrophic (like *Phytium*), hemibiotropic (like *Phytophthora infestans*) and biotrophic (like *Bremia lactucae*) pathogens. For potato, lettuce and spinach these oomycetes are major limiting factors in cultivation as these pathogens are able to overcome recognition and resistance by new *R*-genes often already within a few years after introduction and can cause complete yield losses. The quick adaption of these pathogens to new resistances in their host or to fungicides are due to a successful combination of their large population size, both sexual and asexual reproduction and high gene flow (McDonald and Linde, 2002).

The importance and scientific interest for this group of pathogens has led to the genome sequencing of different oomycetes since 2006: *Phytophthora sojae, P. ramorum* (Tyler et al., 2006), *Phytophthora infestans* (Haas et al., 2009), *Pythium ultimum* (Lévesque et al., 2010), and *Hyaloperonospora arabidopsidis* (Baxter et al., 2010). Besides full genome sequencing, transcriptome sequencing and analysis of the pathogen effector proteins is being done for different oomycetes like *Phytium ultimum* (Cheung et al., 2008), *Pythium insidiosum* (Krajaejun et al., 2011) and for *Bremia lactucae* over 26.000 ESTs were sequenced (with a contig >500bp) (Stassen et al., 2012). Efforts for genome sequencing of *Bremia lactucae* are on the way (The Bremia genome project: http://bremia.ucdavis.edu/index.php?link=overview).

The genome sequence and / or the gene space of different oomycetes are used to study the biology of the oomycetes, as well as their interaction and adaptation to their plant hosts. Within different oomycete species over hundreds effectors have been detected in the classes Crinkler, RXLR and RXLR –like (reviewed by Stassen and van den Ackerveken (2011). Within the *Phytophthora infestans* genome the effector genes are located in highly dynamic expanding regions within the genome, this probably plays a role in the rapid adaptation and high flexibility of this pathogen (Haas et al., 2009).

B. lactucae has a genome size of about 50 Mb and consists of 7 or 8 chromosome pairs (Francis et al., 1990; Francis and Michelmore, 1993). The pathogen produces great amounts of asexual spores that germinate directly rather than by formation of zoospores. The spores of the pathogen are distributed by water and wind, either rain or splashing water. Because the pathogen requires water for the distribution of its spores the pathogen is mainly a problem in humid regions, climates and seasons. Different fungicides are applied to control the pathogen, but pathogen adaption to fungicides is commonly observed (Brown et al., 2004). In humid conditions fungicides need to be applied frequently which is expensive and undesirable for consumers because of unknown health effects in the long term. Because the frequent application of fungicides is undesirable, plant resistance is preferred.

The use of race-specific *R*-genes to downy mildew in lettuce breeding

In breeding, mostly qualitative resistance is used, and at present more than 30 *R*-genes are known (Michelmore and Wong, 2008). These genes are designated *Dm* genes. Most of the mapped *Dm* genes are located in three resistance-gene clusters on linkage groups: LG1 (five *Dm* genes), LG2 (eight *Dm* genes), and LG4 (six *Dm* genes), and with a single *Dm* gene located at LG3 (Paran et al., 1991; Paran and Michelmore, 1993; Maisonneuve et al., 1994; McHale et al., 2009). *Dm3,* located at linkage group two, was cloned and shown to encode a nucleotide-binding site leucine rich repeat (NB-LRR) resistance protein (Meyers et al., 1998; Shen et al., 2002).
Race-specific *B. lactucae-lactuca* spp interactions are often observed. To describe and designate commercially important individual *B. lactucae* races and their reactions to the different lettuce cultivars, the use of a common differential host set and its complementary reference pathogen races is desired. Separate pathogen populations consisting of different *B. lactucae* races/isolates appear in the major lettuce cultivation areas like Europe and USA (Michelmore and Wong, 2008). Designation initiatives are present both in the USA and Europe (http://www.worldseed.org/isf/ibeb.html, Van Ettekoven and Van der Arend, 1999).

Lactuca species as gene pool for *Bremia lactucae* resistance

The use of crop wild relatives in breeding has increased the last 20 years in terms of traits used, the number of wild species used and the number of crops containing genes from wild relatives (Hajjar and Hodgkin, 2007). To the primary gene pool of *Lactuca sativa* belong, besides the landraces and cultivars of *L. sativa,* different wild species which are cross-fertile: *L. serriola* (most likely ancestor, Figure 2), *L. aculeata*, *L. azerbaijanica*, *L. georgica*, *L. scarioloides* and *L. dregeana* (Lebeda et al., 2007; Lebeda et al., 2009). *L. saligna* and *L. virosa* belong to the secondary or tertiary (under discussion) gene pool (Figure 1). *L. saligna* is (mostly only as mother) cross-fertile with *L. sativa.* However, the cross can require much effort due to a low chance at success, and the level of fertility is low (Vries, 1990). *L. virosa* is only sporadically

cross-fertile with *L. sativa* and F_1 hybrids are often sterile (Maisonneuve et al., 1995). Introgression of *L. virosa* within *L. sativa* requires colchicine treatment, bridge crosses with *L. serriola* or embryo rescue (Thompson and Ryder, 1961; Eenink et al., 1982; Maisonneuve et al., 1995).

Bremia lactucae has a large host range and can infect over 200 species belonging to 40 genera within the Compositae (Crute, 1981; Lebeda et al., 2002a; Lebeda et al., 2008b). There is little evidence of gene flow between the *Bremia lactucae* isolates of different Compositae host species, suggesting that the species consists of a number of host specific *formae speciales.* (Voglmayr et al., 2004; Lebeda et al., 2008a). For *L. sativa* probably only the *Bremia lactucae* isolates present on *Lactuca* species might be a risk for lettuce production (Thines et al., 2010).

Quantitative resistance and qualitative resistance by single dominant *Dm* genes against *Bremia lactucae* has been reported to occur in both the primary as the secondary gene pool (Crute and Norwood, 1981; Spencer-Phillips et al., 2002; Lebeda et al., 2008b). New *Bremia lactucae* races have evolved that rendered the resistance conferred by *Dm* genes ineffective, therefore the resistance based on single *Dm* genes has not proven to be durable, (Michelmore et al., 1984); (IBEB) (http://www.worldseed.org/isf/ibeb.html).

Quantitative resistance is less commonly observed and used. In the primary gene pool it can be found in cultivated lettuce like *L. sativa* cv. Grand Rapids and Iceberg (Crute and Norwood, 1981; Norwood et al., 1983; Grube and Ochoa, 2005; Zhang et al., 2009a; Simko et al., 2013) and in wild relative *L. serriola* (Gustafsson, 1989).

In the secondary gene pool within the species *L. virosa* race specific resistance was found, but 13 out 50 tested accessions were resistant to all 20 tested races (Bonnier et al., 1991). Segregation analysis within populations of interspecific *L. sativa* × *L. virosa* crosses indicated that the resistance was explained by one or more dominant genes (Maisonneuve, 2003). The species *L. saligna* shows complete resistance to *B. lactucae* (i.e. resistance to all tested *B. lactucae* isolates) and is considered as a nonhost (Bonnier et al., 1991; Lebeda and Boukema, 1991) or near nonhost (Petrželová et al., 2011). Quantitative resistance (Jeuken and Lindhout, 2002; Zhang et al., 2009a) and qualitative resistance by race specific *Dm* genes, *R36* (Moreau, 1994), *R37* (Van Ettekoven and Van der Arend, 1999) and a not nominated *R*-gene (Zonneveld et al., 2011) has been detected within this species so far.

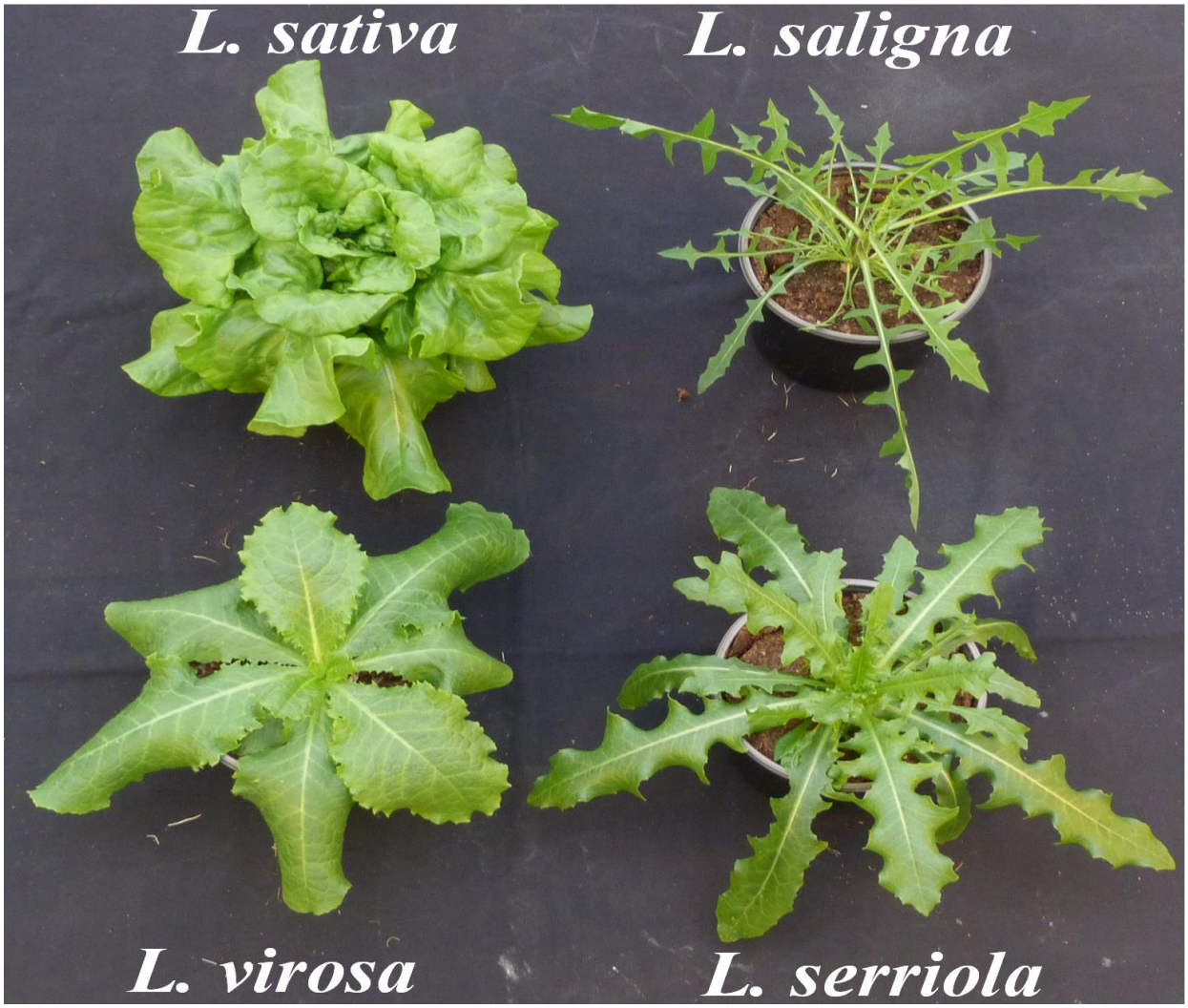


Figure 2. Four *Lactuca* species: *L. sativa* (cv. Cobham Green), *L. saligna* (275-5), *L. virosa* (CGN18634) and *L. serriola* (CGN15735).

Nonhost resistance from *L. saligna*

Since 1976 screenings have been described about the striking resistance spectrum of *L. saligna* (Netzer et al., 1976; Globerson et al., 1980; Norwood et al., 1981). In (1991), Bonnier et al, reported about a large Lactuca germplasm screening for downy mildew resistance in which all 52 tested *L. saligna* accessions were completely resistant to all 20 tested *B. lactucae* races in a leaf disc test.

The absolute resistance (0% sporulation) of *L. saligna* was discussed in Petrželová et al., (2011), because although this species shows always a high level of resistance, 10% of the 1460 interactions (146 accessions × ten tested *B. lactucae* races) showed some sporulation, but only at the seedling stage. Also sparse sporulation (typically about 5% as maximum severity) was

observed under laboratory conditions on the edges of leaf segments of *L. saligna* (Jeuken and Lindhout, 2002). In natural habitats *L. saligna* has never been reported as a host (Lebeda et al., 2001; Lebeda et al., 2002b; Beharav et al., 2008).

Wild species *L. saligna* is cross- fertile with cultivated lettuce *L. sativa*, albeit with a low success rate and reduced fertility in the F_1 and later generations. This cross-ability of a nonhost with a host plant species is exceptional. The interesting absolute resistance of this wild lettuce species is the subject of this thesis.

First attempts of the genetic dissection of *L. saligna* resistance

One of the first reports on the inheritance of the nonhost resistance of *L. saligna* was a study by Lebeda and Reinink (1994) with different *L. saligna* accessions and hybrids between *L. saligna* accessions and *L. sativa*. This study revealed besides race specific resistance within one of the studied *L. saligna* accessions (indicating presence of *Dm* genes), another resistance mechanism that was recessive and affected the development of *Bremia lactucae* at a later stage of the infection cycle.

Over a time course of about 20 years, the breeding industry and pre-competitive research, detected and introgressed some race-specific *Dm*-genes of *L. saligna* (Moreau, 1994; Van Ettekoven and Van der Arend, 1999; Zonneveld et al., 2011). However, these *Dm* genes (individually and stacked) do not provide resistance to all *Bremia lactucae* races (van Treuren et al., 2011), and therefore do not explain the absolute resistance (nonhost status).

As of 1997 research was started at Wageningen University to study the inheritance of nonhost resistance of *L. saligna* with as applied goal to introgress this resistance into cultivated lettuce. An interspecific F_2 population (n=126) was developed from the cross *L. saligna* CGN05271 ×*L. sativa* cv. Olof (cultivar without any known functional *Dm*-genes). F_2 genotype and phenotype data resulted in the first genetic lettuce map with nine linkage groups and the detection of quantitative resistance loci (Jeuken et al., 2001; Jeuken and Lindhout, 2002). For further validation studies and future breeding opportunities, a set of 29 Backcross Inbred Lines (BILs) was developed, covering more than 96% of the *L. saligna* genome, with per BIL mostly one homozygous *L. saligna* introgression in the *L. sativa* 'Olof' background (Jeuken and Lindhout, 2004; Figure 3). Validation by further inbred generations (F_3, F_4, or RIL population) was not feasible as severe reduced vitality and fertility in the F_2 and further inbred generations (F_3 and F_4) was observed. Histology on the *Lactuca – B. lactucae* infection process indicated that the resistance response of *L. saligna* was based mainly on pre-hyphal resistance, as shrivelled hyphae and consequentially no haustorium formation was observed on *L. saligna* (Lebeda et al. 2008b, Zhang et al., 2009b).

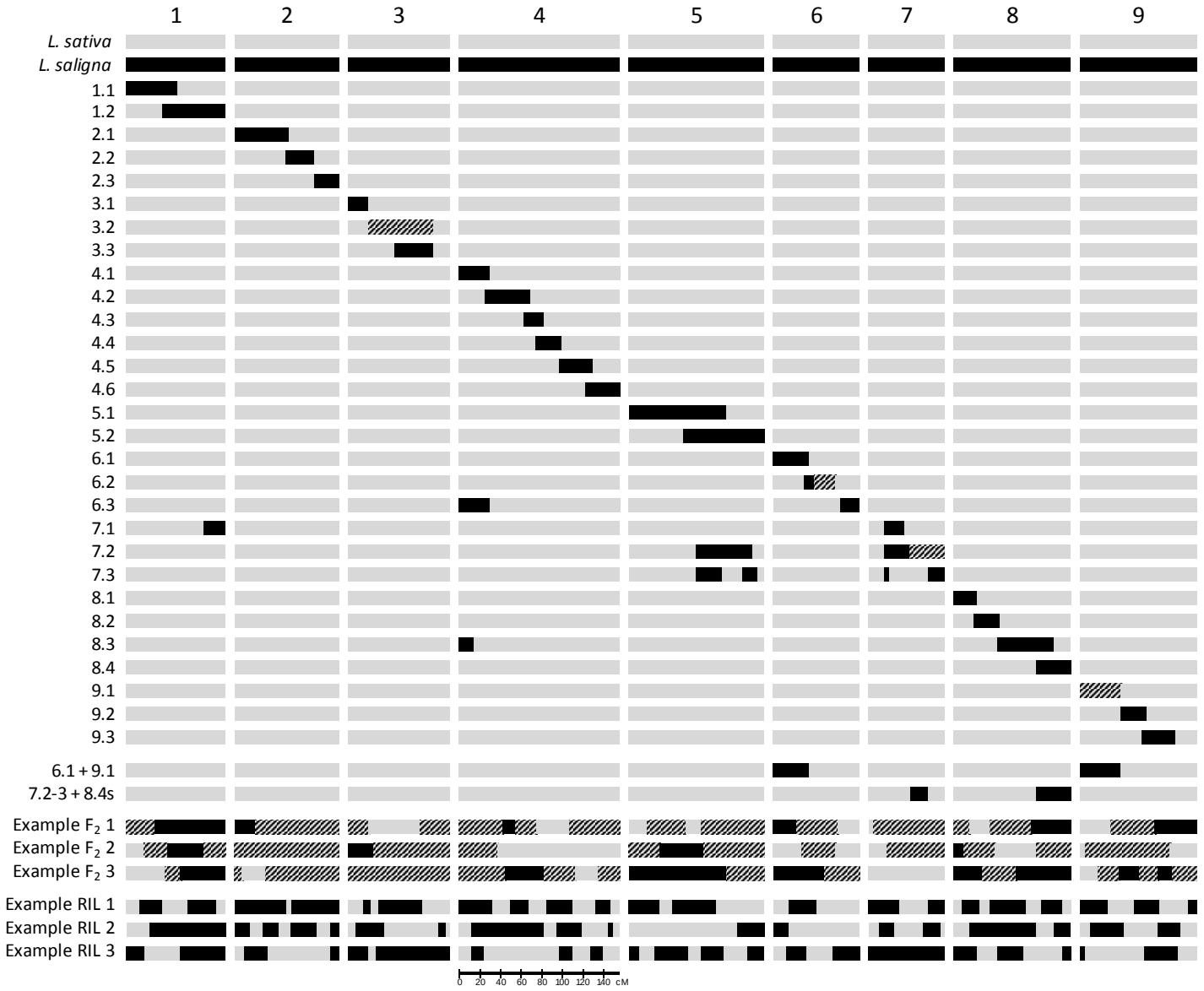
Inheritance of the resistance

The phenotyping of the F_2 and the set of BILs for levels of resistance indicated that the genetic basis of the *L. saligna* resistance is not explained by race specific *Dm* genes but likely by multiple quantitative genes. The F_2 population of 126 plants showed a wide and continuous range of infection levels and identified three minor QTLs, that were effective to both tested isolates (Jeuken and Lindhout, 2002). In addition to those QTLs, one locus for major gene resistance was detected. This gene appeared to be involved in a digenic "hybrid necrosis", based on an interaction between a *Rin4 L. saligna* allele and a potential *R* gene from *L. sativa.* In specific allelic combinations these two genes caused necrotic leaf lesions and quantitative and race specific qualitative resistance (Jeuken et al., 2009). In the set of 29 BILs, 15 lines showed quantitative resistance and based on disease tests with seven different *B. lactucae* isolates we assume that the resistances are race nonspecific (Jeuken and Lindhout, 2004; Zhang et al., 2009a). For most of those 15 BILs the resistance was plant developmental stage dependent except for BIL2.2, 4.2 and 8.2 (Zhang et al., 2009a). BIL 2.2, 4.2 and 8.2 showed an infection reduction of 60-70% at young plant stage and 30-50% at adult plant stage in field situations (most relevant for lettuce cultivation). The position of the *L. saligna* introgressions of these three BILs do not overlap with documented hotspots of monogenic or major resistance genes (*Dm* gene) in lettuce (McHale et al. 2009, Truco et al. 2013).

Hybrid incompatibilities between *L. saligna* and *L. sativa*

Hybrids between closely related species are often unviable or, if they survive, they have phenotypic abnormalities like sterility, low vitality or necrosis (Bomblies and Weigel, 2007). These deleterious hybrid characteristics, collectively called 'hybrid incompatibility', can reduce the exchange of genetic variants between species.

Although *L. saligna* and *L. sativa* are cross-compatible with some efforts, phenotypic abnormalities and severely distorted segregation indicate hybrid incompatibilities (Jeuken and Lindhout, 2002; Jeuken et al., 2009). In a set of 28 backcross inbred lines (BILs), with single *L. saligna* introgressions in *L. sativa* background, seven regions are presumed to be associated with hybrid incompatibility (indicated by absence of a homozygous *L. saligna* introgression). If traits of interest of *L. saligna* are closely linked to regions associated with hybrid incompatibility, introgression into *L. sativa* can be difficult.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9
L. sativa
L. saligna
1.1
1.2
2.1
2.2
2.3
3.1
3.2
3.3
4.1
4.2
4.3
4.4
4.5
4.6
5.1
5.2
6.1
6.2
6.3
7.1
7.2
7.3
8.1
8.2
8.3
8.4
9.1
9.2
9.3
6.1 + 9.1
7.2-3 + 8.4s
Example F2 1
Example F2 2
Example F2 3
Example RIL 1
Example RIL 2
Example RIL 3
0 20 40 60 80 100 120 140 cM

Figure 3.

Figure 3. Genotype representation of a set of 29 Backcross Inbred Lines that cover more than 96% of the *L. saligna* genome. Genotypes classes are: homozygous *L. sativa* in grey, homozygous *L. saligna* in black and diagonal stripes indicate a heterozygous genotype. Additional genotypes are: (top) recurrent parent *L. sativa* cv. Olof and introgression parent *L. saligna* CGN05271; (bottom) two lines in which a hybrid incompatibility region is neutralized by another introgression segment (lines 6.1+9.1 and 7.2-1+8.4; the dependent region is underlined); (bottom) six imaginary example F_2 and RIL plants. This figure is an updated version of the BIL genotype presentation in Jeuken and Lindhout (2004).

Screening for pathogen effector recognition in plants as a strategy for resistance breeding

Oomycete effectors

Oomycetes secrete molecules that target plant molecules and manipulate the host immunity system to enable or enhance pathogen growth within their host. These molecules are called effector proteins. The pathogen needs to deal first with biochemical barriers in the apoplast. Three types of apoplastic effectors are known to manipulate the host: inhibitors of host enzymes, RGD (Argine-Glycine_Aspartic acid) -containing proteins, and toxins (like PcF/SCR or Nep1-like proteins) that lead to host cell death. Besides manipulation of the host in the apoplast the pathogen secretes effectors carrying host-translocation signals and these are transported into the host cell and interfere with the host intercellular. Two classes of these effectors, RXLR and Crinklers, are known in oomycetes, (reviewed by Stassen and van den Ackerveken (2011), Oliva et al., (2010) and by Thines and Kamoun (2010).

Pathogen effectors can be used as an alternative for disease tests to identify resistances. By agrobacterium infiltration (transient assays) of effector proteins into a host plant, protein recognition can be studied. If the effector protein is recognised by the host, it will initiate a hypersensitive response, which is macroscopically visible as a necrotic leaf area. A successful example is the study from Vleeshouwers et al., (2008) who studied the recognition in potato of effectors from *Phytophthora infestans*. By transient assays the effect of a single effector can be observed instead of the sum of effects from a mix of effectors (*B. lactucae* possibly delivers more than 100 effectors into host plant cells). Therefore effector-approach by transient assays might be faster and more effective to identify new *Dm* genes than classic disease tests with downy mildew races. In classical disease tests different resistances triggered by separate effectors mask each other's effects and many potential resistances remain hidden (reviewed in Vleeshouwers et al., (2011)).

Scope and outline of thesis

The main and scientific aims of the research were to 1) gain insight in the inheritance of the nonhost resistance of *L. saligna* to *Bremia lactucae*; 2) determine the precise position of resistance QTLs within BIL-introgression segments and to investigate whether these genes can be employed in breeding; 3) determine whether stacking resistance QTLs from different BILs leads

to near-complete resistance; 4) determine if identification and mapping of resistance genes by an effector-approach by transient assays on large *Lactuca* collection is feasible.

Based on previous research at Wageningen UR Plant Breeding (described in the above paragraphs), we hypothesize that the *L. saligna* resistance can be explained by the joined effect of a certain number of previously identified quantitative resistances, which have been mapped in the introgressions of specific BILs.

This thesis was performed as a part of a larger TTI-Green Genetics research project entitled 'Novel approaches for resistance breeding using pathogen effectors and their host plant targets: towards durable resistance to *Bremia* in lettuce'. Two research groups were involved. Wageningen UR Plant breeding focused on the plant side and the van den Ackerveken group from the University of Utrecht focused on the pathogen side. Their part of the research aimed to identify and functionally analyse effectors of downy mildews.

In the first chapters of this thesis we focus on the resistances within the introgressions of specific BILs. In later chapters we describe alternative strategies for resistance gene identification.

In Chapter 2, we fine mapped resistance QTLs at young and adult plant stage in three introgression segments of *L. saligna* by a substitution mapping approach.

In Chapter 3, the potential of stacking the quantitative resistances of BIL and SUB-BIL introgressions to increase the resistance level under field conditions was analysed. The combined resistance effects of ten developed homozygous lines with two introgression segments (double-combinations) were tested. For one combination conferring a highly increased level of resistance (in young and adult plant stage), we determined the loci responsible for a high level of resistance at young, but not at adult plant stage.

In Chapter 4 we studied a digenic hybrid incompatibility that was detected within one of the BILs that was used for stacking, BIL[4.1+6.3]. We fine-mapped the two loci and studied the genetic basis of the interaction causing transmission distortion and absence of specific genotypes.

As in Chapter 2 and 3 we did not identify the loci responsible for complete resistance of *L. saligna*, we started a new approach.

In Chapter 5, we used multiple segregating *L. saligna* × *L. sativa* populations (F_2 and BC_1) involving three *L. saligna* accessions to identify the nonhost loci of *L. saligna.* Selective genotyping on F_2 plants, that were selected for the phenotypic extremes, i.e. absolutely resistant and highly susceptible plants, revealed interactive resistance loci.

In Chapter 6 a large set of *Lactuca* accessions was screened for their ability to recognise *Bremia lactucae* effector candidates. For the two effector proteins that were recognised by *Lactuca* lines the genetic regions responsible for the recognition were identified. We also determined whether response of *Lactuca* to certain effectors correlated with a resistance reaction to the isolates carrying such effectors.

In Chapter 7 all the results from previous chapters are discussed regarding to: the inheritance of the nonhost resistance in *L. saligna*, best future approach to use for further unravelling the nonhost resistance, and whether Agrobacterium-mediated transient expression of effectors are an effective alternative approach to screen for *Dm*-genes. Furthermore the current status and possibilities regarding downy mildew resistance will be discussed.

In this thesis diverse genetic plant materials, in terms of population types and introgression lines are used and described. For a better overview and understanding, the genetic compositions of several population types are illustrated in Figure 4.

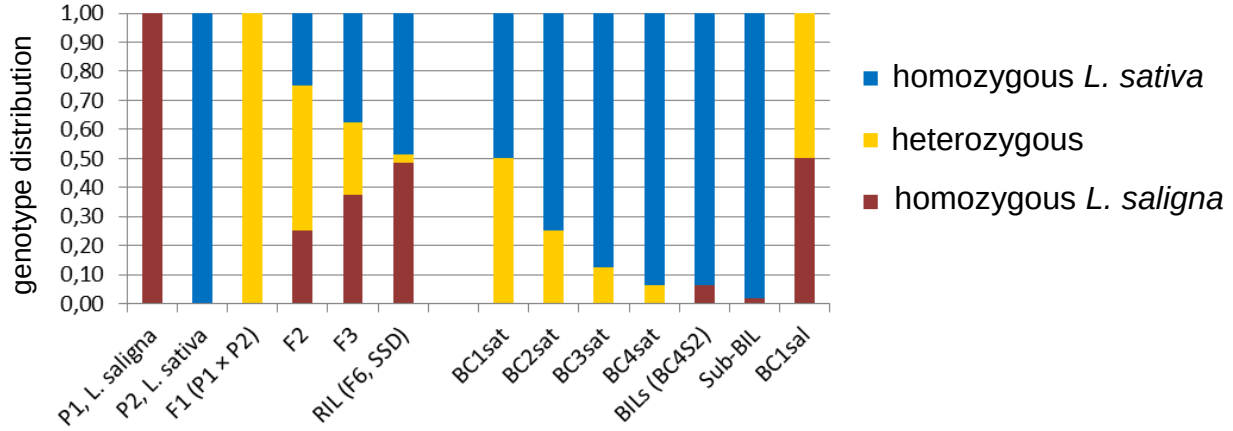

Figure 4. Theoretical genotype distributions per population type or line, assuming Mendelian segregation. P1 and P2 refer to the mother and father of the cross. RILs refer to recombinant inbred lines developed by single seed descent (SSD) until F_6. A BIL represents a backcross inbred line (Jeuken et al. 2004), and a sub-BIL is a line with a smaller introgression segment than the BIL of which it is derived (Chapter 2). 'sat' and 'sal' refer to the *L. sativa* and *L. saligna* backcross parent. Codes for generation of offsprings are explained at page 7.

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CHAPTER 2

Fine mapping quantitative resistances to downy mildew in lettuce revealed multiple sub-QTLs with plant stage dependent effects reducing or even promoting the infection

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Key message:

Three regions with quantitative resistance to downy mildew of non-host and wild lettuce species, *Lactuca saligna*, disintegrate into seventeen sub-QTLs with plant-stage-dependent effects, reducing or even promoting the infection.

Abstract

Previous studies on the genetic dissection of the complete resistance of wild lettuce, *Lactuca saligna*, to downy mildew revealed fifteen introgression regions that conferred plant stage dependent quantitative resistances (QTLs). Three backcross inbred lines (BILs), carrying an individual 30 to 50 cM long introgression segment from *L. saligna* in a cultivated lettuce, *L. sativa*, background, reduced infection with 60-70% at young plant stage and with 30-50% at adult plant stage in field situations. We studied these three quantitative resistances in order to narrow down their mapping interval and determine their number of loci, either single or multiple. We performed recombinant screenings and developed lines (sub-BILs) with smaller overlapping *L. saligna* introgressions than the BIL of which they were derived from (substitution mapping). In segregating introgression line populations recombination was suppressed up to seventeen fold compared to the original *L. saligna* × *L. sativa* F_2 population. Recombination suppression depended on the chromosome region and was stronger suppressed at the smallest introgression lengths. Disease evaluation of the sub-BILs revealed that the resistance of all three BILs was not explained by a single locus but by multiple sub-QTLs. The seventeen *L. saligna*-derived sub-QTLs had a smaller and plant stage dependent resistance effect, some segments reducing, others even promoting downy mildew infection. Implications for lettuce breeding are outlined.

Keywords: non-host resistance, backcross inbred lines (BILs), substitution mapping, plant stage dependent resistance, recombination frequency suppression, QTL

Introduction

Most knowledge on resistance in plants to (hemi)-biotrophic specialized plant pathogens exists on race-specific qualitative resistance that is conferred by *R* genes with the NBS-LRR (nuclear binding site and leucine-rich repeat) motifs of which many are nowadays cloned (Gururani et al. 2012). This resistance by *R* genes relies on the direct or indirect recognition of pathogen-delivered effectors. Much less is known about the genes underlying polygenic and/or quantitative and/or non-race specific resistance, of which very few genes are cloned like e.g. *mlo, Yr36, Pi21, Lr34* and *Rgh4* (Büschges et al. 1997; Fu et al. 2009; Fukuoka et al. 2009; Krattinger et al. 2009; Liu et al. 2012). The resistance conferred by genes like *Lr34, Lr46* and the *mlo* gene, which are not classical *R*-genes, seems to be durable as the resistance remains effective for a long time (Fu et al. 2009; Fukuoka et al. 2009; Jørgensen 1992; Kolmer 1996; Lillemo et al. 2008; Risk et al. 2012; William et al. 2003).

Bremia lactucae causes downy mildew in lettuce (*Lactuca sativa*), which is a devastating foliar disease causing high losses in lettuce cultivation. Resistance breeding focuses on the deployment of classical *R*-genes (named *Dm* genes). But *Dm*-genes are only effective for a short term because the resistance is often broken by new races of *B. lactucae* soon after release of cultivars carrying a new *Dm* gene (Lebeda and Zinkernagel 2003). The wild lettuce species *Lactuca saligna* is completely resistant to all *B. lactucae* races and might be an source of resistance that is more durably effective than resistance conferred by the classical *Dm*-genes (Bonnier et al. 1991; Jeuken and Lindhout 2002; Lebeda and Boukema 1991; Petrželová et al. 2011). The genetics and mechanism of the non-host resistance of *L. saligna* can be studied because of its cross compatibility with cultivated lettuce *L. sativa*. For the present, our aim is to unravel the genetic architecture behind the complete (non-host) resistance of wild lettuce, *L. saligna*, to lettuce downy mildew.

Earlier research included histological and genetic studies. Histology on the infection process indicated that the resistance response of *L. saligna* was based mainly on pre-hyphal resistance (Zhang et al 2009b). Research on a small F_2 population of 126 plants revealed three resistance QTLs, each explaining 12 to 26% of phenotypic variance, and a resistance caused by a digenic interallelic interaction that leads to hybrid necrosis (Jeuken and Lindhout 2002; Jeuken et al. 2009). Fertility limitations of the F_2 hampered further inbreeding, and prompted us to develop a set of 29 backcross inbred lines (BILs) (Jeuken and Lindhout 2004). Those homozygous introgression lines, with *L. saligna* introgression segments from 20 to 80 cM in a *L. sativa* background, represented together 96% of the *L. saligna* genome. The BILs were tested in four types of disease test, namely on seedlings (SDT), young plants (YDT), adult plants in the greenhouse (ADT_G) and adult plants in the field (ADT_F) (Zhang et al. 2009a). Fifteen BILs with quantitative resistance were detected. The three F_2-QTLs were not confirmed in this set of BILs (Jeuken et al. 2008). Of the fifteen resistant BILs, only two BILs, 2.2 and 4.2, showed resistance at all plant stages. BIL8.2 showed resistance in young and adult plant tests, but not in seedling tests (Zhang et al. 2009a). BIL 2.2, 4.2 and 8.2 showed an infection reduction of 60-70% at young plant stage and 30-50% at adult plant stage

in field situations (most relevant for lettuce cultivation). Those three BILs were selected for further fine mapping (Zhang et al. 2009a). A preliminary study on stacking resistances, showed that some combined introgression segments of BIL2.2, 4.2 and 8.2 led at young plant stage to an increased level of resistance compared to their respective individual segments (Zhang et al. 2009b). Stacking large *L. saligna* introgressions implies that also more genes for undesired traits are introgressed. Therefore, lines with smaller introgressions with only the gene conferring the resistance trait from *L. saligna* are preferred for further stacking strategies in resistance breeding.

Major objectives of this study were: 1) to fine map the resistance QTLs on the introgressions in the three BILs at the young plant stage under controlled conditions and at adult plant stage in the field (most relevant for commercial application); 2) to detach the resistance from undesired plant morphology traits for breeding (linkage drag).

Materials and methods

Plant material

L. sativa cv. Olof was the susceptible recurrent parent. The BILs with quantitative resistances were BIL 2.2, 4.2 and 8.2, which have a singular *L. saligna* CGN05271 introgression of 30-50 cM, within a *L. sativa* cv. Olof background (Jeuken and Lindhout 2004). Additional susceptible control lines were: BIL2.1, BIL2.3, BIL4.1, BIL4.3 and BIL8.1 (Zhang et al 2009a). These lines contain an introgression that partly overlaps with the introgression of BIL 2.2, 4.2, or 8.2. Further control lines were *L. sativa* cv. Iceberg (CGN04619) that shows a strong quantitative resistance in the field (Grube and Ochoa 2005) and BIL 4.4 that is super susceptible (Zhang et al. 2009a).

Genetic map

An extended genetic map from a F_2 population (n=126) between *L. saligna* CGN05271 × *L. sativa* cv. Olof is available and contains about 1000 markers. The markers are rather evenly spread over the linkage groups without clear clustering (Jeuken et al. 2001). The set of 29 BILs was previously genotyped with 780 markers, and *L. saligna*-derived alleles were only detected on the expected BIL segments, therefore it is unlikely that the BILs contain besides the original selected introgression segment additional *L. saligna* introgression segments.

Map saturation

To saturate the three BIL introgression regions with markers, additional markers were developed and initially their positions were mapped on the F_2 population (n=126), (Jeuken et al. 2001). New amplified fragment length polymorphism (AFLP) assays with two primer combinations E48M59 (selective nucleotides CAC and CTA) and E33M59 (selective nucleotides AAG and CTA) were performed. EST markers were developed on lettuce EST sequences from the *Compositae* Genome Project Database (CGPDB,

compgenomics.ucdavis.edu) (McHale et al. 2009) and on EST sequences provided by R. Michelmore (Davis, California, USA). Additionally, SSR markers were developed and mapped by Syngenta Seeds B.V, The Netherlands.
To saturate our three target introgression regions with EST based markers, we aligned our F_2 map with the latest version of the RIL (Salinas × *L. serriola*) map (CGPDB) and selected and tested the EST sequences in intervals between common markers within the introgression segment regions for polymorphisms.

Recombinant screening and line development

Recombinant screenings were performed to obtain plant genotypes with smaller, overlapping *L. saligna* introgressions than in the parental BILs. To select recombinant plants that have a crossover site within the introgression, we used the selfed segregating populations from the original heterozygous recombinant backcross-plant (also called preBIL) that was used to develop the homozygous BIL. Two co-dominant PCR-markers nearest to the ends of the introgression were used to genotype the plants and screen for recombinants. Per introgression segment an initial recombinant screening on 200-400 plants was performed, and for the 8.2 introgression additional recombinant screenings on 5148 plants were performed on earlier detected recombinant plants.

Genotyping and selection for homozygous *L. saligna* genotypes in the offspring of the recombinant plants resulted in lines with shorter *L. saligna* introgressions than the original BIL, which we call sub-BILs. Every appointed sub-BIL is derived from a single recombinant plant with its unique recombination event. The sub-BILs were genotyped by markers to determine the marker interval in which the recombination event took place. PCR-markers, number of populations, recombinants, and sub-BILs are shown in Table S1. For the recombinant screening in the 8.2 introgression region we also used a few presub-BILs (heterozygous recombinant backcross-plant).

DNA extraction and genotyping

For genotyping, DNA was isolated in three different ways: a low quality, high-throughput NaOH method (Wang et al. 1993), and two high quality methods: a modified CTAB method described by Jeuken et al. (2001) and by Kingfisher using sbeadex maxi plant kit (LGC Genomics GmbH, Berlin, Germany) and Kingfisher mL magnetic particle processor (Thermo Labsystems) following the manufacturers' protocol.

The polymorphisms of the PCR products from the EST and SSR markers were initially visualised by size differences on agarose gels (directly or after enzymatic digestion) as previously described (Jeuken et al. 2008) and later visualized by high-resolution melting curve differences on a LightScanner System (Idaho Technology). 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 shown in Table 1. Three to nine independent young plant disease tests (YDT) were performed on each line as described (Jeuken et al. 2008; Zhang et al. 2009a). In each experiment six plants per line were used and at 8 to 10 dpi the infection severity level (ISL) as percentage of sporulating area was evaluated on two leaves. We applied *B. lactucae* race Bl:14 on all YDT, except on one experiment in 2009 and two experiments in 2010 where we applied Bl:21.

Table 1. Disease evaluated lines and their replicates at young (YDT) and adult plant stage (ADT_F) experiments

Lines used per introgression region	YDT		ADT_F	
2.2 introgression	Year exp.[a]	Replicates[b]	Year exp.[a]	Replicates[c]
L. sativa Olof, BIL2.2, sub-BIL2.2-01 to sub-BIL2.2-11	08	3x6 = 18	08	18
BIL2.1 and BIL2.3	08	3x6 = 18	N.D.	N.D.
4.2 introgression				
L. sativa Olof, BIL4.2, sub-BIL4.2-01 to sub-BIL4.2-11	08	3x6 = 18	08	18
BIL4.1 and BIL4.3	08	3x6 = 18	N.D.	N.D.
8.2 introgression				
L. sativa Olof, BIL8.2, sub-BIL8.2-01, 02, 06	08;09;10	9x6 = 54	08;09;10;11	76
BIL8.1	08;09;10	9x6 = 54	09;10;11	58
Sub-BIL8.2-03	08;09	6x6 = 36	09	22
Sub-BIL8.2-04, 09, 10, 11	08	3x6 = 18	08;09	40
Sub-BIL8.2-05	08;09	6x6 = 36	08;09	40
Sub-BIL8.2-07	08;09	6x6 = 36	08;09;10;11	76
Sub-BIL8.2-08	08;09;10	9x6 = 54	08;09	40
Sub-BIL8.2-12	08	3x6 = 18	09	22
Sub-BIL8.2-13, 14, 15 to 21, 24, 26	09	3x6 = 18	09	22
Sub-BIL8.2-14, 22, 23, 25, 27	09;10	6x6 = 36	09;10	40
Sub-BIL8.2-28, 29, 33, 37, 38, 39, 40, 41, 44, 49, 52, 56, 58, 60, 62, 64, 65, 66, 70, 71, 75, 79	10	3x6 = 18	10	18
Sub-BIL8.2-81	N.D.	N.D.	10;11	36
Sub-BIL8.2-80, 82, 83	N.D.	N.D.	10	18
Sub-BIL8.2-59, 63, 73, 84, 85	N.D.	N.D.	11	18
Sub-BIL8.2-201 to Sub-BIL8.2-217	N.D.	N.D.	11	18

Bremia lactucae race Bl:14 was applied on all YDT experiments, except on one experiment in 2009 and two experiments in 2010 where we applied Bl:21.

[a] Experimental year, in 20th century, (08 = year 2008).

[b] Number of experiments × number of replicates = total number of replicates.

[c] Number of replicates in the field, depending on year and experiment. Per year experiment × replicate: 2008: 3×4+1×6; 2009: 3×6+1×4; 2010: 3×6 and 2011: 3×6.

Fourteen adult plant disease tests in the field (ADT_F) were performed by breeding companies at seven locations in the autumn of 2008, 2009, 2010 and 2011 (Table 2). Artificial or natural infection or both occurred. Symptoms of *B. lactucae* infection were recognized by at least two independent and experienced observers. Infected leaf material was collected and the isolates were tested for their resistance spectrum on a differential set. The following *B. lactucae* races were identified: Bl:18, 22, 24, 25, 26 and four mixtures. The resistance spectrum of these four

mixtures was complex and not informative enough to lead to the identification of a mix of individual races or of possibly novel isolates. The number of randomized replications, plants per replicate (8 to 25 plants), the location of the field test, the *B. lactucae* infection (natural or artificial and detected races), and the plant age at time of observation for each experiment are shown in Table 2. For the 2.2 and 4.2 introgression one set of sub-BILs was tested in 2008. For the 8.2 introgression four different sets of sub-BILs were tested in 2008, 2009, 2010 and 2011. The following 8.2 sub-BILs were tested in all four sets (years): sub-BIL8.2-01, 02, 06 and 07 (more details in Table 2). Within each year identical sets were tested in all locations. The ISL per replicate was evaluated as an average infection score for whole plants in a scale from zero (no infection symptoms) to nine (maximum infection symptoms) on adult plants.

Data analysis of disease tests

To improve data normality the percentage data of the YDT was arcsine root transformed. For data analyses of individual YDT and ADT_F a one-way ANOVA was employed (with as fixed factor genotype and as block factor replicate). The predicted mean ISL value per line were compared in a Duncan test ($\alpha = 0.05$) and divided the lines in different infection severity groups. Per year correlations between YDT experiments and between ADT_F experiments were tested by a Pearson correlation test. In case of reasonable to high correlations data were pooled per year (set of sub-BILs). The pooled data of the YDT and the pooled data of the ADT_F were analysed employing a linear mixed model, as described in Zhang et al., (2009a) with some small modifications. Predicted means were calculated by this linear mixed model with fixed factors: genotype, experiment and genotype × experiment; and as random factor 'block nested within experiment'. A Duncan test ($\alpha = 0.05$) was applied for multiple comparisons among all the tested lines. Within each set of sub-BILs (year), we mapped the position of the QTLs by the pairwise comparison of the sub-BILs with each other and with the parental lines. The colinearity of the results (infection level individual lines and position of QTLs) was inspected between locations within a year and between the years.

For a visualization of the results the ISL (absolute values) were presented as Relative Infection Severity levels (RIS), which means that the infection levels are converted as relative to the infection level of the susceptible parent *L. sativa* cv. Olof. For visual comparison between 8.2 sub-BILs tested in different experiments, the average infection level of the 8.2 sub-BILs was adjusted to their relative position towards *L. sativa* cv. Olof and BIL8.2. This adjustment was required to standardize the infection levels of those lines that were not tested in all experiments because the experiments had different infestation levels (Figure 1).

Another analysis was executed in which at each marker locus the average ISL were compared between the group of lines with the homozygous *L. sativa* genotype and the group with the homozygous *L. saligna* genotype using the same mixed model analysis as described above. *P-value* s from this analysis were graphed as – log (*P*) or as log (*P*) when the homozygous *L. saligna* or homozygous *L. sativa* genotype respectively showed the lowest

Table 2. Information about disease evaluated field tests (ADT_F). Infection severity level (ISL) was scored on whole plants with a scale from 0 (no infection) to 9 (completely infected).

	ADT_F 2008				ADT_F 2009			
Introgression:	2.2, 4.2 and 8.2				8.2			
Location ADT_F / experiment:	Fijnaart	Etten-Leur	Voorst	Zeewolde	Fijnaart	's-Gravenzande	Voorst	Zeewolde
Experiment code:	2008-01	2008-02	2008-03	2008-04	2009-01	2009-02	2009-03	2009-04
Replications/blocks:	4	4	4	6	6	6	4	6
Plants in replicate/block:	8	25	20	10	8	20	24	9
Soil type:	clay	sand	sand	clay	clay	clay	sand	clay
Natural or artificial infection:	natural	both	natural	artificial	natural	artificial	natural	natural
Bremia lactucae race:[a]	Bl:24	Bl:22, 24, 25, 26	Bl:18 and mix.	Bl:25	mixture	Bl:24 and Bl:26	mixture	Bl:25
Sowing date:	11 July	29 July	18&25 July	28 July	13 July	28 July	16&23 July	29 July
Planting date:	28 July	15 Aug	8&14 Aug	19 Aug	27 July	14 Aug	3&10 Aug	19 Aug
Observation date:	12 Sept	9 Oct	1 Oct	17 Oct	11 Sept	8 Oct	23&28 Sept	14 Oct
Infection severity level Olof:	6.5	7.5	6.5	8.5	4.5	7.8	8.9	7.1
Infection severity level BIL8.2:	4.3	4.8	4.3	6.7	2.4	4.4	7.0	5.7
Quotiënt (ISL BIL8.2 / ISL Olof):	0.66	0.64	0.66	0.79	0.53	0.56	0.79	0.80
Experiment code:	2008-01	2008-02	2008-03	2008-04	2009-01	2009-02	2009-03	2009-04
Correlation (r) with exp. -01:[b]	-	0.9, 0.6, 0.7	0.8, 0.8, 0.6	0.8, 0.6, 0.7	-	0.9	0.8	0.8
Correlation (r) with exp. -02:[b]	0.9, 0.6, 0.7	-	0.7, 0.8, 0.7	0.8, 0.9, 0.8	0.9	-	0.8	0.8
Correlation (r) with exp. -03:[b]	0.8, 0.8, 0.6	0.7, 0.8, 0.7	-	0.6, 0.7, 0.7	0.8	0.8	-	0.8
Correlation (r) with exp. -04:[b]	0.8, 0.6, 0.7	0.8, 0.9, 0.8	0.6, 0.7, 0.7	-	0.8	0.8	0.8	-

	ADT_F 2010			ADT_F 2011		
Introgression:	8.2			8.2		
Location ADT_F / experiment:	Oud Gastel	's-Gravenzande	La Ménitré (FR)	Oud Gastel	's-Gravenzande	Zeewolde
Experiment code:	2010-01	2010-02	2010-03	2011-01	2011-02	2011-03
Replications/blocks:	6	6	6	6	6	6
Plants in replicate/block:	12	20	20	16	25	11
Soil type:	sand	clay	sand	sand	clay	clay
Natural or artificial infection:	natural	natural	artificial	natural	artificial	artificial
Bremia lactucae race:[a]	mixture	Bl:22, 24, 25, 26	Bl:26	Bl:24	Bl:22, 24, 25, 26	Bl:25
Sowing date:	13 July	27 July	12 Aug	08 July	20 July	29 July
Planting date:	28 July	16 Aug	1 Sept	2 Aug	4 Aug	19 Aug
Observation date:	9 Sept	5 Oct	20 Oct	9 Sept	5 Oct	13 Oct
Infection severity level Olof:	7.1	7.5	6.4	7.1	8.2	5.2
Infection severity level BIL8.2:	6.0	6.0	4.2	6.1	6.6	2.4
Quotiënt (ISL BIL8.2 / ISL Olof):	0.85	0.80	0.66	0.85	0.80	0.47
Experiment code:	2010-01	2010-02	2010-03	2011-01	2011-02	2011-03
Correlation (r) with exp. -01:	-	0.9	0.8	-	0.7	0.5
Correlation (r) with exp. -02:	0.9	-	0.8	0.7	-	0.6
Correlation (r) with exp. -03:	0.8	0.8	-	0.5	0.6	-

[a] 'mix.' or 'mixture' means that individual races could not be determined because of a complex mixture of races or due to presence of not described and/or new isolates.
[b] In 2008 the correlations are shown individually for the different sets of sub-BILs from the 2.2, 4.2 and 8.2 introgression segments respectively.

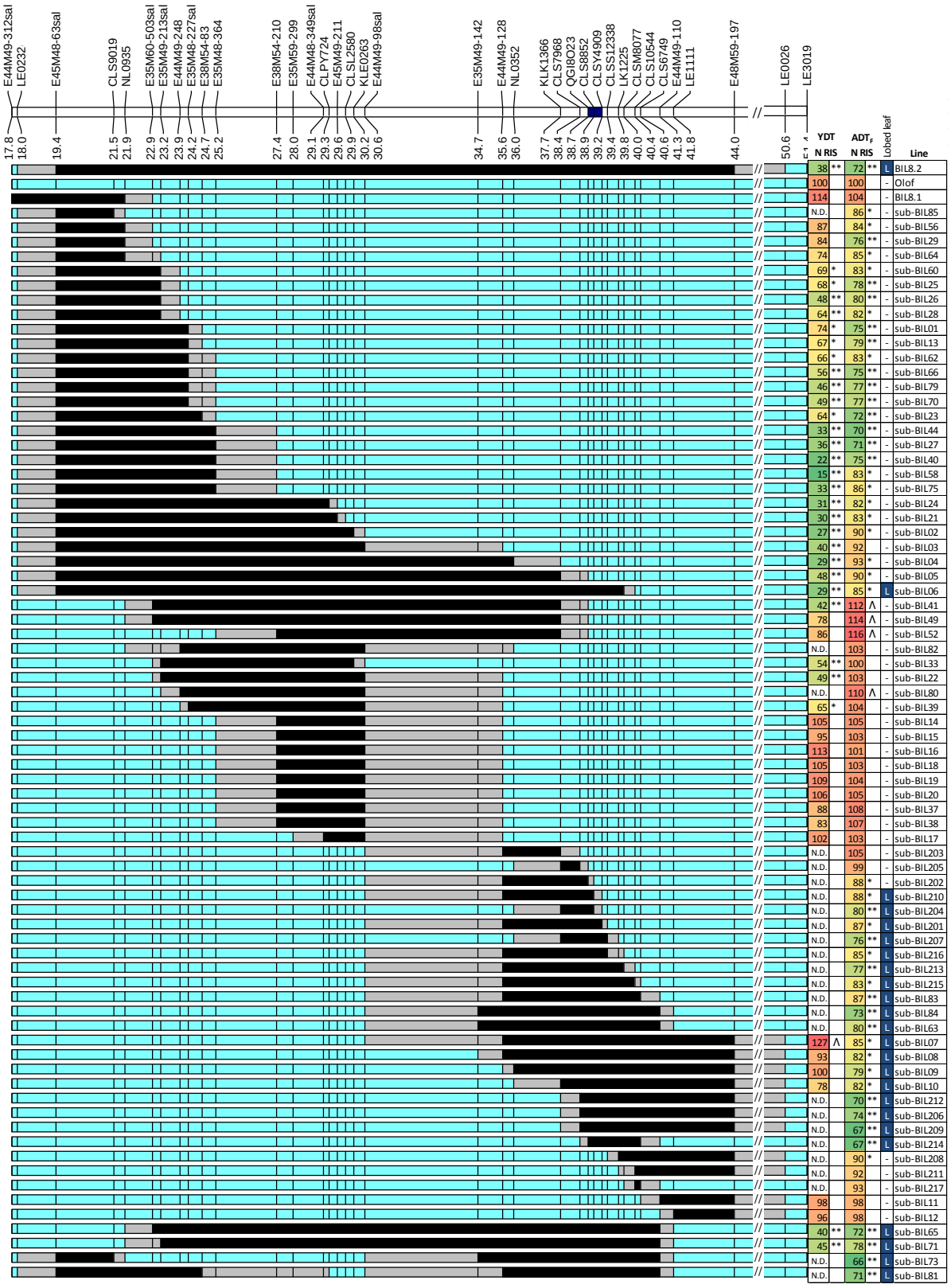
average ISL (Figure 2, S1 and S2). Therefore the highest or lowest peaks, that exceed the threshold levels at α=0.01 = –LOG 2 and LOG -2, indicate the most likely QTL positions and indicate the allele associated with the resistance (Monforte and Tanksley 2000). All statistics was calculated by statistical package IBM SPSS statistics version 19 or GenStat 14.

Results

Recombinant screening and development of sub-BILs

The first recombinant screenings for 2.2, 4.2 and 8.2 resulted in 11, 11 and 12 sub-BILs, respectively (Table 3). First YDT results on those 34 sub-BILs indicated that the resistance within the 2.2 and 4.2 introgression segment was explained by multiple QTLs, while the resistance for the 8.2 introgression seemed to be explained by a single QTL between marker NL0935 and E44M49-97sal (Zhang et al. 2008). The suppression of the recombination frequency within the 2.2, 4.2 and 8.2 introgression was 15, 17 and 2 times compared to the same region in the original F_2 population (Table 3). Because of the possibility of a single gene explaining the QTL effect in 8.2 and because of the lower suppression of recombination we focused for further fine mapping on the 8.2 introgression. Our ADT_F results on the first 12 sub-BILs from 8.2 indicated a second gene for resistance in the region (data not shown). Therefore, we performed an additional recombinant screening within the 8.2 introgression in two different regions. In total 99 additional recombinants were detected and 62 were selected to be developed into homozygous sub-BILs. The selection was based on uniqueness of recombination interval and/or regions where we expected resistance loci on the basis of previous experiments. All sub-BILs were genotyped extensively to determine the different crossover positions (Figure 1, S1 and S2). Some groups of sub-BILs, for example 8.2 sub-BILs 44, 27, 40, 58 and 75, have an identical marker profile, but not an identical genotype, as all sub-BILs are derived from independent recombinants (Figure 1). Therefore, in case there is a relevant resistance gene in the marker interval where a recombination occurred, that resistance gene may occur in some recombinants, but not in others.

The recombination frequency in 8.2-presub-BILs was 2 to 3.5 times more suppressed than in the preBIL8.2, suggesting that the smaller the introgression, the larger the suppression of recombination (Table 3). A nine times higher recombination frequency was observed for the double recombinant presub-BIL8.2-73 with a ~10 cM *L. sativa* segment between two *L. saligna* segments of 3.0 and 8.5 cM, compared to its counterpart presub-BIL8.2-6 with an 21.5 cM introgression with equal outermost introgression extremities and no intermediate *L. sativa* segment (Table 3). The recombination frequency within presub-BIL8.2-73 was even two times higher than in the *L. saligna* × *L. sativa* F_2 population (Table 3). This increased recombination frequency suggests that crossovers occur at much higher frequency in a homozygous segment (from *L. sativa*) than in a chromosome stretch that is heterozygous and non-homologous (one homologue from *L. sativa* and one from *L. saligna*).


E44M49-312sal
LE0232
E45M48-63sal
CLS9019
NL0935
E35M60-503sal
E35M49-213sal
E44M49-248
E35M48-227sal
E38M54-83
E35M48-364
E38M54-210
E35M59-299
E44M48-349sal
CLPY724
E45M49-211
CLSL2580
KLE0263
E44M49-98sal
E35M49-142
E44M49-128
NL0352
KLK1366
CLS7968
QGI8023
CLS8852
CLSY4909
CLSS12338
LK1225
CLSM8077
CLS10544
CLS6749
E44M49-110
LE1111
E48M59-197
LE0026
LE3019
17.8
18.0
19.4
21.5
21.9
22.9
23.2
23.9
24.2
24.7
25.2
27.4
28.0
29.1
29.3
29.6
29.9
30.2
30.6
34.7
35.6
36.0
37.7
38.4
38.7
38.9
39.2
39.4
39.8
40.0
40.4
40.6
41.3
41.8
44.0
50.6
YDT
N RIS
ADT$_F$
N RIS
Lobed leaf
Line
BIL8.2
Olof
BIL8.1
sub-BIL85
sub-BIL56
sub-BIL29
sub-BIL64
sub-BIL60
sub-BIL25
sub-BIL26
sub-BIL28
sub-BIL01
sub-BIL13
sub-BIL62
sub-BIL66
sub-BIL79
sub-BIL70
sub-BIL23
sub-BIL44
sub-BIL27
sub-BIL40
sub-BIL58
sub-BIL75
sub-BIL24
sub-BIL21
sub-BIL02
sub-BIL03
sub-BIL04
sub-BIL05
sub-BIL06
sub-BIL41
sub-BIL49
sub-BIL52
sub-BIL82
sub-BIL33
sub-BIL22
sub-BIL80
sub-BIL39
sub-BIL14
sub-BIL15
sub-BIL16
sub-BIL18
sub-BIL19
sub-BIL20
sub-BIL37
sub-BIL38
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sub-BIL205
sub-BIL202
sub-BIL210
sub-BIL204
sub-BIL201
sub-BIL207
sub-BIL216
sub-BIL213
sub-BIL215
sub-BIL83
sub-BIL84
sub-BIL63
sub-BIL07
sub-BIL08
sub-BIL09
sub-BIL10
sub-BIL212
sub-BIL206
sub-BIL209
sub-BIL214
sub-BIL208
sub-BIL211
sub-BIL217
sub-BIL11
sub-BIL12
sub-BIL65
sub-BIL71
sub-BIL73
sub-BIL81

Figure 1.

Figure 1. Genotypes and disease evaluations at young plant (YDT) and adult plant stage (ADT_F) of lettuce sub-BILs (with smaller *L. saligna* introgressions than in BIL8.2. Genetic map of Chromosome 8, 17-51 cM and genotype graphs of tested lines. Light blue bars represent homozygous *L. sativa*, solid black bars represent homozygous *L. saligna* and grey bars represent marker intervals containing a recombination event. In the disease evaluation table ''N RIS'' means "normalized relative infection severity of each line compared to *L. sativa* cv. Olof and BIL8.2". Gradual color scale is used to visualize differences in N RIS values. In total 9 independent YDT and 14 independent ADT_F were performed with four different sets of sub-BILs (details see Table S1). Significant differences (α=0.01, LSD test): * ISL different from BIL8.2 and *L. sativa* cv. Olof, ** ISL different from *L. sativa* cv. Olof and not from BIL8.2, ^ ISL line was significantly higher than *L. sativa* cv. Olof. N. D. indicates "not determined''. Lines showing leaf morphological trait 'lobed leaf ' are indicated with an 'L' and its map position is indicated in blue in the genetic map.

Table 3. Summary of recombination screenings and their recombination frequencies
Rec. = Recombinant; Rec. freq. = recombinant frequency; Rec. suppr. = times recombination suppression compared to the F_2 population.

Parental plant	Chr.	Fig. *L. saligna* intr. and marker pos.[a]	*L. saligna* intr. length # cM	Dist. betw. rec. scr. markers # cM	Plants #	Rec.[b] #	Rec. freq. per cM BIL/sub-BIL	Rec. freq. per cM F_2	Rec suppre. BIL/sub-BIL vs. F_2[c]
preBIL2.2	C2	8 9	28.5	20.8	442	11	0.0006	0.0091	15.2 × * †
preBIL4.2	C4	10 11	49.2	29.3	258	8	0.0005	0.0092	17.3 × * †
preBIL8.2	C8	3 7	28.6	11.6	361	29	0.0035	0.0074	2.1 × *
presub-BIL8.2-03[d]	C8	2 3	14.4	8.3	1267	45	0.0021	0.0077	3.6 × *
presub-BIL8.2-05	C8	2 4	19.5	14.1	348	20	0.0020	0.0077	3.8 × *
presub-BIL8.2-07,10,83	C8	5 6	10.8[e]	2.9	3047	17	0.0010	0.0070	7.2 × * †
presub-BIL8.2-06[d]	C8	2 4	21.5	14.1	359	17	0.0017	0.0077	4.6 × * † ^
presub-BIL8.2-73[f]	C8	1 4	3.0+8.5	14.5	127	65	0.0176	0.0081	0.5 × * † ^

[a] Schematic presentation of introgression characteristics on scale. The *L. saligna* introgression is presented as a bar. Black means 'heterozygous'-genotype. The transitional region to homozygous *L. sativa*- genotype, where a recombination event resides, is indicated in gray. The position of markers, used for recombinant screening, is indicated by a number: 1 = CLS_S3_9019, 2= NL0935, 3 = KLE0263, 4 = NL0252, 5 = KLK1366, 6 = CLS_S3_6749, 7 = LE1111, 8 = LE4034, 9 = LE1114, 10 = LE0351 and 11 = LsB104.

[b] all recombinants were single cross-overs except for three double recombinants in the offspring of presub-BIL8.2-73.

[c] Fisher's exact test on recombination suppression differences between populations, α= 0.05: Recombinant frequency per cM is significantly different between preBIL/ presub-BIL offspring and F_2 population *; between offspring preBIL/ presub-BIL and preBIL8.2 †; between offspring presub-BIL8.2-73 ^ and presub-BIL8.2-06.

[d] Combined recombinant screening on the offspring of the presub-BIL and a few lines with almost similar but not identical introgression lengths and positions.

[e] Average introgression length from the three lines, with an introgression segment of 14.2, 10.5 and 7.7 cM respectively.

[f] The presub-BIL8.2-73 progeny segregation revealed that presub-BIL8.2-73 has two cross-over events in coupling phase (cis), originating from one recombinant gamete with two cross-over events instead of two recombinant gametes with each one different cross-over event (trans).

The 4.2 and 8.2 introgression segments conferred aberrant leaf morphology. Introgression 4.2 caused leaves to be long, twisted, and dark green and plants to be non-heading (Jeuken and Lindhout 2004). Introgression 8.2 conferred a lobed leaf shape. These plant morphological traits could be fine mapped to a particular marker interval on the original introgression (Figure 1, S1 and S2). Lobed leaf shape was mapped to a 0.5 cM interval within the 8.2 segment (Figure 1). 'Non-heading and long narrow leaf' (Ln) was mapped to a 1.2 cM interval and 'dark green and twisted leaves' (T) was mapped to a 5.0 cM interval within the 4.2 segment (Figure S2).

Disease evaluations at young and adult plant stage

The three BILs, the recurrent parent *L. sativa* cv. Olof and additional control lines showed in all YDT and ADT_F a similar relative infection level as in previous experiments (Figure 2, Zhang et al. 2009a). Between YDT experiments the new sub-BILs showed similar relative infection levels (RIS). For ADT_F the new sub-BILs showed similar relative infection levels (RIS) between locations within a year and between the years. Similar preliminary QTL positions were observed between individual experiments of YDT and between individual experiments of ADTF, with occasional variances in the strength of the effect (details of final QTL positions of pooled data are described in the next paragraphs).

Some infection level differences were observed between ADT_F experiments, but those were mainly due to the evaluation moment. Low infestation levels resulted in large differences, and high infection levels in smaller differences between susceptible and quantitatively resistant lines (Table 2). In all experiments significant differences between the sub-BILs, BIL and *L. sativa* cv. Olof were observed. Between the different experiments within YDT and within ADT_F the ISL of the lines were significantly correlated (r = 0.5-0.9, Table 2), even though different *B. lactucae* races were used. Based on these and earlier field experiments with six different isolates (Zhang et al. 2009a), we assume that the resistances are race nonspecific . When comparing natural or artificial infections for ADT_F, similar correlations were observed between natural × natural (average r= 0.8, n=4), natural × artificial (average r= 0.7, n=9) and artificial × artificial (r=0.7, n=1), which implies no inoculation method effects (Table 2). The significantly correlated experiments allowed us to pool the data from the different experiments with the same lines within YDT and within ADT_F. The correlation of the ISL of the common sub-BILs between the YDT and ADT_F was rather low, 2.2 r = 0.52, 8.2 r = 0.39 and 4.2 r = 0.67. This result suggests that the genes for resistance are growth stage specific in their effect. The data from the two different plant stages (YDT and ADT_F) was not pooled but analysed separately.

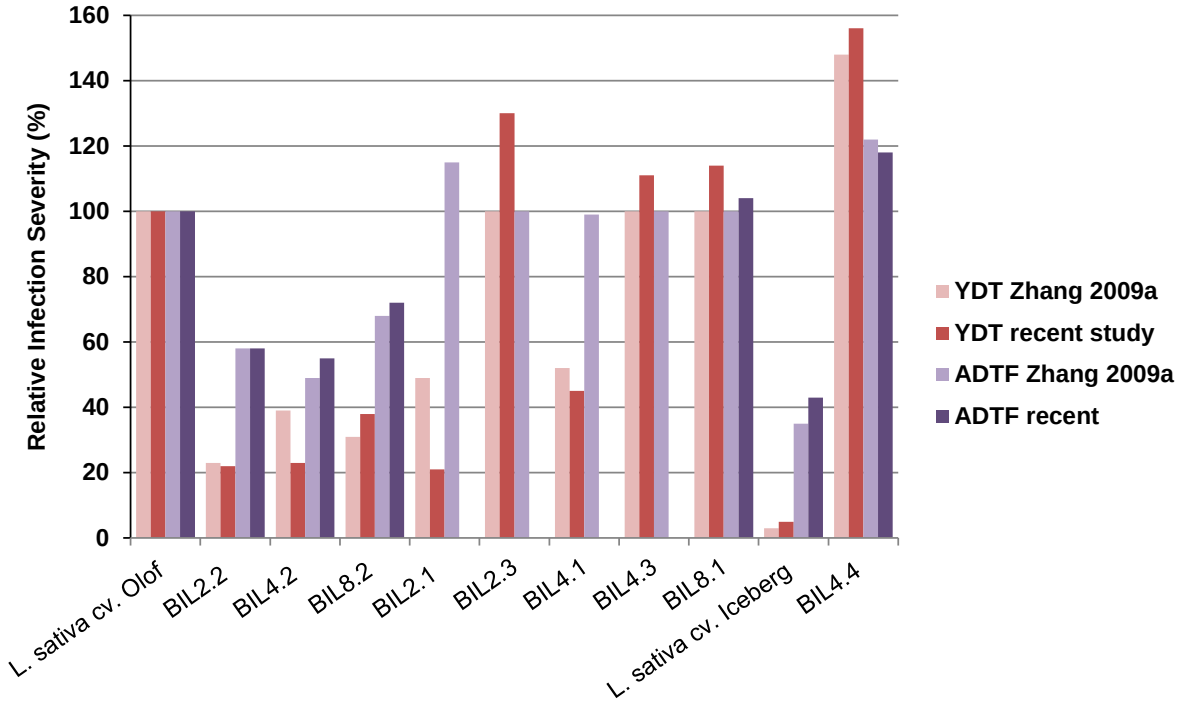

Figure 2. Comparison of average RIS values of common control lines in the recent study (dark color) and a former study by Zhang et al. 2009a (light color). Disease assessments at young plant stage (YDT, red color) and adult plant stage in the field (ADT_F, purple color) are shown. For field test data of Zhang et al. 2009a, the $ADT_{F\text{-}C}$ dataset (11 locations) of Table 1 was used. No recent field test data were available for BIL2.1, 2.3, 4.1 and 4.3.

Mapping resistance loci within the 8.2 introgression

Preliminary results from YDT (Zhang et al. 2008) and new ADT_F results in 2008 with the first set of 12 sub-BILs indicated two sub-regions involved in resistance. One sub-region was effective at both young and adult plant stage and ranges from ~19 – 30 cM (lower RIS in YDT and ADT_F for sub-BIL01, 02, 03, 04, 05, and 06 in Figure 1). The other sub-region was effective at adult plant stage only and ranges from ~36-41 cM (lower RIS in ADT_F for sub-BIL07, 08, 09 and 10 in Figure 1). Lines with a smaller heterozygous introgression than BIL8.2, overlapping these two sub-regions were used to develop new sub-BILs for further fine mapping. The same procedure was followed in subsequent recombinant screenings. Sub-BILs were numbered in order of the time they were developed (Figure 1). The detected QTL positions in different sets of sub-BILs were always verified in later evaluated sub-BIL subsets.

We tested 49 and 74 sub-BILs in YDT and ADT_F respectively. We distinguished four infection classes: 'resistant' with an ISL as the resistant parental BIL, 'susceptible' with an ISL like *L. sativa* cv. Olof, 'intermediate' with an ISL between the resistant BIL and the susceptible *L. sativa* cv. Olof and 'super susceptible' with an ISL more than *L. sativa* cv. Olof. In young and adult plant stage the infection levels of the majority of the 8.2 sub-BILs were distributed over three classes: resistant, susceptible and intermediate (Figure 1). A minority of one sub-BIL in YDT and four sub-BILs in ADT_F fell in the class 'super susceptible' (Figure 1). The many sub-BILs with an intermediate ISL at young and adult plant

stage suggested that the resistance was explained by multiple sub-QTLs instead of one or two loci. The classification of the sub-BILs in one of the four classes was often not similar between YDT and ADT_F, which suggests plant stage dependent resistance. For example sub-BIL41 is resistant in YDT and super susceptible in ADT_F, while sub-BIL07 is susceptible in YDT and resistant in ADT_F (Figure 1).

The plots of the *P-value* and the average RIS per marker position for the homozygous *L. saligna* genotype indicated which segments of the introgression of *L. saligna* conferred a decrease in RIS, namely where the Log*P* had negative values. For the 8.2 introgression, the resistance in the young plant stage was located at around 23 cM and in the adult plant stage at around 20 and 39 cM (Figure 3). In YDT, the sub-BILs with a longer *L. saligna* introgression coming from the top side extending to 27.4 cM (like sub-BIL 02, RIS = 27%), had a lower RIS than the sub-BILs with shorter introgressions like sub-BIL01 (RIS = 74%) (Figure 1). Multiple comparison (Duncan test, α=0.05) between sub-BILs and control lines indicated presence of at least two sub-QTLs between 18.0 cM to 27.4 cM effective at young plant stage (Figure 1 and 2).

Also for ADT_F certain longer introgressions conferred a lower RIS than short introgressions as illustrated at the top side by sub-BIL01 (RIS = 75%) and sub-BIL85 (RIS = 86%) and at the bottom side by sub-BIL209 (RIS = 67%) and sub-BIL208 (RIS = 90%). At the adult plant stage the resistance was explained by at least two sub-QTLs between 18.0 and 25.2 cM and two sub-QTLs between 38.4 and 41.3 cM (Figure 1 and 3). The magnitude of the infection reduction was around 10% for the four individual sub-QTLs and around 30% for both two linked sub-QTLs compared to *L. sativa* Olof. Two sub-QTL at the top are present in sub-BIL01 (RIS 75%) and two sub-QTLs at the bottom are present in sub-BIL209 and sub-BIL214 (both RIS 67%). The resistance was probably not associated with leaf morphology trait ‘lobed leaf’ because sub-BIL202 and sub-BIL208 showed resistance but did not have the *L. saligna* allele for ‘lobed leaf’ (Figure 1).

Neutralising effect genes in ADT_F

Four sub-BILs, sub-BIL41, 49, 52 and 80, showed at the adult plant stage a higher ISL than susceptible parent *L. sativa* cv. Olof (super susceptible, Figure 1). Furthermore we observed several susceptible sub-BILs with high RIS and a long *L. saligna* introgression that completely overlapped smaller introgressions from sub-BILs that had a lower RIS, for example; sub-BIL02 with 90% and sub-BIL01 with 75 % RIS (Figure 1). The tendency of sub-BILs to be relatively susceptible if they had the 25 to 38 cM region derived from *L. saligna* suggests that in that region *L. saligna* carries a gene conferring susceptibility or neutralising resistance (Figure 1 and 3). This conclusion is also drawn from the *P-value* plot (peak with positive Log *P* values, Figure 3). The resistance gene conferring an 18-22 cM region of the 8.2 introgression (RIS of 86% in sub-BIL85) also occurs in BIL8.1. Still, BIL8.1 was completely susceptible (RIS=104%). This suggests that the *L. saligna* chromosome 8 also contains one or more genes to the left of marker E45M48-63 that neutralise the resistance present in the said segment.

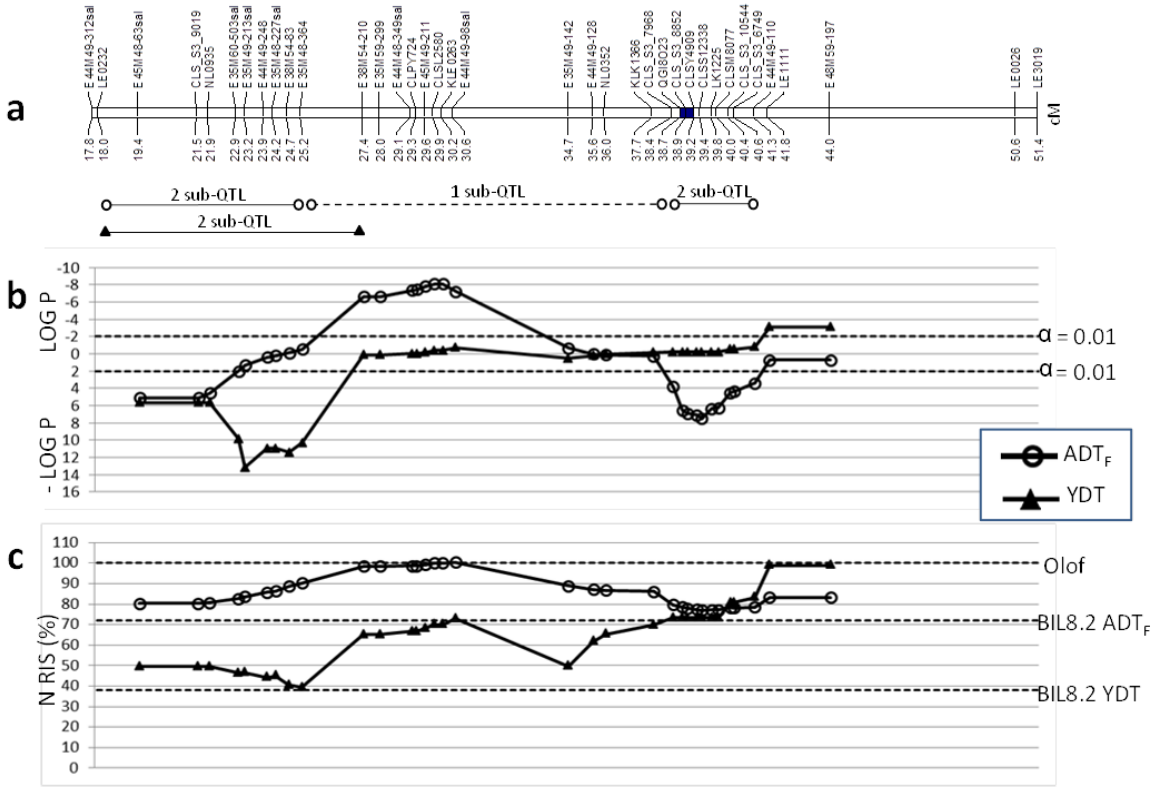

Figure 3. Fine mapping QTLs within the *L. saligna* introgression of BIL8.2 at the young and adult plant stage. Four graphs are aligned according marker positions. From top to bottom: **a**. Genetic map of Chromosome 8, 17-51 cM; Locations of sub-QTL regions for both the ADT_F (lines with open circle) and YDT (lines with triangulars) are indicated. Solid and dashed lines indicate *L. saligna* introgressions reducing and promoting the infection level respectively; **b**. the - LOG transformed probabilities; and **c**. the N-RIS. In **b**, the - LOG transformed probabilities are plotted from a mixed model comparison between the average infection levels of lines with a homozygous *L. sativa* genotype and a homozygous *L. saligna* genotype at each marker position. Probability values were – LOG or LOG transformed, threshold level is set at α=0.01 = –LOG 2 and LOG -2. In **c,** the average N- RIS is plotted per marker locus for the lines that were homozygous *L. saligna* for that marker. With dotted lines the average relative infection level of *L. sativa* cv. Olof (RIS = 100%), BIL8.2 at the adult plant stage (RIS = 72%) and BIL8.2 at the young plant stage (RIS = 38%) are indicated. In blue the fine mapped position of leaf morphology trait 'lobed leaf' is indicated.

Mapping QTLs within the 2.2 and 4.2 introgression

Within both the 2.2 (Figure S1) and 4.2 (Figure S2) introgression the infection levels of eleven sub-BILs were evaluated at the YDT and ADT_F. The infection levels of the 2.2 sub-BILs at the YDT and ADT_F and 4.2 sub-BILs at YDT were distributed over three ISL classes: resistant, susceptible or intermediate. At the adult plant stage the eleven 4.2 sub-BILs were distributed over the two ISL classes resistant or intermediate and none was susceptible (Figure S1 and S2). In both introgressions we did not observe a single locus explaining the resistance, but several loci seem to be responsible and the majority of the loci seem to be plant stage dependent (Figure S1 and S2). The magnitude of the infection reduction ranged between 15 and 35% for the individual sub-QTLs at field situations. A resistance neutralising gene was also detected in YDT in 4.2 introgression and ADT_F in 2.2 (Figure S1 and S2).

Within the 4.2 introgression plant morphological traits long-narrow leaf and non-heading co-localise with each other and with a resistance locus that may or may not explain both the

resistance and plant morphology. Plant morphological traits dark green and twisted leaf co-localise with each other but not with resistance because sub-BIL4.2-11, has no dark green and twisted leafs but is moderately resistant.

Discussion

Disintegration of the resistance

The resistance of all three investigated BIL introgressions, at both young and adult plant stage, fell apart in multiple (linked) sub-QTLs (Figure 3, S1 and S2). The individual effects of those sub-QTLs were smaller than the resistance of the whole BIL introgression segment. The extensively fine mapped region 8.2, causing 30% infection reduction in the field, fell apart in four sub-QTLs, linked per two, with individual effects of almost 10% each. Linked (sub-) QTLs were detected in other studies. In rice fine mapping of a quantitative grain weight gene *qTGWT1-1*, detected in a RIL population, revealed that the effect of the QTL was explained by two tightly linked sub-QTLs, *Gw1-1* and *Gw1-2* (Yu et al. 2008). Also in rice, fine mapping by substitution mapping of a flowering time QTL *dth1.1* revealed two sub-QTLs (Maas et al. 2010; Thomson et al. 2006). However, in most published fine mapping studies with resistance QTLs in plants, the QTLs did not fall apart in multiple sub-QTLs. Tomato QTLs *lb4, lb5b,* and *lb11b* for resistance to *Phytophthora infestans* did not fall apart in sub-QTLs (Brouwer and St. Clair 2004); fine mapping with substitution mapping of *Rphq2*, barley QTL for resistance to leaf rust (*Puccinia hordei*), in a window of 0.11 cM also did not indicate sub-QTLs (Marcel et al. 2007).

Plant stage dependent QTLs

Of the seventeen suggested sub-QTLs in all three BIL introgression segments together, probably only two sub-QTLs might explain resistance at both plant stages (a sub-QTL in 8.2, Figure 3; a sub-QTL in 4.2, Figure S2).The resistance levels of the complete 2.2, 4.2 and 8.2 introgression segments at both plant stages might be explained by interactions among the detected plant stage dependent sub-QTLs or by epistatic interactions among unknown loci. The detection of plant stage dependent sub-QTLs within the three BIL introgression segments corresponds with the result of the set of 29 BILs within the whole lettuce genome, in which the majority of the 15 resistant BILs showed plant stage dependent resistance (Zhang et al. 2009a). Developmental plant stage dependent quantitative resistance has been found in multiple studies, in multiple plant species (Castro et al. 2002; Eenink and Jong 1982; Mallard et al. 2005; Monteiro et al. 2005; Prioul et al. 2004; Qi et al. 1998; Wang et al. 2010).

Neutralizing effect genes

In all three introgression segments (2.2, 4.2 and 8.2) a sub-region was detected that had a negative or neutralizing effect on the resistance level. This effect can be caused by infection promoting genes from *L. saligna* or by absence of possible resistance genes from *L. sativa*.

One of the resistance sub-QTLs within the 8.2 introgression was also located within the overlapping *L. saligna* introgression of BIL8.1. BIL8.1, which is as susceptible as *L. sativa* cv. Olof, should contain therefore besides the shared resistance sub-QTL with BIL8.2 also at least one negative or neutralizing QTL. This fact suggests that the 13 BILs which in the study of Zhang et al. (2009b), were at all plant stages (at least) as susceptible as *L. sativa* cv. Olof may contain QTLs for resistance that are neutralized by genes with an opposite effect within the same BIL introgression.

Recombination suppression

Recombination suppression was observed in the recombinant screenings on lines with heterozygous introgressions (preBILs and presub-BILs). The level of recombination suppression varied from 2 to 17-fold (compared to the F_2 population) and depended on the region and on the size of the heterozygous introgression. Smaller introgressions showed more suppression. Recombination suppression in plants that were heterozygous for a donor introgression was also described in interspecific introgression lines of tomato (Brouwer and St. Clair 2004; Paterson et al. 1990) and barley (Johnston et al, 2013), but not in intra-specific near isogenic lines of maize (Graham et al. 1997) and rice (Wissuwa and Ae 2001). These findings suggest that recombination frequencies tend to get lower, when the introgressed parent species is rather distantly related from the recurrent parent. Brouwer and Clair (2004), Johnston et al., (2013) and Canady et al., (2006) also reported a stronger suppression of recombination within smaller sizes than within larger sizes of introgression segments.
The nine times increased recombination frequency of double recombinant presub-BIL73 compared to that of its counterpart presub-BIL06 suggested that: 1) There is less recombination in a segment that is heterozygous for DNA from different species than in a homozygous segment. 2) As an interstitial segment is homozygous and is accompanied at both sides by heterozygous regions, recombination events accumulate in the interstitial homozygous segment. Similar findings were observed in an *Lycopersicon esculentum* × *Lycopersicon pennellii* F_2 population (Canady et al. 2006).

Gene cloning perspectives

Fragmentation of the resistance into mostly smaller effects and into plant developmental stage specific sub-QTLs makes cloning of the genes probably very difficult and of limited use. Most sub-QTLs conferred only a reduction in field infection severity of around 10%, which requires many replications within disease tests to conclude differences in resistance phenotype between lines with and without the quantitative resistance allele.
In some studies substitution mapping of a QTL might lead to the ultimate cloning of the responsible gene like for *Pi21, Yr36, Lr34* and *Rgh4* (Fu et al. 2009; Fukuoka et al. 2009; Krattinger et al. 2009; Liu et al. 2012). But in our case the genetics was more complex and further attempts for gene cloning of sub-QTLs with effects of about 10% seems not useful at this moment.

Breeding perspectives

Although the resistance within the BILs fragmented into multiple sub-QTLs, some sub-BILs showed a similar effect as the parental BIL and without undesired plant morphological traits, like sub-BIL44, of which the 10 cM introgression length is only a third of the 8.2 introgression. Sub-BIL214, of which the introgression length is only 3 cM, also shows a similar effect as the parental BIL at adult plant stage but it also contains the lobed leaf *L. saligna* allele. If the lobed leaf trait can be implemented as a positive morphological trait of a lettuce variety, sub-BIL214 can be a very interesting quantitative resistance donor in breeding. The resistances of sub-BIL44 and sub-BIL214 have been proven to be functional against four of the newest *B. lactucae* races under different environmental conditions in the field. The similar resistance levels of sub-BILs, like sub-BIL44 and 214, to the parental BIL line, was explained by the presence of a part of the sub-QTLs for resistance and the absence of negative or neutralizing *L. saligna*-introgression regions. For resistance breeding the effects of the single sub-QTLs alone are too small to be of practical interest. By stacking multiple sub-QTLs of smaller introgressions within or among 2.2, 4.2 and 8.2 introgressions, it might be possible to obtain lines with a higher or complete resistance and without undesired morphological traits. The effect of stacking has to be studied in detail to elucidate if and which specific combination of (sub-) QTLs can explain the nonhost resistance of *L. saligna* and to assess its value for practical use.

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Supplemental information

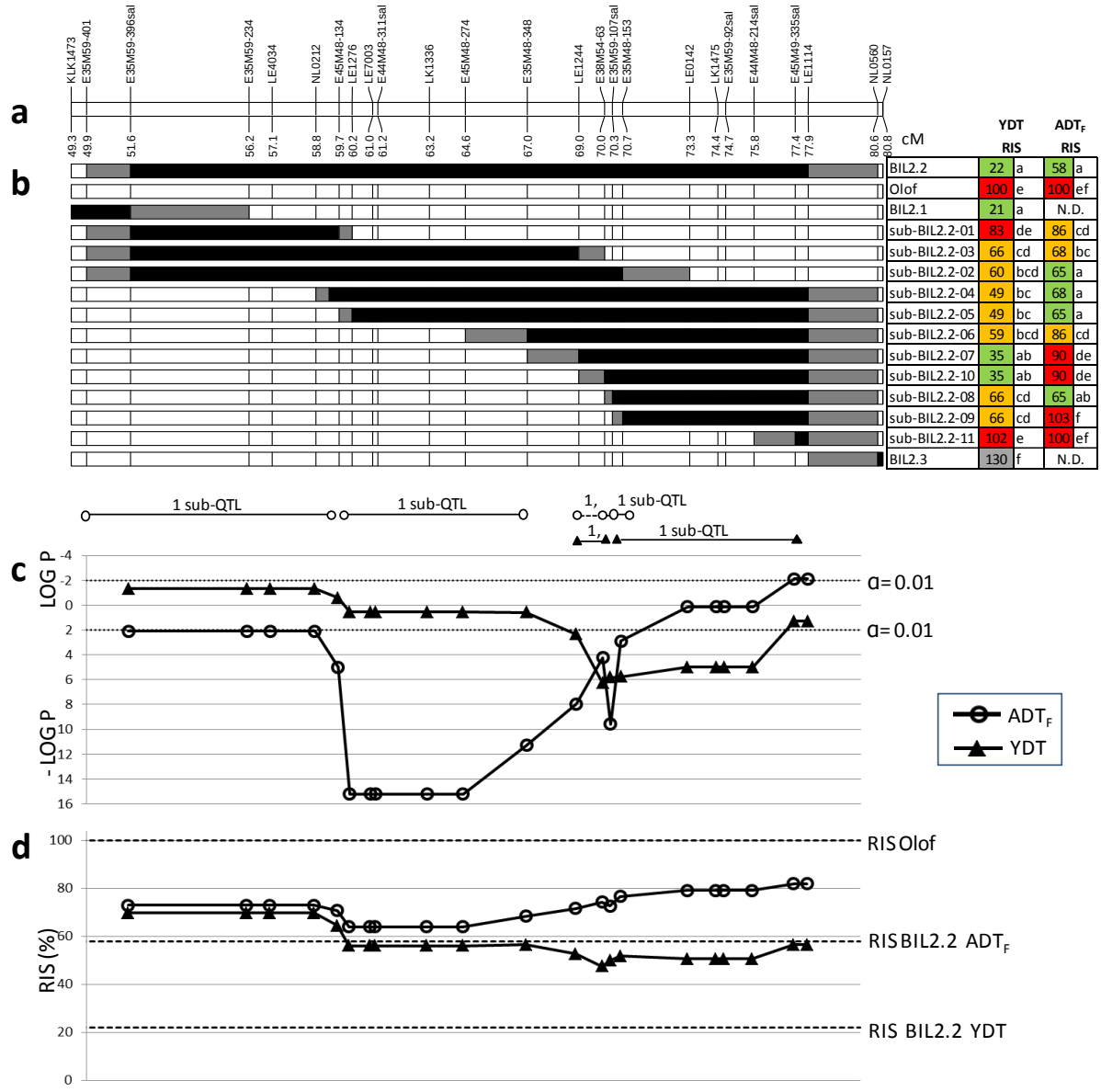

Figure S1. Genotypes and disease evaluation at young plant and adult plant stage of lettuce sub-BILs covering BIL2.2 introgression, the neighboring BILs and the reference lines. From top to bottom: **a**. Genetic map of Chromosome 2, 49-81 cM; **b**. the genotypes and the RIS of the disease evaluated lines; Locations of sub-QTL regions for both the ADT_F (lines with open circle) and YDT (lines with triangulars) are indicated. Solid and dashed lines indicate *L. saligna* introgressions reducing and promoting the infection level respectively; **c** the - LOG transformed probabilities; and **d**. the RIS graphs.
In **b**, left the *B. lactucae* evaluated lines are presented and right the infection scores are presented relative from *L. sativa* cv. Olof. White represents homozygous *L. sativa*, solid black bars represent homozygous *L. saligna* and grey bars represent marker intervals containing a recombination event. Significant differences of the infection scores (Duncan test α=0.05) are indicated by a different letter and by color: RIS different from BIL8.2 and *L. sativa* cv. Olof = orange; RIS different from *L. sativa* cv. Olof and same as BIL2.2 = green; RIS higher than *L. sativa* cv. Olof = gray and the same RIS as *L. sativa* cv. Olof and higher than BIL2.2 = red. (α=0.05, Duncan test). In **c**, the - LOG transformed probabilities are plotted from a mixed model comparison between the average infection levels of lines with a homozygous *L. sativa* genotype and a homozygous *L. saligna* genotype at each marker position. Probability values were – LOG or LOG transformed, threshold level is set at α=0.01 = –LOG 2 and LOG -2. In **d**, the average RIS is plotted from the lines which had the *L. saligna* genotype at the marker positions. In the graph the RIS of *L. sativa* cv. Olof (Olof = 100%), BIL2.2 at the adult plant stage (BIL2.2 ADT_F = 58%) and BIL2.2 at the young plant stage (BIL2.2 YDT = 22%) are indicated by a dotted line.

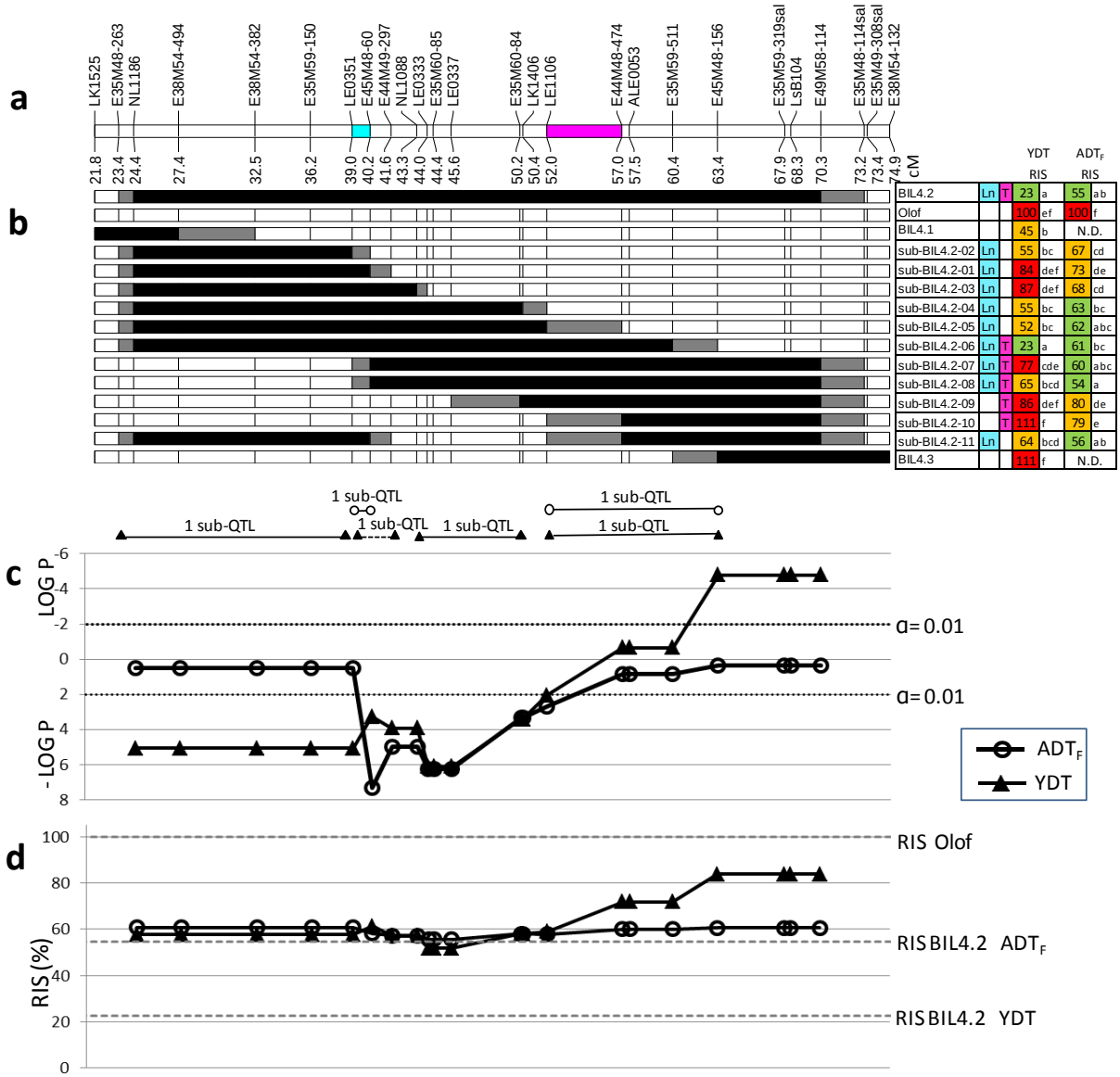

Figure S2. Genotypes and disease evaluation at young plant and adult plant stage of lettuce sub-BILs covering BIL4.2 introgression, the neighboring BILs and the reference lines. From top to bottom: **a**. Genetic map of Chromosome 4, 22-75 cM; **b**. the genotypes and the RIS of the disease evaluated lines; **c** the - LOG transformed probabilities; and **d**. the RIS graphs. In the genotype, LOG P, and plotted RIS value graphs, and the table with RIS values the explanations refer to Figure S1. Lines which showed morphological trait non-heading and long narrow leaf are indicated with an 'Ln' (in blue) behind their name and the lines with dark green and twisted leaves are indicated by a 'T' behind their name (in pink), the mapped position of those traits are indicated in blue and pink respectively, in the genotype graph. In the graph the RIS of *L. sativa* cv. Olof (RIS = 100%), BIL4.2 at the adult plant stage (RIS = 55%) and BIL4.2 at the young plant stage (RIS = 23%) are indicated by a dotted gray line.

Table S1. DNA-markers used for genotyping recombinants

Marker	Chr.	cM	Marker type[a]	Foreward primer	Reverse primer
KLK1473	2	49.3	dCAPs, HhaI	aatcggaactccaccacaa	gtggtttacaaatagggtgattacagcg
LE4034	2	57.1	SCAR	aatctctgacatgaaatcggc	tgccctcttccaagattatca
NL0212	2	58.8	SSR	ccagtgaagaaaccaaagg	cttctccttcatcgtcacc
LE1276	2	60.2	SCAR	tttgggttccttcagtttgc	cacagtttgggatgaacacg
LE7003	2	61.0	SCAR, DdeI	ggtctactggttcgcagagc	aagcctcacatgttcttccc
LK1336	2	63.2	CAPS, Eco88I	tgaggagtccatggatacgg	cgatgcaacagcatggatac
LE1244	2	69.0	SCAR	catccgcttcctcttcagtc	acgagcacctgcatctacaa
LE0142	2	73.3	CAPS, HinfI	agcagtggtggatcgatttc	ttggttctgcaagttgcttc
LK1475	2	74.4	SCAR	ggagttcagggcctctgtc	ccgattctgcggttatcttc
LE1114	2	77.9	CAPS, MseI	caagaggtgaatgggaagga	taccacacaaacaagcggaa
NL0560	2	80.6	SSR	ggaagaagtggaggaagaag	gatccataaggaggaaggg
NL0157	2	80.8	SSR	attgatccatggctacgac	gagcctatttcatccatgc
LK1525	4	21.8	CAPS, HaeIII	cagacgtccacctggaattg	attcagtgcgtctgttgcag
NL1186	4	24.4	SSR	aggggctgatgatgatatg	agtacatacttgtgtcttgtgg
LE0351	4	39.0	CAPS, NlaIV	gaatatgcggcggagataag	aatcacatgaatggatgcaaa
NL1088	4	43.3	SSR	atttgaaagccatggaaac	ttgcttcaaattttccacc
LE0333	4	44.0	SCAR	ggaccgggtttttaagtcgt	tttctctgtatatatgcaatctccatt
LE0337	4	45.6	CAPS, ScrfI	ccatggctaaaaagcaaacc	acattagccaagcgacaaca
LK1406	4	50.4	CAPS, RsaI	caccaccctcaccttagctc	accgttgaatatcggacacg
LE1106	4	52.0	SCAR	tgattatggaggcgaagagg	cacaaagattcattacttgccatc
ALE0053	4	57.5	CAPS, AvaI	tacccctaaagcccacctct	cggtggtgaagattcgtttt
LsB104 [b]	4	68.3	SSR		
LE0232	8	18.0	dCAPS, HinfI	ccatcgcgtaaacatgcccgggagt	taaaggtcgattagggcacg
CLS_S3_9019	8	21.5	HRM	tctaccatgggcaagaccac	ccattcagaagtcgctccag
NL0935	8	21.9	SSR	gtgaaccaatgagtggagg	gaacatccacttggtccag
CLPY724	8	29.3	HRM	gcgttccacgtctttttgat	ctggtggctctgatggaagt
CLSL2580	8	29.9	HRM	ctgcctgtaaaaaccggtca	tggttcgacgtcttcttgct
KLE0263	8	30.2	CAPS, MnlI	caacctcaccggagttttgt	gccggaaagtttgttgttgt
NL0352	8	36.0	SSR	aagctaagcaatatccccc	caaacaatcaccccaaaag
KLK1366	8	37.7	CAPS, MnlI	gaatcgctcaggcaaacaat	tggcctctcaagcagatttt
CLS_S3_7968	8	38.4	HRM	cattggtcagccactttcct	cacctgctggaatgatgatg
QGI8O23	8	38.7	HRM	taactcaaatgggcctctgg	gtgcagtgcagtgagcttgt
CLS_S3_8852	8	38.9	HRM	cttccccacttccacatgct	gggatcctggaaggcctagt
CLSY4909	8	39.2	HRM	cggaatcaacaaacaaatcca	cctcaatagttgaaagccacca
CLSS12338	8	39.4	HRM	tctctcaatcatccccaagc	cagtgcagccaatgtcaaag
LK1225	8	39.8	SCAR	cgcagtgaacattacgaacg	ccacgtatgaacacgtcagc
CLSM8077	8	40.0	HRM	tggagattctttgggtgctc	tcctgggctaaaatgattgc
CLS_S3_10544	8	40.4	HRM	tcttgcctttgtcaccatca	cgcagattgaagctttcgtt
CLS_S3_6749	8	40.6	HRM	ctccgccattgattctttgt	tcggaagaacctgaagcaaa
LE1111	8	41.8	SCAR	aattcactccaccaccgaag	ctacgtcagtgcctatgcca
LE0026	8	50.6	CAPS, MseI	aggtattttccggcgaactt	ctttgtgcctcaaacccaat
LE3019	8	51.4	CAPS, ClaI	attgctggagtcgtggtttc	ctttgtgcctcaaacccaat

[a] For the (d)CAPS markers the used restriction enzyme is given. HRM means that polymorphisms were visualized by a High Resolution Melting curve analyzer (see M&M).
[b] This SSR marker refers to Van de Wiel et al. (1999).

CHAPTER 3

Effects of stacked quantitative resistances to downy mildew in lettuce do not simply add up

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Key message:

In a stacking study of eight resistance QTLs in lettuce against downy mildew, only three out of ten double combinations showed an increased resistance effect under field conditions.

Abstract

Complete race nonspecific resistance to lettuce downy mildew, as observed for the nonhost wild lettuce species *Lactuca saligna*, is desired in lettuce cultivation. Genetic dissection of *L. saligna*'s complete resistance has revealed several quantitative loci (QTL) for resistance with field infection reductions of 30% to 50%. To test the effect of stacking these QTL, we analyzed interactions between homozygous *L. saligna* CGN05271 chromosome segments introgressed into the genetic background of *L. sativa* cv. Olof. Eight different backcross inbred lines (BILs) with single introgressions of 30 to 70 cM and selected predominately for quantitative resistance in field situations were intercrossed. Ten developed homozygous lines with stacked introgression segments (double-combinations) were evaluated for resistance in the field. Seven double-combinations showed a similar infection as the individual most resistant parental BIL, revealing epistatic interactions with 'less-than-additive' effects. Three double-combinations showed an increased resistance level compared to their parental BILs and their interactions were additive, 'less-than-additive' epistatic and 'more-than-additive' epistatic, respectively. The additive interaction reduced field infection by 73%. The double-combination with a 'more-than-additive' epistatic effect, derived from a combination between a susceptible and a resistant BIL with 0% and 30% infection reduction respectively, showed an average field infection reduction of 52%. For the latter line, an attempt to genetically dissect its underlying epistatic loci by substitution mapping did not result in smaller mapping intervals as none of the 22 substitution lines reached a similar high resistance level. Implications for breeding and the inheritance of *L. saligna'*s complete resistance are discussed.

Keywords: quantitative resistance, stacking, pyramiding, additivity, epistasis

Introduction

Improving plant genotypes by breeding requires crossing and selection of the most desirable plants with the best combination of genes. The plants with the preferred genotype contain genes for desirable qualitative and quantitative traits from several parents stacked together. Stacking is often recommended when quantitative trait loci (QTLs) are identified that have a too small individual effect to be of interest for breeding. It is therefore relevant to study the effect of stacking genes for quantitative traits on the level of that trait. These studies are scarce, mostly on conventional segregating populations (F_2, RIL) or on backcross inbred populations (with maximally 5 generations $BC_{2\text{-}3}S_{1\text{-}2}$), and report that QTL × QTL interactions may play a role. Additive as well as epistatic interactions have been reported in quantitative resistance (Castro et al. 2003; Marcel et al. 2007; St.Clair 2010) and in other agronomical traits (Breen et al. 2012; Carlborg and Haley 2004; Eshed and Zamir 1996; Lin et al. 2000). In conventional segregating populations, conclusions on epistatic effects between QTLs are often imprecise and complicated due to different frequencies of genotype classes (Ding et al. 2010; Yano and Sasaki 1997). To better understand the interactions between QTLs, more knowledge is required on the effects of stacked QTLs in sub-BILs that have almost identical genetic backgrounds and allow comparisons of two-locus genotypes in balanced frequencies and many replicated measurements.

In this study we employed a set of 29 lettuce Backcross Inbred lines (BILs), each carrying a single wild lettuce (*Lactuca saligna*, CGN05271) introgression segment in an otherwise identical background of cultivated lettuce (*Lactuca sativa*, cv. Olof). This set of BILs represents 96% of the wild species genome (Jeuken et al. 2008; Jeuken and Lindhout 2004) and has been tested for downy mildew resistance at several plant stages (Zhang et al. 2009a).

In lettuce cultivation, downy mildew infections, caused by the oomycete *Bremia lactucae*, lead to high yield losses and is the most problematic disease in lettuce cultivation. Introgression of the complete resistance from wild non-host lettuce *L. saligna* is considered as an interesting strategy, since that resistance may be more durable than the commonly used classical race specific *R*-genes (Bonnier et al. 1991; Jeuken and Lindhout 2002; Lebeda and Boukema 1991).

In previous research, we detected in an *L. saligna* CGN05721× *L. sativa* cv. Olof F_2 population no race-specific *R*-genes, but instead three QTLs and a resistance caused by a digenic interallelic interaction that leads to hybrid necrosis (Jeuken and Lindhout 2002; Jeuken et al. 2009). In the series of 29 BILs we found an abundance of 15 introgressions that conferred plant stage dependent quantitative resistance against all six tested *B. lactucae* isolates (Zhang et al. 2009a). The genetic size of these *L. saligna* introgressions ranged from 20 to 80 cM (Jeuken et al. 2008). At the, most relevant, adult plant stage in the field (ADT_F) eight BILs showed reduced infection levels, but their 30 to 50% infection reduction is not sufficient for practical application in commercial breeding and cultivation (Jeuken and Lindhout 2004; Zhang et al. 2009a). In

search for a desired race nonspecific very strong or absolute resistance in the field, we studied the potential of stacking the quantitative resistances of BILs. For the purpose of the present paper, each introgression segment conferring quantitative resistance is considered as one QTL, because for the majority of the resistant BILs no information on the number and position of resistance gene(s) within the introgressions is available. For three of the introgression segments used in this study, namely, 2.2, 4.2 and 8.2, information from substitution mapping revealed the presence of multiple linked sub-QTL (Chapter 2).

It was the objective of the present study to find an indication of the genetic control of complete resistance of *L. saligna*. As a pragmatic approach, we tested the null hypothesis that 'the complete nonhost resistance of *L. saligna* to *B. lactucae* is due to the cumulative and additive effects between several quantitative resistance genes (QTLs)'. In that case we assume that stacking several of the introgression segments that confer quantitative resistance should result in (near-) complete resistance. If the null hypothesis is rejected, complex genetics with epistatic gene action(s) might be an alternative explanation for the complete resistance of *L. saligna*. In a preliminary stacking study with six double combinations disease tested at the young plant stage, significantly further decreased infection levels were observed for certain stacking combinations (Zhang et al. 2009b). We extended this study by the development of additional combinations and by multiple independent field evaluations at the adult plant stage to determine the effects of stacked introgressions conferring quantitative resistance in the field. We determined for the stacked introgression segments their interaction type (additive, epistatic) and the direction and magnitude of their effects on the resistance level.

Materials and methods

Stacking of introgressions from BILs

Fifteen out of twenty-eight disease evaluated BILs showed reduced infection in at least one plant stage (seedling, young or adult plants) (Jeuken and Lindhout 2004; Zhang et al. 2009a). For intercrossing, we selected the BILs with introgressions that were effective at adult plant stage in the field: BIL 1.2, 2.2, 4.2, 4.6, 5.2 and 8.2. BIL9.2 was not included, because of its low vitality and dwarf phenotype. BIL7.1, with an introgression on Chromosome 7 and on Chromosome 1, was not included, because its effective introgression is covered in the introgression segment of BIL1.2. Instead, BIL[4.1+6.3] was selected for its very low infection levels at young plant stage and adult plant stage in greenhouse tests, although it was susceptible at adult plant stage in field tests (Zhang et al. 2009a). BIL4.1 was included as a control line for BIL[4.1+6.3]. The square brackets around the introgression segments of BIL[4.1+6.3] and of some of its derived sub-BILs (which are lines with smaller donor segments than the parental BIL), indicates that plants carrying a *L. saligna* segment on Chromosome 6 always require a *L. saligna* segment on

Chromosome 4. In other words, for BIL[4.1+6.3] the 6.3 *L. saligna* introgression segment does not segregate independently from the 4.1 introgression segment, which indicates at a hybrid incompatibility between the donor segment at Chromosome 6 and the recurrent *L. sativa* alleles at Chromosome 4.

Zhang et al. (2009b) intercrossed BIL2.2, 4.2, [4.1+6.3] and 8.2 to obtain six lines with two stacked introgressions segments, in which [4.1 + 6.3] are counted as one introgression segment. Subsequent intercrossing of lines with two stacked segments resulted in four lines with three stacked segments and one line with four stacked segments. In the present study we included most of these lines except for combinations 4.2+[4.1+6.3] and 2.2+4.2+[4.1+6.3], because of limited numbers of available seeds for field tests. We intercrossed five BILs, 1.2, 4.1, 4.6, 5.2 and 8.2, and obtained F_1 plants for the following crossings: 1.2×8.2, 4.1×8.2, 4.6×8.2, and 5.2×8.2. Lines with two or more homozygous introgressions were developed as described by Zhang et al. (2009b). An overview of the intercrossed BILs that resulted in lines with two stacked introgressions is shown in Figure 1, with characteristics on introgression segment length and relative infection severity levels (RIS) in previous studies.

Development and stacking of smaller introgression segments

For the 8.2 introgression (30 cM segment), sub-BILs, which are lines derived from a parental BIL with a smaller donor segments than the parental BIL, were already available (Chapter 2). For stacking of smaller introgressions, we selected four sub-BILs with predominately overlapping introgressions, 8.2-01 (7 cM segment), 8.2-02 (12 cM segment), 8.2-07 (~18 cM segment) and 8.2-81 (2 segments of ~10 and ~8 cM) for intercrossing with genotypes carrying smaller introgressions from [4.1+6.3] (Chapter 2, Figure 1c). The overlap of introgressions between sub-BIL 8.2-02 and 8.2-07 and 8.2-81 in the interval of 30.2 to 35.6 cM is uncertain due to lack of markers. We designated a line that contains two or more stacked donor introgressions derived from either BIL or sub-BIL a ‘combi-line’. As no sub-BILs for the [4.1+6.3] introgression were available yet, a recombinant screening was performed. We focused mainly on the 6.3 introgression because at adult plant stage in a greenhouse test (ADT_G) it conferred quantitative resistance by reducing the relative infection severity (RIS) to 38% compared to the susceptible reference and the 4.1 introgression did not (RIS 94%) (Jeuken et al. 2008; Zhang et al. 2009a).

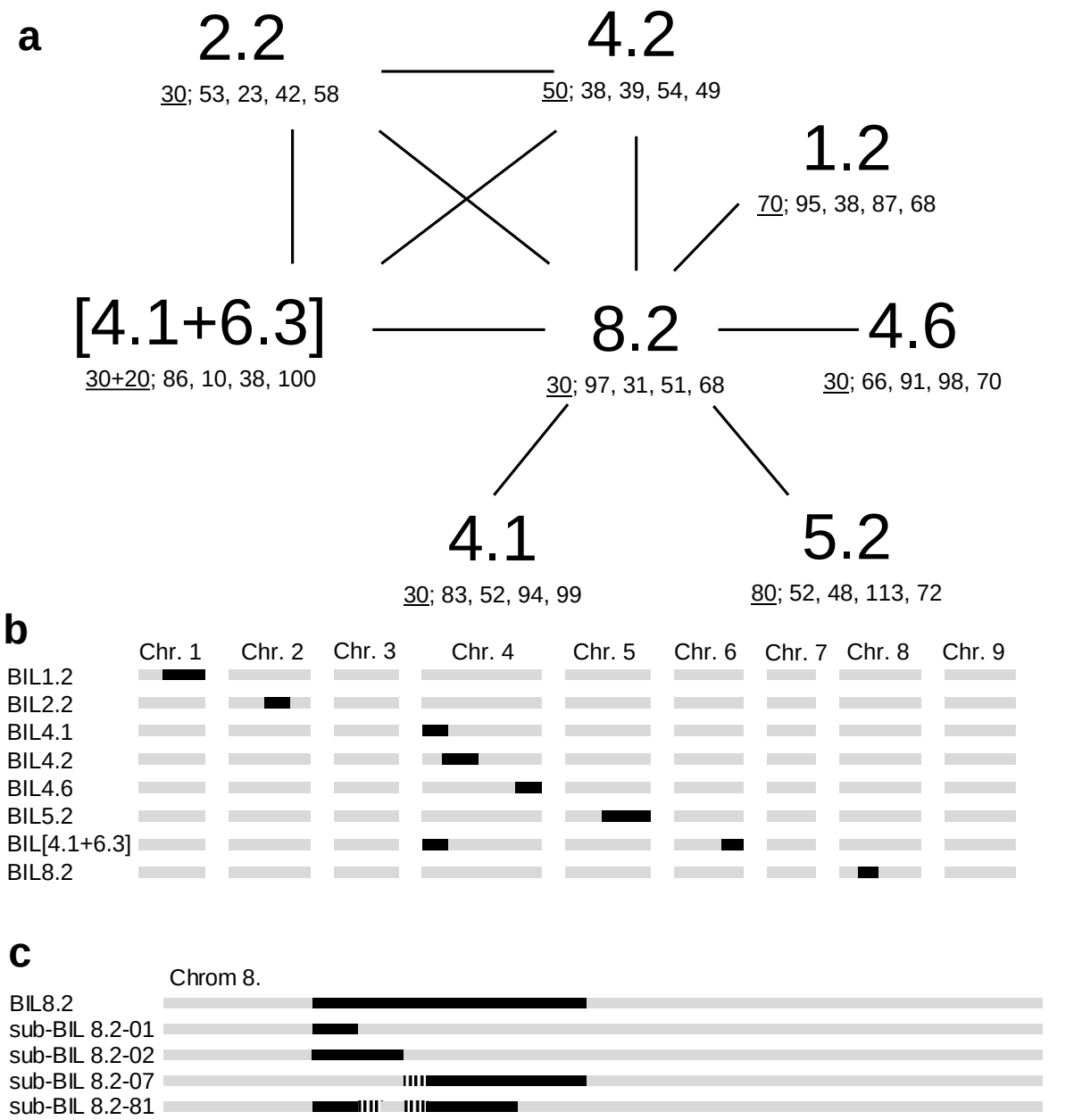

Figure 1 Overview of intercrossed backcross inbred lines (BILs) and their genotype.
1a: Connecting lines indicate successful stacking of introgression segments from two BILs. Per BIL five consecutive numbers indicate: the length of the *L. saligna* introgression segments in cM (underlined and rounded to tens); relative infection severity level (relative to *L. sativa* cv. Olof, which is set at 100% infection) in a seedling disease test (SDT), young plant disease test (YDT), adult disease test in greenhouse (ADT_G) and adult disease test in field (ADT_F) (from 11 locations in the Netherlands and France $ADT_{F\text{-}C}$) respectively as reported in Zhang et al. (2009a).
1b: A schematic genotype presentation is shown per BIL; bar colors in Figure1b and Figure1c represent the genotype: black: homozygous *L. saligna*; gray: homozygous *L. sativa*; vertical striped: unknown genotype.
1c: A schematic genotype presentation is shown for four used sub-BILs of BIL8.2. Exact marker genotypes are shown in Chapter 2.

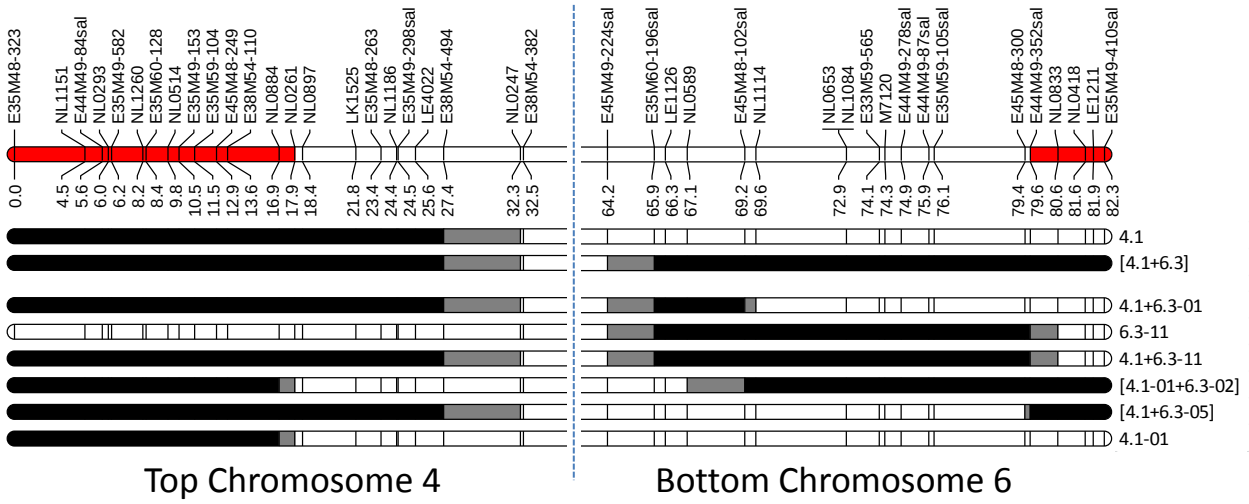

Figure 2. Genotypes of sub-BILs derived from BIL[4.1+6.3]. Black bar represents genotype homozygous *L. saligna*, white means homozygous *L. sativa* and gray represents marker intervals where a recombination event resides. Square brackets around introgression segments, like for BIL[4.1+6.3], indicates that the 6.3 introgression segment cannot be uncoupled from 4.1 segment and they inherit as one unit. The red marker intervals show the map intervals of the hybrid incompatibility loci.

An inbred progeny of 2100 plants from preBIL[4.1+6.3] (heterozygous for both introgressions) was screened for recombinants using markers NL1151 and NL0897 at 4.1 and LE1126 and LE1211 at 6.3 (Table S1, Figure 2). Fourteen hundred plants were screened for 6.3 alone and 700 plants were screened for 4.1 and 6.3. During the process of recombinant screening and development of homozygous lines (sub-BILs), we observed that certain recombinants in 6.3 were not dependent on the presence of 4.1 introgressions segments. Sub-BILs of these specific recombinants allowed us to fine map the hybrid incompatibility loci on 6.3 and 4.1 as well as the locus for resistance on 6.3. For the stacking of smaller introgressions from 4.1, 6.3 and 8.2, a few sub-BILs per introgression segment were selected. We followed the same stacking procedure as described above for the combination of BIL introgressions.

Genotyping

DNA was isolated by NaOH method (Wang et al. 1993) or modified CTAB method (Jeuken et al. 2001). Co-dominant DNA- markers were used to screen for recombinants, to determine the position of the recombination, to distinguish between inbred and outcrossed progeny and for selection of stacked homozygous *L. saligna* introgressions in the combi-lines. Primer sequences for markers covering the 1.2, 4.1, 4.6, 5.2 and 6.3 BIL introgressions are listed in Table S1 and markers for 2.2, 4.2 and 8.2 introgressions are described in Chapter 2. SSR markers were kindly provided by Syngenta BV and EST markers were developed on lettuce EST sequences of the *Compositae* Genome Project Database (CGPDB), (http://compgenomics.ucdavis.edu) and McHale et al., (2009). Polymorphisms between PCR products of *L. saligna* and *L. sativa* alleles

were visualized by high-resolution melting curve differences on a LightScanner System (Idaho Technology) or by size differences on agarose gels (directly or after enzymatic digestion) as described previously (Jeuken et al. 2008).

Assessment of resistance to *B. lactucae*

To determine the effects of stacked (sub)-introgressions on resistance levels in the field, all combi-lines were tested at adult plant stage in the field (ADT_F) in the presence of control lines. Control lines were: parental BILs and sub-BILs, susceptible control *L. sativa* cv. Olof and reference resistant control *L. sativa* cv. Iceberg (with an average RIS of 40% compared to the severity on susceptible controls in field tests, reported by Grube and Ochoa (2005) and Chapter 2). ADT_F were performed in three years (2009, 2010 and 2011) with three to five experiments (locations) per year and four or six replications per experiment (Table S2). Artificial or natural infection occurred. Symptoms of *B. lactucae* infection were recognized by at least two independent and experienced observers. Infected leaf material was collected to isolate the pathogen strains. These isolates were applied to the differential set and tested for their virulence spectrum. The following *B. lactucae* races were identified: Bl:22, 24, 25, 26 and three mixtures. The virulence spectrum of these three mixtures was complex and not informative enough to lead to the identification of the constituent races or of possibly novel races. The number of randomized replications, plants per replicate (8 to 25 plants), the location of the field test, the *B. lactucae* infection (natural or artificial and detected races), and the plant age at time of observation for each experiment are shown in Table S3. The infection severity level (ISL) per replicate was evaluated as an average infection score for whole plants in a scale from zero (no infection symptoms) to nine (maximum infection symptoms) on adult plants as described in Zhang et al. (2009a).

To pinpoint the interactive loci responsible for the increased resistance level of combi-line[4.1+6.3]+8.2 by substitution mapping, a selection of sub-BILs and combi-lines of stacked sub-BILs was tested in ADT_F in 2010 and 2011 and at young plant stage (YDT). In YDT the ISL of the lines was evaluated quantitatively in four independent experiments; once with *B. lactucae* race Bl:14, twice with Bl:21 and once with Bl:26 (inoculated with $2\text{-}4 \times 10^5$ spores per ml). To improve data normality the percentage data of the YDT was arcsine root transformed. Data analysis of YDT and ADT_F employing a linear mixed model was as described in Zhang et al. (2009a) with some small modifications. Multiple comparison of disease evaluated lines for both YDT and ADT_F data was performed by a Duncan's multiple range test, $\alpha=0.05$ and the correlations between experiments were calculated by a Pearson correlation test. Relative infection severity (RIS) levels were calculated as percentage relative to the severity on the susceptible parent *L. sativa* cv. Olof.

Gene action across loci analysis

For combi-lines that showed a significantly lower ISL compared to both individual parental lines, we determined whether effects of stacked (sub)-introgressions suggested additivity or epistasis. RIS levels (in Figure 3 and Table 1) were transformed into reduction in RIS (RRIS) (in Table 2) by calculating the reduction of the ISL from each line relative to the severity on the susceptible parent *L. sativa* cv. Olof. Assuming complete additivity between the combined introgression segments, the RRIS effect of the combi-line should be equal to the sum of the reduction of the two parental lines (expected value). The difference between the expected additive effect and observed RRIS of the combi-line was determined independently for each combination with a linear mixed model (LSD test α=0.05, statistical package SPSS 19.0). When the expected (additive) RRIS for the combi-line was higher than 100% we did not test if the gene action was additive, as the infection level of the combi-line cannot be lower than 0%. In the statistical model 'effect and experiment' were used as fixed factors. For the factor effect, the observed RRIS of the combi-line was compared with the expected additive effect. To determine the similarity between the experiments the effect × experiment interactions were measured. If the observed RRIS was not different from the expected additive effect, the gene action across loci was concluded to be additive and if it was significantly different, the gene action across loci was concluded to be epistatic. About the direction of gene action across loci: if the infection is further decreased (i.e. more resistant), the epistasis is positive; if the infection is increased (more susceptible), the epistasis is negative. About the magnitude of the effects: the additive effect is the sum of its individual components; the magnitude of positive epistatic effects are described as 'less-than-additive' or 'more-than-additive', respectively, as used in Eshed and Zamir (1996).

Results

Infection levels of stacked segments with quantitative resistance

In addition to the nine combi-lines developed and tested by Zhang et al. (2009b), five new combi-lines were developed: 1.2+8.2, 4.1+8.2, 4.6+8.2, 5.2+8.2 and 5.2-01+8.2. The latter line was derived from a recombinant plant and harbours about half of the BIL5.2 introgression, from about 90 cM until the end of the chromosome at 122 cM.

The fourteen combi-lines and their parental BILs were tested and evaluated in two sets (of ten and five combi-lines) in field tests in 2009 (four locations) and in field tests in 2011 (three locations) (Table S2). In both years five lines were in common: *L. sativa* cv. Olof, *L. sativa* cv. Iceberg, BIL4.1, BIL8.2 and combi-line[4.1+6.3]+8.2.

Three of the twelve field experiments (locations) had a lower overall infection level, but within the three locations similar differences between lines were observed as in the other nine locations. The average ISL of susceptible control cv. Olof was in nine locations value 7 or higher and in three locations around value 5 (see Table S3). Within each year the field test results were

highly correlated between all the experiments/locations (2009: average r = 0.85; 2011: average r=0.70) and therefore data were combined per year (Table S3). Between years the field test results were also highly correlated for the lines in common (r = 0.97).

In 2011 the differences in ISL between susceptible control cv. Olof and resistant control *L. sativa* cv. Iceberg was 17% smaller than in 2009, as the resistant control showed about 10 percentage points higher RIS in 2011 than in 2009 (Figure 3). As expected, all BILs had a significantly lower infection severity than *L. sativa* cv. Olof, except in both years for BIL4.1 and BIL[4.1+6.3] . These results confirm earlier observations (Zhang et al 2009a, Zhang et al 2009b).

Of the ten combi-lines with two introgressions, seven showed a similar and not significantly lower infection than the individual most resistant parental BIL (Table 1). Effects of these stacked resistances do not simply add up, but are epistatic with 'less-than-additive' effects.

Three combi-lines, 1.2+8.2, 2.2+8.2 and [4.1+6.3]+8.2, showed a significantly lower RIS than the most resistant parental BIL (Figure 3). Combi-line1.2+8.2 had the lowest RIS (27%), which was lower than of the reference resistant control *L. sativa* cv. Iceberg.

Combi-line[4.1+6.3]+8.2 with an average RIS of 48% over three years, had a similar infection level as *L. sativa* cv. Iceberg. The RIS of combi-line2.2+8.2 was only 10 RIS units (percentage points) lower than the RIS of BIL2.2. Stacking three introgressions did not result in significantly lower RIS than for the respective lines with two stacked introgressions, except for the comparison of 2.2+[4.1+6.3]+8.2 with 2.2+8.2. Stacking four introgressions, 2.2+4.2+[4.1+6.3]+8.2, resulted in a very low RIS of 12%, which was significantly lower than for the respective lines with three and two stacked introgressions (Figure 3).

The gene action between the combined introgression segments 1.2 and 8.2 was additive, between [4.1+6.3] and 8.2 was epistatic with a 'more-than-additive' effect, and between 2.2 and 8.2 was epistatic with a 'less-than–additive' effect (Table 2a).

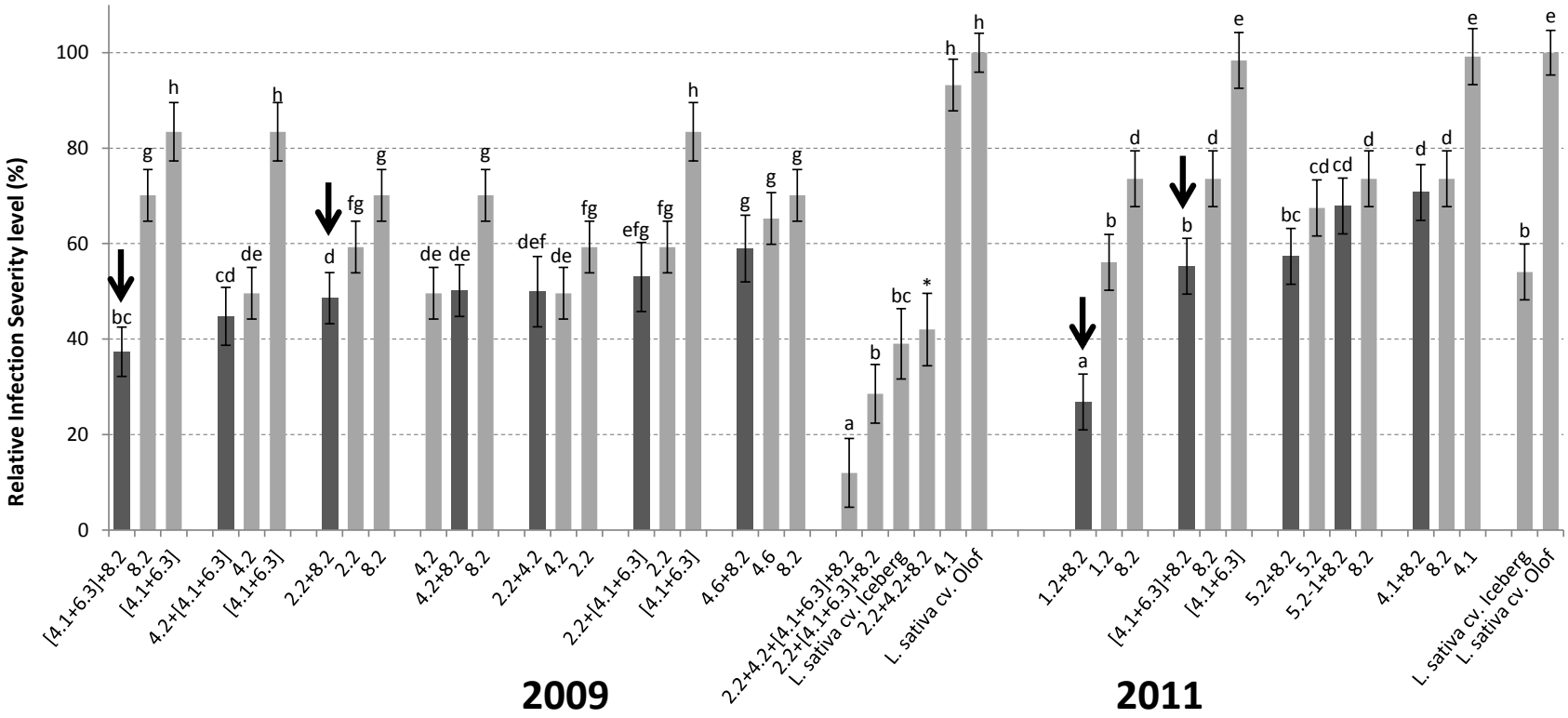

Figure 3. Comparison of relative infection severity level (RIS) of combi-lines (stacked introgressions) with parental BILs (individual introgressions in field tests (ADT_F) in 2009 and 2011.

For ease of comparison combi-line values (dark grey bar) are grouped with their parental line values (light grey bar). Control lines are aligned on the right hand per year. Arrows point to combi-line values that are significantly different from both parental BILs. Susceptible parent *L. sativa* cv. Olof has RIS 100%. Error bars represent 95% confidence intervals and letters in common indicate no significant difference (Duncan's multiple range test, α=0.05). *Combi-line2.2+4.2+8.2 was tested at three locations in 2008, RIS of this line was corrected to RIS in 2009 by its relative position between combi-line[4.1+6.3]+8.2 and *L. sativa* cv. Olof.

Table 1. Infection level comparison of combi-line[4.1+6.3]+8.2 and its derived combi-lines with less and/or smaller introgression segments. Data of Figure 4 are arranged to visualize the effect of stacking introgressions. See legend Figure 4. Gradual color scale is used to visualize differences in RIS values, within each disease test type (from 'green' low infection to 'red' high infection). The susceptible control *L. sativa* cv. Olof has a RIS of 100%. Within each column, RIS values followed by letters in common are not significantly different (Duncan's multiple range test, α=0.05). YDT means young plant disease test, ADT_F means adult plant disease test in the field.

	ADT_F 2011							YDT 2010						
Combi-Line[a]	Line 1 RIS[a]		Line 2 RIS[a]		Combi-Line RIS[b]		Sign. diff. & effect on RIS[c]	Line 1 RIS[a]		Line 2 RIS[a]		Combi-Line RIS[b]		Sign. diff. & effect on RIS[c]
[4.1+6.3]+8.2	100	jkl	75	bc	57	a	reduced	14	cd	31	ef	2	a	reduced
4.1+8.2	98	ijkl	75	bc	70	b								
4.1+6.3-11	98	ijkl	106	lm	89	efghi		42	f	66	gh	18	de	reduced
4.1+8.2-01	98	ijkl	75	bc	83	cdef								
4.1+8.2-02	98	ijkl	97	hijkl	92	fghij								
4.1+8.2-07	98	ijkl	84	cdef	82	cdef								
4.1+8.2-81	98	ijkl	79	bcd	78	bcd								
6.3-11+8.2-01	106	lm	75	bc	88	defgh		66	gh	72	hi	39	f	reduced
6.3-11+8.2-02	106	lm	97	hijkl	104	klm		66	gh	41	f	96	ij	increased
6.3-11+8.2-07	106	lm	84	cdef	80	bcde		66	gh	77	hij	64	gh	
4.1-01+8.2-02	101	jkl	97	hijkl	95	hijk		42	f[d]	41	f	5	bc	reduced[d]
[4.1-01+6.3-02]+8.2-02	93	ijkl	97	hijkl	84	cdefg	reduced[e]	14	cd[e]	41	f	2	ab	reduced[f]
[4.1+6.3-05]+8.2-01	82	ghijk	75	bc	75	bc								
[4.1+6.3-05]+8.2-02	82	ghijk	97	hijkl	91	fghij								
[4.1+6.3-05]+8.2-07	82	ghijk	84	cdef	78	bcd								

[a] Line 1 and 2 represent the parental lines with a single introgression. Their numbering is based on the order in the name of the combi-line.

[b] Combi-line represents the line with the stacked introgression derived from a cross between Line 1 and Line 2, and its name is mentioned in the first column. For example: in combi-Line4.1+8.2, line 1 is BIL4.1 and line 2 is BIL8.2.

[c] If the combi-line was significantly different from both parental lines, its effect on the infection level is shown (reduced or increased). Duncan's multiple range test, α=0.05.

[d] RIS of sub-BIL4.1-01 was not defined. In an earlier experiment the offspring from pre-sub-BIL4.1-01 which contained, besides a homozygous *L. saligna* introgression from top to 17 cM, a segregating introgression at the bottom side of the 4.1 introgression (21 to 32 cM), showed a similar RIS as BIL4.1. Therefore RIS of BIL4.1 is shown and used for comparison.

[e] In the field test of 2010 (Figure 4), combi-line [4.1-01+6.3-02]+8.2-02, and its parental sub-BILs [4.1-01+6.3-02] and 8.2-02 showed no significant differences (RIS 80%, 83% and 84% respectively, Figure 4) and therefore no interaction was observed.

[f] RIS of sub-BIL[4.1-01+6.3-02] was not defined in the described YDT experiments. Earlier YDT experiments in 2008 showed that the infection level was similar to BIL[4.1+6.3] (Chapter 4 in Thesis Zhang, 2008). Therefore RIS of BIL[4.1+6.3] is shown here and used for comparison.

Development of [4.1+6.3] sub-BILs and fine mapping resistance and hybrid incompatibility

In the recombinant screening of the inbred progeny of preBIL[4.1+6.3], we identified 41 recombinants in 4.1 and 32 in the 6.3 introgression. The recombination frequency within the 4.1 and 6.3 introgression was three and ten times suppressed, respectively, compared to the same region in the original *L. saligna* × *L. sativa* F_2 population (Jeuken et al. 2001). Fourteen recombinants were selected to be developed into homozygous 6.3 sub-BILs. The selection was based on uniqueness of recombination interval. Per marker interval (at most) one recombinant was taken to produce a homozygous line with shorter introgression. Figure 2 shows the combination of 4.1 (sub)introgressions and 6.3 (sub) introgressions that were present in the sub-BILs to be phenotyped in disease tests. We selected one recombinant segment for the 4.1 introgression and four different 6.3 sub-introgressions that together covered the complete 6.3 introgression. Two of these sub-BILs were informative to map the hybrid incompatibility between 6.3 and 4.1 introgression, namely, sub-BIL6.3-11 that did not require a 4.1 introgression and sub-BIL[4.1-01+6.3-02] that did require a smaller 4.1 introgression. We mapped the hybrid incompatibility to the intervals 0 - 17.9 cM on Chromosome 4 and 79.6 - 82.3 cM on Chromosome 6 (Figure2).

In previous and recent study BIL[4.1+6.3] showed a low RIS (10 to 14%) at young plant stage (Figure 4b; Zhang 2009a). The newly developed 4.1 and 6.3 sub-BILs allowed us to fine map this resistance.

By comparison of their infection levels at young plant stage (Figure 4c), we mapped this low infection level at Chromosome 6, between 79.4 and 80.6 cM. This conclusion is based on the observation that both combi-lines 4.1+6.3-11 and sub-BIL[4.1+6.3-05] had a similar low RIS (18 and 13% respectively) and overlapping introgression region of 1.2 cM. The resistance on this short interval on 6.3 apparently interacts with a *L. saligna*-derived gene on 4.1, since a low RIS like in [4.1+6.3-05] and 4.1+6.3-11 was not found in the individually tested 6.3-11 (RIS 66%) where the whole chromosome 4 was *L. sativa*-derived (Figure 4c).

Table 2. Overview of additive and epistatic gene action effects of stacked introgression segments of BILs (2a) and sub-BILs (2b)
In general the effects of stacking are only analysed for gene action when the combi-line showed a deviating reduction in infection level from both individual parental lines (shown in Figure 3 and Table 1), except for combi-line2.2+4.2 as an example. YDT means young plant disease test, ADT_F means adult plant disease test in the field.

				Reduction in relative infection severity level					
		Combi-Line (year)[a]	Disease test	Line 1[b]	Line 2[b]	Exp.[c]	Obs.[d]	Obs. vs. ind. lines[e]	Gene action across loci
a	**combi-BILs**	1.2+8.2 (11)	ADT_F	-44	-26	-70	-73	reduced	additive [l]
		2.2+8.2 (09)	ADT_F	-41	-30	-71	-51***	reduced	pos. epistasis, less-than-additive
		2.2+4.2 (09)	ADT_F	-41	-50	-91	-50***	same	pos. epistasis, less-than-additive [f]
		[4.1+6.3]+8.2 (09)	ADT_F	-17 ns	-30	-47	-63*	reduced	pos. epistasis, more-than-additive
		[4.1+6.3]+8.2 (10)	ADT_F	-11	-25	-36	-48**	reduced	pos. epistasis, more-than-additive [j]
		[4.1+6.3]+8.2 (11)	ADT_F	-2 ns	-26	-28	-45*	reduced	pos. epistasis, more-than-additive [i]
		[4.1+6.3]+8.2 (10)	YDT	-86	-69	-155	-98	reduced	N.D.[h]
b	**combi-sub-BILs**	4.1+6.3-11 (10)	YDT	-58	-34	-92	-82	reduced	additive
		6.3-11+8.2-01 (10)	YDT	-34	-28	-62	-61	reduced	additive
		6.3-11+8.2-02 (10)	YDT	-34	-59	-93	-4	increased	negative epistasis
		4.1+8.2-02 (10)	YDT	-58	-59	-117	-95	reduced	N.D.[h]
		[4.1-01+6.3-02]+8.2-02 (11)	ADT_F	-7 ns	-3 ns	-10	-18	reduced	pos. epistasis [g]
		[4.1-01+6.3-02]+8.2-02 (10)	YDT	-86	-59	-145	-98	reduced	N.D.[h]

[a] In brackets experimental year is indicated, in 20th century, (09 = year 2009).
[b] Line 1 and 2 represent the lines with a single introgression. Their numbering is based on the order in the name of the combi-line. For example: in combi-Line1.2+8.2, line 1 is BIL1.2 and line 2 is BIL8.2. Significant reduction in relative infection severity levels (RRIS) (relative in percentage from susceptible parent *L. sativa* cv. Olof) are presented from line 1 and line 2.Non significant reduction is indicated by 'ns' (Duncan's multiple range test, α=0.05).
[c] Exp. = Expected RRIS when gene action across loci is additive (sum RRIS line 1 and line 2).
[d] Obs. = Observed RRIS from combi-line, RRIS is compared to expected and P values from LSD test are indicated: * = <0.05, ** = < 0.01, *** P=<0.001.
[e] Effect on RIS of combi-line compared to the individual lines.
[f] Reduction in infection was not different from both individual parental lines (line 1 and 2).
[g] Both parental lines did not show an significant reduction in infection from *L. sativa* cv. Olof, therefore effect of additivity is not tested.
[h] N.D. means not defined because expected additive effect exceeds absolute resistance (RRIS higher than 100%) and therefore the magnitude of the effect can not be estimated.
[i] An experiment x effect interaction was detected between three locations. For the individual locations the effects were: 1 x additive, 1 x less-than-additive, 1 x more-than-additive.
[j] An experiment x effect interaction was detected between four locations. For the individual locations the effects were: 3 x additive and 1 x more-than-additive.

Genetic dissection of combi-line[4.1+6.3]+8.2

To genetically dissect the epistatic loci of the [4.1+6.3]+8.2 combination, we stacked smaller introgressions of 4.1, 6.3 and 8.2 and subsequently phenotyped the genotypes carrying combinations of these sub-introgressions. We used: one 4.1 sub-BIL (4.1-01), three 6.3 sub-BILs with overlapping introgressions ([4.1-1+6.3-02], [4.1+6.3-05] and 6.3-11) and four 8.2 sub-BILs with overlapping introgressions (8.2-01, 8.2-02, 8.2-07and 8.2-81, Figure 4c, d). Twenty-two lines with shorter introgressions of 4.1 and/or 6.3 and/or 8.2 in various combinations were disease evaluated to fine map resistance and investigate possible interactions for resistance between the introgressions (Figure 4c-g).

At the young plant stage, the infection levels of the tested lines were highly correlated between the four experiments (average, r=0.91, lowest r=0.89 and all correlations highly significant; P=<0.001) and no race × sub-BIL interaction, hence no race-specificity, was observed. Therefore we combined the data of the four experiments for analysis.

There were many interesting interactions between the stacked (sub)-introgressions. For example, one of the most resistant combi-lines in the YDT was combi-line 4.1-01+8.2-02 (RIS 5%), which was obviously more resistant than the line with 8.2-02 alone (RIS 41%) and 4.1 alone (RIS 42%). Strikingly, the resistance conferred by 8.2-02 alone (RIS 41%) was completely cancelled, when combined with 6.3-11 in combi-line 6.3-11+8.2-02 (RIS 96%) (Figure 4d, f). This high susceptibility in combi-line 6.3-11+8.2-02 was at least partly due to a central segment of 8.2, since combi-lines 6.3-11+8.2-01 and 6.3-11+8.2-07, which only differed from combi-line 6.3-11+8.2-02 by shorter introgressions of 8.2, were again medium resistant (RIS 39% and 64% respectively) (Figure 4f).

In 2010 and 2011, the set of lines was evaluated in field tests. The control lines and BILs had an infection level as expected (Zhang et al 2009a). Within each year the field test results were significantly correlated between all locations (all correlations, P=<0.001; 2010: average r = 0.82; 2011: average r=0.66) and therefore combined. Between years the field test results were also significantly correlated for the 18 lines in common (correlation, P=<0.001, r = 0.91). Combi-line[4.1+6.3]+8.2 showed on average a RIS of 48% over three years (2009: 37%, 2010: 52%, and 2011: 57%; Figure 3 and 4a), which is similar to the RIS on our reference resistant line *L. sativa* cv. Iceberg (average RIS 43%).

At the adult plant stage none of the sub-introgression combinations showed a significantly lower RIS than one of its parental lines and none of the lines had a similar low RIS as combi-line[4.1+6.3]+8.2 (Figure 4 and Table 1). Therefore, we were not able to narrow down the high resistance of combi-line[4.1+6.3]+8.2 to smaller marker intervals nor to identify the underlying epistatic loci.

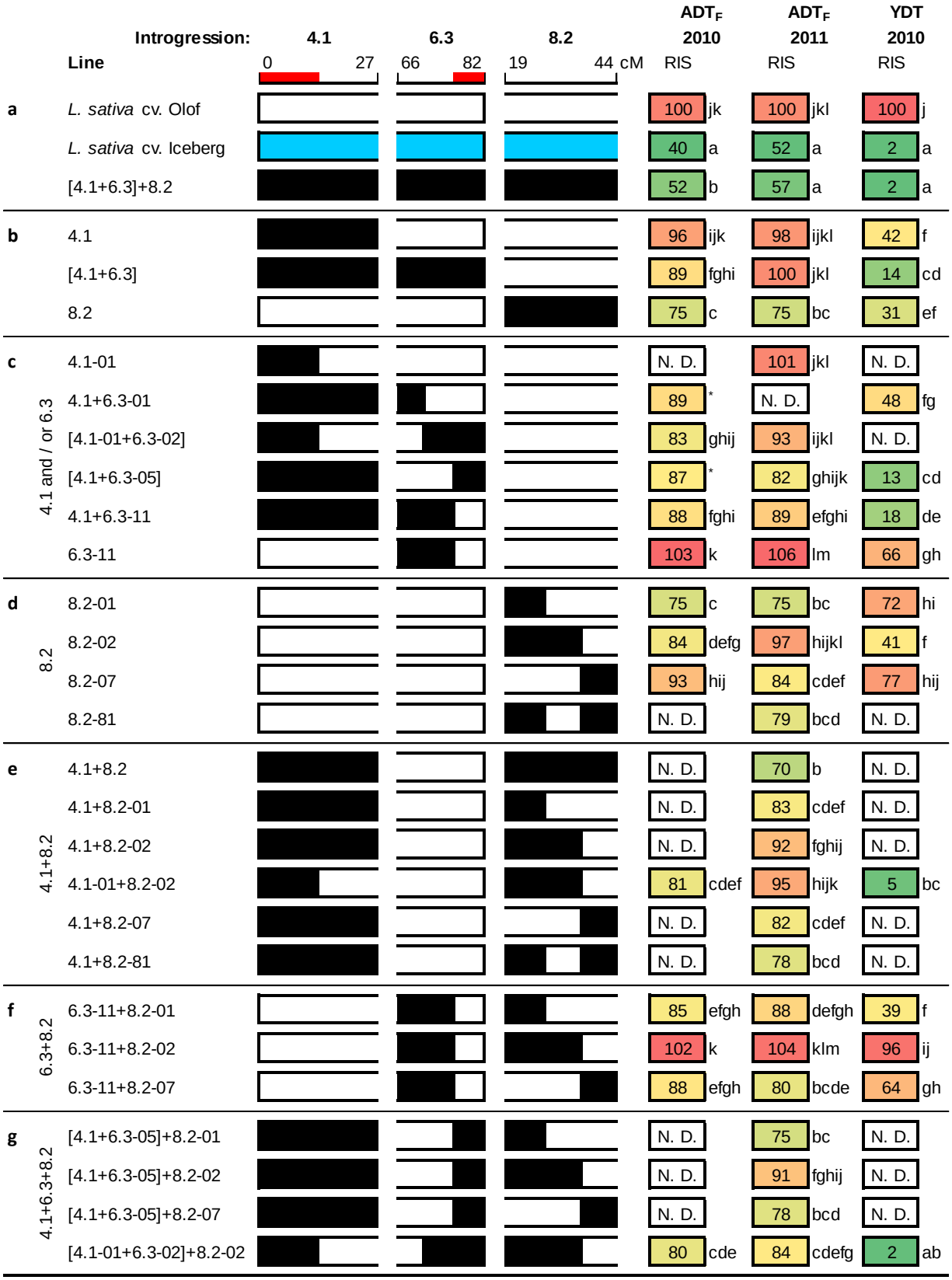
Introgression:
4.1
6.3
8.2
Line
0
27
66
82
19
44 cM
ADT$_F$ 2010 RIS
ADT$_F$ 2011 RIS
YDT 2010 RIS
a
L. sativa cv. Olof
100 jk
100 jkl
100 j
L. sativa cv. Iceberg
40 a
52 a
2 a
[4.1+6.3]+8.2
52 b
57 a
2 a
b
4.1
96 ijk
98 ijkl
42 f
[4.1+6.3]
89 fghi
100 jkl
14 cd
8.2
75 c
75 bc
31 ef
c
4.1 and / or 6.3
4.1-01
N. D.
101 jkl
N. D.
4.1+6.3-01
89 *
N. D.
48 fg
[4.1-01+6.3-02]
83 ghij
93 ijkl
N. D.
[4.1+6.3-05]
87 *
82 ghijk
13 cd
4.1+6.3-11
88 fghi
89 efghi
18 de
6.3-11
103 k
106 lm
66 gh
d
8.2
8.2-01
75 c
75 bc
72 hi
8.2-02
84 defg
97 hijkl
41 f
8.2-07
93 hij
84 cdef
77 hij
8.2-81
N. D.
79 bcd
N. D.
e
4.1+8.2
4.1+8.2
N. D.
70 b
N. D.
4.1+8.2-01
N. D.
83 cdef
N. D.
4.1+8.2-02
N. D.
92 fghij
N. D.
4.1-01+8.2-02
81 cdef
95 hijk
5 bc
4.1+8.2-07
N. D.
82 cdef
N. D.
4.1+8.2-81
N. D.
78 bcd
N. D.
f
6.3+8.2
6.3-11+8.2-01
85 efgh
88 defgh
39 f
6.3-11+8.2-02
102 k
104 klm
96 ij
6.3-11+8.2-07
88 efgh
80 bcde
64 gh
g
4.1+6.3+8.2
[4.1+6.3-05]+8.2-01
N. D.
75 bc
N. D.
[4.1+6.3-05]+8.2-02
N. D.
91 fghij
N. D.
[4.1+6.3-05]+8.2-07
N. D.
78 bcd
N. D.
[4.1-01+6.3-02]+8.2-02
80 cde
84 cdefg
2 ab

Figure 4.

Figure 4. Genetic dissection of the low infection level of combi-line [4.1+6.3]+8.2.
The genotypes and infection severity levels of individual and stacked lines are shown. A schematic genotype presentation is shown for introgression segments 4.1, 6.3 and 8.2 at Chromosomes 4, 6 and 8 respectively; bar color represents the genotype of the introgression segments: black: homozygous *L. saligna*; white: homozygous *L. sativa* cv. Olof. The strong resistance control line *L. sativa* cv. Iceberg is shown in blue. Hybrid incompatibility loci are shown in red map intervals. Relative infection severity levels (RIS) are presented from five adult disease tests in field (ADT_F) in 2010, three ADT_F in 2011 and four young plant disease tests (YDT). Gradual color scale is used to visualize differences in RIS values, within each year (from 'green' low infection to 'red' high infection). Letters in common within each type of disease test, indicate no significant difference between the lines tested in one year (Duncan's multiple range test, α=0.05). N.D. means not determined. * means lines were tested in two out of five ADT_F experiments in 2010 (in spring).

Discussion

The diversity in joint effects of quantitative resistances

Stacking of introgression segments of resistant BILs and sub-BILs resulted in lettuce lines that occasionally showed an additive effect for field resistance. However, deviations from additivity were the more frequent (Table 2a and Table 2b). This indicates that in *L. saligna -Bremia* non-additivity for combined quantitative resistances is more the rule than exception. From a practical perspective, only two of the ten combinations (1.2+8.2 and [4.1+6.3]+8.2) resulted in a substantially increased level of resistance, and might be valuable for breeding.

The stacking of 4.1, 6.3 and 8.2 sub-introgressions resulted in some cases in superior levels of resistance at young plant stage. At adult plant stage in the field these combinations of sub-introgressions did not lead to as high levels of resistance as in combi-line [4.1+6.3]+8.2. Surprisingly, in one combination we found a negative epistatic interaction, i.e. two introgressions that individually conferred resistance but in combination (combi-line 6.3-11 + 8.2-02 in YDT) resulted in high susceptibility. A variety of gene interactions across loci as found in the present study were also reported among quantitative traits in other plant species: QTLs for yield and yield trait components in rice showed both additive and epistatic effects (Zhuang et al. 2002), in rice epistatic interactions between heading date genes *Hd1* and *Hd2*, and *Hd2* and *Hd3* were reported in stacked QTL-near isogenic lines (Lin et al. 2000) and mainly additive effects but also epistatic with 'less-than-additive' effects (28%) were detected in yield associated traits in tomato in 180 studied interactions (Eshed and Zamir 1996). In the scarce studies on stacking quantitative disease resistances also a variety of interactions between stacked QTLs were observed. In a stacking study of three resistance QTL in wheat against *Fusarium* head blight, the best performing line showed a 'less-than-additive' epistatic interaction between two QTLs (Miedaner et al. 2006). Additive gene actions across loci was observed in two studies in barley: by stacking three QTLs to barley stripe rust at the adult plant stage (Castro et al. 2003) and by stacking two QTLs to barley leaf rust (Marcel et al. 2007). No (significant) additional decrease in infestation was observed when two root knot nematode (*Meloidogyne hapla*) resistance QTLs were

combined in potato (Tan et al. 2009). Whether the lack of additive effects in QTLs for resistance as shown in our present study is rule or exception is not yet clear. It is conceivable that studies with no increased resistance effects by stacking have a lower probability to be published because of the negative result.

Based on stacking mutant genes for flowering time in *Arabidopsis*, Coupland (1995) proposed that genes in the same pathway show together no increased effect when combined, while when the genes are in different pathways their combined effect was increased. So the nature of gene interaction across loci might reveal whether genes are involved in the same pathway. In the present study most of the combinations of introgressions did not confer a decrease in infection, suggesting that a large part of our studied introgressions might contain genes involved in the same resistance pathway.

Fine mapping of the resistance within combi-line[4.1+6.3]+8.2

For the resistance at young plant stage, the interactive loci of [4.1+6.3]+8.2 seem to be located at the segments of combi-line 4.1-01+8.2-02 that has three times less *L. saligna* genome than combi-line [4.1+6.3]+8.2 (3% versus 9% based on genetic map lengths) and a similar level of resistance. At young plant stage the 4.1 introgression interacts with other loci, like with the 1.2 cM interval on 6.3 and with 8.2-02, and thereby significantly reduces infection levels.

At adult plant stage in field tests none of the combinations of (sub-) introgressions showed a similar or higher resistance level than combi-line [4.1+6.3]+8.2, and therefore we were not able to narrow down the loci (Figure 4). The inheritance is complex and probably it is based on multiple interactive loci (>3) between and within the 4.1, 6.3 and 8.2 introgressions or possibly copy-number variation (CNV) could play a role. CNVs might increase the dosage of QTL or epistatic effects in the introgression segments to reach the high resistance level of combi-line [4.1+6.3]+8.2. Soybean cyst nematode resistance mediated by the quantitative trait locus *Rhg1* was explained by CNVs that increased the expression of a set of dissimilar genes in a repeated multigene segment (Cook et al. 2012)

In the substitution mapping study of individual introgression segments 8.2, 2.2 and 4.2, similar plant stage dependence and complex inheritance, based on interactions between sub-QTLs or possibly CNVs, was observed (Chapter 2). Plant stage-dependent effectiveness of partial resistance genes has also been reported in other plant pathosystems like in barley- rust and barley - powdery mildew (Aghnoum et al. 2009, Wang et al. 2010).

Implications for breeding

The stacking of the resistance QTLs under study did not lead automatically to substantially increased levels of resistance. The effect of seven out of ten developed double introgression combinations did not deviate from that of individual introgressions (and did not lead to higher levels of resistance). For breeding, the joint effect of additive or epistatic ‘more-than-additive’

QTL effects, which is observed for combi-line1.2+8.2 and combi-line[4.1+6.3]+8.2 respectively, are the most valuable /interesting. The genetic dissection of combi-line[4.1+6.3]+8.2 led to lines with lower *L. saligna* genome percentages and similar low infection levels in young plant stage, but not in field test at adult plant stage. The complex inheritance for field resistance of combi-line[4.1+6.3]+8.2 makes this line unpromising for practical application in breeding.

For breeding, combi-line1.2+8.2 seems the most valuable line, with an additive effect across loci, resulting in a much lower infection level (about half) at adult plant stage than on the resistant reference line *L. sativa* cv. Iceberg. Future substitution mapping of the 1.2+8.2 introgressions must reveal whether individual loci per introgression segment interact additively or complex interactions are responsible. Future stacking studies must reveal a third additive or epistatic locus that, stacked with 1.2 and 8.2, may lead to complete resistance.

It is arguable whether we should merely exploit additive QTL interactions in breeding, as there might be an increased danger that *B. lactucae* may be able to overcome the resistance imparted by these QTL. Preserving some of the genetic complexity of the resistance (epistasis) might increase the chance for a more durable resistance.

Nonhost resistance from *L. saligna*

From the stacking of eight introgression segments in various double and triple combinations and one line with four stacked introgressions, only the latter was nearly completely resistant in the field. Most double and triple combinations showed a similar (and not lower infection) as the individually most resistant parental line.

Additivity in resistance effect was an exception. Instead, various epistatic interactions between introgression segments were observed. Introgressions that conferred no or only a small individual effect interacted with moderately effective loci to enhance the resistance substantially (like [4.1+6.3] on 8.2 introgression in the field). Other introgressions that conferred a large individual effect (like 4.2) did not lead to further reduction in infection when combined with others. Some introgressions even canceled the resistance conferred by another introgression when they are combined (like the sub-BIL 8.2-02 introgression that neutralized the 6.3-11 effect in YDT).

Our proposed hypothesis that the complete nonhost resistance of *L. saligna* CGN05271 to *B. lactucae* is due to the cumulative and additive effects between several quantitative resistance genes from BILs cannot be accepted based on the results in the present study.

The observed diverse interactions between and within (sub-)introgressions on resistance levels of lettuce to *B. lactucae* might suggest the following: 1) The genetic basis of the nonhost resistance from *L. saligna* CGN05271 is very complex, and/or 2) Epistatic and/or additive interactions between yet untested combinations of genes explain non-host resistance, and /or 3) Nonhost resistance of *L. saligna* is caused by a gene(s) of which the action was not discovered in the set of BILs (for instance because of 5% missing *L. saligna* genome in the set of BIL, and/ or due to

close linkage to regions involved in hybrid incompatibilities). The QTLs detected in the BILs and in the present study may affect physiological qualities of the plants, leading to rather marginal variation in levels of suitability as nutrient source for *B. lactucae*.

Overall, the diverse interactions make it hard to prove in a stepwise, logical and deductive way which combination of genes/loci cause the complete resistance of the nonhost species *L. saligna.*

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Supplemental information

Table S1 DNA Markers for genotyping.
Markers printed in bold are used in the recombinant screening.

Marker	Chr.	cM	Marker type[a]	Forward primer	Reverse primer
NL0311	1	48.4	SSR	GTCTTGGAGCAACACCTTC	AATGCCACTTAAGCTTTCAC
LE1176	1	93.9	CAPS, HinfI	GTGAAGAGTGGTGGTTCGGT	GGGAAAGGCCACCATTAACT
NL1151	4	4.5	SSR	TTTGGTATCAAGCCTCTCG	CAAGTCTATCAAGCCCTGG
NL0293	4	6.0	SSR	TCACTACGAGAATGGCCTC	TACAGTCAAGGTCAAGCCC
NL1260	4	8.2	SSR	CTTAGAAAGCTGCCACCAC	GGAGCGATTTTACAGTTCG
NL0514	4	9.8	SSR	CTCTTTCTCCTCCATCCG	TGGGGTAAGAGAAATTAGGG
NL0884	4	16.9	SSR	GTCAAGCAAGTGAAGAGGC	GAGCAGAACAAGGAGCATC
NL0261	4	17.9	SSR	GAGACCAATGAGGTTGAGG	GCAGGTGATCTGGAATGAC
NL0897	4	18.4	SSR	GAAGACAAGAAGTCGACGG	CGATCGAGATAACGAAAGC
LK1525	4	21.8	CAPS, HaeIII	CAGACGTCCACCTGGAATTG	ATTCAGTGCGTCTGTTGCAG
NL1186	4	24.4	SSR	AGGGGCTGATGATGATATG	AGTACATACTTGTGTCTTGTGG
LE4022	4	25.6	SCAR	TGAATGCCAATCTATCTATCGTG	TTCTGTCTAGCAAATTTGAGCTTG
NL0247	4	32.3	SSR	AAAGATGGTGGGAGTGTTG	AGGGATAGAAGGCATAGGAG
LE1162	4	136.5	SCAR	TAAAGAGGATCTCATGGGCG	GAATGCAACATATGCAACCG
NL1036	4	145.8	SSR	ACTTGCAGGTTTACCAACG	CTTTGAATGAGAGGAAGGC
NL1035	4	154.2	SSR	ATGCAATAGACCTTGGTGC	TTGTCCACCTCCCAAATAC
NL0173	5	59.0	SSR	CGCAGAGATAGAGACAGGG	ACGTGCAATAAACCAAACC
LE1221	5	69.2	SCAR	GGAACGTTTCATGGCATTCT	TTGCATATGTGGTGGAGGAA
NL0783	5	79.6	SSR	GTCAATGAACCGGCTAAAC	GAACAAAAACCGTTTACATCTC
NL1220	5	80.4	SSR	TCCCACAGTTTCCTCATTC	AAATCGCCATTTACGACTG
NL0750	5	100.8	SSR	TGTGTATTTTATGCGCACC	TTGCTCTCACTGATCTCCC
NL0889	5	120.5	SSR	GTCGCCATATCAAAAGAGG	GAGCAAACATGCAAATAGG
LE1126	6	66.3	CAPS, AluI	CTTTGCTCCAATTCCTCTCG	AATGCCATAGTGAAGCTGGG
NL0589	6	67.1	SSR	AACGAATGTATACCGCAGC	ACGATTGGTCAAGGAAGTG
NL1114	6	69.6	SSR	AAGGCCATTGTAGGTGATG	GCTTCACTTGCTCTTGGAC
NL0653	6	72.9	SSR	TCTCAATCCTGTGGCTTTC	GCGAATGATCGAGAAGAAG
NL1084	6	72.9	SSR	CAACAGCAACAATCTGCAC	AGCACTTCCAAATTTCAGC
M7120	6	74.3	CAPS, SSiI	ACAGCAACAGCCGACCG	CGCACATTATTCGGCTCAAA
NL0833	6	80.6	SSR	ATGTCTAGAGGCGCAACAG	CTTGTTCCTCCCATGACTC
NL0418	6	81.6	SSR	AAGCCCAAAGAAGAAGAGG	ATGCATTTGGATTCTCGTC
LE1211	6	81.9	CAPS, HinfI	CGGGTGATTACATCGGCTAT	CGCAACCAACCAAATTTACC

[a] For the CAPS markers the restriction enzyme is given.

Table S2 Overview of disease evaluated lines and their replicates at young (YDT) and adult plant stage (ADT_F) experiments. For the readability line names are alternately displayed in bold. The map position of large introgression segments of BILs and smaller donor segments of its derived sub-BILs are visualized in Figure 1 and Figure 4 respectively.

Lines used	YDT		ADT_F	
Stacking BILs	Year exp.[a]	Replicates[b]	Year exp.[a]	Replicates[c]
L. sativa cv. Olof, ***L. sativa* cv. Iceberg**, BIL4.1, **[4.1+6.3]**, 8.2, combi-line [4.1+6.3]+8.2	N.D.	N.D.	09; 11	40
BIL2.2, **4.2**, 4.6, Combi-line 2.2+4.2, **2.2+[4.1+6.3],** 2.2+8.2, **4.2+[4.1+6.3],** 4.2+8.2 [4.1+6.3]+8.2, **2.2+[4.1+6.3]+8.2**, 2.2+4.2+[4.1+6.3]+8.2, **4.6+8.2**	N.D.	N.D.	09	22
BIL1.2, **5.2** Combi-line1.2+8.2, **5.2+8.2**, 5.2-01+8.2, **4.1+8.2**	N.D.	N.D.	11	18
Fine mapping resistance of combi-line[4.1+6.3]+8.2				
L. sativa Olof, ***L. sativa* cv. Iceberg**, BIL4.1, **[4.1+6.3]**, 8.2, sub-BIL 6.3-11, **8.2-01**, 8.2-02, **8.2-07**, Combi-line [4.1+6.3]+8.2, **4.1-01+8.2-02**, 6.3-11+8.2-01, 6.3-11+8.2-02, **6.3-11+8.2-07**, [4.1-01+6.3-02]+8.2-02, **4.1+6.3-11**,	10	4x6 = 24	09; 10; 11	70
sub-BIL[4.1-01+6.3-02]			10; 11	48
sub-BIL4.1-01, **8.2-81**, Combi-line 4.1+8.2, **4.1+8.2-01**, 4.1+8.2-02, **4.1+8.2-07**, 4.1+8.2-81 [4.1+6.3-05]+8.2-01, **[4.1+6.3-05]+8.2-02**, [4.1+6.3-05]+8.2-07			11	18
Combi-line 4.1+6.3-01	10	4x6 = 24	10[d]	12[d]
sub-BIL[4.1+6.3-05]	10	4x6 = 24	10[d]; 11	42[d]

[a] Experimental year, in 20th century, (08 = year 2008).

[b] Number of experiments × number of replicates = total number of replicates.

[c] Number of replicates in the field, depending on year and experiment. Per year experiment × replicate: 2009: 3×6+1×4; 2010: 5×6 (two experiments in spring and three in autumn) and 2011: 3×6.

[d] These lines were evaluated in the two experiments in spring.

Table S3 Characteristics and description of disease evaluated field tests (ADT_F) All locations are in the Netherlands except for La Ménitré. 'FR' stands for France.

	ADT_F 2009 autumn				**ADTF 2010 spring**	
Location ADT_F / experiment:	Fijnaart	's-Gravenzande	Voorst	Zeewolde	Etten-Leur	's-Gravenzande
Replications/blocks:	6	6	4	6	6	6
Plants in replicate/block:	8	20	24	9	12	20
Soil type:	clay	clay	sand	clay	sand	clay
Natural or artificial infection:	natural	artificial	natural	natural	artificial	artificial
Bremia lactucae race:[a]	mixture	Bl:24 & Bl:26	mixture	Bl:25	Bl:24	Bl:24 & Bl:26
Sowing date:	13 July	28 July	16 & 23 July	29 July	7 April	4 March
Planting date:	27 July	14 Aug	3 &10 Aug	19 Aug	28 April	8 April
Observation date:	11 Sept	8 Oct	23 & 28 Sept	14 Oct	1 July	9 June
Infection severity level Olof:[b]	4.5	7.8	8.9	7.1	4.3	8.8
Infection severity level BIL8.2:[b]	2.4	4.4	7.0	5.7	2.3	7.0
Quotient (ISL BIL8.2 / ISL Olof):	0.5	0.6	0.8	0.8	0.5	0.8
Experiment code:	2009-01	2009-02	2009-03	2009-04	2010-01	2010-02
Correlation (r) with exp. -01:[c]	-	0.8	0.9	0.8	-	0.9
Correlation (r) with exp. -02:	0.8	-	0.8	0.8	0.9	-
Correlation (r) with exp. -03:	0.9	0.8	-	0.9	0.7	0.9
Correlation (r) with exp. -04:	0.8	0.8	0.9	-	0.8	0.9
Correlation (r) with exp. -05:	-	-	-	-	0.9	0.8

	ADT_F 2010 autumn			**ADT_F 2011 autumn**		
Location ADT_F / experiment:	Oud Gastel	's-Gravenzande	La Ménitré (FR)	Oud Gastel	's-Gravenzande	Zeewolde
Replications/blocks:	6	6	6	6	6	6
Plants in replicate/block:	12	20	20	16	25	11
Soil type:	sand	clay	sand	sand	clay	clay
Natural or artificial infection:	natural	natural	artificial	natural	artificial	artificial
Bremia lactucae race:[a]	mixture	Bl:22, 24, 25, 26	Bl:26	Bl:24	Bl:22, 24, 25, 26	Bl:25
Sowing date:	13 July	27 July	12 Aug	08 July	20 July	29 July
Planting date:	28 July	16 Aug	1 Sept	2 Aug	4 Aug	19 Aug
Observation date:	9 Sept	5 Oct	20 Oct	9 Sept	05 Oct	13 Oct
Infection severity level Olof:[b]	7.1	7.5	6.4	7.1	8.2	5.2
Infection severity level BIL8.2:[b]	6.0	6.0	4.2	6.1	6.6	2.4
Quotient (ISL BIL8.2 / ISL Olof):	0.8	0.8	0.7	0.9	0.8	0.5
Experiment code:	2010-03	2010-04	2010-05	2011-01	2011-02	2011-03
Correlation (r) with exp. -01:	0.8	0.8	0.9	-	0.7	0.7
Correlation (r) with exp. -02:	0.9	0.9	0.8	0.7	-	0.7
Correlation (r) with exp. -03:	-	0.9	0.6	0.7	0.7	-
Correlation (r) with exp. -04:	0.9	-	0.7	-	-	-
Correlation (r) with exp. -05:	0.6	0.7	-	-	-	-

[a] Mixture means that the individual races could not be determined because of a complex mixture of races or due to presence of not described and/or new isolates. [b] Infection severity scale from 0 (no infection) to 9 (completely infected).[c] Shown correlations were calculated between all experiments within one year.

Chapter 4

Sex-independent non-transmission of a gametophyte with a hybrid genotype is responsible for a di-genic reproductive barrier between wild and cultivated lettuce species

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Key message

In an inbred progeny of *L. saligna – L. sativa* hybrids a pair of transmission ratio distortion loci was observed at Chromosome 4 and 6, which is is indicative for a hybrid incompatibility. Segregation analyses of new inbred and backcross populations indicated a non-transmission of a particular hybrid gametophyte (male and female).

Glossary:

F2_1997_FR = Reference mapping population of 126 F_2 plants derived from the cross between *L. saligna* CGN05271 × *L. sativa* cv. Olof (Jeuken et al. 2001). The number '1997'refers to the year in which F_2 plants were grown and DNA was isolated. The letters 'FR' refer to country of origin of the *L. saligna*-parent.

Genetic glossary:

a = *L. sativa* allele
b = *L. saligna* allele
aa= homozygous *L. sativa*
ab = heterozygous
bb= homozygous *L. saligna*

Abstract

Hybrids between species are sometimes inviable, sterile or show other, less extreme, negative phenotypes. This so-called 'hybrid incompatibility' (HI) is of evolutionary interest for its role in speciation as a reproductive isolating barrier. Its genetic architecture is predominantly due to interactions between at least two genes (known as the Bateson–Dobzhansky–Muller interaction model). HI can severely arrest exchange of genetic variants between species, resulting in transmission ratio distortion (TRD).

In research activities on interspecific progenies between cultivated (*Lactuca sativa*) and wild lettuce (*L. saligna*), we have observed several loci for TRD (TRDL), indicative for HI. One pair of TRDL involves the bottom of Chromosome 6 and the top of Chromosome 4. Plants carrying a *L. saligna* segment at the bottom of Chromosome 6 require a *L. saligna* segment at the top of Chromosome 4 to result in a vital plant (but not vice versa). Unraveling the mechanism and the loci underlying this HI was the subject of our study. Digenic transmission ratio distortion in a segregating population revealed that three of the nine expected genotypes were absent. Those missing genotypes indicated the absence of the zygotic product of a gametophyte with a particular hybrid genotype. Hypotheses tested on observed and expected segregation ratios indicated a sex-independent non-transmission of this hybrid gametophyte genotype. This non-transmission in both female and male gametophtes was confirmed in reciprocal backcross populations. The hybrid incompatibility regions were narrowed down to3 cM intervals at both chromosomes. Further discussion involves potential biological explanations for the observed TRD and gives suggestions to determine the exact moment of transmission distortion in the expected prezygotic phase.

Keywords: hybrid incompatibility, gametophyte non-transmission, transmission ratio distortion, distorted segregation, pre- and postzygotic barrier

Introduction

Hybrids between or within species are sometimes lethal or, if they live, they have phenotypic abnormalities like sterility (Coyne and Orr, 1998). These deleterious hybrid characteristics, collectively called 'hybrid incompatibility' (HI), can reduce the exchange of genetic variants between species. Evolutionary biologists and geneticists are interested in HI because of its role in speciation as a reproductive isolating barrier and its unusual genetic and evolutionary properties. Breeders are hindered by HI as it limits them to fully exploit wild species for their crop/livestock improvements (Tuberosa et al. 2014).

Hybrid incompatibilities usually arise by improper interactions between two (or more) genes according to the Bateson–Dobzhansky–Muller interaction (BDMI) model (Orr, 1996). This model is agnostic regarding which evolutionary divergence processes are involved but focuses instead on the incompatibility, which is a byproduct of that divergence.
Recent studies in plants and animals have identified some of the interacting genes that underlie hybrid incompatibility. These genes represent a wide array of functions, including those involved in oxidative respiration, nuclear trafficking, DNA-binding, and plant defense (Johnson, 2010).
HI in hybrid plants is phenotypically often recognized by reduced fitness like sterility, necrotic leaf lesions, wilting, retarded growth or lethality (Bomblies and Weigel 2007). This may lead to non-Mendelian segregation of alleles and genotypes (segregation distortion or transmission ratio distortion, TRD) in hybrid progenies. Locations of transmission ratio-distorted loci (TRDL) have been used as indicators of genetic incompatibilities in several plant species: Mimulus (Fishman et al. 2001), rice (Harushima et al. 2001), Eucalyptus (Myburg et al. 2004), tomato (Moyle and Graham 2006), Ceratodon moss (McDaniel et al. 2008), Ceratopteris fern (Nakazato et al. 2007), *Arabidopsis thaliana* (Salomé et al. 2011) and *Arabidopsis lyrata* (Leppälä et al. 2013).
We study HI in interspecific lettuce populations to get an insight in the genetic barriers of the secondary gene pool for cultivated lettuce. Crossed species are wild lettuce, *Lactuca saligna*, and cultivated lettuce, *L. sativa*, which both are diploid autogamous species. Hybrids of these have been created to study the inheritance of downy mildew resistance. Lettuce downy mildew (*Bremia lactucae*) is one of the most devastating diseases in lettuce production. The nonhost species *L. saligna* is completely resistant to this disease and still crossable with *L. sativa*.

From the specific cross *L. saligna* CGN05271 × *L. sativa* cv. Olof, we obtained an F_2 population and created an Introgression Line library of Backcross Introgression Lines (BILs). This set of BILs is derived from $BC_{4\text{-}5}S_{1\text{-}2}$ plants (recurrently backcrossed to *L. sativa*). It contains 29 lines the majority of which has one homozygous *L. saligna* introgression in *L. sativa* background. The introgressions together represent 96% of the wild species genome (Jeuken et al. 2008; Jeuken and Lindhout 2004). Severely distorted segregation ratios have been observed in the F_2 population and in the $BC_{4\text{-}5}S_{1\text{-}2}$ progenies for development of BILs (Jeuken et al. 2001, Jeuken and Lindhout 2004). Six TRDL with a preference for *L. sativa* alleles were identified in

both population types. After extensive genotype analysis of $BC_{4\text{-}5}S_{1\text{-}2}$ progenies of minimal 50 plants, these six TRDL could not be obtained as a homozygous *L. saligna* introgression segment in a plant with a *L. sativa* background (BIL). This result pinpointed six hybrid incompatibilities that form a reproductive barrier between the two *Lactuca* species (Jeuken and Lindhout 2004). For one of these six HI, which was associated with 'hybrid necrosis' symptoms, we have unraveled its HI mechanism. The digenic incompatibility was caused by a *L. saligna* allele of the *Rin4* gene at the top of Chromosome 9 and a *L. sativa* allele of a potential *R*-gene in the middle of Chromosome 6 (Jeuken et al. 2009).

In this report we focus on the mechanism of non-Mendelian inheritance in the severely distorted region of the hybrid genome at the bottom of Chromosome 6. At this region a TRDL was detected in the original F_2 mapping population (referred to as 'F2_1997_FR') as well as in the $BC_{4\text{-}5}S_{1\text{-}2}$ progenies for the development of BILs. Hybrid plants carrying a *L. saligna* segment on the bottom of Chromosome 6 required a *L. saligna* segment on the top of Chromosome 4 to result in a vital plant. We selected BIL[4.1+6.3] that contains two homozygous *L. saligna* introgression segments, one segment '6.3' of almost 20 cM at the bottom of Chromosome 6 and another segment '4.1' of almost 30 cM at the top of Chromosome 4 (Jeuken and Lindhout 2004). The square brackets in 'BIL[4.1+6.3]' indicate that the 6.3 *L. saligna* introgression segment does not segregate independently from the 4.1 *L. saligna* introgression segment.

In a previous study the digenic HI was roughly mapped to an interval of 3 cM within the 6.3 region and an interval of 18 cM within the 4.1 region (Chapter 3).
Within BIL[4.1+6.3] a resistance QTL, *rbq6*, with a major effect against downy mildew at the young plant stage is mapped at the 6.3 region. Its mapping interval partly overlaps with the HI mapping interval (Zhang et al. 2009a, Chapter 3). To introgress *rbq6* into cultivated lettuce without further linkage drag by genes at the 4.1 or 6.3 introgression, uncoupling between resistance and HI is desired.

The primary objectives of this study are to validate the HI indication of BIL[4.1+6.3], elucidate the HI genetic architecture and to postulate a model for the genetic conflict. The secondary objectives are to fine map the HI loci and to find recombinants between the resistance locus and the locus for HI.

Materials and methods

Materials

Plant material to elucidate the genetic model behind the hybrid incompatibility:

- Control lines: BIL[4.1+6.3] contains two homozygous *L. saligna* introgressions in the *L. sativa* cv. Olof background: a 4.1 introgression segment of almost 30 cM and a 6.3 segment of almost 20 cM. BIL4.1contains de 4.1 introgression segment. Sub-BIL6.3-11 contains a large sub-introgression of 6.3 and no introgression on Chromosome 4. Sub-BIL[4.1+6.3-05] and sub-BIL[4.1-01+6.3-02] contain smaller sub-introgression(s) of BIL[4.1+6.3] (Chapter 3). The square brackets indicate that the *L. saligna* sub-introgression segment at Chromosome 6 does not segregate independently from the *L. saligna* introgression segment at Chromosome 4.
- Segregating population: Inbred progeny of preBIL[4.1+6.3] that contains identical introgressions as BIL[4.1+6.3], but in a heterozygous state (the prefix 'Pre' refers to the heterozygous state of introgression segments).

Segregating populations to determine the genotype and allele frequencies of the C4 and C6 locus individually (without segregation of the other HI locus):

- C4 locus. An inbred progeny of a genotype with a heterozygous 4.1 introgression and another heterozygous introgression (8.2, which is irrelevant for the here studied HI; the preBIL4.2+8.2 is described in Chapter 3 in a *L. sativa* cv. Olof background.
- C6 locus. An inbred progeny of a line with two introgression segments (n=545) in a *L. sativa* cv. Olof background: one homozygous *L. saligna* introgression covering the C4 HI locus, and one heterozygous introgression covering the C6 HI locus (progeny is described in Chapter 3 as preBIL4.1s+6.3-2).

Plant material to narrow down the mapping intervals of the HI:

- Mapping population of 126 F_2 plants derived from the cross between *L. saligna* CGN05271 × *L. sativa* cv. Olof, referred to as ' F_2_1997_FR' (Jeuken et al. 2001). The number '1997'refers to the year in which F_2 plants were grown and DNA was isolated. The letters 'FR' refer to country of origin of the *L. saligna*-parent. The original F_2 map (Jeuken et al. 2001) and genotype profile has been extended from 488 (mainly AFLP markers) to about 1000 markers (predominantly supplemented with EST-based markers).
- Inbred progenies (n= 16-32; referred to as F_3 families) of fourteen plants with a recombination in one of the former HI mapping intervals (within in 4.1 or in 6.3 region). These recombinant plants were identified from the genotyping of the preBIL[4.1+6.3] inbred progeny. F_3 family numbers are 01, 02, 03, 15, 17, 18, 19, 21, 22, 33, 45, 46, 50 and 59.

Genotyping

DNA was isolated by NaOH method (Wang et al. 1993) or modified CTAB method (Jeuken et al. 2001). Newly and previously developed co-dominant DNA- markers were used to genotype the inbred progeny of preBIL[4.1+6.3] and mapping population F_2_1997_FR. Primer sequences for 21newly developed markers covering the 4.1 and 6.3 BIL introgressions are listed in Table 1 and previously developed markers are listed in Chapter 3. SSR markers were kindly provided by Syngenta BV and EST markers were developed on lettuce EST sequences of the *Compositae* Genome Project (http://compgenomics.ucdavis.edu), McHale et al., (2009) and the reference lettuce genome V3.2 (https://lgr.genomecenter.ucdavis.edu/). Polymorphisms between PCR products of *L. saligna* and *L. sativa* alleles were visualized by high-resolution melting curve differences on a LightScanner System (Idaho Technology) or by size differences on agarose gels (directly or after enzymatic digestion) as described previously (Jeuken et al. 2008). The average marker density for both introgression regions is around 1 marker per 0.7 cM.

Genetic nomenclature

L. sativa and *L. saligna* are diploid autogamous species (2n=18) with estimated genome sizes of about 2.6 Gbp. In this study their alleles are presented as follows: 'a' = *L. sativa* allele and 'b' = *L. saligna* allele. Genotypes at 4.1 and 6.3 region are indicated by its chromosome number (4 and 6) and its alleles. For example: ' 4ab6aa' means that the genotype is heterozygous at C4 and homozygous *L. sativa* at C6 locus.

Characterization of genetic architecture of HI

Seven-hundred plants of the inbred progeny of preBIL4.1+6.3 (4ab6ab) were genotyped with markers spanning the previous HI intervals at the introgression segments 4.1 (markers NL1151 and NL0897) and segment 6.3 (markers LE1126, M7120 and LE1211) (Chapter 3). These 700 progeny plants were categorized according C4 and C6 genotypes. We excluded recombinant plants and plants with missing marker scores for one of the loci. Categorisation of genotypes, from two independent loci with each three genotype classes ('aa,' 'ab' and 'bb'), would under Mendelian segregation result in nine genotype categories.

Hypotheses for the hybrid incompatibility were defined based on reproduction barrier assumptions, pre-zygotic (i.e. in the gametophytes) or post-zygotic (i.e. occurring in the fertilised egg cell or in the embryo), and were tested by a Chi-square test ($\alpha = 0.05$) on observed and expected segregation ratios. To validate non-rejected hypotheses, reciprocal crosses were made between preBIL[4.1+6.3] (plant with a heterozygous introgression at both C4 and C6, genotype 4ab6ab), of which gametophytic genotypes segregate for both loci, and BIL4.1 (genotype 4bb6aa) of which the gametophytic genotype is fixed and does not segregate. Segregation ratios of this BC_1 offspring were analyzed by Chi-square tests.

Mapping of the hybrid incompatibility at the C4 and C6 loci

To narrow down the HI map intervals, we compared newly developed genotypes of plants with a recombination near or in one of the previously identified 18 cM and 3 cM HI interval regions at C4 and C6 respectively (Chapter 3).

Twelve and nine newly developed markers in the 3 and 18 cM intervals were developed (Table 1) and mapped in F2_1997_FR. Old and new markers were tested on: 1. A selection of seventy plants of the F2_1997_FR population that are recombinant in at least one of the previous HI intervals; 2. Sub-BIL6.3-11, because this line has a relatively large homozygous *L. saligna* sub-introgression of 6.3 segment but it is not dependent on the presence of the homozygous *L. saligna* 4.1 segment; and 3. Inbred families (n= 16-32) of fourteen plants selected out of seven-hundred genotyped plants of the preBIL4.1+6.3 inbred progeny (referred to as F_3 families). The selected plants were recombinant in the 4.1 and/or 6.3 HI regions and potentially informative if they show TRD in the next inbred generation (see description Phenotyping HI). HI map intervals were narrowed down by combining genotype information with HI phenotype information.

Phenotyping HI

In a normally segregating inbred progeny of 4ab6ab, the following 6bb genotypes would be observed: 4aa6bb, 4ab6bb, or 4bb6bb. It appeared from our observations (see Results) that plants with two *L. saligna* alleles at C6, always carriedtwo *L. saligna* alleles at C4 (4bb6bb). Recombinant plants at C4 and/or C6 in homozygous condition 4b'b'6bb or 4aa6b'b' (in which b' is the shortened *L. saligna* introgression segment), would indicate the approximate position of the HI loci.

A second approach that appeared from our observation (see Results), is that progeny from 4ab6ab plants after selfing, resulted in non-Mendelian, odd segregation. Segregation analysis on progeny from selfed 4ab'6ab or selfed 4ab6ab' would indicate whether or not the present b' segment carries the HI locus. If the segregation would be normal, b' would not carry the HI locus anymore, in case the segregation is odd, the b' would carry the HI locus.

Table 1 DNA markers

Marker name	EST sequence[1]	Ch[1]	LGR[1] Lg	LGR[1] Mbp	Forward primer	Reverse primer	Amplicon[2] length	Amplicon[2] type
CLSX10758	CLSX10758.b1_K01.ab1	4	4	2.0	AGCACCCTTGAAACATTTGG	GTTCCAATCTCCCGTAGTGC	240	intron
CLLY8178	CLLY8178.b1_D05.ab1	4	4	7.9	GGTAATCAAGCGGAAGTGGA	CCACCAATACACTTGCAAGAAA	211	intron
CLSS5115	CLSS5115.b1_F07.ab1	4	4	23.9	GCCACCGTCATTCTCGTATT	GCAAGAACAGCCATGAGTGA	168	exon
CLS_S3_Contig2928	CLS_S3_Contig2928	4	4	29.9	CACCGACACCGCTCTTAAAT	GCACAGCATGAAATTCCTCA	234	exon
CLS_S3_Contig2219	CLS_S3_Contig2219	4	4	37.9	GCAGAAGGACTTGGTGAAGC	CATTGGGGGTCGATAAACTG	341	intron
QGG20I02	QGG20I02.yg.ab1	4	4	41.9	TTGTAGTGTAAATGCGTCATTGG	ACAGCTGTTGACATTTTACTTTTG	215	exon
CLSL2433	CLSL2433.b1_B09.ab1	4	4	50.0	CCTGGTGGAGGATTAACTCG	AACGAATTTGTTTCCCATGA	298	intron
CLLY11908	CLLY11908.b1_G01.ab1	4	4	52.8	TTAAGTTCCCCTGCCCTTG	TTGGACCAATGTGTCCTTCA	246	intron
CLS_S3_Contig7077	CLS_S3_Contig7077	4	4	53.8	TTCCTGTAAAAATGCCACCA	CACCCTCCATCGGAACTTAT	224	intron
CLS_S3_Contig4465	CLS_S3_Contig4465	4	4	58.0	CAGCATCAACTGCATCCAAA	CACCGTAATGGCCTACGTCT	230	intron
CLS_S3_Contig5375	CLS_S3_Contig5375	4	4	60.4	CTGTCACTGTGCCTCACACA	TGGAGCATTTTCCTCCTTCA	190	intron
LSS_S3_Contig1360	LSS_S3_Contig1360	4	4	61.9	GATCTTTACGGTGGGTGCAG	ATTCAGTGCGTCTGTTGCAG	231	intron
CLS_S3_Contig6649	CLS_S3_Contig6649	6	8	3.8	TCGATGGGTGGATTGCTAGT	AAAGGGGTTGCGATGGTTAT	232	intron
Y4646	Y4646.b1_K09.ab1	6	8	7.8	TGCAATTAGCATCTGCATCC	CGGAATCCTTGAGGGTACAA	225	exon
CLS_S3_Contig3369	CLS_S3_Contig3369	6	8	10.0	TGGCCTCGGTATCCTATTTG	CGTCATGATCCCAGTCTTCA	225	intron
CLS_S3_Contig1511	CLS_S3_Contig1511	6	8	14.3	ATTCCCATTTTCCCTCCATC	GCACAAATGTCGGTGTCATC	205	intron
CLSM10904	CLSM10904.b1_P13.ab1	6	8	17.9	GGGAAAAGAATTTGCTCGAC	GCTCATCCCCAGTAATTCCA	197	intron
QGA7F22	QGA7F22.yg.ab1	6	8	22.0	GGGAGGAAATGATGTGCCTA	TACCGGAGGTTTACCACCAC	173	exon
CLLY9820	CLLY9820.b1_G08.ab1	6	8	26.0	ATGGAAGCTCCAATGGTTTG	CAACATTCAGGGCCAAATCT	161	exon
CLS_S3_Contig4263	CLS_S3_Contig4263	6	8	31.9	GATTTGGCTGGGATTTTCAA	CTCTCGGTGGAGCACGTATT	200	intron
CLS_S3_Contig7223	CLS_S3_Contig7223	6	8	34.0	ACCGCATCAAAATCTTCGAC	GGAACTTGGTAGCTGGGTGA	174	exon

[1] EST sequences are derived of the LGR (=lettuce genome resource), GBrowse Lattuga version 3.2 (https://lgr.genomecenter.ucdavis.edu/). 'Ch' means Chromosome of our genetic map, 'Lg' means linkage group in LGR and 'Mbp' is million base pairs. Our genetic map versus LGR: our Chromosome 4 aligns with Linkage group 4, our Chromosome 6 aligns with the inversed Linkage group 8.

[2] The PCR amplicon product length is given in base pairs. Type refers to an amplicon product over an intron or within an exon.

Results

Characterization of the genetic architecture of HI

A segregating offspring of inbred preBIL[4.1+6.3], that was heterozygous for both introgressions, allowed us to study the segregation ratio of the two introgression segments 4.1 and 6.3. Most strikingly, three of the nine expected genotypes were absent in an offspring of 635 plants (Figure 1a). The non-observed genotypes were 4aa6ab, 4aa6bb and 4ab6bb. Consequently, plants that were homozygous *L. saligna* at the 6.3 introgression were always homozygous *L. saligna* at the 4.1 introgression, genotype 4bb6bb. The segregation ratio of the 635 plants was significantly different from a normal digenic segregation (Hypothesis 1, Figure 1d).

Observed segregation ratios

Segregation C4 and C6 loci

a) inbred offsp. 4ab6ab

C4 \ C6	aa	ab	bb	Tot:	freq:
aa	75	0	0	75	0.12
ab	126	130	0	256	0.40
bb	80	151	73	304	0.48
Tot:	281	281	73	635	
freq:	0.44	0.44	0.11		

Segregation individual loci

b) inbred offspr. 4ab6aa

C4	obs.	1:2:1
aa	41	30
ab	58	59
bb	19	30
Tot:	118	119
	P=0.02, rejected	

c) inbred offspr. 4bb6ab

C6	obs.	1:2:1
aa	137	136
ab	271	273
bb	137	136
Tot:	545	545
	P=0.99	

Expected segregation ratios according hypotheses

d) H1: P=<0.01, rejected

C4 \ C6	aa	ab	bb	Tot:
aa	40	79	40	159
ab	79	159	79	317
bb	40	79	40	159
Tot:	159	317	159	635

e) H2: P=<0.01, rejected

C4 \ C6	aa	ab	bb	Tot:
aa	33	33	9	75
ab	113	113	29	255
bb	134	135	35	304
Tot:	280	281	73	634

f) H3: P=<0.01, rejected

C4 \ C6	aa	ab	bb	Tot:
aa	58	0	0	58
ab	115	231	0	346
bb	58	115	58	231
Tot:	231	346	58	635

g) H4: P=<0.01, rejected

C4 \ C6	aa	ab	bb	Tot:
aa	53	53	0	106
ab	106	159	53	318
bb	53	106	53	212
Tot:	212	318	106	635

h) H5: P=<0.01, rejected

C4 \ C6	aa	ab	bb	Tot:
aa	64	0	0	64
ab	127	191	0	318
bb	64	127	64	255
Tot:	255	318	64	637

i) H6: P=0.2-0.5

C4 \ C6	aa	ab	bb	Tot:
aa	71	0	0	71
ab	141	141	0	282
bb	71	141	71	283
Tot:	283	282	71	636

j) offspr. 4ab6ab x 4bb6aa

informative for female transmission

C4C6	obs.	exp.H6	exp.H1
4ab6ab[a]	2	0	29
4ab6aa	31	39	29
4bb6ab	47	39	29
4bb6aa	37	39	29
Tot:	117	117	116
	P=0.18		P=<0.01, rejected

k) offspr. 4bb6aa x 4ab6ab

informative for male transmission

C4C6	obs.	exp.H6	exp.H1
4ab6ab	0	0	29
4ab6aa	46	39	29
4bb6ab	32	39	29
4bb6aa[b]	62[b]	39	29
Tot:	140	117	116
	P=0.11		P=<0.01, rejected

Figure 1.

Figure 1 Hypothesis testing for transmission ratio distortions due to a hybrid incompatibility interaction between the 4.1 and 6.3 region.
Genotypes at 4.1 and 6.3 region are indicated by its chromosome number (4 and 6) and its alleles: 'a' = *L. sativa* allele and 'b' = *L. saligna* allele. For example: 4ab6aa means that the genotype is heterozygous at C4 and homozygous *L. sativa* at C6. To visualize ratios, genotype plant numbers are gradually colored from low (red) to high (green) numbers within a progeny.
1a, 1b, 1c: Observed segregation ratios of inbred progenies segregating for the C4 and/or C6 introgressions are shown. **1a:** an inbred progeny of genotype 4ab6ab.**1b:** an inbred progeny of genotype 4ab6aa. **1c:** an inbred progeny of 4bb6ab (the segregating locus is underlined). In **1d-1i**, the observed segregation in **1a** was compared against expected two-loci segregation ratios according six hypotheses by a chi-square test. The segregation could only be tested between the genotype groups containing at least one plant in the expected genotype group. Expected plant numbers are rounded up in tables, but actual, not rounded up, numbers are used for the chi-square test. The actual probabilities from the chi-square test are shown when P values were larger than 0.01.Abbreviations: Tot. = total number of plants, freq = genotype frequency, obs. = observed, exp. = expected, offspr. = offspring. Mendelian segregations ratio's: '1:2:1' stands for a single locus genotype ratio in an F_2.
1d: H1 = Hypothesis 1, shows the expected segregation ratio under the assumption of independent segregation of two loci with a Mendelian 1:2:1 segregation per locus (both loci have allele frequencies of 0.5).
1e: H2 = Hypothesis 2, shows the expected segregation ratio under the assumption of dependent segregation between C4 and C6. Segregation ratios were calculated by using the observed allele frequencies from the 4ab6ab offspring in **1a**: Locus C4, *L. sativa* allele 'a' = 0.32 and *L. saligna* allele 'b' = 0.68; Locus C6, *L. sativa* allele 'a' = 0.66 and *L. saligna* allele 'b' = 0.34.
Segregation ratio of H3 to H6 are based on an independent (Mendelian, 1:2:1) segregation for the two loci which was adapted to the individual hypothesis. These segregation ratios were compared with the observed segregation ration presented in **1a**.
1f: H3 = Hypothesis 3, shows the expected segregation ratio under the assumption of a postzygotic incompatibility by the lethality of genotypes: 4aa6bb, 4ab6bb and 4aa6ab.
1g: H4 = Hypothesis 4, shows the expected segregation ratio under the assumption of a prezygotic incompatibility by non-transmission of male **or** female gametophytes with a 4a6b gametophyte genotype
1h: H5 = Hypothesis 5, combines Hypothesis 3 and 4.
1i: H6 = Hypothesis 6, showing the expected segregation ratio under the assumption of a prezygotic incompatibility by non-transmission of 4a6b male **and** female gametophytes.
1j, 1k: To validate the non-rejected H6 for non-transmission of male (2k) and female (2j) 4a6b gametophytes per gametophyte type, we analysed the segregation ratios in backcross populations of the reciprocal cross 4ab6ab × 4bb6aa (and also tested it for H1, Mendelian segregation 1:1:1:1). The presumed gametophyte genotypes derived from the double heterozygous parent are underlined for convenience of tracking.
[a] In **1j**) two plants with 4ab6ab genotype were observed, that are probably due to an selfing of the mother plant (gametophytes 4a6a + 4b6b) instead of an backcross. Also four plants with 4aa6aa genotype were detected which only can be derived from an selfing of the mother plant. The other three observed genotypes can contain besides BC_1 plants also some selfings.
[b] In **1k**) the plant number of the 4bb6aa genotype is much higher than the expectations for both hypotheses. This observation is explained by the fact that 4bb6aa is the only genotype in which the number of backcross plants can be overestimated due to occurrence of selfings of the mother plant. Because the actual number of BC_1 plants cannot be distinguished from plants generated by selfing, this genotype class is excluded from the chi-square test.

In other segregating populations, which were fixed for one of the introgressions (4.1 or 6.3) and segregated for the other introgression, the 6.3 region individually showed a normal segregation (*L. saligna* allele frequency of 0.50, Figure 1c) and the 4.1 region showed a skewed segregation with a preference for *L. sativa* alleles (*L. saligna* allele frequency of 0.41, Figure 1b). When both loci were segregating (Figure 1a), a preference for *L. saligna* alleles at the 4.1 region and for *L. sativa* alleles at the 6.3 region was observed and reflected in *L. saligna* allele frequencies of 0.68 for C4 and 0.34 for C6 (based on Figure 1a). We tested a second hypothesis, in which the segregation ratio was based on these calculated allele frequencies from the observed segregation. However this second hypothesis was rejected (H2, Figure 1e). The seed germination rate of the population segregating for both interacting loci (selfed offspring from preBIL[4.1+6.3]) was normal (>95%) and no seedling lethality was observed. This observation indicated that the absence of three genotypes was caused by the absence of seeds with those genotypes rather than by lethality of embryos in seeds. The rejection of a third hypothesis based on lethality of the three genotypes (H3, Figure 1f) is consistent with our seed germination observations.

The three absent genotypes have in common that they all are a product of at least one copy of the hybrid gametophytic genotype, namely a *L. sativa* allele at 4.1 and a *L. saligna* allele at 6.3, further referred to as '4a6b' (numbers refer to chromosome numbers of the introgressions, and 'a' is a *L. sativa* allele and 'b' is *L. saligna* allele, see Figure 1). We tested segregation ratios according three hypotheses based on non-transmission of the 4a6b gametophytes: one-parental (H4, Figure 1g), one parental and in combination with lethality of the three non-observed genotypes (H5, Figure 1h) and di-parental (H6, Fig 2i).

Hypothesis 6, in which maternal and paternal 4a6b gametophytes are absent, was not rejected (Figure 1i). Non-transmission of gametophytes with 4a6b genotypes leads to absence of the three mentioned genotypes and halves the plant number of double heterozygotes (4ab6ab genotype), as the latter genotype can now only be produced from fusion of gametophyte 4a6a with 4b6b. To validate the maternal and paternal effects, segregation ratios were analysed of a reciprocal cross, between a plant with a fixed gametophyte genotype, BIL4.1 with only 4b6a gametophytes, and the double heterozygote preBIL[4.1+6.3], a plant that normally would segregate for all gametophyte genotypes (4a6a, 4b6b, 4a6b and 4b6a). Indeed in both reciprocal progenies one of the four expected genotypes was absent. The double heterozygote (4ab6ab genotype) did not occur, or was observed in a very low frequency (probably due to some selfings), which implies that the 4a6b gametophytes were not transmitted (Figure 1j and Figure 1k). Overall these results suggest that the observed distorted segregation ratio between 4.1 and 6.3 is explained by the male and female 4a6b gametophytes not participating in the reproduction.

In our original F_2_1997_FR population (n=126) we observed a similar inter-locus segregation distortion by absence of the three particular genotypes. This population has a *L.*

saligna cytoplasm, while preBIL[4.1+6.3] and its selfed progeny has a *L. sativa* cytoplasm, suggesting that cyto-nuclear interactions do not play a role.

Mapping of the hybrid incompatibility at the C4 and C6 loci

Recombinant plants at C4 and/or C6 (or their inbred progenies) were used to map the HI loci by combining the genotype information with the HI phenotype information. The HI map intervals were narrowed down by using two HI phenotype approaches (detailed description in M&M). A first approach focused on the fact that existence of a plant with a homozygous *L. saligna* introgression on C6 depends on the presence of a homozygous *L. saligna* segment on C4 (genotype 4bb6bb). An example is F_2 plant 95 (of F2_1997_FR) which had a homozygous *L. saligna* introgression segment covering the previous HI interval at C6 and a homozygous *L. saligna* introgression segment at C4 that was shorter than its previous HI interval (Figure 2b). This genotype reduced the HI mapping interval at C4 with 8 cM.

A second HI phenotype approach that appeared from our observation , is that inbred progeny from 4ab6ab plants showed a non-Mendelian, odd segregation, and resulted in absence of three plant genotypes 4a<u>a</u>6a<u>b</u>, 4<u>aa</u>6<u>bb</u>, 4<u>a</u>b6<u>b</u>b (see former paragraph and Figure 1; the non-transmitted hybrid gametophyte is underlined). Recombinant versions of these three genotypes are informative for presence/absence of HI loci. If such recombinant genotypes were present, then the HI loci were not situated at the remaining introgressions. If such recombinant genotypes were absent for particular introgression combinations, then the HI loci should be located at the smallest interval where these genotypes were absent. Using this second HI phenotype approach, the most informative genotypes for the HI locus at C4 were one plant of F2_1997_FR plant (123) and three genotypes of two F_3 families (Figure 2c, with detailed evidence descriptions). Among the most informative genotypes for the HI locus at C6 were three genotypes of one F_3 family and sub-BIL6.3-11 (Figure 2d, with detailed evidence descriptions).
By these two HI phenotype approaches the HI mapping intervals were reduced from 18 to 3 cM at C4 and from 4.4 to 3 cM at C6 (Figure 2c and Figure 2d).

In a previous study the HI interval at C6 overlapped with a resistance QTL, *rbq6*, interval (Chapter 3). Due to the new genotype information of sub-introgression 6.3-11, resistance QTL, *rbq6*, was mapped to a 1.6 cM interval, which overlaps with the HI interval for only 0.1 cM (Figure 2d). Because sub-BIL6.3-11 does not show HI (it is independent of the genetic composition on the C4 HI locus), and the 6.3-11 introgression is associated with *rbq6* resistance, it can be concluded that both traits are uncoupled.

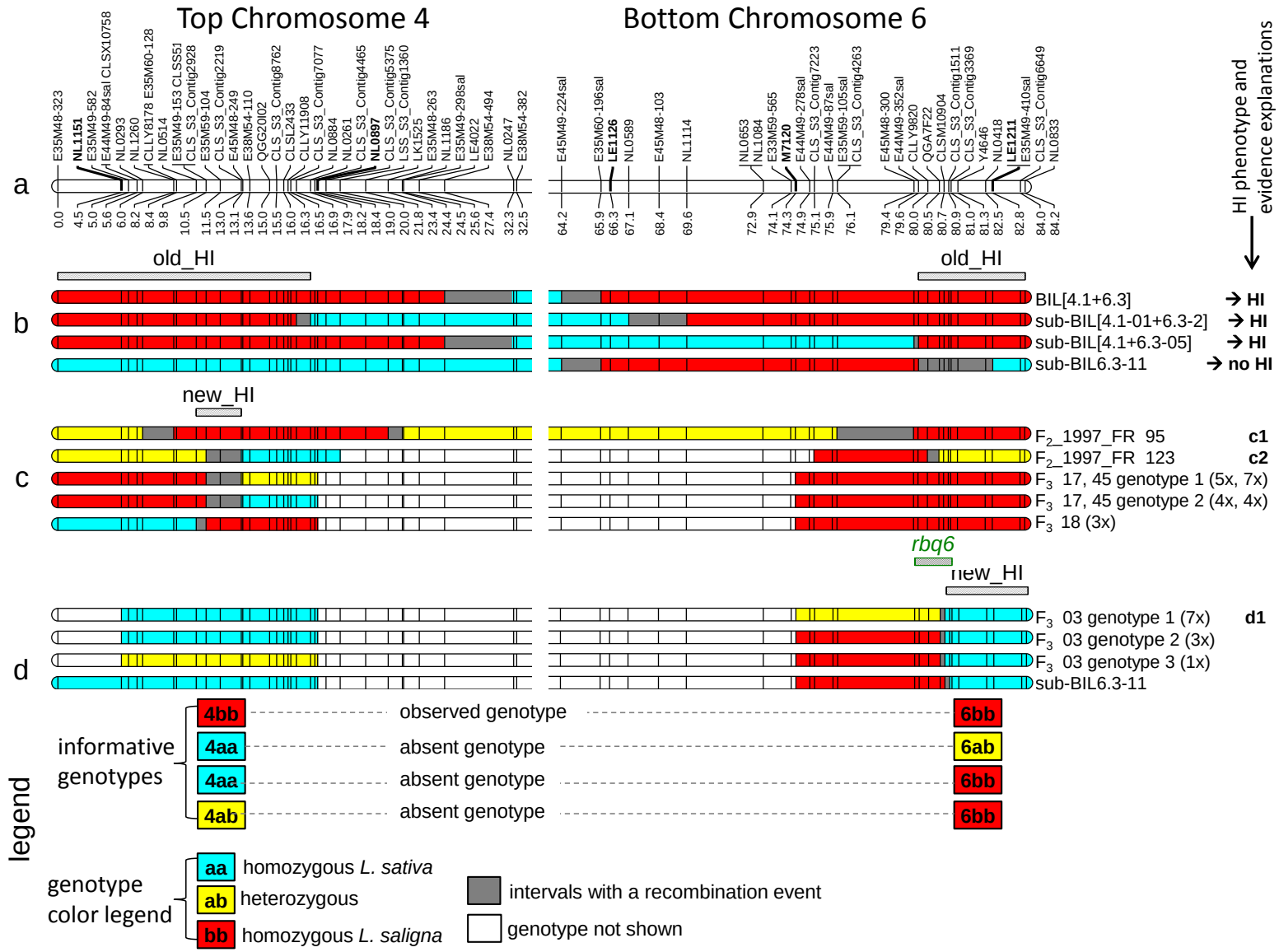
Top Chromosome 4
Bottom Chromosome 6
HI phenotype and evidence explanations
a
b
c
d
old_HI
new_HI
rbq6
BIL[4.1+6.3] → HI
sub-BIL[4.1-01+6.3-2] → HI
sub-BIL[4.1+6.3-05] → HI
sub-BIL6.3-11 → no HI
F2_1997_FR 95 c1
F2_1997_FR 123 c2
F3 17, 45 genotype 1 (5x, 7x)
F3 17, 45 genotype 2 (4x, 4x)
F3 18 (3x)
F3 03 genotype 1 (7x) d1
F3 03 genotype 2 (3x)
F3 03 genotype 3 (1x)
sub-BIL6.3-11
legend
informative genotypes
4bb observed genotype 6bb
4aa absent genotype 6ab
4aa absent genotype 6bb
4ab absent genotype 6bb
genotype color legend
aa homozygous L. sativa
ab heterozygous
bb homozygous L. saligna
intervals with a recombination event
genotype not shown

Figure 2.

Figure 2. Map intervals of Hybrid Incompatibility loci associated with 4.1 and 6.3 *L. saligna* introgression segments.
Legend shows genotype codes combined with colors and informative genotypes for mapping the HI loci

a. Genetic maps derived from population F2_1997_FR. Markers in bold were used to genotype 700 plants of preBIL4.1+6.3 inbred progeny.
b. Previous HI intervals (referred to as 'old_HI', Chapter 3) and its four informative genotypes. Previous segregation analysis of inbred progenies ($BC_{4\text{-}5}S_{1\text{-}2}$) led to conclusions about presence/absence of 'HI' (Chapter 3).
c. New HI interval at C4 based on the most informative genotypes found in segregating populations: two genotypes of population F2_1997_FR and three genotypes of three F_3 families. For F_3 families, the family number is presented (nr. 17, 45 and 18) and the number of times that the visualized genotype was observed. The tested progeny sizes were 16 for all three F_3 families.
d. New HI interval at C6 and downy mildew resistance QTL interval, *rbq6*, are based on most informative genotypes: three genotypes of F_3 family 03 tested progeny size was 32) and the new genotype of sub-BIL6.3-11.

Examples of evidence explanation:

Part b
→ **HI** = selfing of the hybrid between this genotype with *L. sativa*, resulted in distorted odd segregation, i.e. 6bb was only present if at C4 the plant was 4bb.
→ **no HI** = presence of this genotype as such in a (segregating) population indicates that the HI locus does not reside on the remaining *L. saligna* segment on C6.

Part c and d
c1. This plant is 6bb for the HI relevant part of the C6 introgression. It is 4ab for the left part of the C4 introgression, and 4bb for the right part of the C4. Our genetic HI-observations suggest that 4ab6bb would not have been observed, so the HI locus at C4 should be located at the right part of the C4 introgression.
c2. This plant is 6ab for the HI relevant part of the C6 introgression. It is 4ab for the left part of the C4 introgression, and 4aa for the right part of the C4. Our genetic HI-observations suggest that 4aa6ab would not have been observed, so the HI locus at C4 should be located at the left part of the C4 introgression.
d1. This plant is 4aa for the HI relevant part of the C4 introgression. It is 6ab for the left part of the C6 introgression, and 6aa for the right part of the C6 introgression. Our genetic HI-observations suggest that 4aa6ab would not have been observed, so the HI locus at C6 should be located at the right part of the C6 introgression.

Discussion

Hybrid incompatibility by interaction between 4.1 and 6.3 regions

In an inbred progeny of preBIL[4.1+6.3] (n=700), that was heterozygous for the 4.1 and 6.3 introgression segments, we did not detect plants that were homozygous *L. saligna* for 6.3, except when homozygous *L. saligna* for 4.1 (excluding recombinants). This result confirmed the hybrid incompatibility that was suggested from previous studies on BIL[4.1+6.3], where some part of the 6.3 *L. saligna* introgression needs to be accompanied by some part of the 4.1 *L. saligna* introgression in order to be viable.

Genotypic observations on this inbred population of 700 plants and on 126 plants of the original F2_1997_FR population, showed a distorted segregation ratio with absence of three out of nine expected genotypes, irrespective of the cytoplasmic genome (*L. saligna* or *L. sativa*) and therefore not depending on cyto-nuclear interactions. The maternal and paternal non-transmission of one particular hybrid gametophyte, '4a6b' with a *L. sativa* at C4 and a *L. saligna* allele at C6, could explain the distorted segregation ratio. Genotypic observations on reciprocal backcross populations was in agreement with the hypothesis of sex-independent transmission distortion (so that it can be explained by the non-transmission, non-viability or non-functionality of gametophytes with 4a6b hybrid genotype).

Transmission ratio distortion due to gametic dysfunction has been frequently detected in inter- and intraspecific hybrids of plants (Crow, 1991; Lyttle, 1991; Morishima et al, 1992; Harushima et al. 2001). Dysfunction of gametophytes occurs in either male (Cameron & Moav, 1957; Loegering & Sears, 1963; Sano, 1983; Mizuta et al. (2010)) or female gametes (Maguire, 1963; Scoles & Kibirge-Sebunya, 1983; Yang et al. (2012)) or in both male and female gametophytes (Rick, 1966; Endo & Tsunewaki, 1975; Sano et al, 1979; Finch et al, 1984; Koide et al. 2008).

Most literature on defective gametophytes refer to either maternal or paternal effects (Grossniklaus et al. 1998; Ding et al. 2012; Fishman and Willis 2001; Moyle and Nakazato 2008; Song et al. 2005; Wang et al. 2012) and scarce literature is available on genes affecting functionality of both male and female gametophytes (Tan et al. (2010)).

Whether the male and female gametophytes with the 4a6b gametophyte genotype are either not formed in meiosis, or lethal, or dysfunctional for fertilisation, needs to be further studied. If the gametophytes are formed but are lethal, we might be able to test this for the female gametophytes by counting and comparing the number of achenes ("seeds") per capitula and for the male gametophytes by microscopical observations of the pollen (like pollen vitality and tube growth, Peterson et al. 2010). Alternatively, the gametophytes with the 4a6b haplotype may be present and vital, but may be unable to fuse with any other gametophyte to form together the embryo and/or the endosperm. Little is known about the process of gametophyte fusion in plants and even in animals (Snell 2012).

In general it would be more likely that the non-transmission of male and female 4a6b gametophytes is obstructed by one and the same process (i.e. a defective meiosis) than in two different processes (i.e. in microsporogenesis and in macrosporogenesis).

A transmission ratio distortion caused by a defective meiosis may lead to meiotic drive. Meiotic drive is defined as any alteration of the normal process of meiosis that leads to nonrandom inclusion of chromosomes or alleles in the products of meiosis (Sandler and Novitski 1957). Alternatively it can be described as a non-Mendelian inheritance phenomenon in which certain selfish genetic elements skew sexual transmission in their own favour. Examples of meiotic drive in plants are found in maize (Buckler et al. 1999; Birchler et al. 2003) and in

monkeyflower (Fishman & Willis 2005). In those cases a chromosomal region (in maize) or several loci (in monkeyflower) were found to occur at much higher frequencies than expected.

In our TRD example two interactive loci at different chromosomes are involved. If 4a6b gametophytes would not be formed in meiosis, this gametophyte genotype would be the non-preferential one over the other three possible gametophyte genotypes. In that case, we hypothesize that instead of presence of a selfish gene one could speak of a case of 'joint fate' or 'fatal combination': allele 4a and allele 6b ending up in the same meiotic product cause their own downfall. In a future microscopic study on the anther tissue of flower buds of genotype 4ab6ab, we might be able to observe defective meiosis in 50% of the tetrads as tetrads containing two empty cells.

Some other weaker indications for preference (meiotic drive) among, the three transmitted gametophytes 4a6a, 4b6a and 4b6b seem present from the reciprocal backcrosses (Figure 1j and Figure 1k). The numbers of these transmitted gametophytes show that there is a '20% more' preference for 4b6b gametophytes than for 4a6a and 4b6a gametophytes by female transmission (Figure 1j) and a '18% more' preference for 4a6a than for 4b6b gametophytes by male transmission (Figure 1k, calculations for 4b6a gametophytes are excluded, see legend). This observation suggests a sex-dependent preference for the non-hybrid gametophyte genotypes. However, at the moment these ratios can still be coincidence due to the relatively low numbers of tested plants. A new progeny with a larger size is needed to validate if these gamete genotype preferences are significant.

In future studies functional tests need to be executed to exactly specify the moment of non-transmission. Possible approaches and methods have been suggested above.

Implications for breeding

At Chromosome 6 the resistance QTL, *rbq6*, is uncoupled from the HI locus. Therefore *rbq6* cannot be the cause for the HI.

The hybrid incompatibility loci were fine mapped to 3 cM regions at Chromosome 4 and 6. These genetic intervals relate to physical distances of maximal 30 Mbp at C4 and 17.9 Mbp at C6 based on the reference lettuce genome (Lattuga Gbrowse version 3.2; estimation of lettuce genome estimation is 2.6 Gb).

To elucidate the genes responsible for this genetic barrier between *L. saligna* CGN05271 and *L. sativa* cv. Olof a further fine mapping approach should be followed. In case the HI is due to a compromised meiotic process, the underlying genes may lead to insight into genes that are required for a successful meiotic process.

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CHAPTER 5

Extreme selection strategy in a resistance mapping study of wild lettuce, *Lactuca saligna*

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Key message:

To unravel the nonhost resistance loci of *L. saligna* against downy mildew, a selective sampling was applied in large F_2 populations for phenotypic extremes of infection, i.e. 'absolute resistant' or 'highly susceptible'. QTL analysis revealed three epistatic loci with a joint major effect and a single locus with an individual major effect.

Abstract

The wild lettuce species, *Lactuca saligna,* is absolute resistant to the downy mildew pathogen, *Bremia lactucae* and this resistance might be an interesting resistance source for cultivated lettuce. Previous research on a *L. saligna* × *L. sativa* F_2 population and a set of backcross inbred (BIL) lines with *L. saligna* introgression segments in a *L. sativa* background, indicated that the resistance from *L. saligna* inherits polygenically as it disintegrates into multiple quantitative loci (QTL). QTL × QTL studies by stacking introgressions with QTLs showed that effects of stacked quantitative resistances were not simply additive. A few additive and merely epistatic interactions with different size of effects, and even negative, appeared. Overall, we did not find a combination with stacked introgression that shows absolute resistance in the field. In this study we applied a selective sampling of phenotypically extreme F_2 individuals (also referred to as 'selective genotyping") to increase detection power for unraveling the probably epistatic inheritance of the absolute resistance of *L. saligna*. We developed segregating populations (F_2, BC_1) of crosses between three *L. saligna* accessions and a common susceptible *L. sativa* parent. Hybrid incompatibilities, which cause low vitality, sterility and segregation distortion, were observed in all populations. Out of 1455 germinated F_2 seeds vital plants with extreme disease phenotypes were selected (62 highly resistant and 25 highly susceptible). In genotype comparisons between the resistant and susceptible F_2 groups, we identified four resistance loci (nominated 'bott_C1', 'mid_C6', 'mid_C7' and 'bott_C9') of which only bott_C9 showed an individual major effect and only bott_C9 was *L. saligna* accession specific. Multi-locus interaction studies between the other three identified loci showed epistatic interactions with large effects in three out of four tested segregating populations (two F_2 and two BC_1sativa). Implications of our extreme sampling strategy are discussed.

Introduction

Susceptibility to pathogens and pests is an exception in the plant kingdom, most plant species are resistant (nonhost) to most plant pathogens. Nonhost resistance is interesting because it is the most prevalent and durable form of disease resistance (Heath 1981; Niks 1988). Genetic studies on nonhost resistance are however scarce, mostly because nonhost species are not crossable with host species. Alternative options to study the genetic basis of nonhost resistance are studies on exceptional susceptibility found in some accessions within a normally resistant plant species or caused by mutations. For instance inheritance of nonhost resistance in *Hordeum vulgare* (barley) to *Puccinia* rust fungi was studied by crossing a barley accession with exceptional susceptibility to the wheat leaf rust fungus (*Puccinia triticina*) with normal, resistant, barley varieties (Atienza et al. 2004; Jafary et al. 2006). A study that made use of mutants showed that *pen1* and *pen2* mutants increased the entry rate in Arabidopsis of two powdery mildew fungi that in nature only colonise grass and pea species. (Lipka et al. 2005). In contrast to these studies, in the lettuce-downy mildew pathosystem there is a nonhost species, *L. saligna* that is sufficiently crossable with cultivated lettuce *L. sativa* to allow studies on transmission, inheritance and level of resistance.

Bremia lactucae causes downy mildew in lettuce (*Lactuca sativa*) which leads to high yield losses in commercial lettuce cultivation. Breeding for resistance by single dominant resistance (*R*) genes is only effective as a short term solution, because those genes are easily broken by the pathogen. For a durable solution, the wild lettuce species *L. saligna* might be a good source as this species is highly to absolutely resistant to all *B. lactucae* races (Bonnier et al. 1991; Lebeda and Reinink 1994; Lebeda and Zinkernagel 2003; Petrželová et al. 2011) and cross fertile with *L. sativa*. To introduce this resistance into the cultivated *L. sativa* we need to acquire more knowledge about the genetic basis of this resistance.

Previous research did not lead to identification of genes or loci that alone or together confer the absolute resistance of *L. saligna* to *B. lactucae*. A small F_2 population of 126 plants (hereafter referred to as F_2_1997_FR) showed a wide and continuous range of infection levels and identified three QTLs, which did not show race specificity to the two tested isolates (Jeuken and Lindhout 2002). Besides those QTLs, one locus was detected, which later appeared to be an digenic "hybrid necrosis" case between a *Rin4 L. saligna* allele and a potential *R* gene from *L. sativa*, causing necrotic lesions, quantitative and race specific resistance (Jeuken et al. 2009). Besides this genetic hybrid incompatibility, severely distorted segregations, plants with low vitality and sterility indicated additional cases of genetic incompatibilities within the F_2 population of this cross.

To investigate the inheritance of the *L. saligna* resistance further, a set of 29 backcross inbred lines (BILs) was developed, covering 95% of the *L. saligna* genome. Most lines had a homozygous single introgressed chromosomal segment from *L. saligna* in the *L. sativa*

background and 15lines showed quantitative resistance (Jeuken and Lindhout 2004; Zhang et al. 2009a). For most of those 16 BILs the resistance was plant developmental stage dependent and probably race non-specific (Zhang et al. 2009a). The infection reduction at adult plant stage in field tests was 30 to 50%. Because these resistances were not absolute, the introgression segments from BILs that showed resistance at most plant stages or had a high field resistance were stacked in pairs. Only two of the ten pairs showed a deviating and much higher resistance level (50% and 70% reduction from the control *L. sativa* cv. Olof) compared to both single-introgression BILs (This thesis, Chapter 3). Occasionally additive interactions, but mostly positive epistatic interactions with 'less or more than additive' effects, and even negative epistatic interactions appeared. Fine mapping of quantitative resistance in three BIL introgressions resulted in disintegration of the responsible genetic factor(s) into multiple, mostly plant stage dependent, QTLs with smaller effects (Chapter 2).

Previous research suggests that not a single locus, nor the combination of two introgressions each conferring quantitative resistance explain the absolute resistance of *L. saligna* to *B. lactucae*. We hypothesize that multi-locus interactions with additive and/or epistatic effects explain the absolute resistance. We embarked on a new analysis using multiple segregating *L. saligna* × *L. sativa* populations involving three *L. saligna* accessions. We used BC_1 populations backcrossed with both parents and large F_2 populations in which we selected for the phenotypic extremes, absolutely resistant and highly susceptible plants. Here we describe and discuss the results of this alternative approach. We show that the epistatic interaction of one locus with two other loci might explain the observed absolute resistance in two *L. saligna* accessions

Materials and methods

Plant material

We crossed *L. sativa* cv. Olof as a father with three geographically distinct *L. saligna* accessions as mothers, CGN15705 [1], CGN05271 [1], 275-5 [2] that have been collected from Georgia (GEO), mainland France (FR) and the island Corsica (CO) in France respectively (kindly provided by [1]Centre for Genetic Resources, the Netherlands, http://www.cgn.wur.nl and [2] provided by A. Beharav from the University of Haifa and collected by Prof. Nevo). F_1 plants were crossed with one or both parents and /or selfed to generate segregating populations (Figure 1). A backcross on the F_1 with *L. sativa* is indicated 'BC_1sativa' and with *L. saligna* as 'BC_1saligna'. BC_1 populations were further inbred.

Phenotypic selection process on F_2

From one F_1_GEO, five F_1_FR and eleven F_1_CO plants we sowed 1779 F_2 seeds. From the germinated F_2 seeds we selected only plants that were very susceptible or highly resistant at

young (2-3 weeks old, 1 to 3 expanded leafs) and adult plant stage (6-8 weeks) and that were vital (not malformed or necrotic) (Figure 2, flow chart). For the "resistant" group we used the criteria 0% at young and maximum 15% infection at adult plant stage. For the "highly susceptible" group we selected the five and fifteen most susceptible plants per population (GEO, FR, CO) from two quantitatively scored disease tests at the young plant stage (YDT). In the second YDT test we selected three times more susceptible plants (15=3 x5), as based on the selection in the first YDT experiment we came across a shortage of susceptible plants after the adult plant stage tests. At adult plant stage we used the criteria: infection severity level (ISL) higher than 35% for Bl:21 or Bl:16 and higher than 15% for both isolates.

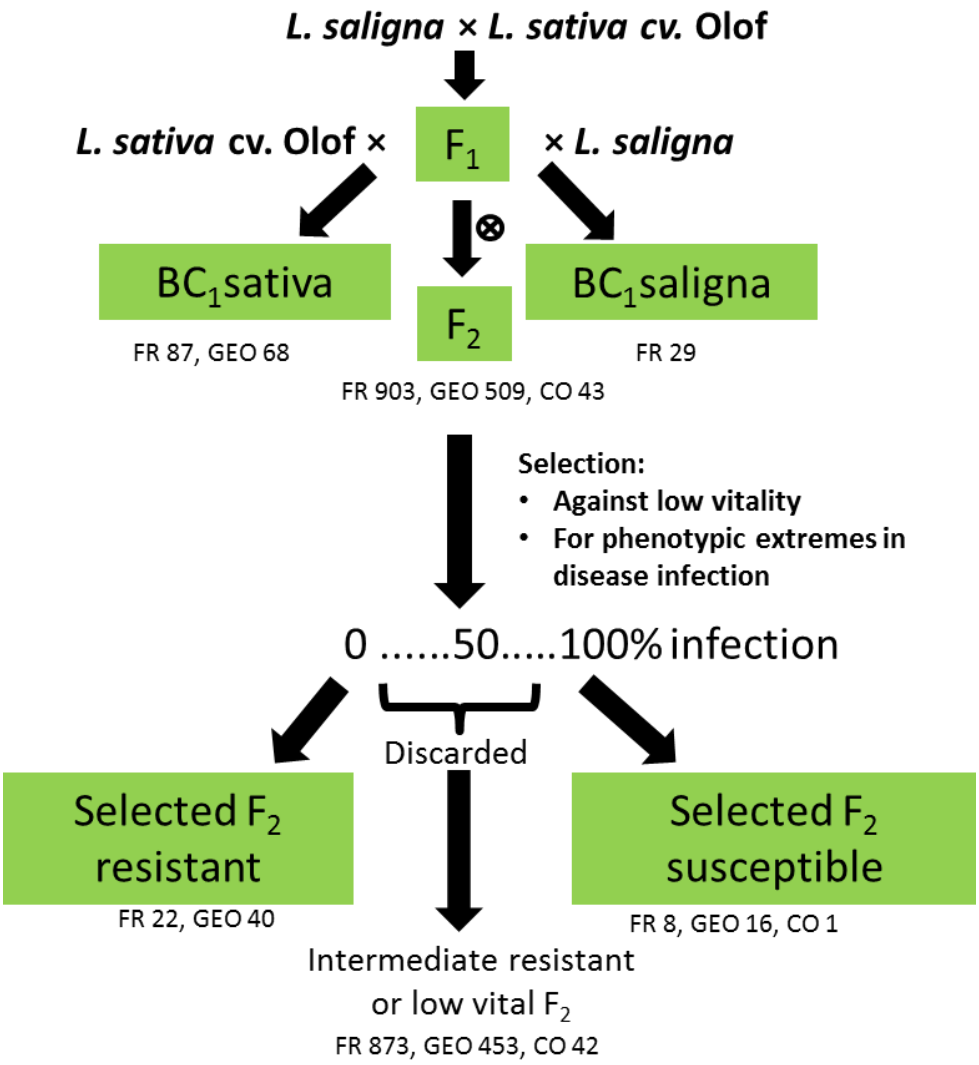

Figure 1. Crossing scheme for the genetic dissection of the downy mildew resistance in three *L. saligna* accessions, CGN05271 (FR), CGN15705 (GEO) and 275-5 (CO). Green boxes indicate that the population is phenotyped for resistance. The number of phenotyped plants is shown under the green boxes per *L. saligna* accession cross.

We phenotyped the BC_1sativa, BC_1saligna and F_2 plants during their whole lifetime for 'low vitality'. We qualified 'low vitality' traits as: necrosis on leaves, malformed leaves, stunted growth or bleaching. Hybrid necrosis led in a previous study to quantitative temperature-dependent race non-specific resistance and to temperature independent race specific resistance (Jeuken et al. 2009). Plants showing 'low vitality' phenotype were excluded from further phenotyping, genotyping and analysis.

Disease assessments

The following control lines were included in disease tests: parental *L. saligna* accessions; strong resistant controls combi-BIL[4.1+6.3]+8.2 and *L. sativa* cv. Iceberg (Grube and Ochoa 2005; Zhang et al. 2009b), moderately resistant BIL8.2; and susceptible controls *L. sativa* cv. Olof and *L. sativa* cv. Cobham Green.

In three independent experiments Young plant Disease Tests (YDT) were performed on 2-3 weeks old F_2 plants as described by (Jeuken et al. 2008; Zhang et al. 2009a). Vital plants with one to three full expanded leafs were inoculated with isolate Bl:21 (3 to 4×10^5 spores / ml). In all three experiments F_2 plants were categorized in absence or presence of infection, based on observations from 8 until 14 dpi. In two experiments (1 and 2) also the level of the infection was scored on the most infected leaf in percentage of leaf area covered with sporulation at 8 and 9 dpi.

Adult plant disease tests in the greenhouse (ADT_G) were performed on 6-8 weeks old plants from F_1, BC_1sativa, BC_1saligna and on selected F_2 plants populations in four independent experiments as described by Jeuken and Lindhout (2002) with some modifications. Plants were phenotyped for *B. lactucae* resistance before bolting to exclude the possibility that bolting has an influence on the resistance score. In all ADT_G experiments four leaf squares (~2×2 cm) from four fully extended leaves were collected per plant and inoculated with 3 to 6×10^5 spores / ml with race Bl:21 and occasionally with Bl:16 (selected_F_2) and Bl:24 (BC_1sativa_GEO). The Infection Severity Level (ISL) was scored as percentage of sporulating leaf area between 8 and 14 dpi. Different progenies were assayed in different independent but linked tests (selected_F_2 in exp. 1 and 2; BC_1saligna_FR plants in exp. 3a and 3b, F_1 and BC_1sativa_FR plants in exp. 4a and 4b; BC_1sativa_GEO exp. 5a (Bl:24), 5b and 5c (Bl:21)). Isolate Bl:21 was applied, because this isolate was considered one of the most virulent on *L. saligna* seedlings (Petrželová et al. 2011).

Phenotyping for early bolting and hybrid sterility

*L. saligna*_FR and *L. saligna* _GEO bolt about 30-40 days earlier than *L. sativa* Olof under spring conditions (unpublished data, M. J.W. Jeuken). Early bolting in the selected $F_2$was indirectly quantified by measuring the stem length at 11-13 weeks in the winter. For the BC_1sativa the early bolting was scored qualitatively (absence / presence) between 7-8 weeks in the summer for the BC_1sativa_FR and for BC_1sativa_GEO between 7-9 weeks in the winter.

Hybrid sterility level was assessed in BC_1sativa by determination of the average number of achenes per capitula (abbreviated as ACHNUM). We counted five capitula per plant.

Genetic maps and genotyping

A genetic map from the F_2_1997_FR population is available (Jeuken et al. 2001). The original F_2 map with 488 AFLP markers has been continuously saturated with additional markers based on EST sequences as part of the Compositae Genome project (http://compgenomics.ucdavis.edu/; (McHale et al. 2009). We selected 85 EST based markers that were evenly distributed over the linkage groups with an average marker interval of 11 cM (Table S1, see supplemental information). The selected F_2 plants and the BC_1sativa and BC_1saligna progenies were genotyped with the framework map of 85 markers by high-resolution melting curve differences on a LightScanner System (Idaho Technology). For genotyping, DNA was isolated in two different ways: a low quality, high-throughput NaOH method (Wang et al. 1993) and a high quality method according to the Kingfisher protocol and as described in Chapter 2.

Mapping loci and QTLs

Kruskal-Wallis test (α=0.005) from MapQTL4.1 package (Van Ooijen 2009) was performed on independent and pooled experiments to detect association of marker alleles with trait levels for F_2 and BC_1- populations. The QTL mapping on F_2_1997_FR by (Jeuken et al. 2001) was re-analyzed with 123 markers, from which 85 markers were in common with the framework map that was used for the selected F_2_GEO and F_2_FR populations. Furthermore a Fisher's Exact test (α=0.01; http://vassarstats.net/index.html) was used to map loci within the selected F_2 by analyzing the difference of genotype segregation ratio's per marker between the selected resistant and susceptible F_2 plants. Calculation of gene action for the detected loci in the selected F_2 plants was performed on the fraction of susceptible plants.

Multi-locus interaction analysis

Markers identified by the Kruskal-Wallis test to be associated with a trait level were further analyzed for multi-locus interactions. For the selected F_2, we compared the fraction of susceptible plants (#s / #s+#r) between different genotype classes using a Fisher's Exact test (α=0.05). For F_2_1997_FR, BC_1sativa_FR and BC_1sativa_GEO, multiple comparison of the ISL between the different genotype classes was done by a univariate analysis of variance and a Duncan test (α=0.05), using the general linear model package IBM SPSS Statistics 19.

Results

F_2 selection

From the 1779 sown seeds 1455 germinated with variable germination percentages between the F_2 populations (91, 80 and 46% for GEO, FR and CO, Figure 2). However, 525 plants (36%) had low vitality (17% hybrid necrosis phenotype and 19% malformed) and were discarded. The hybrid necrosis phenotypes were observed in plants from the F_2_FR and F_2_CO, but not from F_2_GEO.

At young plant stage 930 F_2 plants were tested with a very high disease pressure, resulting in a relatively high ISL of 69% for the strong resistant lines *L. sativa* cv. Iceberg and combi-BIL[4.1+6.3]+8.2 and an ISL of 93% for *L. sativa* cv. Olof. Quantitative scores from 781 plants showed a wide range of infection levels, with an overall F_2 average ISL of 20% (Figure 3). From the 781 plants, 169 plants (22%) showed 0% infection and 121 plants (15%) had an ISL higher than 50%. Based on our criteria (see M&M) we selected 116 highly resistant and 45 highly susceptible F_2 plants for tests at adult plant stage. Ninety-eight percent of the young resistant F_2 plants were still highly resistant at adult plant stage. But only 40% of young susceptible plants were still highly susceptible at adult plant stage. Selection resulted in 62 highly resistant and 25 highly susceptible F_2 plants. Two thirds of the F_2 plants came from the GEO and one third from the FR crosses. Only one susceptible plant from the CO cross could be selected and was included.

Downy mildew resistance of different populations at adult plant stage

We compared infection severity levels to *B. lactucae* between different populations at adult plant stage (Figure 4, dot plot). The infection levels from the same plants were correlated between the independent experiments with Bl:21: experiment 3a and 3b with BC_1saligna_FR plants: r=0.67; experiment 4a and 4b with BC_1sativa_FR plants: r=0.66; and experiment 5b and 5c with BC_1sativa_GEO plants: r=0.67), therefore the data from the same plants between experiments were pooled. The correlation of between Exp. 5a Bl:24 and the Bl:21 experiments (Exp. 5b and 5c) was lower (r= 0.57) and therefore analysed individually.

BC_1saligna

A population of 26 BC_1saligna_FR plants had on average an ISL of 0.5% (Figure 4). This infection level was similar to that on the *L. saligna* accessions and much lower than on the strong resistant controls combi-BIL[4.1+6.3]+8.2 and Iceberg (ISL 13% and 15% respectively, Exp. 3). The BC_1saligna_FR plant with the highest infection level had an ISL of 5%.

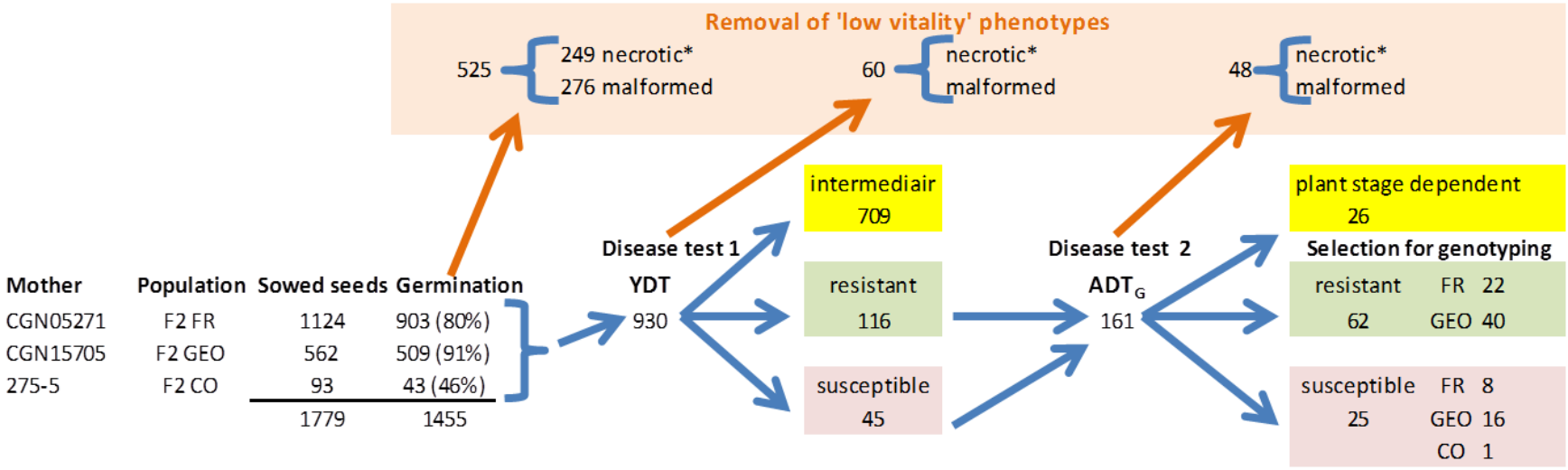

Figure 2. Flowchart of F_2 selection for highly resistant and susceptible plants in three F_2 populations. Codes for mothers: *L. saligna* accessions: FR = France, CGN05271, GEO = Georgia, CGN15705, CO = Corsica, 275-5. Father was always *L. sativa* cv. Olof. * Necrotic plants were only observed in F_2 populations derived from *L. saligna* accessions from FR and CO.

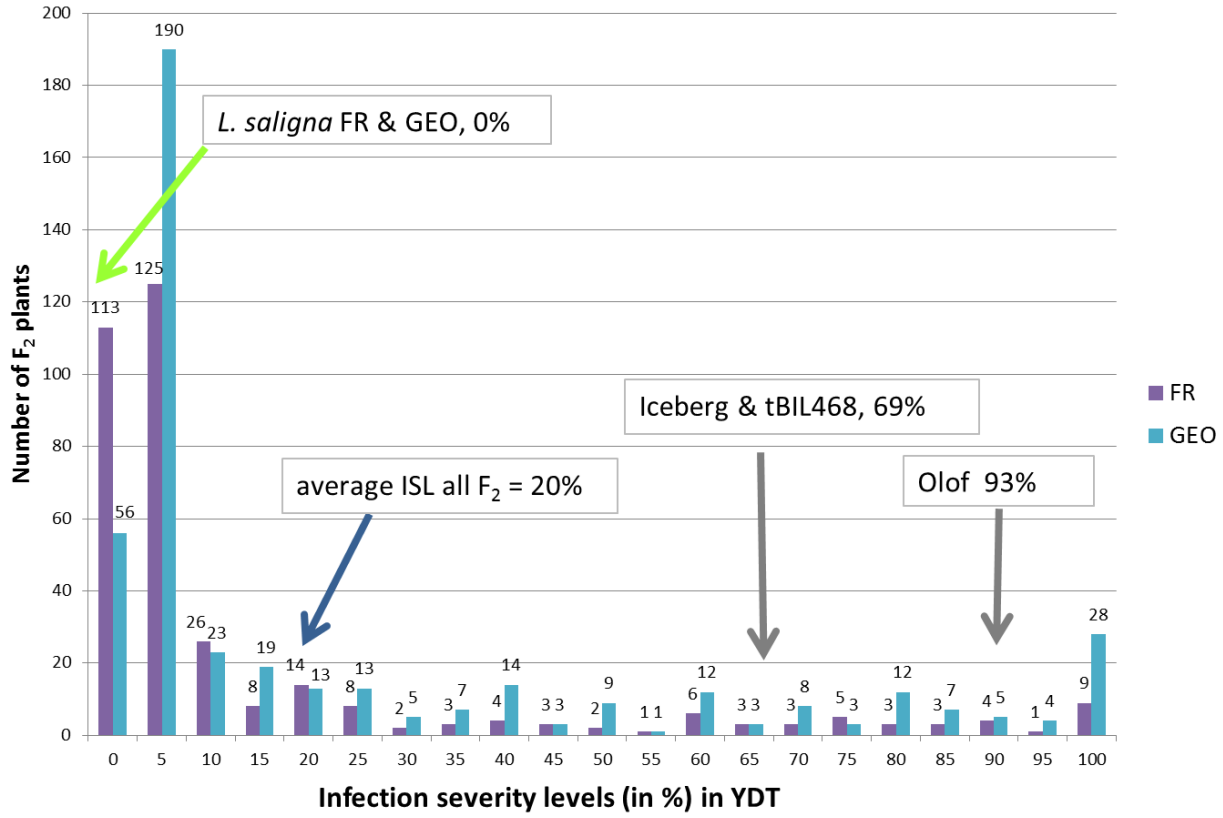

Figure 3. Distribution of 781 F_2 plants over infection severity level classes at young plant stage. From the cross, *L. saligna* CGN05271 (FR) × *L. sativa* cv. 'Olof', 346 F_2 plants are shown and from the cross, *L. saligna* CGN15705 (GEO) × *L. sativa* cv. 'Olof', 435 F_2 plants are shown. Plants that were vital and did not show sporulation (ISL 0%) or were highly susceptible (ISL 60 to 100%) were selected for further phenotyping at adult plant stage. Control lines are presented by arrows that point to their equivalent infection severity class.

F_1

F_1 _FR and F1_GEO plants showed an average ISL of 2 and 4%, which is closer to the levels of *L. saligna* accessions (0%) and BC_1saligna_FR (0.5%) than to the levels of strong resistance controls combi-BIL[4.1+6.3]+8.2 and *L. sativa* cv. Iceberg (ISL 8% and 10% respectively, Exp. 4).

BC_1sativa

The BC_1sativa_FR and BC_1sativa_GEO populations showed a wide and continuous range of ISL (Exp. 4 and exp. 5 respectively). Of the 76 BC_1sativa_FR plants 16 plants had an equal or higher ISL than the *L. sativa* parent (average ISL: 63%) and 6 plants had a similar ISL (2-11% ISL) as the strong resistant control lines combi-BIL[4.1+6.3]+8.2 and *L. sativa* cv. Iceberg (average ISL: 8%). Only one plant nearly reached a similar ISL as the *L. saligna* parents or the BC_1saligna population. Of the 63 BC_1sativa_GEO plants three plants had an equal or higher ISL than the *L. sativa* parent (average ISL: 77%) and thirteen plants showed a similar ISL (1-4%) as the strong

resistant control lines combi-BIL[4.1+6.3]+8.2 and *L. sativa* cv. Iceberg (average ISL: 4% and 1%, respectively).

Detection of resistance loci

QTL mapping and genotype segregation analysis on 56 selected F_2_GEO plants showed four loci, at the bottom of C1 (bott_C1), in the middle of C6 (mid_C6), in the middle of C7 (mid_C7), and at the bottom of C9 (bott_C9) (Figure 5 and 6). In further analyses the mid_C7 locus was observed to contribute to resistance in the populations of selected F_2_FR (n=30) and F_2_1997_FR (n=126), mid_C6 in selected F_2_FR and BC_1sativa_FR and bott_C9 in BC_1sativa_GEO (Figure 5 and Table 1).

Bott_C1 locus had in selected F_2_FR an effect on the resistance level when the fraction of susceptible and resistant plants was compared between presence and absence of at least one copy of *L. saligna* allele (dominant) (Fisher's Exact test, P=0.03). Therefore, we assume that the bott_C1 locus was effective in both F_2_FR and F_2_GEO. In the F_2_GEO the bott_C9 locus shows the highest association with resistance in both tests with race Bl:21 and in the test with Bl:24, but this locus does not show an effect in the French progenies (F_2 and BC_1sativa). In BC_1_GEO other loci associated with resistance were detected, but only in individual experiments and therefore not further considered as resistance loci.

The gene action of the four loci (bott_C1, mid_C6, mid_C7 and bott_C9) was calculated for the selected F_2 populations and indicated a more dominant than additive effect (Table 1).

Interactions of resistance loci

Potential interactions between the four loci detected in the selected F_2_GEO were studied. In F_2_GEO the mid_C6 locus seems to contain a key gene for resistance that interacts with both bott_C1 and mid_C7. The fraction of susceptible plants was only reduced significantly by an *L. saligna* allele at mid_C6 when there was an *L. saligna* allele present at bott_C1 or / and at mid_C7. Presence of mid_C6 from *L. saligna* did not result in a lower proportion of susceptible plants if C1 and C7 both carried the *L. sativa* allele in homozygous condition. Similar but not always significant interactions between the same pairs of loci were observed for the smaller selection F_2_FR (n= 30). Because the interactions in both populationswere similar and between the same three loci, data were combined, analysed and presented together (Figure 7 and Table S2).

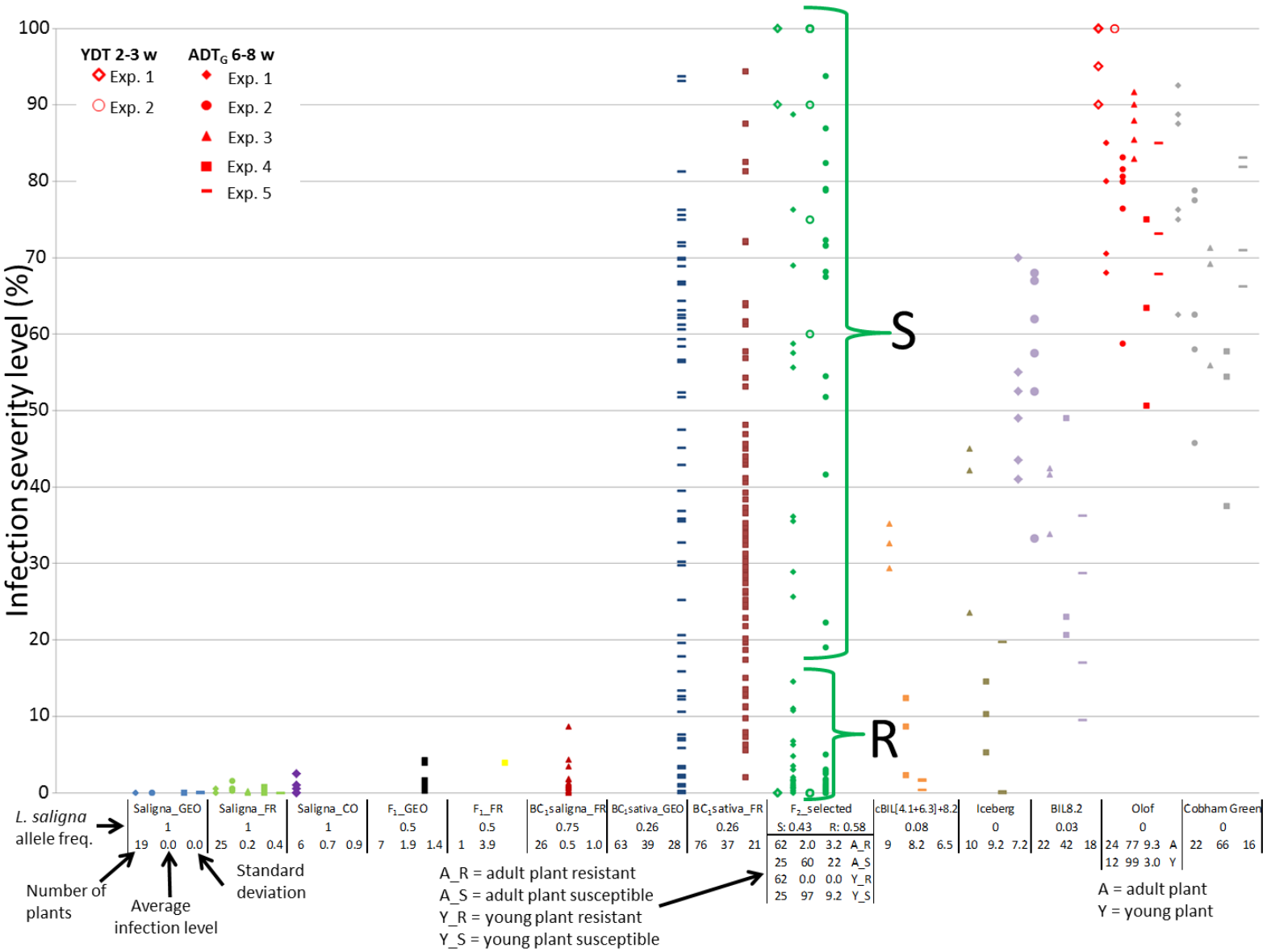
YDT 2-3 w
Exp. 1
Exp. 2
ADT$_G$ 6-8 w
Exp. 1
Exp. 2
Exp. 3
Exp. 4
Exp. 5
Infection severity level (%)
100
90
80
70
60
50
40
30
20
10
0
S
R
L. saligna allele freq.
Saligna_GEO 1 19 0.0 0.0
Saligna_FR 1 25 0.2 0.4
Saligna_CO 1 6 0.7 0.9
F1_GEO 0.5 7 1.9 1.4
F1_FR 0.5 1 3.9
BC1saligna_FR 0.75 26 0.5 1.0
BC1sativa_GEO 0.26 63 39 28
BC1sativa_FR 0.26 76 37 21
F2_selected S: 0.43 R: 0.58
62 2.0 3.2 A_R
25 60 22 A_S
62 0.0 0.0 Y_R
25 97 9.2 Y_S
cBIL[4.1+6.3]+8.2 0.08 9 8.2 6.5
Iceberg 0 10 9.2 7.2
BIL8.2 0.03 22 42 18
Olof 0 24 77 9.3 A
12 99 3.0 Y
Cobham Green 0 22 66 16
Number of plants
Average infection level
Standard deviation
A_R = adult plant resistant
A_S = adult plant susceptible
Y_R = young plant resistant
Y_S = young plant susceptible
A = adult plant
Y = young plant

Figure 4.

Figure 4. Infection severity plots from control lines and populations in seven experiments (two YDT and five ADT_G). Plants with the hybrid necrosis phenotype are not included. For the F_2 population R and S mean highly resistant and susceptible respectively. Only the selected and genotyped F_2 plants are shown from all three *L. saligna* × *L. sativa* populations, derived from the following *L. saligna* accessions as mother; CGN05271 (FR), CGN15705 (GEO) and 275-5 (CO) crossed with *L. sativa* cv. Olof. Each line / population is visualized by a different color. Per experiment the control lines and the population(s) are visualized by a different symbol.

We did not have enough informative genotypes to study interaction between the bott_C9 locus and the other three loci in the selected_F_2_GEO. Only one of sixteen susceptible plants had a *L. saligna* allele and only three out of forty resistant plants had no *L. saligna* alleles on the bott_C9 locus (Table S2A). However, from the effects of the four loci individually bott_C9 had the largest effect (Figure 7). A significant mid_C6 × bott_C1 interaction was only detected in the BC_1sativa_FR when the mid_C7 locus was homozygous *L. sativa* (Table S3). No significant interactions were detected within the BC_1sativa_GEO population (Table S3).

When checking the haplotypes of the loci in the selected F_2, the four loci in F_2_GEO and three loci in selected F_2_FR explained almost always the resistant or susceptible phenotype. There were only four exceptions, of which one plant had a recombination event next to the peak marker at mid_C6.

When checking the haplotypes of BC_1sativa plants, in the BC_1sativa_FR progeny the three loci together explained an infection level reduction of about 50% between complete absence and presence of a copy of the *L. saligna* allele at the three loci (Table S3). The six highly resistant BC_1sativa_FR (< 11 % ISL) showed the following haplotypes for the three loci: two plants had one *L. saligna* allele at mid_C6 and bott_C1, two plants had one *L. saligna* allele at mid_C6 and mid_C7 and two plants had one *L. saligna* allele at all three loci.

From the 43 BC_1sativa_FR plants with an ISL higher than 30%, only seven plants had a copy of a *L. saligna* allele at mid_C6 and bott_C1 or/and mid_C7. However one plant had a recombination close to the mid_C6. Therefore, the combination of the three loci mostly results in high resistance.

When checking the haplotypes of the BC_1sativa_GEO, eleven plants had one *L. saligna* allele at the bott_C9 locus and were highly resistant against Bl:21 and Bl:24 (average ISL 4%). In the other 52 plants we observed that the mid_C7 locus explained an (Bl:21) infection level reduction of almost 50% (Table S3).

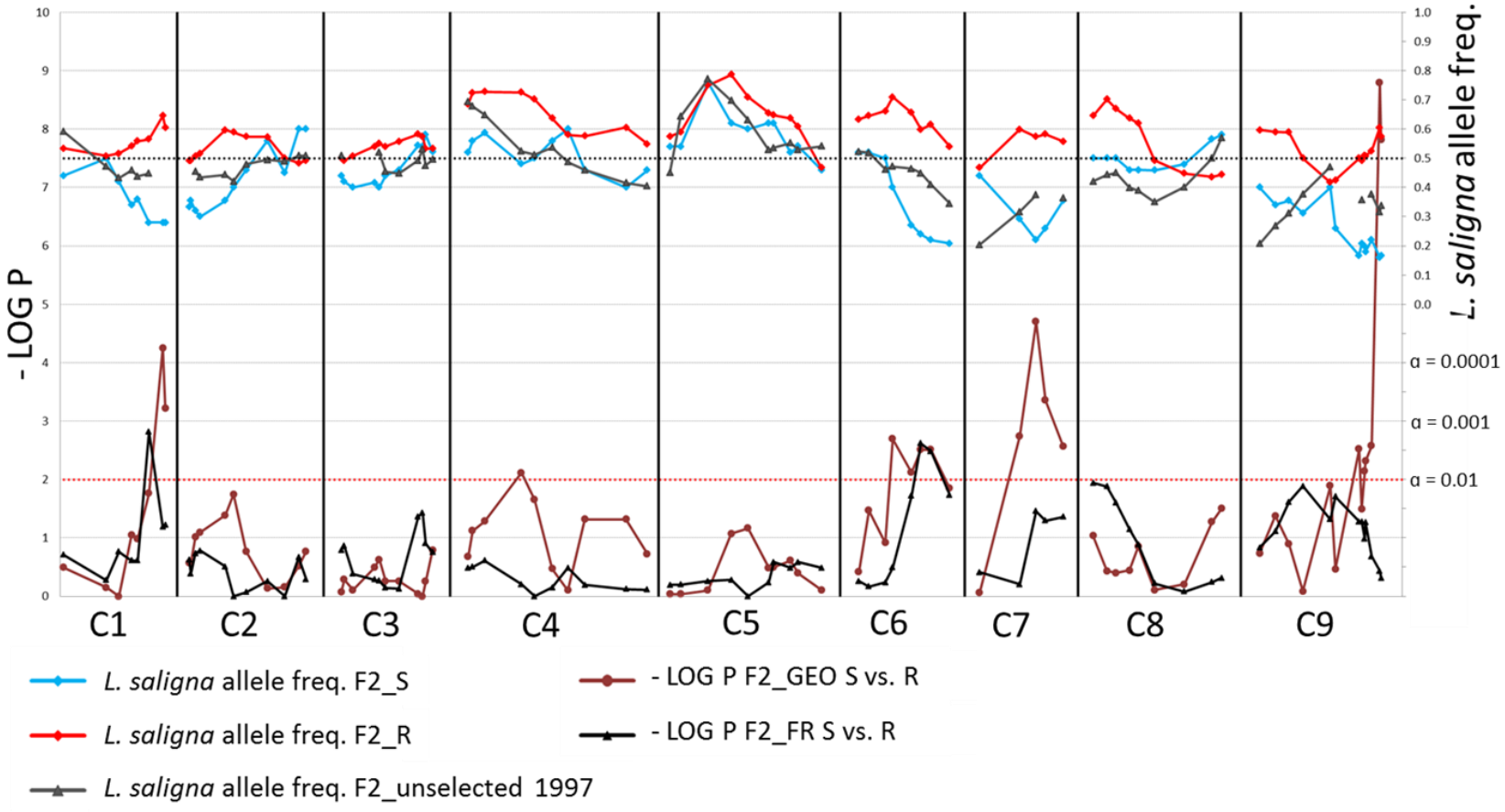

Figure 5. Graphs of genotype segregation ratio differences (lower graph) and *L. saligna* allele frequencies (upper graph) between groups of selected resistant and selected susceptible F_2 plants over the nine lettuce chromosomes (85 markers). Selected F_2 plants from *L. saligna* accessions CGN05271 and *L. saligna* CGN15705 are indicated by F_2_FR and F_2_GEO, respectively. Fisher's Exact test (α=0.01) was performed per marker to test difference/similarity of genotype segregation ratios between the groups of susceptible and resistant F_2 plants. P values from the Fisher's Exact test were – LOG transformed and plotted for each F_2 population. The *L. saligna* allele frequency is plotted from the groups of selected susceptible and selected resistant F_2 plants (F_2_FR and F_2_GEO together) and from an unselected F_2 population from 1997 (Jeuken and Lindhout, 2002).

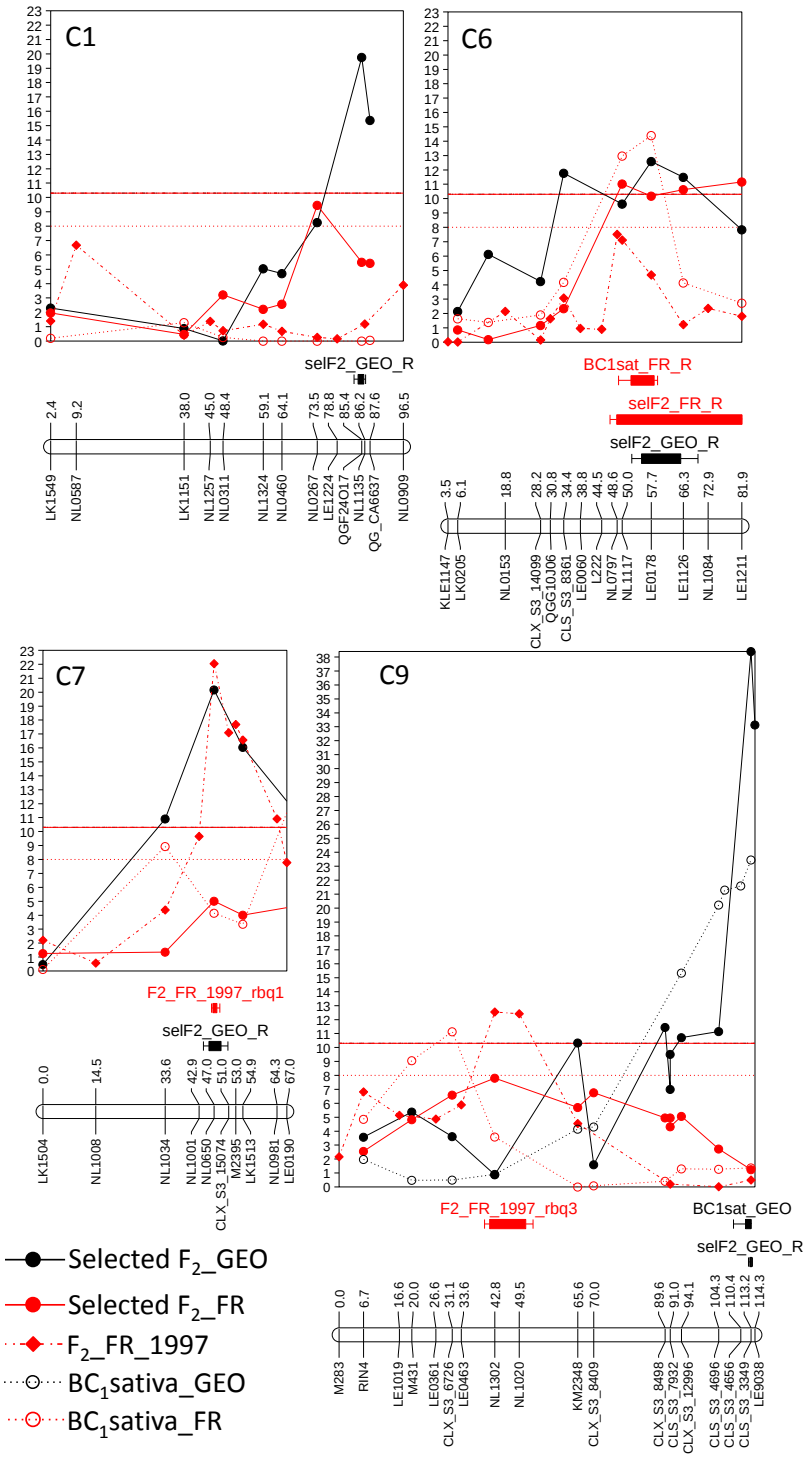

Figure 6. QTL profiles of infection severity levels to *B. lactucae* in five (sub-) populations (F_2_FR_1997, BC_1sativa_FR, BC_1sativa_GEO and selected F_2_FR and selected F_2_GEO) at Chromosomes 1, 6, 7 and 9. Kruskal-Wallis test results (K values) are plotted for linkage groups in which significant peak values were found. Threshold values with a α=0.005 are indicated as horizontal red lines, solid for F_2 and dotted for BC_1sativa. K values for populations derived from a *L. saligna* CGN05271 (FR) are shown in red and from CGN15705 (GEO) in black. For BC_1sativa_GEO population K values are only shown for Chromosome 9, because significant K values at other Chromosomes were identified only in individual experiments and were not confirmed in the other two experiments.

Table 1. Overview of loci for observed traits and distorted segregation ratios in six different populations. Shown are loci with a significant effect on the following phenotypes: resistance from *L. saligna*, early bolting from *L. saligna* and hybrid sterility.

Phenotype / Chromosome	Position cM	Detected in population[1]			Sign.[3]
Resistance			additive[2]	dominance[2]	
1	85.4	A	-0.30	-0.31	***
6	57.7	A, b, d	-0.29	-0.13	*
7	47.0	a, b, C	-0.44	-0.54	**
9	107.0	A, e	-0.42	-0.37	****
Early bolting			**Early bolting by allele**		
7	54.9	A, b, d, e	*L. saligna*		****
Sterility			**Sterility by genotype**		
5	3.0	D	heterozygous		*
6	66.3	D	heterozygous		**
9	89.6	D	heterozygous		***
Distorted segregation ratio			**Allele preference**		
4	8.2	a ,C, d, e	*L. saligna*		****
5	25.5	A ,b, c, d, f	*L. saligna*		****
9	107.0	C, e, f	*L. sativa*		****

[1] Populations with a significant effect (Kruskal-Wallis test, α=0.005) on the loci are indicated by a letter: a = F_2_GEO, b = F_2_FR, c = F_2_1997_FR, d = BC_1sativa_FR, e = BC_1sativa_GEO and f = BC_1saligna_FR. A capital letter indicates the population with the strongest effect, [2] for the population with the strongest effect the additive and dominance effect is given, the values are based on the resistance : susceptibility frequencies not on averages of infection levels, and [3] the significance level. 3 * = α = >0.005, ** = α = >0.001, *** = α = >0.0005, **** = α = >0.0001 based on Kruskal-Wallis test (MapQTL v4.1). Only the loci conferring a significant effect are shown (Kruskal-Wallis test, α=0.005) detected within the new populations (not F_2_1997), except for the distorted segregation loci, for which we show only the loci that were detected in at least 3 populations. For the detected loci the results for the marker with the most significant effect is shown.

Other phenotypic observations

Early bolting was mapped at 54.9 cM at Chromosome 7 and was located at about 8 cM distance from the mid_C7 resistance locus (Table 1). Compared to the fertility of *L. sativa* cv. Olof (ACHNUM = 14 achenes), the average fertility of the BC_1sativa plants was nearly as low (ACHNUM = 1.5 achenes for FR and 1.2 for GEO) as of the F_1 plants (ACHNUM = 0.8 achenes for FR and 1.0 for GEO). Four out of the 84 evaluated BC_1sativa_FR plants and null out of 39 evaluated BC_1sativa_GEO plants had more than five achenes per capitula. In BC_1sativa_GEO no sterility QTLs and in BC_1sativa_FR three sterility QTLs were identified at, C5 (3.0 cM), C6 (66.3 cM) and C9 (89.6 cM) in which the heterozygous genotype was associated with sterility.

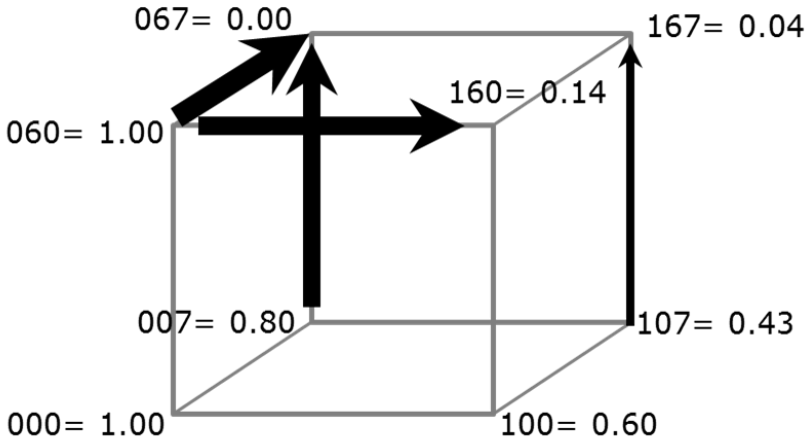

Figure 7. Comparison of fraction susceptible F_2 plants (FR and GEO) between eight genotype groups of three resistance loci in a three-way interaction. The figure is a three-dimensional visualization of data in Supplemental Table S2c. The cube shows an infection severity landscape of three loci, where the nodes are genotypes. Genotypes are shown by numbers in order of the locus chromosome number, '1', '6' and '7' = heterozygous or homozygous *L. saligna* at bott_C1, mid_C6 locus and mid_C7 locus respectively; '0'= homozygous *L. sativa*. Genotype of the three loci is based on the marker profiles from: QGF24O17 for bott_C1, LE0178 for mid_C6, and NL0650 for mid_C7. Fraction of susceptible plants for each genotype group is indicated (= #.##). Significant differences (Fisher's Exact test, α=0.05) are indicated by arrows in the direction of the genotype with the lowest fraction of susceptible plants and thickness of arrow indicates the size of decrease/reduction.

Segregation distortion

Over de six populations (3 F_2, 2 BC_1sativa and 1 BC_1saligna) twelve regions with distorted segregation were observed with preferences for *L. saligna* or *L. sativa* alleles. Most regions with distorted segregation ratios differed between the population, except for three regions, C4 (8.2 cM), C5 (25.5 cM), C9 (107.0 cM) (Table 1). The region on C4 and C5 had a consistently distorted segregation with a strong *L. saligna* allele preference (*L. saligna* allele frequency of 0.68 and 0.77 respectively in the unselected F_2_1997_FR). Bott_C9 distorted in three populations (F_2_1997_FR, BC_1saligna_FR and BC_1sativa_GEO) with a strong *L. sativa* allele preference (*L. sativa* allele frequency of 0.68 in the unselected F_2_1997_FR).

Discussion

From the three *L. saligna* × *L. sativa* crosses, the F_2_FR and F_2_CO segregated for the hybrid necrosis phenotype that results in necrotic lesions on the leaves and a quantitative race nonspecific resistance that influences the resistance phenotype. The F_2_GEO population did not segregate for the hybrid necrosis phenotype which makes the Georgian F_2 population more ideal for genetic dissection of the nonhost resistance to *Bremia lactucae.*

On average F_1 _GEO, F_1_FR and BC_1saligna_FR plants were highly resistant (Figure 4, dot plot). These highly resistant F_1 observations contrast with earlier reports in which *L. saligna*

× *L. sativa* F_1 plants from three different *L. saligna* accessions had more tendency to susceptibility as their ISL values ranged between those of *L. sativa* cv. Iceberg and those of the susceptible parent (Lebeda and Reinink 1994). The high resistance level of the F_1 and BC_1saligna indicated that at least a large part of the absolute resistance depends on dominant *L. saligna* alleles, and/or on a multi-locus interaction between additive or epistatic alleles.

At the young plant stage the F_2 populations from FR and GEO showed a wide range of infection levels, but 68% of the plants had an infection level of less than 10% (Figure 3). At the adult plant stage in the F_2_1997_FR population a wide and continuous range of infection levels, with equal distributions over the classes, was observed (Jeuken and Lindhout 2002). Possibly at young plant stage more QTLs or QTL × QTL interactions are present than at adult plant stage. This phenomenon is reflected in our disease screening on the set BILs, where we detected twice more resistant BILs in young plant stage than adult plant stage (Zhang et al. 2009a).

The 76 BC_1sativa_FR plants and 63 BC_1sativa_GEO plants had a wide and continuous range of infection levels including low numbers of plants (6 and 13 respectively) with a similar or lower ISL as the strong resistant control lines combi-BIL[4.1+6.3]+8.2 and *L. sativa* cv. Iceberg. The low percentages of plants, 8% and 21%, with a strong resistance level reduces the possibility of the resistance being due only to the presence of one major resistance gene in the *L. saligna* parent, because one dominant resistant gene in a BC_1sativa would result in 50% resistant progeny, if under Mendelian segregation.

Analysis on several populations (BC_1sativa and multiple F_2) revealed three loci (bott_C1, mid_ C6 and mid_ C7) that together contribute to absolute resistance and one accession specific (GEO-cross) locus, bott_C9, with an individual strong resistance effect. Most loci seemed dominant, which is in agreement with the very high resistance level of the F_1 and BC_1saligna_FR.

The four loci (bott_C1, mid_C6, mid_C7 and bott_C9) are not located in the documented hotspots of monogenic or major resistance genes (*R* gene) in lettuce (McHale et al. 2009, Truco et al. 2013). In our previous studies quantitative resistances have been ascribed to regions that overlap with the mid_C7 and bott_C1 loci. The mid_C7 locus coincides with the earlier identified *Rbq1* in the F_2_1997_FR population (Jeuken and Lindhout 2002). This locus also coincided with a hybrid incompatibility region and therefore it was only possible to obtain the introgression segment in homozygous state when it was combined with the fourth introgression on chromosome 8 (BIL8.4 introgression segment). However, testing this line (with *L. saligna* introgression mid_C7+8.4) under field conditions showed that it was susceptible (unpublished data). The bott_C1 locus coincides with the 25 cM overlapping Chromosome 1 introgression regions of BIL1.2 and BIL7.1 that show quantitative resistances in field tests (Jeuken et al. 2008; Zhang et al. 2009a; Chapter 3). The bott_C9 has not been associated with resistance previously, but recently we mapped a *Bremia*-effector response gene within a 4 cM window in *L. saligna* CGN05271-*L. sativa* hybrids (Chapter 5) that coincides with the bott_C9 locus of the Georgian population. The locus mid_C6 was not implicated previously with *B. lactucae* resistance. Both

mid_C6 and bott_C9 chromosome regions were associated with hybrid incompatibilities, because BILs carried the respective introgression segments only in heterozygous state and in many segregating populations a strong preference for *L. sativa* alleles was observed for bott_C9.

The advantages of the extreme phenotype selection are obvious: reductions in genotype costs and in labor, and increased amounts of genotyped plants with phenotypic extremes. If the phenotype variation is normally distributed, this method increases the detection power of QTLs. The advantage of the increased detection power by the extreme selection strategy can however be neutralized or decreased by presence of a major QTL elsewhere as this causes a higher phenotypic variation elsewhere (Sen et al. 2009) and by interacting loci with asymmetric allele frequencies as their mutual phenotype comparisons are less reliable (Allison et al. 1998). In our case, in the selected_F_2 the bott_C9 locus seems to have a major effect in the Georgian population and it has an asymmetric allele frequency in three of the six tested populations (Table 1). This implies that some of the QTLs we detected in the three tested F_2 populations could be false positives or that we have missed some important QTLs which are involved in the absolute resistance of *L. saligna*. Therefore we validated our results in three other populations (BC_1sativa_GEO, BC_1_sativa_FR and unselected F_2_1997_FR) in which we did not use the extreme selection method.

Interaction

Of the four identified loci, mid_C6 showed an epistatic interaction with two loci, bott_C1 and mid_C7. In the selected F_2_GEO and F_2_FR populations the presence of a plant in the susceptible or resistant group could be explained by the mid_C6 × bott_C1 or / and mid_C7 interaction. Also in the BC_1sativa_FR, resistance or susceptibility seems for a large part explained by this interaction. Within the BC_1sativa_GEO these interactions seem to play no role.

Although in the selected F_2_FR population the presence of a plant in the susceptible or resistant group could be explained by the mid_C6 x bott_C1 or / and mid_C7 interaction this was not so clear in the BC_1sativa_FR and BC_1sativa_GEO. Therefore we cannot exclude the possibility that an additional locus is /loci are is required for absolute resistance. Validations in inbred families of resistant BC_1sativa plants are required to confirm our candidate epistatic interactions.

These data on multi locus interactions align with recent findings about a more prevalent role of epistasis in the inheritance of traits than was thought before (Breen et al. 2012; Flint and Mackay 2009; Stower 2012; Zhuang et al. 2002).

Prospects and consequences for breeding

Epistatic interactions between mid_C6 with bott_C1 and mid_C6 with mid_C7 explained in the selected F_2 plants always resistance, except for three plants (Table S2). These two interactions were present in both the French and Georgian populations, suggesting that these interactions

might explain the genetic basis of the absolute resistance of the species *L. saligna*. Because of the nature of the resistance in *L. saligna* we expect the introgressed resistance to be durable as well.

The strong resistance conferred individually by the bott_C9 locus implies a classic race specific *R*-gene, but it does not explain the absolute nonhost resistance of the species *L. saligna* because this locus is not identified in the French population.

The four detected resistance loci are interesting for breeding for *B. lactucae* resistance if they can be uncoupled from undesired hybrid incompatibilities and undesired phenotypes (early bolting, sterility). Only bott_C1 is not linked to a hybrid incompatibility nor an undesired phenotype and could therefore be the easiest for introgression. Introgression of the other loci might involve more efforts, but is not unfeasible, as we have been already successful in the genetic dissection of two cases of hybrid incompatibility (Jeuken et al 2009, Chapter 3 about a C4-C6 interaction). Resistant BC_1_sativa plants with the desired loci can be further backcrossed with *L. sativa* to study the resistance loci in a *L. sativa* background without undesirable traits from *L. saligna*. The set of 29 BILs (Jeuken and Lindhout 2004) can be used to stack the desired resistance loci in a *L. sativa* background and validate the results.

Further implications

With the selected extremes of F_2 populations, resistant and susceptible, we were able to efficiently detect epistatic interactions on the basis of a relatively small number of genotyped plants out of a large number of phenotyped plants. Detection of such interactions between *L. saligna*-derived introgressions in combinations of the 29 available BILs (Jeuken et al. 2008), would not have been feasible, since it would require hundreds of doubleBILs and thousands of triple BILs to be developed and tested.

The genetic basis of the resistance in the *L. saligna*_FR material is, except for the bottom C9 locus which had only an effect in the F_2_GEO population, very similar to that of the *L. saligna*_GEO material: same loci and same interactions. This result is what we expected, as the species *L. saligna* is absolute resistant to all *B. lactucae* races and therefore we expect a common genetic basis.

The present study suggest that the mid_C6 x bott_C1 or / and mid_C6 x mid_C7 interactions play a role in the nonhost resistance from *L. saligna*. These loci and their interactions are interesting targets to study their practical use for resistance breeding and to investigate their molecular basis.

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Supplemental Table S1. Primer sequences, contig and map position.

Marker name	Contig or publication	Chr.	Position (cM)	Primer forward	Primer reverse
LK1549	QG_CA_Contig5046	1	2.4	aggattcgcgactgaatttg	agtgctgcttcccagtcttc
LK1151	QG_CA_Contig1246	1	38.0	ccaagttcttgagcctccac	tctgcaggagcgatttcac
NL0311		1	48.4	gtcttggagcaacaccttc	aatgccacttaagctttcac
NL1324		1	59.1	cataaccggaagcttgttc	tgcataaagaaatatgcaaaac
NL0460		1	64.1	cgattttcatacactctgcc	ttgattgcctctgtgtttg
NL0267		1	73.5	ggcagtgggtgtaaatgac	tgactggatcagcagattg
QGF24O17	QGF24O17.yg.ab1	1	85.4	gtttctccccctttcagctt	tcccaaacatggtggatctt
QG_CA6637	QG_CA_Contig6637	1	87.6	cattgtgctcgttgcagagt	gcaccaacctcgttcaattt
SCW09	Paran & Michelmore[1]	2	-5.3	gtgaccgagtagtcttaacctagt	gtgaccgagtgtaacaacgtaaat
SCV12	Paran & Michelmore[1]	2	-4.0	acccccccactaccatatcaatctc	acccccccacttgtcctgcaacttt
LK210	QG_CA_Contig4663	2	0.0	attccatccaccgatgttgt	aaattggcaagcatctcagc
NL1283		2	3.8	atcgggttttgtgattttg	aatttcgacgaaccaacac
NL0736		2	24.8	gagaaatgtccgaaactgc	ctcaagtcctttgcctgac
NL0593		2	31.9	gaagcagaagttgtgaggc	caaagcacttggatccttc
NL0967		2	42.2	aagaagtgacattccggtg	ttccacattcgtcaagattc
LE1276	QG_CA_Contig7689	2	60.2	tttgggttccttcagtttgc	cacagtttgggatgaacacg
LK1475	QG_CA_Contig7099	2	74.4	ggagttcagggcctctgtc	ccgattctgcggttatcttc
NL0842		2	86.2	ttctatccgtttgggaatg	tgctgctgatttaccacac
NL0319		2	92.3	gctgactggatttaggacg	gtctgactgtccattttgttg
NL0561		3	4.4	tacagtcgacgtttcttgc	gggtaaagacggagaaacc
QG_CA5854	QG_CA_Contig5854	3	6.8	attcgattatcgccgctatg	cgattctcaaacaagcgaca
QGA8B05	QGA8B05.yg.ab1	3	14.2	tggatgatgtgccgataaga	cctgttgccttgaaattggt
QG_CA1077	QG_CA1077	3	32.1	tagggcctcctcttccattt	aaacagcttcggcttcaaaa
LE1169	QG_CA_Contig3570	3	36.2	gatgagccgacgaatcattt	caacgtgtcacagcctgatt
NL1124		3	41.4	ccgttactttagtccgctg	cttccacctttttgagacg
NL1049		3	52.5	gccatttaacagatttgtgtg	ctgaagtgtctttttattaccac
NL1174		3	68.5	gagcatctgatctccgttc	tgattggaattgggcttac
LE3092	QG_CA_Contig4490	3	72.2	tggccaaagtctccaaagat	ggcattgctccaagttttgt
NL0870		3	74.7	agactttcaccatggttcg	aaatggtttccagcaacac
NL0117		3	80.8	gtacaatggagatggtggg	tctgatctgaaatcccgac
NL1151		4	4.5	tttggtatcaagcctctcg	caagtctatcaagccctgg
NL1260		4	8.2	cttagaaagctgccaccac	ggagcgattttacagttcg
NL0897		4	18.4	gaagacaagaagtcgacgg	cgatcgagataacgaaagc
NL1088		4	43.3	atttgaaagccatggaaac	ttgcttcaaattttccacc
LK1527	QG_CA_Contig7732	4	48.9	aagttcttccgatcccttcc	ccctgtttcctcctttaccc
NL0531		4	59.9	gtcggtatcaaattaggcg	aggcagagattggatgatg
NL0207		4	75.0	atatccgtcattgtcgtcg	caaagcccatatgaaaaatg
NL1337		4	87.9	cttcgtggaaggttttcag	atcttgtgccatggtaagc
LE1233	QG_CA_Contig5723	4	102.0	caggattcttcaggagcagc	cccaatctcgtccactgttt
LE1162	QG_CA_Contig345	4	136.5	taaagaggatctcatgggcg	gaatgcaacatatgcaaccg
NL1035		4	154.2	atgcaatagaccttggtgc	ttgtccacctcccaaatac
LE0354	CLX_S3_Contig7850	5	-6.0	ggatgcggttaaagaagcaa	ccccattaaacggaattgtg
NL1090		5	3.0	actcaatgcacgattctcc	tcagtgtaagtgcctgtgg
NL0103		5	25.5	acacaaatcaagggaatgc	tccctgactgagtggagtc
NL0853		5	45.2	ttcctgtgtttgtgtcagg	cggaattacaaccaacattac
NL0173		5	59.0	cgcagagatagagacaggg	acgtgcaataaaccaaacc
NL0871		5	76.4	tttattcatgggtcaagcc	gaaccgaaggatttcacac
NL1220		5	80.4	tcccacagtttcctcattc	aaatcgccatttacgactg
NL1159		5	94.5	caaatcgtacttccgcttc	acacctggagattttgtgc
NL0750		5	100.8	tgtgtattttatgcgcacc	ttgctctcactgatctccc
NL0889		5	120.5	gtcgccatatcaaaagagg	gagcaaacatgcaaatagg

Marker name	Contig or publication	Chr.	Position (cM)	Primer forward	Primer reverse
LK0205	QGA13L14.yg.ab1	6	6.1	gtgtgatcctgcatcccaat	tagtcgcgccctgtttacat
LK1471	QG_CA_Contig6524	6	14.3	tggcatggaatttgaatcag	ccttgacactcctcccattc
CLX_S3_14099	CLX_S3_Contig14099	6	28.2	agaagcaagctccatgagga	tagtcggaaaacgccagttc
CLS_S3_8361	CLS_S3_Contig8361	6	34.4	tctgcaaccatctgaagcac	cccatttgcaccatacacag
NL1117		6	50.0	actgtctccaccgaagatg	ttggttacagggattttgg
LE0178	QGG26M07.yg.ab1	6	57.7	ttgctgacataagagaagtttcaa	gtatcatccacatcgtctaga
LE1126	QG_CA_Contig1905	6	66.3	ctttgctccaattcctctcg	aatgccatagtgaagctggg
LE1211	QG_CA_Contig4578	6	81.9	cgggtgattacatcggctat	cgcaaccaaccaaatttacc
LK1504	QG_CA_Contig1477	7	0.0	gcatcaggaaatccgagtgt	ccgcctagggttcttcctac
NL1034		7	33.6	gaacaggaacaaaacccag	acctgtgtgggtctcaaag
NL0650		7	47.0	gggaaacgtaatagaacgg	aatctcgtggcaaatatgg
LK1513	QGF20P01.yg.ab1	7	54.9	cgaagacaaagcctggaaag	ttgcagtacacagcaacacg
LE0190	QG_CA_Contig1870	7	69.9	cgtccctactgtgagcaaca	atacgagagaccgcgtgcta
NL0851		7		tcttgagaagaaaccacgg	gagatgttgaacgcgatg
QGG16B23	QGG16B23.yg.ab1	8	0.0	agcctcccacatcatttgaa	aaagcccagcaactaccaca
LE1065	QG_CA_Contig2688	8	11.6	gtgaaaaccagccctaccaa	aggcttcccaacattcacac
CLS_S3_9019	CLS_S3_Contig9019	8	19.0	tctaccatgggcaagaccac	ccattcagaagtcgctccag
NL0935		8	21.9	gtgaaccaatgagtggagg	gaacatccacttggtccag
KLE0263	QGH6L10.yg.ab1	8	30.2	caacctcaccggagttttgt	gccggaaagtttgttgttgt
KLK1366	QGI7O15.yg.ab1	8	37.7	gaatcgctcaggcaaacaat	tggcctctcaagcagatttt
LE3019	QG_CA_Contig2149	8	51.4	attgctggagtcgtggtttc	ctttgtgcctcaaacccaat
NL0455		8	76.0	gacaagctcaaggcaactc	tgatcatctacatagcttcactg
CLS_S3_6304	CLS_S3_Contig6304	8	99.0	ctgtatgtggtccggcaagt	tcatcccgccataaccataa
NL0159		8	107.3	atgtgtaaccagtcggagg	cctgaacgcaataacttcc
RIN4	Jeuken et al, 2009	9	6.7	cgagcagggaagagaatgag	tagagggagtcccatggcta
M431	QG_CA_Contig6010	9	20.0	gatcgatcgttcatcgttctc**t**ca	ttgttgaaacaagttcactatttgg
CLX_S3_6726	CLX_S3_Contig6726	9	31.1	ggacgatggttttggagcta	acgagcagcttcacgatttt
NL1302		9	42.8	tttccagatgaaatccctg	atcaatggcttcctgtgtc
KM2348	QG_CA_Contig2348	9	65.6	taaacttcgggacgaaccac	gccaaaatgcgaaagttgc
CLX_S3_8409	CLX_S3_Contig8409	9	70.0	tcccgataaagaccctgatg	aggaggaactgaacgatgga
CLX_S3_8498	CLX_S3_Contig8498	9	89.6	ggataggaggaggtgggaag	ggtcaccggctaatacctca
CLS_S3_7932	CLS_S3_Contig7932	9	91.0	acacaaaaccctgctcaacc	cgatcgaattgacgaccttt
QGC23M07	QGC23M07.yg.ab1	9	91.0	cgggatcttgaaaaggcata	cgcgtaacaaaacacattcg
CLX_S3_12996	CLX_S3_Contig12996	9	94.1	tcttggcctctcattgatcc	ccaacggggaacacaaatac
CLS_S3_4696	CLS_S3_Contig4696	9	104.3	aatctccagcttcgggtttt	actacgaaacgacccattgc
CLS_S3_4656	CLS_S3_Contig4656	9	110.4	ccgtatgccgttcatcttct	gcactccaattgaatgatcg
CLS_S3_3349	CLS_S3_Contig3349	9	113.2	ctttttggaaggcaatctgg	tccagggaaaaccatctttg
LE9038	QGD7H11.yg.ab1	9	114.3	ggatcaccatcatagtcagcttgt	gatggagcgtccgatcagtgtctg

[1] I. Paran and R. W. Michelmore, 1993. All markers starting with NL are SSR markers, other markers are EST based markers. Markers presented in grey and black are tested only in selected F_2 and BC_1sativa_FR respectively.

Table S2. Comparison of fraction of susceptible F_2 plants between the genotype groups of four resistance loci, bott_C1,mid_C6, mid_C7 and bott_C9, individually (**a**), in a two-way (**b1, b2, b3**) and in a three-way interaction (**c**).

a

Loci	F_2_GEO aa	F_2_GEO ab/bb	F_2_FR aa	F_2_FR ab/bb
bott_C1	.73	.12	.67	.17
	4:11 b	36:5 a	2:4 b	20:4 a
mid_C6	.64	.17	.75	.09
	5:9 b	35:7 a	2:6 b	20:2 a
mid_C7	.65	.09	.67	.22
	7:13 b	32:3 a	1:2 a	21:6 a
bott_C9	.83	.03	.40	.20
	3:15 b	37:1 a	6:4 a	16:4 a

b1

mid. C6	bott. C1 aa	bott. C1 ab/bb
aa	.86	.43
	1:6, b	4:3, b
ab/bb	.63	.06
	3:5, b	32:2, a

b2

mid. C6	mid. C7 aa	mid. C7 ab/bb
aa	.78	.40
	2:7, b	3:2, b
ab/bb	.55	.03
	5:6, b	29:1, a

c

mid_C6	bott_C1 aa / mid_C7 aa	bott_C1 aa / mid_C7 ab/bb	bott_C1 ab/bb / mid_C7 aa	bott_C1 ab/bb / mid_C7 ab/bb
aa	1.00	.80	.60	.43
	0:5 d	1:4 cd	2:3 bcd	4:3 bcd
ab/bb	1.00	.00	.14	.04
	0:6 d	5:0 ab	6:1 ab	43:2 a

b3

bott. C1	mid. C7 aa	mid. C7 ab/bb
aa	1.00	.20
	0:10, b	4:1, a
ab/bb	.30	.07
	7:3, a	28:2, a

Genotype of the four loci is based on the marker profiles from: QGF24O17 for bott_C1, LE0178 for mid_C6, NL0650 for mid_C7 and CLS_S3_3349 for bott_C9. The resistance, conferred by *L. saligna* alleles, was mainly dominant, therefore only the genotype groups with absence (homozygous *L. sativa*, "aa") and presence (heterozygous "ab" or homozygous *L. saligna*, "bb") of *L. saligna* alleles for each locus are shown. Gradual color scale is used to visualize differences in fraction of susceptible plants (susceptible / all plants) (green: higher proportion of resistant, to red: higher proportion of susceptible plants in the genotype class). Under each colored box the fraction of susceptible plants is indicated. Within sub-table **a** per individual locus, letters in common indicate that the fraction of susceptible plants between the two genotype combinations are not significantly different (Fisher's Exact test, α=0.05). For sub-tables **b1**, **b2**, **b3** and **c** the comparison between the 4 and 8 genotypes can be made within each table.

Table S3. Comparison of the average infection severity level of selected genotypes within populations BC_1sativa_FR and BC_1sativa_GEO (infection score in percentage). Genotypes of four resistance loci are compared per individual locus (**a**), in a two-locus (**b1**and **b2**) and in a three-locus interaction (**c**).

a

Loci	BC_1_FR aa	BC_1_FR ab	BC_1_GEO aa	BC_1_GEO ab
bott_C1	38	36	39	39
	39 a	33 a	22 a	35 a
mid_C6	44	28	36	41
	38 b	34 a	29 a	31 a
mid_C7	42	32	50	27
	34 b	38 a	29 b	33 a
bott_C9	39	33	45	4
	45 a	27 a	52 b	11 a

b1

mid_C6	bott_C1	BC_1_FR	BC_1_GEO
		aa	aa
aa	aa	44	36
		17 b	8 a
ab	aa	32	52
		22 ab	10 a
aa	ab	44	48
		21 b	13 a
ab	ab	21	43
		12 a	15 a

b2

mid_C6	mid_C7	BC_1_FR	BC_1_GEO
		aa	aa
aa	aa	50	57
		20 b	13 b
ab	aa	31	61
		14 a	11 b
aa	ab	38	30
		18 ab	9 a
ab	ab	27	34
		20 a	16 a

b3

bott_C1	mid_C7	BC_1_FR	BC_1_GEO
		aa	aa
aa	aa	47	51
		16 b	10 ab
ab	aa	38	60
		18 ab	14 b
aa	ab	31	36
		23 a	9 a
ab	ab	33	32
		15 ab	14 a

c

mid_C6	mid_C7	bott_C1	BC_1_FR	BC_1_GEO
			aa	aa
aa	aa	aa	52	39
			8 c	4 a
ab	aa	aa	42	64
			8 bc	5 a
aa	ab	aa	38	32
			9 bc	4 a
ab	ab	aa	27	39
			14 ab	5 a
aa	aa	ab	49	61
			12 c	8 a
ab	aa	ab	16	59
			6 a	6 a
aa	ab	ab	38	28
			9 bc	5 a
ab	ab	ab	26	33
			6 ab	8 a

Genotype of the four loci is based on the marker profiles from: QGF24O17 for bott_C1, LE0178 for mid_C6, NL0650 for mid_C7 and CLS_S3_3349 for bott_C9. For BC_1sativa_FR plants with hybrid necrosis symptoms were excluded. For BC_1sativa_GEO, the plants that were heterozygous at bott_C9 were excluded from the interaction analyses (**b1, b2, b3, c**). Within sub table **a** per individual locus, letters in common indicate that the average infection severity level between the two genotype combinations within each population are not significantly different (Fisher's Exact test, α=0.05). For sub-tables **b1**, **b2** and **c** the comparison between the 4 and 8 genotypes can be made within each population and within each table. Gradual color scale is used to visualize differences in average infection severity level. Under each colored box the number of plants within each genotype group is indicated.

CHAPTER 6

Specific *in planta* recognition of two GKLR proteins of the downy mildew *Bremia lactucae* revealed in a large effector screen in lettuce

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Key message :

A screen for 34 *B. lactucae* candidate effectors in a collection of *Lactuca* spp revealed two *in planta* recognitions, for effectors BLG01 and BLG03. BLG01 recognition was observed in the majority of the tested *L. saligna* accessions and mapped to Chromosome 9. BLG03 recognition was observed in two *Dm2*-containing *L. sativa* lines and mapped to the *RGC2*-cluster at Chromosome 2.

Abstract

Breeding lettuce (*Lactuca sativa*) for resistance to the downy mildew pathogen *Bremia lactucae* is mainly achieved by introgression of dominant downy mildew resistance (*Dm*) genes. New *B. lactucae* races quickly render *Dm* genes ineffective, possibly by mutation of recognized host-translocated effectors or by suppression of effector-triggered immunity. We have previously identified 34 potential RXLR (-like) effector proteins of *B. lactucae* that were here tested for specific recognition within a collection of 129 *B. lactucae*-resistant *Lactuca* lines. Two effectors triggered a hypersensitive response: BLG01 in 52 lines, predominantly *L. saligna*, and BLG03 in two *L. sativa* lines containing *Dm2* resistance. The N-terminal sequences of BLG01 and BLG03, containing the signal peptide and GKLR variant of the RXLR translocation motif, are not required for *in planta* recognition, but function in effector delivery. The locus responsible for BLG01 recognition maps to the bottom of lettuce Chromosome 9, whereas recognition of BLG03 maps in the RGC2 cluster on chromosome 2. *Lactuca* lines that recognize the BLG effectors are not resistant to *B. lactucae* isolate Bl:24 that expresses both BLG genes, suggesting Bl:24 can suppress the triggered immune responses. In contrast, lettuce segregants displaying *Dm2*-mediated resistance to *B. lactucae* isolate Bl:5 are responsive to BLG03, suggesting that BLG03 is a candidate Avr2 protein.

Introduction

The lettuce downy mildew pathogen, *Bremia lactucae*, causes large losses in susceptible host plants and has been classified as a pathogen with a high risk of quick adaptation to lettuce resistance traits and chemical control (Brown et al. 2004; McDonald and Linde 2002). Its large effective population size, high gene flow and mixed (both sexual and asexual) reproductive system contribute greatly to this risk. *B. lactucae* belongs to the order Peronosporales, an order of the oomycetes that includes downy mildew- and *Phytophthora* species. The downy mildews are obligate biotrophs that are found on many plant species, including Arabidopsis (*Hyaloperonospora arabidopsidis*), cucurbits (*Pseudoperonospora cubensis*), grapevine (*Plasmopara viticola*), and sunflower (*Plasmopara halstedii*). Obligate biotrophs depend on the living host for their growth and reproduction (Kemen and Jones 2012; Spanu 2012). Therefore, for successful infection it is of prime importance that biotrophs cope with inducible defences of the host, which can be described as consisting of two overlapping layers of plant immunity (Boller and He 2009; Jones and Dangl 2006; Thomma et al. 2011). The first is triggered by the recognition of pathogen-derived molecules termed pathogen-associated molecular patterns (PAMPs) by transmembrane pattern recognition receptors (PRRs), and is referred to as PAMP-triggered immunity (PTI). Though pathogens can avoid inducing PTI (e.g. by masking PAMPs), a more common mechanism is the suppression of PTI within the host cell (Jones and Dangl 2006; O'Connell and Panstruga 2006). Pathogens can achieve this by translocating proteins (effectors) into host intracellular compartments where they can manipulate cellular processes (e.g. the suppression of plant defence responses), leading to effector-triggered susceptibility (ETS). Gram-negative bacterial pathogens deploy a type III secretion system to bring effectors into the host cytoplasm by means of a pilus-like structure (reviewed by Büttner and He, 2009). Oomycetes get in close contact with host cells by penetrating the plant-cell wall and invaginating the plant cell membrane to form haustoria, feeding structures that are thought to contribute to pathogenicity (Avrova et al. 2008; Kemen and Jones 2012; Whisson et al. 2007). From the haustoria, effectors are secreted from the pathogen before they cross the host membrane (Whisson et al. 2007). In the case of the order Peronosporales, two main classes of effectors that enter host cells have been defined: Crinklers and RXLR proteins (reviewed by Stassen and Van den Ackerveken 2011). The canonical RXLR effector contains an N-terminal signal peptide, and a translocation domain that contains an RXLR amino acid motif and, optionally, a dEER motif. The C-terminal part of the protein, following the translocation domain, is referred to as the effector domain. Variations in the presence and exact sequence of motifs in the translocation domain have been observed, e.g., QXLR motifs in *P. cubensis* effectors (Tian et al. 2011) or the presence of an EER motif but not of an RXLR motif in *H. arabidopsis* ATR5 (Bailey et al. 2011).

A second layer of plant defence is triggered when host cells recognise pathogen effectors. This effector-triggered immunity (ETI) is mediated by resistance (R) proteins, which recognize

effectors or their activity on host targets. Most known R-proteins belong to the family of cytoplasmic nucleotide binding (NB) and leucine-rich repeat (LRR) proteins (Dangl and Jones 2001; Jones and Dangl 2006; Takken and Goverse 2012). Defence triggered by recognition of effectors by R-proteins is often associated with the hypersensitive response (HR) that is visible as programmed cell death of host tissue. Effectors that are recognised by host R-proteins and trigger HR are termed avirulence proteins (AVRs). All AVRs cloned from oomycete pathogens thus far are RXLR and RXLR-like effectors (Na et al. 2013; Song et al. 2013; Stassen and Van den Ackerveken 2011; Wang et al. 2011), with the exception of ATR5 (Bailey et al. 2011). RXLR effectors are predicted to be present in large numbers in the genomes of oomycetes belonging to the order Peronosporales, from 134 in *H. arabidopsidis* (Baxter et al. 2010) to 563 in *P. infestans* (Haas et al. 2009). RXLR effectors are highly diverse between species (Jiang et al. 2008) and can also be differentially present within different isolates of a pathogen species (Cabral et al. 2011; Wang et al. 2011). Oomycete pathogens rapidly evolve to overcome R-protein-mediated recognition or ETI by (i) amino acid substitutions in the effector protein (Armstrong et al. 2005; Gilroy et al. 2011), (ii) by down-regulation, loss, or silencing of the effector gene (Na et al. 2013; Qutob et al. 2009) or premature stop codons in the sequence (Song et al. 2013), or (iii) by suppression of ETI by other effectors (Fabro et al. 2011; Guo et al. 2009; Wang et al. 2011). Therefore, resistance is the outcome of a complex network of interactions between effectors and components of the host's defence machinery. Unravelling such a network requires knowledge about the individual interactions between effectors, R-proteins and host targets.

The interaction between lettuce and *B. lactucae* has been extensively studied as a host-pathogen model for gene-for-gene interactions (Michelmore and Wong 2008). More than 40 major downy mildew-resistance (*Dm*) genes are known, as well as minor-effect resistance genes that may confer partial or field resistance. A single *Dm* gene, *Dm3*, has been cloned and is part of a large locus of several megabases known as *R* gene candidate *2* locus (*RGC2* locus, (Meyers et al. 1998) that contains at least 30 other NB-LRR genes (Kuang et al. 2004).

Cultivated lettuce (*Lactuca sativa*) can be crossed with some difficulty, with wild lettuce species that include *Lactuca* species that are considered *B. lactucae* non-hosts (e.g., *L. saligna*). These wild lettuce species provide a pool of genetic material from which new *Dm* genes and resistance QTLs have been identified (Jeuken and Lindhout 2002; Van Treuren et al. 2011; Zhang et al. 2009a). Dominant resistance genes have been extensively used to breed *B. lactucae*-resistant lettuce cultivars. However, turnover of *Dm* genes has been rapid, because *B. lactucae* is quick to adapt to newly introduced resistance genes. To understand the molecular basis of ETI in the lettuce-*B. lactucae* interaction and to identify new *R*-genes for resistance breeding, we deployed a selection of 34 *B. lactucae* candidate RXLR and RXLR-like effectors, which were previously identified by transcriptome sequencing (Stassen et al. 2012), to screen a large collection of lettuce breeding lines for new recognition specificities. We discovered two proteins,

containing the newly found RXLR-like variant GKLR, that are recognised *in planta*, one of which is recognised by *L. sativa* cultivars containing the *Dm2* gene.

Materials and Methods

Cloning and sequencing

Effector predictions were sequence verified from PCR product using flanking primers and sequenced by Macrogen Inc (Seoul, Korea). All primers used in this study are listed in Supplementary Table 4. Verified effector candidates were TOPO-cloned into the pENTR vector using the pENTR/D-TOPO Cloning kit (Invitrogen, Carlsbad, CA, U.S.A.) according to manufacturer's instructions starting from the sequence after the predicted signal peptide cleavage site of the effectors, unless otherwise indicated, and were preceded by a newly introduced start codon. Constructs were then recombined into the pK2GW7 vector (Plant-Systems Biology VIB, Ghent, Belgium). Clones were electro-transformed into Agrobacterium tumefaciens strain C58C1 (pGV2260). Clones were electro-transformed into *Agrobacterium tumefaciens* strain C58C1_pGV2260. The PsojNIP and YFP constructs are described by Cabral and associates (2012). For sequencing of effector alleles, DNA was PCR amplified using primers flanking the coding sequence, blunt-end ligated into pJET1.2 (Fermentas, Vilnius, Lithuania), and transformed into *Escherichia coli* DH5α by heat-shock. Plasmid isolation and sequencing was carried out by Macrogen Inc.

Lettuce lines for the effector recognition screen

Lettuce lines were chosen on the basis of the differential sets EU-A and EU-B proposed by the International Bremia Evaluation Board (IBEB, http://www.worldseed.org/isf/ibeb.html), a set of resistant accessions proposed by Michelmore and Ochoa (1994), parents of RIL-populations segregating for *B. lactucae*-resistance (Grube and Ochoa 2005; Hand et al. 2003; Jeuken et al. 2001; Truco et al. 2007), and other known sources of *B. lactucae*-resistance (Hagnefelt and Olsson 1999; Lambalk et al. 2000; Van Treuren et al. 2011). Also one resistant *L. aculeata* and two resistant *L. altaica* accessions (Van Treuren et al. 2011) were included to widen genetic diversity.

Agrobacterium-mediated transient transformation assay

A. tumefaciens strains containing the 35S-effector T-DNA were grown overnight in selective media at 28°C and 220 rpm. Cells were spun down at 2500 g for 10 minutes and resuspended in induction medium (1x M9 salts, 1% glucose, and 50μM acetosyringone) with appropriate antibiotics. After growth for 4 h at 28°C and 220 rpm, cells were spun down at 2500 g for 10 min and re-suspended in infiltration medium (0.5x Murashige and Skoog salts, 10 mM morpholinoethanesulfonic acid (MES), 0.5% fructose, 0.5% sucrose, 150 μM acetosyringone).

Strains were then pressure infiltrated into leaves using a needleless syringe. Responses in lettuce lines were scored 8 dpi, unless otherwise indicated. To test whether any of the 34 effectors are *recognised in planta*, we pressure-infiltrated suspensions of the *Agrobacterium* strains carrying the effector constructs into leaves of the selected lettuce lines. Visual responses to transient expression of effector candidates were scored 5 to 8 days after *Agrobacterium* infiltration. We included strains carrying a *YFP*- or *GUS*-containing vector as a negative control, and a necrosis-inducing protein (*NIP*) gene-containing vector as a positive control. Expression of *YFP* is not expected to elicit visible cell death; therefore, any response seen after infiltration with *Agrobacterium* carrying the *YFP* construct is considered background. The *NIP* gene encoding *PsojNIP* that is derived from *Phytophthora sojae* induces a cell death response in lettuce that is visible 1 to 2 days after infiltration of the *Agrobacterium* strain and develops into a dark necrotic lesion after 48 hours.

Time course q-PCR

For time-course experiments, one-week-old *L. sativa* cv. 'Olof' seedlings were spray-inoculated with 150 spores μl^{-1} until runoff was imminent. Seedlings were grown under high humidity (closed tray with transparent lid) at 17°C with 9 h of light (100 $\mu E/m^2/s$) and kept under these conditions for the duration of the experiment. Samples were taken and snap-frozen immediately after spraying and every 24 h until 5 dpi. Total RNA was extracted using the Spectrum plant total RNA Kit (Sigma Life Science, St. Louis) and treated with DNAse (Fermentas). cDNA was synthesised using RevertAid H minus Reverse Transcriptase (Fermentas) and Oligo(dT)15. Cycle thresholds (C_T) were determined in triplicate per transcript using the ABI PRISM 7900HT or the Life technologies ViiA7 system using SYBR Green as reporter dye. Expression levels were determined as the number of qPCR cycles required for the abundance of each amplicon to reach C_T level, and were normalised to *L. sativa* actin or *B. lactucae* actin (resulting in ΔC_T values).

Effector translocation assay

The method for testing the delivery of the effector domain of Avr3a (originally from *P. infestans*) from transgenic *P. capsici* into cells of *N. benthamiana* was performed essentially as described by Schornack and associates (2010). The coding sequences of BLG01 and BLG03, encoding the N-terminal region of de proteins containing the signal peptide and GKLR-DER domain, were amplified from cDNA and cloned into the pTOR: CRN16-AVR3a vector (Schornack et al. 2010) digested with ClaI/AscI to replace the CRN16 sequence with the BLG sequences. Transformation of *P. capsici* strain LT3112 was performed as described by Huitema and associates (2011). *P. capsici* transformants were inoculated on detached *N. benthamiana* leaves in the form of agar plugs from the border of radially grown colonies on V8 agar plates, and scored 4 to 7 days post inoculation. The reduction in the percentage of spreading lesions on R3a plants relative to that on

wild-type plants was calculated as 1- (% spreading lesions R3a / % spreading lesions WT) × 100, and was compared using analysis of variance (ANOVA) and *t* tests.

Codon-based test for positive selection

Insertions and deletions were removed from sequences and sequences with internal stop codons were removed. Synonymous (dS) and non-synonymous (dN) substitution rates were calculated using Nei and Gojobori's method (Nei and Gojobori 1986), using MEGA4 (Tamura et al. 2007). Standard error was determined by 500 bootstrap replications. The null hypothesis of no selection (dN = dS) versus the positive selection hypothesis (dN > dS) were determined using the Z-test: $Z = (dN-dS)/ \sqrt{[Var(dS)+Var(dN)]}$.

Materials for mapping the BLG01 response

Two crosses of *L. saligna* and *L. sativa* were previously made: cross 1 of *L. saligna* CGN05271 × *L. sativa* cv. 'Olof' and cross 2 of *L. saligna* CGN11341 × L. sativa cv. 'Norden' (Jeuken et al. 2001). Materials for assays 1, 2, and 3 were as follows. Assay 1: three replicates of 28 BILs derived from cross 1 that, together, cover 96% of the *L. saligna* CGN5271 genome (Jeuken and Lindhout 2004), and the parental lines of Cross 1 and Cross 2. Assay 2: F_3, F_4 and BC_1S_1 plants derived from cross 1 and cross 2. Assay 3: parental lines of cross 1 and cross 2, F_1 offspring of cross 1, and F_3 offspring of F_2 plants of both cross 1 and cross 2, with a recombination near the C9 locus.

Marker development and genotyping

For fine mapping, new markers were developed and selected to saturate the region. Based on alignment of our F_2 map (improved version of (Jeuken et al. 2001) with the *L. sativa* x *L. serriola* RIL map by the Lettuce SFP Chip Project (http://chiplett.ucdavis.edu) and the Compositae Genome Project Database (CGPDB) (http://compgenomics.ucdavis.edu), we selected candidate EST sequences and markers. Primers were developed, tested, and, in case of polymorphism, run on the segregating populations for mapping. Polymorphisms between *L. sativa* and *L. saligna* PCR products of the EST markers were visualized by high-resolution melting curve differences on a LightScanner System (Idaho Technology, Salt Lake City, UT, U.S.A.). All plants with a cell death response in transient assay 2 and all the plants from assay 3 were genotyped using the new markers.

Disease test on adult plants

A detached-leaf assay was conducted on adult plants (37 days after sowing) according the protocol of ADT_G, as previously described (Jeuken et al. 2008). Two genotyped BC_1S_2 populations from BC_1S_1 plant that showed a cell death response towards BLG01 were tested with

B. lactucae Bl:24. From each plant, at least six leaf squares were collected (2.5 by 2.5 cm). Leaf squares were inoculated with inoculum from *B. lactucae* isolate Bl:24 containing 4 x 105 spores per ml. At 9 dpi, the percentage of the area of each leaf square that showed *B. lactucae* sporulation was determined. One-way ANOVA was used to analyse the data, with line (offspring from one parent with genotype at C9 locus) as fixed factor and each different plant as block factor. For comparison between the heterozygous and homozygous *L. sativa* genotypes from each BC_1S_1 parent with each other, a Duncan's multiple range test ($\alpha = 0.05$) was performed with GenStat (14th edition) software.

Results

Screening Lactuca accessions for recognition of 34 B. lactucae effectors

Previously, we predicted 77 potential RXLR and RXLR-like effectors from our *B. lactucae* transcriptome data (Stassen et al. 2012). From these, we cloned a set of 16 full-length effector candidates and, from previous *B. lactucae* transcript assemblies, we cloned and verified 12 potential effectors. Finally, we obtained the full coding sequence of six potential effectors that were not full-length in the assembled 454-transcript data by using short reads of spore-derived genomic DNA or by 3' rapid amplification of cDNA ends. The coding sequences of all 34 B. lactucae effector candidates were cloned in a GATEWAY ENTRY plasmid and confirmed by Sanger sequencing (see Supplemental Information 1). A summary of features of the candidate effector proteins is given in Table 1 for 30 *B. lactucae* RXLR (BLR) proteins that contain the canonical RXLR motif and 4 candidates with an alternative RXLR-like motif that were predicted based on their similarity to effectors of other oomycetes and by comparison with a hidden Markov model based on the amino acid sequence surrounding the RXLR motifs of the BLRs. In three of these RXLR-like motifs, the first residue is not an arginine but a glycine (named BLG01, BLG02 and BLG03), and in the fourth RXLR-like protein this residue is a glutamine (named BLQ01). EER-like domains (rich in E,Q,D or N residues, preferably ending in R or K) were found in the 25 of the 34 effector candidates, including in the BLG proteins that have a conserved DER sequence. The length of the predicted effector proteins varied from 65 to 463 amino acids, with an average of just under 180 amino acids.

Table 1: Overview of cloned *B. lactucae* effector candidates. The first RXLR or RXLR-like motif and position within 100 amino acids from the start codon are indicated. The first EER or EER-like motif and position of which the first amino acid is within 20 amino acids of the first amino acid of the RXLR motif are shown. Source: M = main assembly, 3 = 3' rapid apmpification of cDNA ends, E = transcript extended with SOLiD data, P = preliminary assemblies.

				RXLR-like		EER-like	
ID	**Source**	**Contig ID**	**length**	**start**	**motif**	**start**	**motif**
BLR01	P	-	86	42	RKLR	52	EQK
BLR02	P	-	146	85	RLLR		
BLR03	P	-	141	48	RFLR	59	EEER
BLR04	P	-	76	45	RELR	60	DIK
BLR05	P	-	97	32	RALR	58	DED
BLR06	P	-	281	46	RCLR		
BLR07	P	-	253	47	RALR	68	EEER
BLR08	P	-	135	38	RLLR		
BLR09	P	-	112	37	RRLR	81	EER
BLR10	P	-	112	37	RRLR	81	EER
BLR11	P	-	463	46	RRLR	57	DESER
BLR12	P	-	123	49	RYLR	61	ELEK
BLR13	M	16131	363	44	RRLR	55	EER
BLR14	M	29191	75	46	RKLR		
BLR15	M	50216	102	47	RSLR	60	DEER
BLR16	M	32917-1	98	47	RSLR	60	NDER
BLR17	M	18684	282	50	RRLR	64	DAEK
BLR18	M	48006	92	46	RALR	55	NEDR
BLR19	M	31910	160	45	RLLR	54	DNNEER
BLR20	M	45396	130	53	RLLR	69	DEAD
BLR21	M	33962	65	39	RILR		
BLR22	M	43968	185	33	RGLR		
BLR23	M	24965	107	57	RSLR	62	DENR
BLR24	M	43687	91	55	RSLR	74	ELEQ
BLR25	M	48013	82	55	RALR		
BLR26	E	16394	187	46	RRLR	59	QNDER
BLR27	E	38529	434	40	RQLR		
BLR28	3	08983	279	49	RRLR		
BLR29	3	43449	311	35	RMLR	46	EES
BLR30	3	50216	101	47	RSLR	60	DEER
BLQ01	3	59265	79	49	QLLR	61	DEEQR
BLG01	M	25695	336	44	GKLR	57	DER
BLG02	M	31920	233	44	GRLR	57	DER
BLG03	M	23857	243	42	GKLR	55	DER

Recognition of effectors *in planta* can be efficiently tested by transient expression of the corresponding *B. lactucae* coding sequences using *Agrobacterium tumefaciens* for delivery to plant cells. Because all known RXLR(-like) effectors that are recognized by plant R proteins are host-translocated, we engineered the coding sequences such that the signal peptide sequences were removed (ΔSP) and replaced by new start codons in front of the predicted signal peptide cleavage sites. Infiltration of lettuce leaves with *Agrobacterium* species carrying a T-DNA vector with the ΔSP coding sequences under control of the 35S *Cauliflower mosaic virus* promoter

results in production of the ΔSP-effector protein in the plant cell cytoplasm, the same location at which the protein is thought to arrive when secreted from *B. lactucae* and translocated into the host cell. Intracellular recognition of effectors by *R*-gene encoded proteins is expected to induce a clearly visible cell death response, also referred to as the hypersensitive response (HR). Because no *B. lactucae* effectors are known, thus far, that trigger HR in *Lactuca spp.,* we made use of the cell-death-inducing protein PsojNIP of the oomycete *P. sojae* that was previously used as a positive control for transient expression in *L. sativa* (Jeuken et al. 2009). Necrotic lesions developed, starting from 2 days post *Agrobacterium* species infiltration, in response to transient expression of *PsojNIP* but not in response to expression of either yellow fluorescent protein (YFP*)* or β-glucuronidase (GUS). Results were similar in all other *Lactuca* species tested, e.g. in *L. saligna* and *L. acaleata* (Supplemental Figure 1).

In total, 129 *Lactuca* accessions or lines were selected, representing a broad range of *B. lactucae* resistances, including dominant *Dm* genes and partial or quantitative resistance traits, as well as additional genetically unknown forms of resistance to *B. lactucae* (see Supplemental Table 1). The collection of 129 *Lactuca* lines was screened for effector recognition by *Agrobacterium* infiltration of the 34 different effector constructs. *Agrobacterium*-mediated transient expression was robust in nearly all tested lines, as can been seen from the cell death response that is induced by *PsojNIP* expression. None of the tested lines showed a strong response to *Agrobacterium* with the *YFP* construct. Average scores were given per lettuce-effector combination based on the presence of no or few visible symptoms (0), strong yellowing of the leaf (1) or cell death (2). The responses of the *Lactuca* lines to each of the 34 effectors, defined as an average score from at least two replicates, are summarized in Supplemental Table 2. Two of the 34 effectors, BLG01 and BLG03, induced clear cell death responses (average symptoms scores ≥ 1.3) in several *Lactuca* lines. Four other effectors were found to induce cell death in a single line in one experiment; however, the specific recognition could not be confirmed for these combinations.

Recognition of the RXLR-like proteins BLG01 and BLG03

A variant of the mature (without signal peptide) protein, $BLG01^{E}$ - based on an allele from *B. lactucae* isolates NL519 and F703, was found to induce a stronger response than the protein encoded by our reference strain Bl:24 ($BLG01^{A}$). Of the 129 tested lines 41 gave a strong cell death response to $BLG01^{E}$. Weak or inconsistent responses to $BLG01^{A}$ and $BLG01^{E}$ were observed for 16 and 8 *Lactuca* lines, respectively (reviewed in Supplementary Figure S2). The higher number of $BLG01^{E}$ -responsive lines is likely due to a stronger response that is more easily detected, because most lines that showed a weak response to $BLG01^{A}$ showed a strong response to $BLG01^{E}$. Additionally, the response of 16 lines is only visible with $BLG01^{E}$ but not with $BLG01^{A}$. Most of the *Lactuca* lines in our collection that showed a response to BLG01 were of the *L. saligna* species. BLG01 recognition in a few responsive *L. serriola* and *L. virosa* lines

could not be confirmed in additional transient expression experiments (Supplementary Table S3), suggesting that BLG01 recognition is specific for *L. saligna*.

A second effector, BLG03, was specifically recognised in two *L. sativa* lines, Amplus and UCDM2 (Figure 1). In all other lines BLG03 did not induce responses that could be distinguished from the GUS negative control (e.g., in Olof, Figure 5). The set of cultivated lettuce lines (*L. sativa*) included in the screen contains differential lines that provide a wide range of genetically known *R*-genes and can be used to determine *R*-gene specificities. Both Amplus and UCDM2 contain the *Dm2* resistance specificity that is absent in all other *L. sativa* lines tested. Therefore, *Dm2* could mediate the recognition of BLG03.

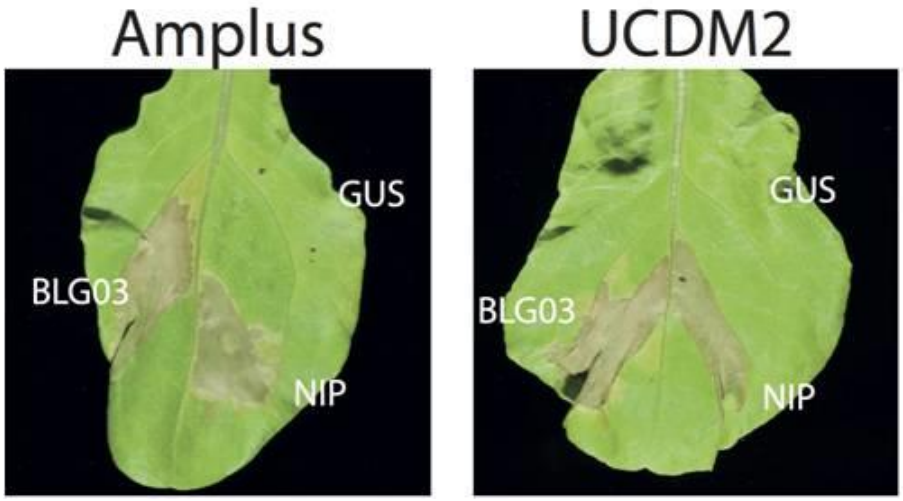

Figure 1. Hypersensitive response triggered by BLG03 in *L. sativa* cultivars Amplus and UCDM2. β-Glucuronidase (GUS) and PsojNIP (NIP) serve as controls for responses to *Agrobacterium* and successful T-DNA transfer, respectively. Pictures were taken 6 days post inoculation.

Strikingly, BLG01 and BLG03 both contain the RXLR-like motif GKLR. In addition, the signal peptide- and GKLR-containing N-termini of BLG01 and BLG03 are 63% similar (Figure 2). The N-terminus of a third *B. lactucae* protein, BLG02, also shows homology to these effectors. However, BLG02 is not recognised in any of the lettuce lines tested. The GKLR and DER motifs are identical in BLG01 and BLG03, whereas BLG02 has a GRLR variant of the RXLR motif. The effector domains (C-terminal of the DER motifs) do not share the high level of similarity that is seen for the signal peptides and $G^{K}/_{R}$LR-containing N-termini, but are only 45% similar.

BLG01, BLG02 and BLG03 do not have significant homology to any sequences in the National Center for Biotechnology Information non-redundant protein database (e-value < 1e-3), nor are there any significant matches to Pfam domains (Finn et al. 2010). The best BLAST matches in a combined database of oomycete proteins (*H. arabidopsidis*, *P. infestans*, *P. ramorum*, *P. sojae*, *Pythium ultimum* and *Saprolegnia parasitica*) were to a putative *P. infestans* RXLR effector (PITG_15128, e-value 0.037) for BLG01, a *P. sojae* RXLR effector (Ps_133875, e-value 0.81) for BLG03, and a *P. ramorum* RXLR effector (Pr_97351, e-value 0.032) for BLG02 (Supplemental Information 2).

```
          <--Signal peptide --><--------Translocation domain-------->
BLG01     MVRVYVAALTGFLALSASASATLQLTSVNESLADAYDSTAPARGKLRAYAATNVESDERA 60
BLG02     MVRIYVAALTVVLAFSASCSATSPLTLAKATPVTANGSGGRAQGRLRAHTTTNVEIDERS 60
BLG03     MVRVYVAALAAIFALSA--SATLHLTLANASSVTTEEGDGRPQGKLRVNAATNVESDER- 57
          ***:*****: .:*:**  ***  ** .: : . :  . . .:*:**. ::**** ***

BLG01     FSNLLEHLKTLNVLFSPLSKERMEAAISKSDSSVVKHLSEDGAVNEKKLGIGRDGVKAFE 120
BLG02     ISDVIYAIARQVADHRSNLVQAPKRTYTTYLATLDLSLDKVLEQNWSELRLLY--VKQTK 118
BLG03     FLDGLKALVRGFYDLTPFAPRFQKDTYSNLLLKLNLSFEQFSGKDTLQLRRLYDAARRHN 117
          : : :  :        .   .  : : :.   .:   :.:    :  :*      .:  :

BLG01     PAKVSKVKMILEDIFRKPYNKILLKVLSKANGGERNLVRNLAQAEMLGYKVFFLKSTLAS 180
BLG02     RKRPKDFVSMYELLVKK------------------------------YGLLKLTDMMKK 147
BLG03     KVNPTNPVSVYTGLVEK------------------------------YGEFEIVNMVYS 146
            . ..   :   :..*                                *  : : . : .

BLG01     KWERDGVSLMDVWSYICKDVKAPTEEEMKIFSHWCGYAFVLSAKGKFSAEETVIEKTLLK 240
BLG02     PDYISLSET-DNFKYLYDEASVYWR-KNSVLK---QYASELYDSKGNSKTFDVTDFESLQ 202
BLG03     LKHAQSSKSRDVLKRLGKEEKWYWKDKKDAGK---MYAEALDLGNEFTVKNMVSKLSKLE 203
              .  .  *  . : .: .   . : .  .    **  *      :    * .   *:

BLG01     VHNDNKNKKGKLAINMLAQIHLSRKFKVDANFFELYDPSPSSLKKLLLELEKSPRACDLD 300
BLG02     PFFERIGRRGD-----YLEITAAEHEKWKT---NKLRPT--------------------- 233
BLG03     QFLNRIDQKASEEQLKALVVADIERVKTKK---ESVSPSDVEM----------------- 243
           . :. .::..        :   .: * .    :   *:

BLG01     VELYRLLKQAVTDDKFMKGIWDRSTKKPEQPNAKRN- 336
BLG02     -------------------------------------
BLG03     -------------------------------------
```

Figure 2. Alignment of the amino acid sequence of candidate effectors BLG01, BLG02 and BLG03. Signal peptide and translocation domains are indicated above the alignment and the GXLR and DER motifs are underlined in the alignment. Symbols under the alignment indicate the degree of conservation of the above residues and indicate identical (*) residues, the presence of conserved substitutions (:) or semi-conserved substitutions (.). Numbers to the right of the alignment indicate the residue number of the last residue in the column counted from the start of the protein, skipping gaps.

BLG01 and *BLG03* expression

In order to be recognized *in planta* these $G^K/_RLR$ effectors need to be expressed during the infection process. Expression *in planta* was already observed, because the effector transcripts were previously identified by transcriptome sequencing of infected lettuce leaves (Stassen et al. 2012). To determine the changes in expression during the different stages of infection, we analysed a time-series by quantitative polymerase chain reaction (qPCR) (Figure 3). *L. sativa* cv. Olof seedlings were spray-inoculated with spores of *B. lactucae* isolate Bl:24, after which samples were taken every 24 hours, starting immediately after spraying. Within the first 24 hours, the majority of spores germinated and *B. lactucae* had penetrated the epidermis. Substantial *B. lactucae* hyphal growth and formation of haustoria in mesophyll cells occurred over the next four days. At six dpi, conidiophores formed and sampling was stopped. The expression of *B. lactucae* actin relative to *L. sativa* actin shows the substantial relative growth of *B. lactucae* throughout the

entire time course (Figure 3). Expression of *BLG03* increases immediately after inoculation and appears to be stable from one dpi onwards. *BLG01* gene expression decreases slightly during the course of infection, with expression levels comparable to those of *BLG03* at the later stages of infection. *BLG02* shows an increase of expression at the first day after inoculation similar to that of *BLG03*. After one dpi, expression levels of *BLG02* decline, approaching the level seen immediately after inoculation. Similar expression in time was detected in an independent biological replicate. These results confirm that the three *BLG* genes are expressed from 0 to 5 dpi suggesting that the effector proteins are produced at all stages of infection.

The GKLR domains of BLG01 and BLG03 are required for translocation, but not for effector recognition

The recognition of the BLG01 and BLG03 effectors is thought to occur in the host cell cytoplasm. To test whether the GKLR and DER motifs are needed for effector recognition, we investigated whether the effector domains of *BLG01* and *BLG03* are sufficient for recognition when expressed in lettuce. Using the same *Agrobacterium*-mediated transient transformation system as used to screen the collection of lettuce lines, we expressed BLG01$^{60\text{-}336}$ in the responsive *L. saligna* line CGN05271, and BLG03$^{58\text{-}243}$ in the responsive UCDM2 line. Cell death responses in the zones infiltrated with BLG01$^{60\text{-}336}$ did not differ from those infiltrated with BLG01 with translocation domain (BLG01$^{22\text{-}336}$) (Figure 4), indicating that the translocation domain (BLG01$^{22\text{-}60}$) is not required for *in planta* recognition in CGN05271. Similarly, the recognition of BLG03 is also independent of the translocation domain (BLG03$^{20\text{-}58}$), as shown by the cell death response induced by BLG03$^{58\text{-}243}$ (Figure 5).

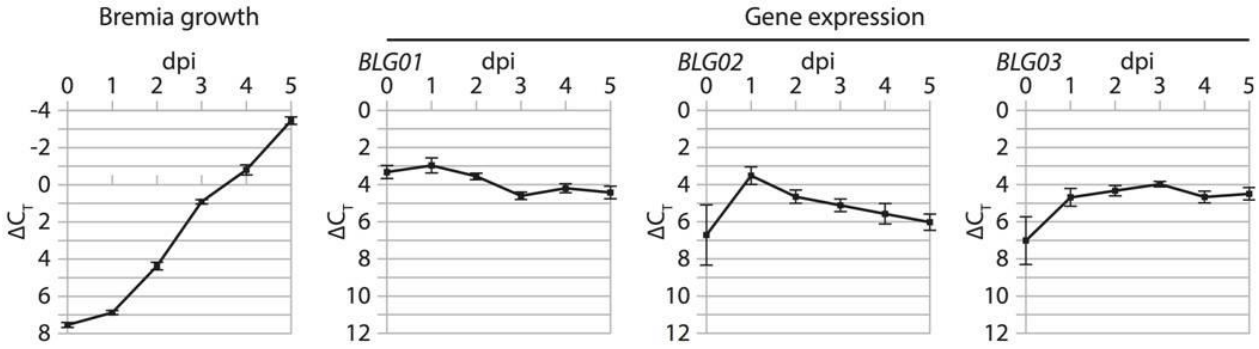

Figure 3. *B. lactucae* isolate Bl:24 growth and effector gene expression during infection of *L. sativa* cv. 'Olof'. Growth is inferred by the increase of *B. lactucae* actin relative to lettuce actin throughout the time course calculated as cycle threshold (ΔC_T). Effector gene expression is determined relative to *B. lactucae a*ctin. The difference in C_T required to reach threshold is given; because lower values indicate higher expression, the y axis has been reversed to ease interpretation.

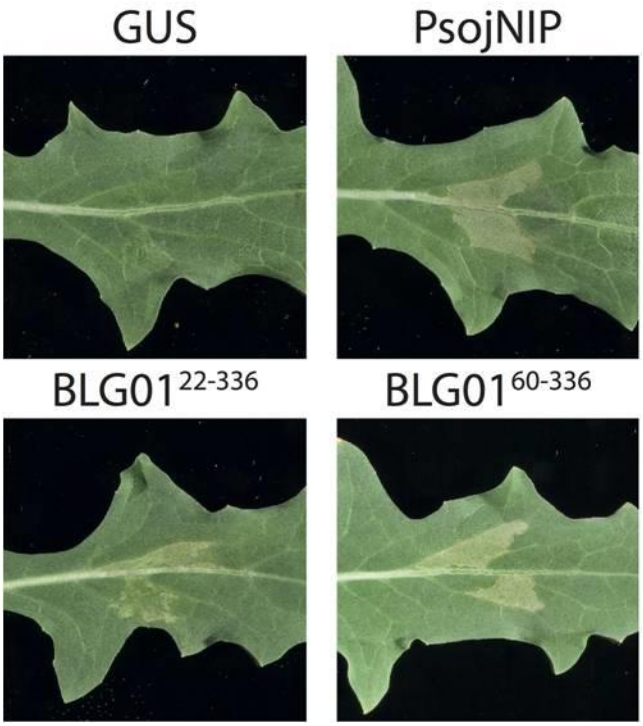

Figure 4. Recognition of BLG01 without signal peptide (BLG01$^{22-336}$) and without signal peptide and translocation domain (BLG01$^{60-336}$) in *L. saligna* CGN05271. β-Glucuronidase (GUS) and PsojNIP serve as controls for responses to *Agrobacterium* and successful T-DNA transfer, respectively. Pictures were taken 8 days post inoculation.

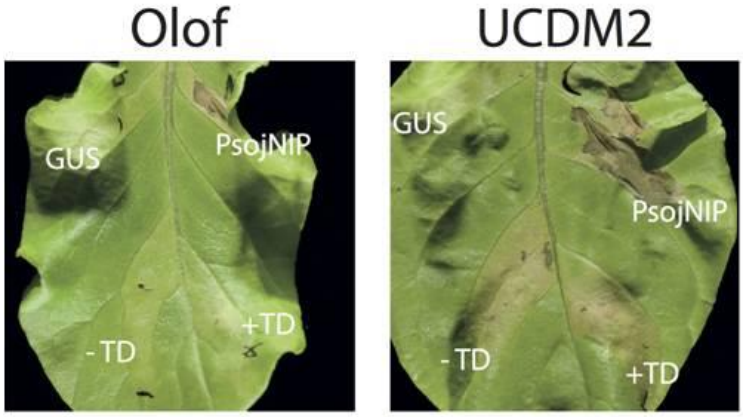

Figure 5. Recognition of BLG03 with (+TD; BLG03$^{20-243}$) and without translocation domain (-TD; BLG03$^{58-243}$) in *L. sativa* cv. 'UCDM' but not in *L. sativa* cv. 'Olof'. β-Glucuronidase (GUS) and PsojNIP serve as controls for responses to *Agrobacterium* and successful T-DNA transfer, respectively. Pictures were taken 6 days post inoculation.

To test whether the GKLR- and DER-containing domains mediate translocation of the effectors, we tested the translocation domain of BLG01 and BLG03 in the AVR3a- translocation assay in *P. capsici* (Schornack et al. 2010). The assay is based on recognition of transgenic *P. capsici* strains by *N. benthamiana* plants expressing R3a. The *P. capsici* strains express a fusion of the signal peptide and translocation domain of interest to the effector domain of AVR3a, which is

recognised intracellularly by R3a, only if the translocation domain is able to mediate translocation. *P. capsici* transformed with an empty vector was able to spread from the initial infection site in 100% of wild-type *N. benthiamana* plants and 70% of R3a expressing plants (Figure 6).

The CRN16:Avr3a fusion (Schornack et al. 2010) was used as positive control and transformants containing this construct caused spreading lesions from 95% of infection sites on wild-type plants, but only in 12.5% of infection sites on R3a expressing plants. *P. capsici* transformants expressing BLG01:AVR3a and BLG03:AVR3a fusions spread from 58% and 46% of infection sites, respectively, on wild-type plants. By contrast, no spreading lesions were observed on R3a-expressing *N. benthamiana* plants, strongly suggesting that the BLG01 and BLG03 GKLR and DER-containing domains mediate translocation. Transformants expressing BLG01:AVR3a and BLG03:AVR3a constructs in which the GKLR motif was substituted by AAAA caused more spreading lesions on R3a plants than the non-mutated BLG:AVR3a transformants, suggesting that disruption of the GKLR motif prohibits efficient translocation (Supplemental Figure 3).

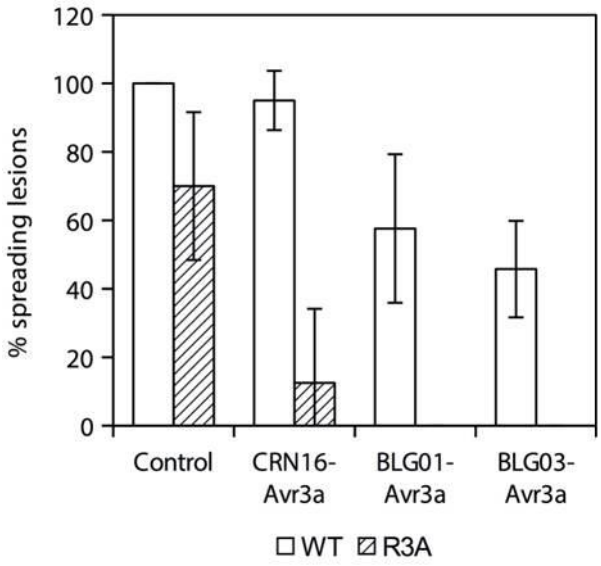

Figure 6. N termini of BLG01 and BLG03 allow translocation of AVR3a from *P. capsici* into *N. benthamiana* cells. Mycelial plugs of *P. capsici* strain LT3112 (control) and transformants thereof carrying the AVR3a fusion constructs were inoculated onto *N. benthamiana* wild-type (WT) and R3A leaves. Inoculation sites were scored for lesion formation 7 days after inoculation, and the percentage of spreading lesions was determined. Data represent three independent experiments. The reduction in the percentage of spreading lesions on R3a plants relative to that on WT plants was similar for CRN16-AVR3a, BLG01-AVR3a and BLG03-AVR3a, whereas the reduction in the percentage of spreading lesions was significantly lower in control ($p < 0.05$).

Allelic diversity of GXLR effectors

Allelic diversity of the three $G^K/_R$LR effectors in a selection of *B. lactucae* isolates was investigated to test if the proteins show signs of selection. A set of eight *B. lactucae* isolates was chosen as a group with high diversity based on their *R*-gene-specificities. Because *B. lactucae* is diploid, a single isolate can contain different alleles. To determine possible alleles, PCR products were cloned and, from each isolate, at least eight clones per gene were sequenced. Seven different alleles were found for BLG01 and BLG03, and six alleles for BLG02. The distribution of alleles is represented in Table 2 and the protein translations of the different alleles can be found in Supplemental Information 3.

Table 2: Distribution of alleles of *BLG01*, *BLG02* and *BLG03* over eight *B. lactucae* isolates.

Race	*BLG01*	*BLG02*	*BLG03*
Bl:5	C,D^x,F^y,G^z	A,E	B,G
Bl:16	A,B	A,B^3	A
Bl:17	na^w	A,C	D,F^2
Bl:24	A	A,B^3	A,C
NL519	E^1	B^3,C	A,D
F703	E^1	C,D	na
CA3	C	A,F	A
CA6	C	A	A,E

Abbreviation: na = not amplified.
[x] Premature stop codon before translocation domain
[y] Premature stop codon after translocation domain
[z] Encodes same protein sequence as A.

Two striking observations can be made regarding BLG01. First, as can be seen from the allele distribution, isolate Bl:5 appears to possess 4 alleles of this effector, which could be due to gene duplication or polyploidy. Secondly, the allele sequences reveal that no functional BLG01 proteins are encoded in isolates NL519 and F703 due to a nonsense mutation in the fifth codon (TAC→TAA, Y→stop). The same stop is found in one of the alleles of Bl:5, whereas two other Bl:5 alleles have premature stops at other positions. In one of these alleles the stop (CAG→TAG, Q→stop) is at amino acid position 24, the third residue after the predicted signal peptide cleavage site (SA|TL); in the other case, a two-nucleotide deletion in the effector domain (amino acid position 286) induces a frame shift that reads into a stop after two amino acids. In contrast to NL519 and F703, a full-length copy of the effector is present in Bl:5. For most isolates two different alleles of BLG02 and BLG03 were found. $BLG02^A$ and $BLG02^B$ encode the same protein sequence and only have synonymous nucleotide variants. All amino acid differences encoded in BLG02 alleles were found in the effector domain. This is in contrast to BLG03, in which amino acid polymorphisms are also found in the signal peptide and translocation domain. Although one allele has an insertion of two amino acids in the signal peptide, all *BLG03* alleles are predicted to encode a signal peptide. $BLG03^F$, only present in Bl:17, has a premature stop

codon in the effector domain. Furthermore, BLG03^{G}, found in Bl:5, is more sequence divergent from the Bl:24 reference sequence than all other BLG03 alleles. None of our effectors are predicted to be under positive selection to maintain amino acid diversity based on the ratio of synonymous to non-synonymous substitutions (codon-based test of positive selection averaging over all sequence pairs: BLG01: Z-score = 0.05790, p = 0.47696; BLG02: Z-score = 0.15217, p = 0.43965; BLG03: Z-score = -1.31461, p = 1.0). However, the many different alleles and, in particular, the nonsense alleles suggest that *BLG01* and *BLG03* have been under selective pressure.

The different *BLG01* alleles were next used to make constructs of the effector without signal peptide for *in planta* expression. Constructs with the effector domains of all *BGL01* alleles except for F (see Supplemental Information 1) were tested in *L. saligna* CGN5271 to determine their potential to trigger cell death. BLG01^{D} and BLG01^{G} did not appear to induce cell death in CGN5271. Strikingly, the effector domain of *BLG01^{E}* triggered the strongest responses when expressed in *L. saligna* CGN05271 and, as a result, provided more clear-cut scoring (Supplemental figure 4). However, because there is a premature stop in the signal peptide of BLG01^{E} this protein will not be produced by the *B. lactucae* isolates F703 and NL519.

BLG01 recognition in *L. saligna* CGN05271 is not linked to *B. lactucae* resistance

To genetically map the locus responsible for the response to BLG01, backcross inbred lines (BILs), which cover 96% of the *L. saligna* CGN05271 genome in a *L. sativa* Olof background (Jeuken and Lindhout 2004), were tested by transient *Agrobacterium*-mediated expression of the *BLG01* alleles A and E. None of the BILs showed a cell death response to BLG01 (Table 3). Because 4% of the *L. saligna* genome is absent in the set of 28 BILs, the locus could be located in one of the four chromosomal regions (bottom Chromosome 3, top Chromosome 5, top Chromosome 7, bottom Chromosome 9) that are not represented in the BILs (Jeuken and Lindhout 2004; Jeuken et al. 2008). From the original *L. saligna* CGN05271 x *L. sativa* cv. Olof F_2 mapping population (Jeuken et al. 2001) viable F_3, F_4 and BC_1S_1 families were obtained from selfed parental lines that were heterozygous or homozygous *L. saligna* at one of the four regions that were not represented in the BILs. Several families were found to segregate for cell death in response to both allele *BLG01^{A}* and allele *BLG01^{E}* (Table 3). Most F_3 plants that were responsive to the stronger BLG01^{E} allele were also responsive to the BLG01^{A} allele. Only six plants responsive to BLG01^{E} were not visually scored as responsive to BLG01^{A}, probably because of the weaker response to BLG01^{A}. This segregation was also observed in F_3 plants from a cross between the responsive *L. saligna* parent CGN11341 and the non-responsive *L. sativa* parent cv. Norden (Table 3). Comparing the parental genotypes from the families that showed cell-death revealed that the response to BLG01 was linked to a region at the bottom of Chromosome 9 that was always homozygous or heterozygous *L. saligna*, indicating that the trait is dominant. This was confirmed in four F_1 plants obtained from a cross between CGN05271 and Olof that were all

responsive to the effector (Table 3). The position of the locus on chromosome 9 was further confirmed and more precisely positioned using F_3 families, of which the F_2 parent had a recombination event near the candidate region. The locus could thus be mapped to a region of 4.4 cM between markers CLSX3110 and CLS_S3_4656 as shown by the genotype of four informative F_3 plants (Figure 7).

The BLG01-triggered response resembles ETI and, therefore, was expected to be causally linked to *B. lactucae* resistance. The responsive *L. saligna* line CGN05271 is a non-host for *B. lactucae*, because of multiple quantitative resistance loci (Zhang et al. 2009a; Zhang et al. 2009b); therefore, we tested segregating families for linkage of the response to BLG01 and the *B. lactucae* resistance phenotype. Both BC_1S_2 families obtained from BC_1S_1 plants 90_2 and 90_6 that were responsive to BLG01 were as susceptible to isolate Bl:24 as the *L. sativa* cv. 'Olof' parent (Table 4). We conclude that the locus for responsiveness to BLG01 does not confer resistance to *B. lactucae* isolate Bl:24 that expresses the recognized effector protein.

Table 3: Response to *BLG01* transient expression in *L. saligna* and *L. sativa* parental lines and progeny. Numbers between brackets indicate the number of families.

Lines/Populations	Assay 1 and 2			Assay 3		remark
	# plants tested	BLG01^E # cell death	BLG01^A # cell death	# plants tested	BLG01^E # cell death	
L. sativa cv. 'Olof'	6	0	0	3	0	Nonresponsive parent
L. saligna CGN05271	6	6	6	3	3	Responsive parent
Set of 28 BILs	84	0	0			
BC_1S_1	5	2 (1)	1 (1)			
F_3	48	15 (11)	13 (9)	74	19 (8)	
F_4	3	3 (1)	2 (1)			
F_1				4	4	
L. sativa cv. 'Norden'	4	0	0	2	0	Nonresponsive parent
L. saligna CGN11341	7	7	7	3	3	Responsive parent
F_3	14	7 (4)	5 (4)	3	3 (2)	

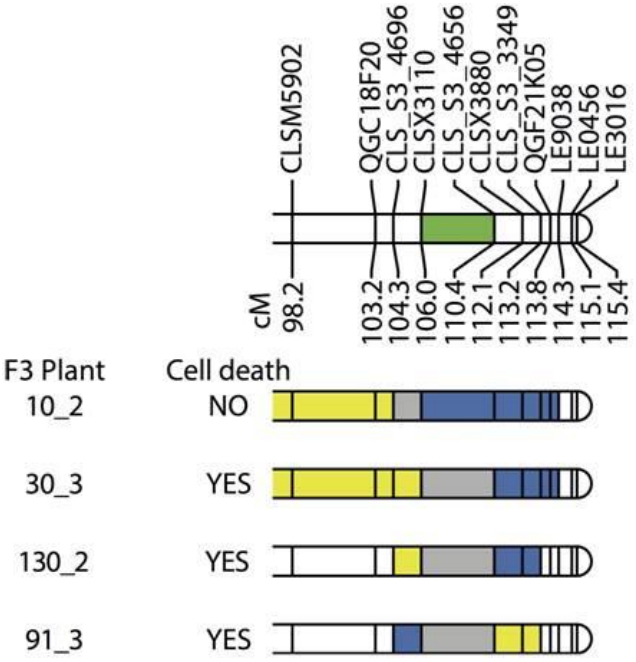

Figure 7: Locus of the BLG01 cell-death response in *L. saligna* CGN05271 at the bottom of Chromosome 9. Genotype graphs for four F_3 plants with the closest recombination are shown. Blue is homozygous *L. sativa* cv. Olof, yellow is heterozygous, grey represent intervals with a recombination event, and white means unknown genotype. Green indicates the smallest region in which the cell-death response to BLG01 is fine mapped.

Table 4: Disease assessment by detached leaf assay at adult plant stage, ADT_G with *B. lactucae* Bl:24 on genotyped BC_1S_2 populations from two BC_1S_1 plants that showed a cell-death response to $BLG03^E$.

plant	genotype bottom C9	# plants tested	# leaf segments	race Bl:24 ISL
BC_1S_2 90_2	homozygous *L. sativa*	3	18	84
	heterozygous	6	36	75
BC_1S_2 90_6	homozygous *L. sativa*	3	18	75
	heterozygous	6	36	92
L. sativa cv. 'Olof'	homozygous *L. sativa*	3	27	84

BLG03 recognition and *B. lactucae* resistance are linked to the *Dm2* gene

BLG03 is recognised by lettuce lines Amplus and UCDM2 (Supplemental Table 2 and Figure 1), which carry known resistance loci to *B. lactucae*. Amplus contains two *R*-genes, *Dm2* and *Dm4*, whereas UCDM2 contains a single locus, *Dm2*. Therefore, these two lines have the *Dm2* locus in common, which is absent from the other tested lines. This raised the possibility that the *Dm2*-encoded R-protein mediates BLG03 recognition. We investigated whether resistance to *B. lactucae* isolate Bl:5, to which lettuce *Dm2* confers resistance, co-segregated with the ability to recognise BLG03 in an F_2 population of a UCDM2 × Cobham Green cross, in which the *Dm2* resistance locus segregates. The response to BLG03 was determined by *Agrobacterium*

infiltration in leaves, and resistance to *B. lactucae* Bl:5 was determined in a leaf disc assay. Of 143 tested F_2 plants, all but 28 developed a clear cell death, in response to transient expression of BLG03 (four sites per plant), indicating that the ability to recognise the effector is a dominant trait. All plants that recognised BLG03 were resistant to Bl:5. Conversely, all plants that did not recognise BLG03 were susceptible to Bl:5. This indicates that *Dm2* or a closely linked gene from the *Dm2* background is required for recognition of BLG03.

To investigate the link between BLG03 recognition and *Dm2* further, we tested all available BLG03 alleles on 'Olof' (negative control), UCDM2, and Amplus. Alleles A to E were able to induce cell death responses in UCDM2 and Amplus. Alleles F and G did not induce a cell death response in any of the tested lines.

Because Amplus and UCDM2 are susceptible to *B. lactucae* isolate Bl:24, from which we initially cloned *BLG03*, we set out to investigate whether any of the other effectors cloned from Bl:24 could suppress the cell death response induced by BLG03. We inoculated a mixture (total optical density at 600 nm = 0.8) of *BLG03* and individual other effector genes at ratios of 1:2 (UCDM2) or 1:3 (Amplus). These ratios were determined as the lowest *Agrobacterium-BLG03*/*Agrobacterium-GUS* ratio at which a consistent cell death response could be seen. Using this setup, we did not find any reduction of the cell death responses induced by BLG03 in combination with any of the 33 other effectors compared to the combination of BLG03 and GUS. A similar setup using BLG01 in *L. saligna* CGN05271 also did not reveal any reduction of BLG01-induced cell death responses by other effectors.

Discussion

In planta effector recognition

Two *B. lactucae* RXLR-like effectors were identified that are specifically recognised in *Lactuca* breeding material. A relatively large number of wild lettuce accessions are capable of recognising BLG01 and mounting a cell death response. BLG03, in contrast, is recognised specifically in two cultivated lettuce lines that share the *Dm2* resistance specificity. Our *B. lactucae* effector screen of effectors for *in planta* recognition has uncovered potential gene-for-gene interactions. The screening of 54 effectors of the oomycete *P. infestans* in 10 wild *Solanum* genotypes by transformation with Potato virus X (PVX) uncovered 36 specific interactions (Vleeshouwers et al. 2008). In contrast, screening of 60 effectors of the downy mildew *H. arabidopsidis* in 12 *Arabidopsis thaliana* accessions using *Pseudomonas syringae pv.* DC3000 for delivery did not uncover any HR (cell death) upon effector delivery (Fabro et al. 2011).

Wild *Lactuca* species can be used to introgress *R* genes or QTLs into lettuce cultivars. For example, the *Dm3* resistance gene originates from a *L. serriola* accession but is very rare in natural populations, with only a single accession of 1,033 tested from 49 natural populations having an intact *Dm3* gene (Kuang et al. 2006). *Dm3* is a fast evolving *R* gene from the *RGC2*

locus. This locus encodes two types of *R* gene candidates, a fast evolving type and a type that evolves at a much slower rate and is more conserved among different accessions (Kuang et al. 2004). Recognition of the *B. lactucae* effector BLG01 appears to be relatively common in *L. saligna* species and to only occur sporadically in other species. The wide recognition of BLG01 may indicate recognition by a slowly evolving or more ancient *R* gene. The exact nature of the recognition of BLG01 in *L. saligna* and whether this recognition is dependent on the same gene in all lines remains to be determined.

The recognition of BLG01 in *L. saligna* CGN05271 was mapped at the bottom of Chromosome 9, where no *R* gene clusters to *B. lactucae* in *L. serriola* and *L. sativa* are known thus far (Kesseli et al. 1994; Truco et al. 2007). None of the 23 lettuce EST sequences from the Lettuce SFP Chip Project (http://chiplett.ucdavis.edu) that are mapped within the 4.4 cM region between our flanking markers (CLSX3110 & CLS_S3_4656) show homology to NB-LRR-like resistance proteins. We are aware of only three *R* genes from *L. saligna* that have been introgressed into *L. sativa*, none of which are located on Chromosome 9 (Moreau 1994; Segura et al. 2011; Van Ettekoven and Van der Arend 1999). Interestingly, the cell death response to the *P. syringae* effector AvrPto did map in the same region on Chromosome 9 in a *L. sativa* x *L. serriola* recombinant inbred line (RIL) population (McHale et al. 2009).

BLG03 is recognised in only two lettuce breeding lines of the initial screen. These lines share the *Dm2* resistance locus, which maps in or near the *RGC2* locus (Meyers et al. 1998). The *Dm2* locus provides resistance to Bl:5, and recognition of BLG03 correlated with resistance to Bl:5 in 143 F_2 plants of a UCDM2 × Cobham Green cross, in which *Dm2* segregates. This indicates that the response to BLG03 maps to the RGC2 locus in UCDM2. Sequencing of BLG03 in eight different isolates revealed seven different alleles. Of the sequenced *B. lactucae* isolates, Bl:5 and F703 are unable to successfully infect *Dm2*-containing hosts. We were not able to amplify BLG03 from *B. lactucae* isolate F703, although the quality of DNA was not a problem because we could amplify BLG01 and BLG02. Both Bl:5 BLG03 alleles (B and G) were not found in other sequenced *B. lactucae* isolates. One of the Bl:5 alleles ($BLG03^G$) did not induce a visible response in *Dm2*-containing lines but the other ($BLG03^B$) was recognised in the same lines as the Bl:24 reference allele ($BLG03^A$). The fact that resistance to isolate Bl:5 and recognition $BLG03^B$ are fully linked in a segregating F_2 population strongly suggests that the effector could be the avirulence protein Avr2 that is recognized by the *Dm2* gene.

Effector recognition versus resistance

In most reported cases, effector recognition is linked to resistance. Recognition of BLG01, however, was not linked to resistance to Bl:24 in laboratory assays, and BLG03 was cloned from a *B. lactucae* isolate that can successfully infect the lettuce lines in which BLG03 is recognised. Examples of a lack of correlation between recognition of an effector and resistance to pathogen isolates that express the effector have previously been reported. Screening of 54 *Phytophthora*

infestans effectors in *Solanum* species by PVX expression uncovered two interactions that were not correlated to resistance. In an F_2 population of a cross between a resistant and susceptible *Solanum* species, no correlation between the ability to recognise certain *P. infestans* effectors and resistance to *P. infestans* was found, despite perfect correlation between recognition of other *P. infestans* effectors and resistance (Vleeshouwers et al. 2008). Likewise, not all *A. thaliana* accessions that recognise ATR39-1 or ATR1Emco5 are resistant to isolates that encode these alleles (Goritschnig et al. 2012; Krasileva et al. 2011; Rehmany et al. 2005).
An explanation for the lack of resistance in plants that can recognise individual *B. lactucae* effectors is that *B. lactucae* uses additional effectors to suppress ETI. A study that investigated crosses of virulent and avirulent *B. lactucae* isolates revealed a possible locus that inhibits avirulence triggered by Avr5/8 but no evidence for other inhibitors of avirulence in *B. lactucae* (Ilott et al. 1989). The study highlights that inhibitor loci exist but that they are polymorphic. Since this study was performed, over 20 years of selective pressure on *B. lactucae* has given rise to new isolates that break various resistances. However, none of our 34 tested effectors were able to suppress BLG01- or BLG03-induced cell death. We cannot rule out the existence of suppressors of cell death because our selection of effectors is nonexhaustive and suppressors may even be non-RXLR effectors, which we did not investigate.

Findings described for the *P. infestans* effector AvrSmira2 (Rietman et al. 2012) provide an alternative explanation for an apparent lack of correlation between effector recognition and resistance. *Agrobacterium*-mediated transient transformation of potato cultivar ‘Sarpo Mira’ with *AvrSmira2* induced a cell death response and indicates the presence of a resistance factor named *Rpi-Smira2*. Cell death responses to *AvrSmira2* were found to segregate in offspring of a cross between an *Rpi-Smira2*-containing parent and a universally susceptible parent. As with our *B. lactucae* effector BLG03, no resistance to *P. infestans* strains containing *AvrSmira2* could be scored in plants that show a cell death response towards AvrSmira2 in laboratory assays. Field trials, however, revealed a partial resistance phenotype correlated with the ability to recognise *AvrSmira2*. Therefore, field trials in plants containing the *L. saligna* locus for recognition of BLG01 are important to determine whether the locus confers a partial resistance phenotype and if the locus is of value for breeding *B. lactucae*-resistant lettuce. Overall, our large effector screen in lettuce has demonstrated, in our opinion, that effector-based approaches are applicable to agriculturally important plant-pathogen systems, yielding both fundamental knowledge on recognition and leads for resistance breeding.

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CHAPTER 7

General discussion

Genetic dissection of nonhost resistance from *L. saligna*

Although nonhost resistance is the most common form of resistance, limited information is available about its genetic and molecular basis. Lack of knowledge of the nonhost resistance is mainly due to the fact that most nonhost species are not crossable with host species. Probably the basis of the nonhost resistance differs among plant species and is complex (Fan and Doerner, 2012).

We study the nonhost resistance to *B. lactucae* of *L. saligna* which is cross compatible with cultivated lettuce *L. sativa*. Our main aim of the research was to gain insight in the inheritance of the nonhost resistance of wild lettuce, *L. saligna,* to downy mildew, *B. lactucae*.

Former research with a small F_2 population and a set of BILs derived from one interspecific cross (*L. saligna* CGN05271 × *L. sativa* cv. Olof) indicated that the basis of nonhost resistance is not explained by major *R* genes (*Dm*-genes), but rather might be polygenic and explained by quantitative resistance loci (QTLs) (Jeuken and Lindhout, 2002; Jeuken and Lindhout, 2004; Zhang et al., 2009a). Research on the set of (28) BILs indicated the presence of quantitative resistance in five, eleven, six and nine BILs in the respective plant stages: seedling, young plant, adult plant (greenhouse) and adult plant (field test) (Zhang et al., 2009a). Plant stage dependent resistance was commonly observed and in only two BILs, BILs 2.2 and 4.2, the resistance was effective at all plant stages (Zhang et al., 2009a). At the start of this PhD Thesis (Chapter 1) we hypothesized, based on former research, that the *L. saligna* resistance might be explained by the joined effect of a certain number of previously identified quantitative resistances, which have been mapped in the introgressions of specific BILs. The research aims for this hypothesis were to: 1) determine the precise position of resistance QTLs within BIL-introgression segments and to investigate whether these genes can be deployed in breeding; 2) determine whether stacking of resistance QTLs from different BILs leads to near-complete resistance to *B. lactucae.*

For the first aim we studied the precise position of resistance QTLs within BIL-introgression segments 2.2, 4.2 and 8.2 which were 29, 49 and 29 cM long, respectively (Chapter 2). In previous studies BILs 2.2, 4.2 and 8.2 were most promising as their quantitative resistance was effective at (nearly) all tested plant stages and reduced infection by 60-70% at young plant stage and 30-50% at adult plant stage in field situations (which is most relevant for lettuce cultivation) (Zhang et al., 2009a).

By substitution mapping we mapped not one but multiple resistance loci within all three BIL introgressions. Resistances of the three BILs fragmented into multiple plant stage dependent sub-QTLs. The *L. saligna*-derived sub-QTLs had a smaller and plant stage dependent resistance effect, some segments reducing, others even promoting downy mildew infection. The sub-QTLs were positioned in windows of about 3 to 10 cM (Chapter 2). These findings differ from most published fine mapping studies on resistance QTLs in plants, as in those studies the QTLs do not

fall apart in multiple sub-QTLs. For example QTLs *lb4, lb5b,* and *lb11b* for resistance to *Phytophthora infestans* in tomato were mapped each as a single sub-QTL in a window of 6.9, 8.8 and 15.1 cM, respectively (Brouwer and St. Clair, 2004); QTL *Rphq2*, for resistance to leaf rust (*Puccinia hordei*) in barley in a window of 0.11 cM (Marcel et al., 2007). However linked (sub-) QTLs have been detected for other traits in several other studies. For example in rice a grain weight QTL, *qTGWT1-1,* was explained by two tightly linked sub-QTLs, *Gw1-1* and *Gw1-2* (Yu et al., 2008) and in rice a flowering time QTL *dth1.1* revealed two sub-QTLs (Thomson et al., 2006; Maas et al., 2010). In the stigma exertion (se2.1) QTL fine mapping study in tomato of Chen and Tanksley (2004), five tightly linked sub-QTLs were mapped of which three controlled stamen length, one style length and one affecting anther dehiscence.

To determine whether stacking resistance QTLs from different BILs leads to near-complete resistance to B. *lactucae,* we stacked the introgressions of eight different BILs in combinations of two. The best performing combination in the field, in combi-BIL line 1.2+8.2, showed on average 73% infection reduction, which is relatively high but not considered as (near)-complete resistance (Chapter 3). In contrast, at young plant stage stacking different combinations of intogressions lead in some cases to near-complete resistance (Zhang et al., 2009b, and Chapter 3). Of the ten developed double combination BILs one combination of introgressions indicated an additive effect on resistance level, eight combinations a 'less-than-additive' epistatic effect and one combination a 'more-than-additive' epistatic effect (Chapter 3).

Inheritance of the nonhost resistance of *L. saligna* to *B. lactucae*

In Chapter 1 we proposed the hypothesis that the complete nonhost resistance of *L. saligna* CGN05271 to *B. lactucae* is due to the cumulative and additive effects between several quantitative resistance genes. Based on the results in the present PhD study (Chapter 3) the proposed hypothesis cannot be accepted neither rejected. The hypothesis cannot be confirmed as we did not identify combinations of previously detected loci to explain (near-) complete resistance. The hypothesis is not rejected as we tested only ten double combinations, and potentially other combinations of two or more interactive loci may cause complete resistance.

A striking observation was that BIL[4.1+6.3] that individually did not confer field resistance, in combination with BIL8.2 revealed an 'more-than-additive' epistatic effect with a high resistance level in field situations (Chapter 3). This result demonstrates that introgressions which in combination confer a high resistance level do not need to confer a high level of resistance when they occur alone in a susceptible lettuce genetic background. Nonhost resistance of *L. saligna* might even be based on a combination of resistance loci that individually have no resistance effect. If one would like to study this hypothesis by using the BIL-introgression stacking approach, all 29 BILs should be intercrossed to create double-combinations and after selection of double-homozygotes, these should be intercrossed to create triple and quatro-combinations.

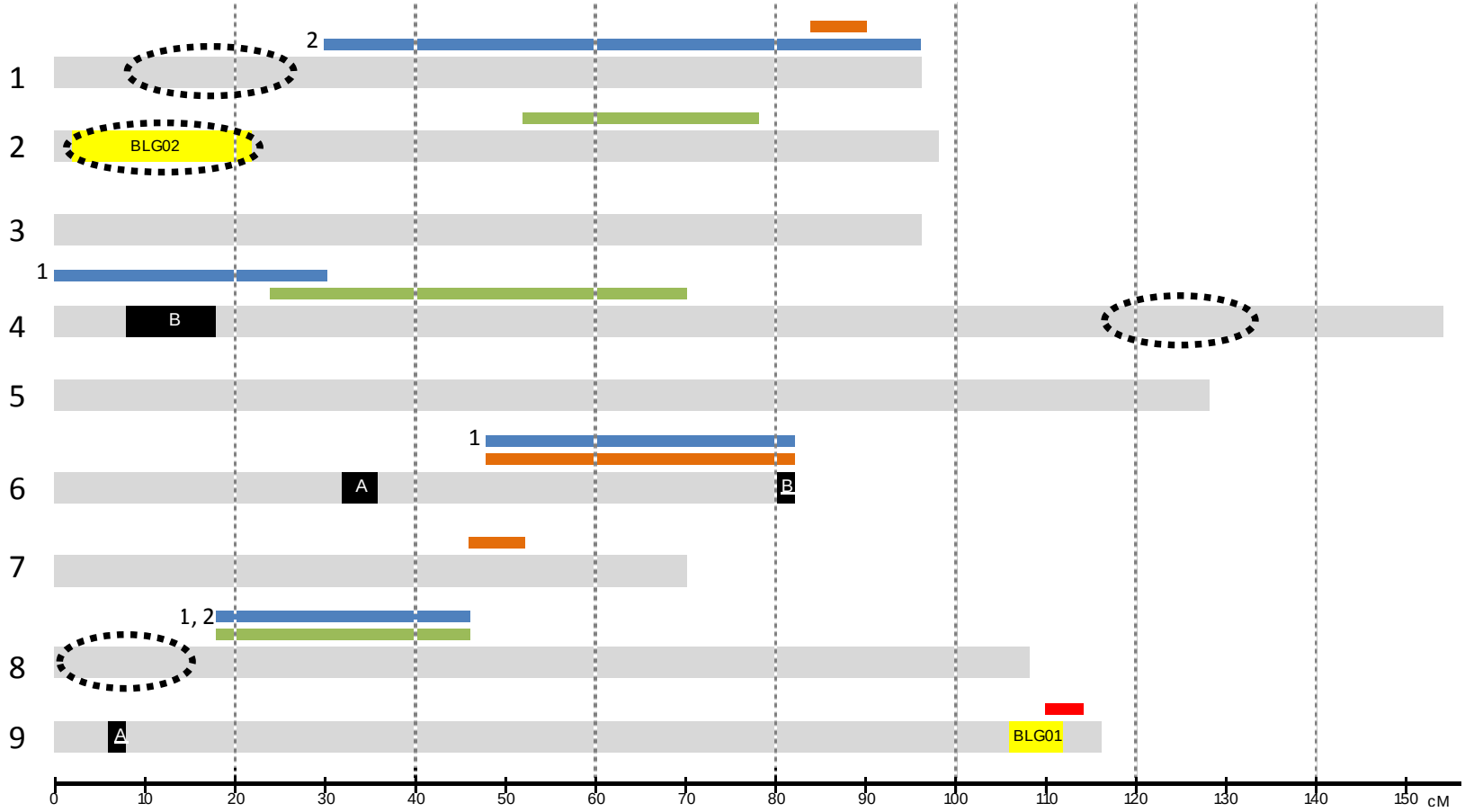

Figure 1. Map position of the following traits that were identified and studied during the PhD thesis: downy mildew resistance, recognition of effector proteins and hybrid incompatibilities.

Green bars: *L. saligna* introgression segments with multiple sub-QTL for resistance (Chapter 2). Blue bars: stacked *L. saligna* introgression segments with significant increased resistance effect (Chapter 3). Yellow region: candidate effector cell death response loci (Chapter 6). Orange bar: interactive quantitative resistance loci from selectively genotyping large F_2 populations (Chapter 5). Red bar: monogenic major resistance from *L. saligna* CGN15705 (Chapter 5). Black region: digenic hybrid incompatibility regions (each interacting region is indicated in black and with the same letter; the dependent region (region which cannot be homozygous *L. saligna* without a *L. saligna* introgression at the other region) is underlined; (Jeuken et al., 2009; Chapter 4; Chapter 5). Dashed ovals: resistance gene clusters in *L. sativa* and *L. serriola* (mainly *Dm* genes; (Kesseli et al., 1994; Truco et al., 2007)).

The formula to calculate all possible combinations is C(n,m)=n!/(m!*(n-m)! in which C=number of combinations; n= number of different introgressions segments; m=number of introgression segments in one line (m= 2, is a double combination).

The development and disease phenotyping of all possible 406 double, all 3654 triple and all 23751 quatro combinations is however hardly feasible. Therefore if epistatic interactions are involved in the nonhost resistance of *L. saligna*, stacking BILs seems not a practically feasible approach.

New genetic hypothesis

The observed diverse interactions between and within (sub-)introgressions of *L. saligna* on resistance levels of lettuce to *B. lactucae* suggest that the genetic basis of the nonhost resistance from *L. saligna* CGN05271 is very complex and/or involving epistatic relationships. Preliminary results from our selective genotyping for phenotypic extremes in large F_2 populations (Chapter 5) also hinted at epistatic interactions to explain the complete resistance of the nonhost parent, although further validations in next plant generations are needed.

Limitations of the present study

The use of wild species in breeding increases genetic diversity in the often narrow genetic basis of domesticated crops. Several difficulties, that are often associated with crosses involving two related species have, however, complicated the use of wild relatives in breeding. Problems which are often observed in populations derived from wide crosses are: linkage drag, suppressed recombination, reduced fertility, reduced vitality or locus specific transmission distortions due to pre- and post-zygotic barriers (Rieseberg and Blackman, 2010; Maheshwari and Barbash, 2011; Grandillo, 2014). For breeding, wild relatives are often successfully used for introduction of monogenic traits like *R*-genes. For polygenic and/or quantitative traits the use of wild relatives is limited because of the above described difficulties. Regarding mapping population type, an exotic library, like our set of 29 BILs with per BIL an average size of the wild donor introgression of 4% of the genetic map, has less of the above mentioned difficulties compared to mapping populations as F_2, F_3 and RILs in which on average the plants consist for half of their genome of wild donor introgressions. Besides, an exotic library has many advantages over biparental mapping populations like: 1) lines of the library have a higher morphological and genetic similarity to each other and to the recurrent parent, which enables more precise estimates of quantitative traits and 2) homozygous lines like BILs can be tested in infinite experiments and replications under different conditions and in that way QTL × environment interactions can be studied more accurately (Zamir, 2001; Jeuken and Lindhout, 2004).

Also in our study the cross between wild lettuce, *L. saligna,* and cultivated lettuce, *L. sativa,* caused hybrid incompatibility problems, including reduced fertility, low vitality, hybrid

necrosis symptoms, and severely distorted segregation ratios (Jeuken and Lindhout, 2002); Chapters 3, 4 and 5). These difficulties were more severe in F_3 than in F_2, and more severe in F_2 than in backcross populations or BILs. Consequently the development of a RIL population was hampered.

In the F_2 population derived from the French *L. saligna* CGN05271, auto-immune symptoms interfered with disease phenotyping, hampering genetic studies on resistance in that population (Figure 1 regions 'A'; Jeuken et al., 2001; Jeuken et al., 2009). Most of these limitations by hybrid incompatibility could be solved/avoided by backcrossing and finally by the development of a set of BILs. Recombinant screenings in five BILs were however hindered by a suppressed recombination within the heterozygous *L. saligna* introgression segments, with suppressions ranging from two up to 17 times compared to the recombination frequencies in those chromosome regions in the F_2 population (Chapter 2 and 3).

Another limitation for unravelling the nonhost resistance, unrelated to the wideness of the cross, was the plant stage dependence of the resistance levels in segregating populations in contrast to the plant stage independent complete resistance of the donor species *L. saligna.* This limitation required that the resistance levels in controlled disease tests at seedling, young or adult plant stage always needed to be validated in field tests at adult plant stage (Zhang et al., 2009a).

Overall the set of BILs had more advantages and fewer limitations than segregating populations like F_2 and BC_1. However when the nonhost resistance is explained by epistatic loci (at independently segregating loci), the detection of these loci is difficult or impossible within the set of BILs. Segregating populations with higher wild donor genome content (like F_2, F_3, BC_1) will be more suitable to detect epistatic interactions.

Hypothetic molecular basis of the nonhost resistance

Within the species *L. saligna* two resistance mechanisms have been identified against *B. lactucae*, one based on qualitative resistance by race specific *Dm*-genes (*R36,* Lebeda and Zinkernagel, 2003; unnamed *R* genes in Moreau, 1994; Van Ettekoven and Van der Arend, 1999; Zonneveld et al., 2011) and one based on multiple race nonspecific quantitative resistance genes (Jeuken and Lindhout, 2002; Zhang et al., 2009a, Chapter 5, Lebeda and Reinink, 1994). Some *L. saligna* accessions are associated with qualitative resistance because of: 1) monogenic race-specific complete resistance was observed in mapping populations (Zonneveld et al., 2011), 2) race-specific resistance was detected at the seedling stage (Petrželová et al., 2011). This race-specific resistance seems to be superimposed on the nonhost resistance, as some *L. saligna* accessions (for example CGN05271) do not react with monogenic resistances and are still completely resistant to all *B. lactucae* isolates, while some *L. saligna* accessions (for example CGN15705) do react with a monogenic major resistance effect and with interactive quantitative genes that together confer complete resistance (Chapter 5). These quantitative resistance genes

that have not been identified yet, (Lebeda and Reinink, 1994; Jeuken and Lindhout, 2002, Chapter 2, Chapter 5). Although monogenic resistances in some *L. saligna* accessions are absent we cannot exclude *Dm*-genes as candidates for the quantitative resistance loci and therefore also as basis of the nonhost resistance, since occasionally classical monogenic race-specific *R*-genes can explain quantitative resistance (Tan et al., 2008; Poland et al., 2009). Also in lettuce some *R*-genes like *Dm*6 give incomplete resistance (Johnson et al., 1977), and some *Dm* genes (*Dm6*, *Dm7*, *Dm11*, *Dm15* and *Dm16*) are less effective at temperatures below 10°C (Judelson and Michelmore, 1992).

Our 15 previously detected quantitative resistance loci were not located within the four *Dm*-gene clusters that were identified in *L. sativa*, with a possibly exception for *rbq13* at BIL2.1 (Truco et al., 2007; McHale et al., 2009; Zhang et al., 2009a). Therefore it seems less likely that these QTLs are explained by *Dm*-genes conferring incomplete resistance, although we are not aware of the similarity/dissimilarity of localization of *Dm*-gene clusters in *L. sativa* and in *L. saligna*. More important, histological studies indicate a different mechanism since the *L. saligna* accessions without *Dm*-genes showed a later effect in arrest of infections than the lines that had *Dm*-gene based resistance (Lebeda and Reinink, 1994).

The multiple quantitative resistance loci associated with introgressions from *L. saligna* probably confer PAMP-triggered immunity. *B. lactucae* effector proteins are probably not able to manipulate plant targets within *L. saligna* which are involved in this PAMP-triggered immunity. Or at those loci the *L. saligna* introgression has replaced a *L. sativa* region that contained a compatibility factor contributing to successful infection of *B. lactucae* on lettuce. Due to a continuous co-evolution between *B. lactucae* and *L. sativa / L. serriola* species, *B. lactucae* effectors are adapted to manipulate the host *L. sativa* and seem not or less able to manipulate to manipulate the targets in the nonhost *L. saligna*. The genus *Bremia* includes, besides *B. lactucae* infecting *L. sativa* and *L. serriola*, different species which infects hosts from three different tribes in the Asteraceae subfamilies Asteroideae and Carduoideae. As *B. lactucae* isolates collected on other species than *Lactuca* are not able or not as good able to infect *Lactuca* species (existence of different *Bremia*, formae speciales) co-evolution seems to play an important role for each *Bremia* species to be able to infects its specific host species.(Thines et al., 2010).

An intriguing question is: why are *Dm*-genes occasionally present in *L. saligna* accessions if they harbor already another resistance mechanism resulting in complete resistance? An explanation might be that *L. saligna* turned into a nonhost after it was a host, due to for instance (near-) extinction of the species *L. saligna* in *B. lactucae*- favorable climates or an extinction of *B. lactucae* in regions where *L. saligna* was growing (due to for instance a climate change) which stopped the co-evolution between the species.

Screening for resistance by recognition of effectors

Nowadays, the search for effector genes becomes more affordable by decreased sequencing costs. Application of effector gene information to search for *R*-genes (effector triggered immunity) or plant targets (PAMP-triggered immunity) for resistance breeding becomes therefore more feasible. Effector-assisted breeding has been successfully used for resistance breeding against both biotrophic as well as necrotrophic pathogens in various crops, reviewed by Vleeshouwers and Oliver (2014). One such successful example was the screen for homologues of the broad-spectrum *RB/Rpi-blb1* gene that provides resistance to *Phytophthora infestans* and originates from *Solanum bulbocastanum* which is not directly cross fertile with cultivated potato. Functional allele mining by potato virus X agroinfection with *AVRblb1* led to the discovery of the functional *RB/Rpi-blb1* homolog *Rpi-sto1* in the directly cross fertile species *Solanum stoloniferum,* which is now used in potato breeding (Vleeshouwers et al., 2008).

For discovery of new *Dm*-genes, disease tests are performed on seedlings. These disease tests are less labor intensive than *Agrobacterium tumefaciens* transient transformation assays (ATTA) in which effector genes are transiently expressed in minimal 4 weeks old plants. As described earlier, the completely resistant species *L. saligna* harbors *Dm* genes besides nonhost-resistance QTLs. These *Dm* genes, with unknown resistance spectrum and unknown genetic position, might be interesting and/or of practical value, but due to the (almost) complete resistance character of the species they are impossible or difficult to identify with regular disease seedling tests. For discovery of *Dm*-genes in highly or completely resistant *Lactuca* species, like *L. saligna* and *L. virosa*, effector assisted screens might be a good alternative. In disease tests multiple resistances can be triggered by separate effectors which mask each other's effects (when delivered by *B. lactucae*, possibly >100 effectors are brought into host plant cells) (Vleeshouwers et al., 2011). In effector assisted screens the effect of a single effector can be observed, rather than the sum of effects from a mix of effectors. Because the effects of single effectors can be studied, more knowledge about the interaction between the pathogen effectors, the host targets and *Dm*-genes will be gained. Further effector allele frequency studies on recent *B. lactucae* populations in the field could reveal insights on the indispendibility of certain effectors. These new knowledges might lead in the future to breeding of a more durable resistance with *Dm*-genes that recognize non dispensable effectors.

Thirty-four potential RXLR (-like) effector proteins from *B. lactucae* race Bl:24 were identified by the Van den Ackerveken group in the University of Utrecht. These were tested for recognition within a collection of 129 *Lactuca* accessions, including 52 *L. saligna* accessions. Two of these effector proteins were recognized since they triggered a hypersensitive cell death response. One effector protein (nominated 'BLG01') was recognized by 47 of the 52 *L. saligna* accessions and another (nominated 'BLG03') was recognized by two *L. sativa* accessions. Strikingly, both recognized effector proteins contained the RXLR-like motif 'GKLR'. In

mapping studies with F_2 populations, no association was found between the recognition of BLG01 or BLG03 and resistance to Bl:24 (Chapter 6). BLG01 recognition mapped to the bottom of Chromosome 9 in the *L. saligna* accession CGN05271 (Chapter 6). Surprisingly, a major resistance effect, observed in F_2 and BC_1 populations of the *L. saligna* accession CGN15705 × *L. sativa* cv Olof cross (Chapter 5), mapped to the same position as the gene for BLG1 recognition in *L. saligna* CGN05271 (Chapter 6, see Figure 1). *L. saligna* CGN15705 did not show a recognition response to BLG01 (personal communication M. Jeuken, 2014)

In numerous studies, the recognition of an effector protein and resistance were correlated, but also examples of absence of correlation are reported. In potato, for example, no association was found between recognition of PexRD28-1 and PexRD46 *Phytophthora infestans* effector proteins and resistance to the pathogen (Vleeshouwers et al., 2008). Similarly, *Arabidopsis thaliana* ecotype Wei-0, that recognizes *Hyaloperonospora arabidopsidis* effector ATR39-1 is not resistant to the isolates that produce this effector protein(Goritschnig et al., 2012). It is possible that cell death response to effectors BLG01 and BLG03 when applied alone was suppressed by other effectors in the situation where the pathogen infected the tissue. In *Phytophthora* species effectors have been demonstrated to suppress effector-induced cell death induced by the pathogens other effectors. For instance, Wang et al., (2011) showed that *Phytophthora sojae* is capable of suppressing effector-induced cell death in soybean. Likewise, *Phytophthora infestans* can prevent host cell death during the biotrophic phase with effector *AVR3a* in potato (Bos et al., 2010). Whether *B. lactucae* is capable to suppress effector-induced cell death responses of BLG01 and BLG03 needs to be further investigated.

Future approaches in dissecting the nonhost resistance

The BILs approach did not allow the fine mapping of genes for nonhost resistance with a sufficiently large effect to offer perspectives to map based cloning. A different approach is needed to genetically dissect the nonhost resistance. The diverse (sub)QTL × (sub)QTL interactions within the BILs, and possibly within F_2 populations, made it hard to prove in a stepwise, logical and deductive way which combination of genes/loci are responsible for the absolute resistance of the nonhost species *L. saligna.*

<u>Alternative genetic approach</u>

Chapter 5 already made a start with an alternative approach to unravel the nonhost resistance. We used a selective genotyping approach in which in segregating populations only the plants with extreme phenotypic infection values (highly resistant and highly susceptible) were genotyped. By focusing on the phenotypic extremes we identified a combination of loci which might explain the complete resistance of *L. saligna*. To identify the responsible genes that form the basis of the nonhost resistance within the whole species *L. saligna*, resistance mapping in more and diverse

population types and from different parental *L. saligna* accessions (preferably from diverse geographic origin) seems required. Therefore, repeating the approach used in Chapter 4 with some more *L. saligna* accessions should confirm which loci are responsible for resistance within the whole species. These resistance genes can be stacked into a *L. sativa* background to validate whether they explain the nonhost resistance. For this confirmation the set of BILs is probably the most suitable material.

Populations with half of the genome from *L. sativa* and *L. saligna,* like F_2, F_3 and RILs, often suffer from hybrid incompatibilities and lack of fertility. Backcross populations like BC_1, which are selfed after backcrossing one or more times (BC_1S_{1-n}) , might be an alternative, as these populations have less of those difficulties (See Figure 3, Chapter 1). Two BC_1 *L. sativa* populations and one BC_1 *L. saligna* population were used in this study (Chapter 5) and confirmed two of the four loci conferring resistance which were detected in the selectively genotyped F_2 populations. Backcross populations have, however, as disadvantage that from one of the parents only a quarter (or less in later generations) of the genome is present and therefore, for studying interactions, larger population sizes or family numbers are required to detect (epistatic) QTLs.

Molecular approach

A molecular approach to unravel the nonhost resistance from *L. saligna* might be: to sequence RNA-transcripts, to determine the PAMP proteins or early expressed effector proteins from a *B. lactucae – L. saligna* interaction and, with these, to find interactive plant protein targets. The most informative time lapse of RNA-transcript sampling would be within 48 hours. In the first 24 hours the infection stages from spore germination to formation of a secondary vesicle occur (Maclean and Tommerup, 1979). Between 24 and 48 hours after inoculation, hypha formation attempts are arrested in *L. saligna* (Lebeda et al., 2008; Zhang et al., 2009b). Sequencing of the *B. lactucae* transcriptome and identification of *B. lactucae* effectors was previously started within our collaboration project by the Van den Ackerveken group of University of Utrecht and described in (Stassen et al., 2012); Chapter 6). Pathogens, such as fungi and oomycetes, secrete effector proteins that interact with and modulate plant proteins to suppress defense, and promote growth of the pathogen in the plant (Kamoun, 2007). The plant proteins that are modulated by effector proteins are called ‘plant targets’. These plant targets can be identified by interaction cloning, using effectors as bait in the yeast two-hybrid system. Using the yeast-two-hybrid system, Bos et al., (2010) showed that effector *AVR3a* from potato blight pathogen *Phytophthora infestans* interacts with and stabilizes host U-box E3 ligase CMPG1, which is required for infestin1-triggered cell death. Unlike effectors, plant targets seem to be conserved (orthologs of the target genes are present among species) between different plant species (Anderson et al., 2012). However, the plant target genes within *L. sativa* might be (slightly) different in *L. saligna* and because of these differences *B. lactucae* is able to suppress the nonhost resistance in *L. sativa* (resulting in host status) but not in *L. saligna*. The molecular basis of the nonhost resistance of *L.*

saligna might be unravelled by comparing *L. sativa* and *L. saligna* alleles of plant target genes. Validation of plant targets revealed by the two-hybrid system might be executed by introgression of the plant targets from *L. saligna* within *L. sativa* and vice versa. Replacement of the candidate plant targets of *L. sativa* with those from *L. saligna* should lead to resistance while replacement of the plant target genes of *L. saligna* with those of *L. sativa* should lead to susceptibility.

Application of resistances from *L. saligna* in breeding

Although the resistance within the BILs fragmented into multiple sub-QTLs and we did not find combinations of *L. saligna* BIL introgressions which explain complete resistance, we found some resistance loci which might be interesting for breeding resistant lettuce. The qualitative resistance that locates at the bottom of Chromosome 9 (Figure 1, Chapter 5) might be interesting because it is located outside the four known *R*-gene clusters (at Chromosome 1, 2, 4 and 8, Figure 1 Chapter 7). This potentially new *Dm*-gene position provides new possibilities/options for breeders. The new qualitative *L. saligna* resistance can be stacked with defeated and/or undefeated introgressed *Dm*-genes in current cultivars, without the need for a recombination event in coupling phase. Pink (2002), reported about a variety of strategies to deploy non-durable 'major' or '*R*' genes for resistance to plant diseases. The major resistance effect at the bottom of Chromosome 9 was observed against both tested races of *B. lactucae*, Bl:21 and Bl:24. Further tests on the resistance spectrum need to be executed to value this resistance gene for breeding purposes.

For breeding with quantitative resistance, the combination of the *L. saligna* BIL introgressions 1.2 and 8.2 might be interesting (Figure 1, Chapter 3), with an additive effect across loci, resulting in a low infection level of about 27% under field conditions against at least four *B. lactucae* races. Future substitution mapping of the 1.2+8.2 introgressions must however reveal if individual loci per introgression segment interact additively or that complex interactions are responsible.

Other promising resistance loci are the three interactive quantitative resistance loci, bott_C1, mid_C6, and mid_C7 that were identified by selective genotyping (Chapter 5). These three loci were identified in mapping populations of two different *L. saligna* accessions and might explain the nonhost resistance from the *L. saligna* species. If their resistance effects can be confirmed in future experiments against multiple *B. lactucae* races, they might be very valuable (Figure 1, Chapter 5).

Future focus, tools and prospective

Besides lettuce transcriptome sequences, also the genome sequence of *L. sativa* cv. Salinas is publicly available since 2012 (https://lgr.genomecenter.ucdavis.edu/). Sequencing the *L. saligna* genome and aligning it with the reference genome might be an option for the near future. This alignment will reveal genetic and structural variation between the two *Lactuca* genomes and it will facilitate the development of new DNA markers, essential for mapping, identification of candidate genes and cloning genes. For unravelling the inheritance of nonhost resistance, the selective genotyping method in different *L. saligna* x *L. sativa* populations, as used in Chapter 5, seems to be the most promising approach.

As the transcriptome from *B. lactucae* is sequenced and the first effectors have been identified (Stassen et al., 2012); Chapter 6) research on the interaction between the *B. lactucae* effectors and their *Lactuca* plant targets is starting up. Identification of the targets of the effector proteins by a two-way hybrid system is on its way in a follow up research project. The use of effectors to screen for *Dm*-genes might be feasible in lettuce as we already found a cell death response for two effectors, albeit without association with resistance. For practical use in breeding, the use of effectors to identify new *Dm*-genes might be the most useful in (near) nonhost species for which seedling disease tests are not discriminative.

After identification of the *L. saligna* genes that confer the nonhost status to *B. lactucae*, their introgression into *L. sativa* may be the next obstacle due to, for instance, hybrid incompatibility, linkage drag or suppression of recombination. In that case, targeted mutagenesis or genome editing (with CRISPR/Cas9 or TALENs) of the *L. sativa* orthologs might be an approach in the future to induce the nonhost resistance within this species. One drawback could, however, be that for application in breeding and commercial lettuce cultivation in Europe, these targeted mutagenesis technologies are currently considered as GMO. If they would be exempted in the future or put under a lighter regulatory regime this could open up perspectives in the future.

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SUMMARY

Cultivated lettuce (*Lactuca sativa*) is susceptible to downy mildew disease under greenhouse and field conditions. Downy mildew (*Bremia lactucae*) causes serious yield losses worldwide and resistance to it is one of the major breeding goals. Breeding lettuce for resistance to *B. lactucae* is mainly achieved by introgression of race-specific dominant resistance (*R*) genes. However, appearance of new *B. lactucae* races quickly renders *R* genes ineffective. The wild lettuce and nonhost species, *Lactuca saligna*, is absolute resistant to downy mildew, is crossable with cultivated lettuce and its resistance genes might be more durable. The identification of genes conferring nonhost resistance is a crucial step in its understanding and usage in breeding.

Previous studies on the genetic dissection of the complete resistance of *L. saligna* accession CGN05271 has revealed no monogenic race-specific *R*-genes but has indicated a polygenic inheritance and a quantitative character. In a set of 29 Backcross Inbred Lines (BILs; single *L. saligna* introgression in a *L. sativa* background) that together covers 96% of the *L. saligna* genome, fifteen introgression regions have been identified that confer plant stage dependent quantitative resistance (QTLs). Three BILs, carrying an individual 30 to 50 cM long introgression segment from *L. saligna*, reduce infection at most plant stages with reduction levels of 60-70% at young plant stage and of 30-50% at adult plant stage in field situations.

In Chapter 2, we studied these three quantitative resistances in order to narrow down their mapping interval and determine their number of loci, either single or multiple. This study entailed a substitution mapping which includes recombinant screenings, the development of lines with smaller overlapping *L. saligna* introgressions (sub-BILs) and subsequent phenotyping. Disease evaluation of the sub-BILs revealed that the resistance of all three BILs was not explained by a single locus but it disintegrated in multiple sub-QTLs. The *L. saligna*-derived sub-QTLs had a smaller and plant stage dependent resistance effect, some segments reducing, others even promoting downy mildew infection. The genetic complexity of many sub-QTL and the small individual effect of about 10% infection reduction in field situations make further attempts for gene cloning not useful.

From the set of 29 BILs, eight BILs confer race nonspecific quantitative resistance against lettuce downy mildew in the field. Individually these introgressions do not provide the complete resistance like *L. saligna* in the field and their infection reduction of 30 to 50% is not high enough for cultivation practice. Possibly a specific combination of these introgressions (QTLs) gives complete resistance and explains the nonhost resistance of *L. saligna*. In search for the genetic control of the nonhost resistance and for a desired race nonspecific very strong or absolute resistance in the field, we studied the potential of stacking the quantitative resistances of BILs per combinations of two (Chapter 3).

Seven out of ten developed double-combinations showed a similar and not significantly lower infection than the individual most resistant parental BIL. Three double-combinations resulted in an increased resistance level compared to their parental individual lines. Of the latter three lines one had additive and two had epistatic interactions between the introgressions (one with 'less-than-additive' and one with 'more-than-additive' effects). Thus, non-additivity is more the rule than exception. The 'additive'combination showed the highest field infection reduction of 70% compared to the recurrent susceptible *L. sativa* cv. Olof parent. The combination with a ´more-than-additive' epistatic effect showed a field infection reduction of 50%. For the latter a substitution mapping was performed by 22 lines, which could not pinpoint interactive loci, probably because the inheritance is complex and/or based on multiple interactive loci.

In conclusion, the QTL substitution mapping results (Chapter 2) and the QTL stacking results (Chapter 3) indicated that the resistance of *L. saligna* is genetically complex and interactions between QTLs/genes might play a role. We hypothesize that multi-locus interactions with additive and/or epistatic effects explain the absolute resistance.

In a new approach to unravel the inheritance of the *L. saligna* nonhost resistance, we applied 'selective genotyping' on the phenotypic extremes of large F_2 populations, in which multi-locus interactions between *L. saligna* alleles are still prevalent (Chapter 5). In this study we used segregating populations (F_2 and BC_1) of crosses between three *L. saligna* accessions and a common susceptible *L. sativa* parent. Out of 1455 germinated F_2 seeds, we selected vital plants with extreme disease phenotypes (62 highly resistant and 25 highly susceptible). From QTL mapping analysis and genotype comparisons between the resistant and susceptible F_2 groups, we identified four resistance loci (nominated 'bott_C1','mid_C6', 'mid_C7' and 'bott_C9') of which only 'bott_C9' showed an individual major effect and only 'bott_C9' was *L. saligna* accession specific. Multi-locus interaction studies between the other three identified loci, indicated presence of epistatic interactions with large effects in three out of four tested segregating populations. These indications for three interactive loci need to be validated in future studies to estimate their value in nonhost / complete resistance to downy mildew.

Hybrids between related species like *L. saligna* and *L. sativa* are sometimes inviable or if they live, they have phenotypic abnormalities. These deleterious hybrid characteristics are referred to as "hybrid incompatibility" (H. I.). During the development of sub-BILs for BIL[4.1+6.3] in Chapter 3, we observed a digenic H.I. Plants carrying a *L. saligna* segment on Chromosome 6 always require a *L. saligna* segment on Chromosome 4. In Chapter 4, we fine-mapped the two loci in an F_2 population and used the F_2 to study the genetic basis of the interaction. The F_2 showed adeviant segregation ratio in which three out of nine expected genotype classes (based on Mendelian segregation) were completely absent. Testing of observed segregation with hypothesized segregations suggested a prezygotic reproduction barrier by non-transmission of one specific hybrid gametophyte (male and female).

In Chapter 6, we describe results that are obtained in cooperation with our partner at the University of Utrecht, within the Technological Top Institute Green Genetics - *Lactuca-Bremia* project. This Chapter focuses on candidate effectors of *B. lactucae*. Thirty-four potential RXLR (-like) effector proteins were identified by transcriptome sequencing and were tested for recognition within a collection of 129 *Lactuca* accessions, including 55 *L. saligna* accessions. One of these effector proteins, nominated 'BLG01', was recognised by most tested *L. saligna* accessions, and triggered a hypersensitive cell death response. BLG01 recognition was mapped on the bottom of Chromosome 9 using the set of 29 BILs and some F_3 families of the cross *L. saligna* CGN05271 x *L. sativa* cv. Olof. The *B. lactucae* isolates which carry BLG01 effector proteins were still able to infect *Lactuca* lines which recognised this effector protein.

In Chapter 7, the results of this thesis are evaluated and we glimpse into future research and applications. The results of different strategies for genetic dissection of the non-host resistance of *L. saligna*, the insight in interspecific reproductive barriers (like hybrid incompatibility) and the uncovering of BLG01 effector recognition in the species *L. saligna* are important steps forward towards understanding the incompatible interaction between *B. lactucae* and *L. saligna* and its future application in resistance breeding.

SAMENVATTING

Cultuur sla (*Lactuca sativa*) is vatbaar voor valse meeldauw (*Bremia lactucae*) in zowel kas-teelt als op het veld. Valse meeldauw (*Bremia lactucae*) veroorzaakt wereldwijd grote opbrengstverliezen en resistentie ertegen is een van de belangrijkste veredelingsdoelen. In de veredeling van sla op resistentie tegen *B. lactucae* wordt vooral gebruik gemaakt van klassieke resistentiegenen, zogenaamde '*R'* genen. Deze *R* genen gedragen zich dominant, geven volledige resistentie en zijn meestal fysio-specifiek. Echter uit de praktijk blijkt dat deze *R* genen snel doorbroken worden door het ontstaan van nieuwe *B. lactucae* fysio's in het veld.

De wilde sla soort, *Lactuca saligna*, is compleet resistent (zogenaamde 'niet-waard' resistentie) en is kruisbaar met cultuur-sla (*L. sativa*). De resistentie uit deze wilde soort is mogelijk anders dan de *R*-gen resistentie en meer duurzaam. De identificatie van de genen, die deze resistentie verklaren, zou een cruciale stap zijn voor inzicht in en het gebruik van deze niet-waard resistentie in de veredeling.

Eerdere studies naar de genetische achtergrond van de volledige resistentie van *L. saligna* (accessie CGN05271) tegen valse meeldauw lieten zien dat de resistentie niet verklaard werd door monogene fysio-specifieke *R*-genen maar door meerdere genen met een kwantitatief karakter. In een set van 29 terugkruisingslijnen (Engelse term: 'backcros inbred lines', afgekort als 'BILs'), die samen 96% van het *L. saligna* genoom vertegenwoordigden, werden vijftien introgressie-gebieden geïdentificeerd die plantstadium afhankelijke, kwantitatieve resistentie vertoonden (Engelse term: 'quantitative trait loci', afgekort als 'QTLs'). Drie BILs, met één enkel introgressie segment (30 tot 50 cM lang) van *L. saligna* in een *L. sativa* achtergrond, vertoonden een verminderd infectie-niveau in de meeste plant stadia, met reducties van 60 tot 70% in het jonge plant stadium en van 30 tot 50% in het volwassen plant stadium in het veld.

In dit proefschrift in Hoofdstuk 2 bestudeerden we deze drie kwantitatieve resistenties om uit te zoeken of de resistenties werden verklaard door één of meerdere genen en waar deze genen liggen op de genetische kaart (een positie op de genetische kaart = 'locus'). Door middel van selectie en inteelt van planten met een recombinatie dicht bij de genen die kwantitatieve resistentie veroorzaakten, ontwikkelden we plant-lijnen met kleinere *L. saligna* introgressie-fragmenten in een *L. sativa* achtergrond. Door middel van ziekte toetsen van deze plant-lijnen achterhaalden we het aantal resistentie-loci. Deze ziektetoets evaluaties wezen uit dat de resistentie van de drie BILs niet werd verklaard door één locus (1 QTL), maar door meerdere loci (meerdere sub-QTLs). Deze sub-QTLs waren plantstadium-afhankelijk en verklaarden ieder een kleiner gedeelte van de resistentie. Daarnaast werden er ook sub-QTLs van *L. saligna* geïdentificeerd die de valse meeldauw infectie juist bevorderden. Door de genetische complexiteit van de vele sub-QTLs en het lage gemiddelde individuele sub-QTL effect van

ongeveer 10% infectie vermindering in veldsituaties, lijkt het verder niet zinvol om de achterliggende genen te kloneren.

Acht BILs van de set van 29 BILs geven een kwantitatieve resistentie in het veld. Hun infectie reductie varieert van 30 tot 50 % en dit is niet hoog genoeg voor commerciële teelt. Mogelijk verklaart een specifieke combinatie van twee of meer *L. saligna* introgressies van deze lijnen samen de complete niet-waard-resistentie van *L. saligna*. Op zoek naar de genetische verklaring van de niet-waard-resistentie en naar een niet-fysio-specifieke, zeer sterke of absolute resistentie in het veld, hebben we de mogelijkheden van het stapelen van de kwantitatieve resistenties van de BILs per verschillende combinatie van twee onderzocht in Hoofdstuk 3.

Zeven van de tien ontwikkelde combinaties van twee *L. saligna* BIL introgressies lieten een vergelijkbare en niet significant lagere infectie zien dan de meest resistente BIL-ouder. De drie andere dubbelcombinaties lieten wel een verhoogde resistentie zien ten opzichte van beide ouder-lijnen. Van deze drie lijnen had de gestapelde resistentie van één lijn een additief effect, en van de andere twee een epistatisch effect (één met een ‘minder-dan-additief’ en één met een ‘meer-dan-additief ’ effect). De ‘additieve’ combinatie toonde met een infectie-reductie van 70% ten opzichte van de vatbare terugkruising ouder (*L. sativa* cv. ‘Olof’) de hoogste infectie-reductie in het veld. De combinatie met een ‘meer-dan-additief ’ epistatisch effect toonde op het veld een infectie reductie van 50%. Voor deze laatste combinatie probeerden we de verklarende resistentie loci te achterhalen door middel van substitutie-kartering met behulp van 22 gedeeltelijk nieuwe lijnen. Echter geen van deze 22 lijnen haalde een infectie-reductie van 50%, waardoor de interactieve loci niet konden worden geïdentificeerd. Waarschijnlijk is de genetische verklaring complex en/of gebaseerd op meerdere interactieve loci.

Concluderend, de resultaten van de fijnkartering en het stapelen van de QTLs (Hoofdstuk 2 en Hoofdstuk 3) suggereren dat de absolute resistentie van *L. saligna* genetisch complex is en dat interacties tussen QTLs / genen een rol kunnen spelen. Onze hypothese is dat multi-locus interacties met additief en / of epistatisch effecten de absolute resistentie verklaren.

In een nieuwe benadering om de niet-waard-resistentie van *L. saligna* te ontrafelen (Hoofdstuk 5), hebben we een selectieve steekproef van fenotypisch extreme individuen toegepast op grote segregerende F_2 populaties (waarbinnen alle *L. saligna* genen uitsplitsen en de verklarende genen-combinatie dus nog kan voorkomen). In deze studie maakten we gebruik van segregerende populaties (F_2 en BC_1) van kruisingen tussen drie *L. saligna* accessies en een gemeenschappelijke vatbare *L. sativa* ouder. Uit 1455 gekiemde F_2 zaden werden alleen vitale planten geselecteerd met extreme ziekte fenotypes, 62 zeer resistente en 25 zeer vatbare planten. Door middel van QTL karteringsanalyses en genotype vergelijkingen tussen de resistente en vatbare F_2 groepen, werden vier resistentie-loci geïdentificeerd, genaamd 'bott_C1' , 'mid_C6', 'mid_C7' en 'bott_C9', waarvan slechts bott_C9 een groot individueel effect toonde en *L. saligna* accessie specifiek was. Interactie studies tussen de loci, 'bott_C1' , 'mid_C6' en 'mid_C7', suggereerden dat de resistentie in drie van de vier geteste populaties (twee F_2 en één BC_1)

verklaard kon worden door epistatische interacties tussen deze drie loci. Toekomstig validatie-onderzoek zal uitwijzen of deze epistatische interacties bevestigd worden en de niet-waard resistentie van *L. saligna* kan verklaren.

Hybriden tussen verwante soorten zoals *L. saligna* en *L. sativa* zijn soms niet levensvatbaar. Deze 'hybride incompatibiliteit' (HI) is vaak het gevolg van een 'genetische incompatibiliteit' in de hybride. Tijdens de ontwikkeling van sub-BILs uit BIL[4.1+6.3] in Hoofdstuk 3 werd een H. I. gevonden, die waarschijnlijk veroorzaakt word door twee genen op verschillende chromosomen. Om levensvatbaar te zijn vereisten planten met een specifiek *L. saligna* introgressie segment op Chromosoom 6 altijd de aanwezigheid van een specifiek *L. saligna* introgressie segment op Chromosoom 4. In Hoofdstuk 4 hebben we deze twee HI-loci onderzocht en fijngekarteerd. Een F_2 populatie is gebruikt om de genetische basis van de interactie te bestuderen. De uitsplitsingsverhouding van de twee HI loci in de F_2 week af van de te verwachte uitsplitsing-verhouding (gebaseerd op berekende allel frequenties). De afwijkende uitsplitsing vertoonde de afwezigheid van drie van de negen verwachte genotypen (op basis van een Mendeliaanse uitsplitsing). Toetsing van de waargenomen segregatie aan hypothetische segregaties suggereerde een prezygotische voortplantingsbarrière door het niet-overdragen van één specifieke hybride gametofyt (zowel vrouwelijk als mannelijk).

In Hoofdstuk 6 beschrijven we de resultaten die zijn verkregen in samenwerking met onze partner van de Universiteit van Utrecht, binnen het TTI-Groene Genetica - *Lactuca-Bremia* project. Dit hoofdstuk richt zich op de kandidaat-effectoren van *B. lactucae.* Vierendertig potentiele effector-eiwitten werden geïdentificeerd door het sequensen van het *B. lactucae* transcriptome. Ze werden getest op 'herkenning' in een steekproef van 129 *Lactuca* accessies, waarvan 55 *L. saligna* accessies. Deze 'herkenning' was zichtbaar als 'necrose' of 'celdood' (een symptoom wat overeenkomstig is met resistentie-reacties door *R*-genen, zogenaamde 'overgevoeligheidsreactie'). Effector eiwit, 'BLG01', werd herkend door bijna al de geteste *L. saligna* accessies. BLG01 herkenning werd gekarteerd op de onderkant van Chromosoom 9 met behulp van de set van 29 BILs en enkele F_3 families. Echter de *B. lactucae* isolaten die het BLG01 effector-eiwit tot expressie brengen, konden nog steeds de *L. saligna* × *L. sativa* hybriden, die deze effector eiwit herkenden, infecteren.

In hoofdstuk 7 worden de resultaten van dit proefschrift geëvalueerd en werpen we een blik op toekomstig onderzoek en toepassingen. De resultaten van de genetische ontledingen van de *L. saligna* resistentie, het inzicht in de voortplantings barrières door H.I. en het vinden van de BLG01 effector herkenning in de soort *L. saligna*, zijn belangrijke stappen voorwaarts voor het begrijpen van de *L. saligna* niet-waard-resistentie en de toekomstige toepassing hiervan in sla-veredeling.

ABOUT THE AUTHOR

Erik den Boer was born on 23 March, 1980 in Dirksland, in The Netherlands. He obtained his bachelor (BSc) degree in HAS Den Bosch in 2005 in Greenhouse Horticulture. After his BSc he started a Master (MSc) program at Wageningen University (specialisation Plant Breeding). In this Master program he participated in a lettuce- *Bremia* research project of Wageningen UR Plant Breeding. It resulted in a co-authorship for an article in the journal 'Plant Cell'. After an internship on corn salad (*Valerianella locusta*) at breeding company Rijk Zwaan, he graduated and obtained his MSc degree in 2008. He accepted an offer to become a PhD student in the lettuce research group of Dr. Marieke Jeuken at Wageningen UR Plant Breeding. The PhD research described was performed as a part of a larger research project funded by Technologisch Topinstituut Groene Genetica (TTI) and was entitled "Novel approaches for resistance breeding using pathogen effectors and their host plant targets: towards durable resistance to *Bremia* in lettuce''. Since January 2013 Erik den Boer is working at the plant breeding company Rijk Zwaan as a Crop Researcher on the following crops: endive, chicory, spinach, beetroot, fennel, rocket and leek.

LIST OF PUBLICATIONS

Jeuken, M.J., Zhang, N.W., McHale, L.K., Pelgrom, K., den Boer, E., Lindhout, P., Michelmore, R.W., Visser, R.G., and Niks, R.E. (2009) *Rin4* causes hybrid necrosis and race-specific resistance in an interspecific lettuce hybrid. Plant Cell **21**:3368-3378.

Joost H.M. Stassen, Erik den Boer, Pim W. J. Vergeer, Annemiek Andel, Ursula Ellendorff, Koen Pelgrom, Mathieu Pel, Johan Schut, Olaf Zonneveld, Marieke J.W. Jeuken, and Guido Van den Ackerveken. (2013) Specific *in planta* recognition of two GKLR proteins of the downy mildew *Bremia lactucae* revealed in a large effector screen in lettuce. Molecular Plant-Microbe Interactions 26. 11: 1259-1270.

Erik den Boer, Ningwen W. Zhang, Koen Pelgrom, Richard G.F. Visser, Rients E. Niks and Marieke J.W. Jeuken. (2013) Fine mapping quantitative resistances to downy mildew in lettuce revealed multiple sub-QTLs with plant stage dependent effects reducing or even promoting the infection. Theoretical and Applied Genetics **126**: 2995-3007

Erik den Boer, Koen Pelgrom, Ningwen W. Zhang, Richard G.F. Visser, Rients E. Niks and Marieke J.W. Jeuken. (2014) Effects of stacked quantitative resistances to downy mildew in lettuce do not simply add up. Theoretical and Applied Genetics **127**: 1805-1816.

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Part of this text is written in English for those who do not understand Dutch and other parts I wrote in Dutch.

Heel veel dank aan Marieke Jeuken, dat je me na mijn Master Thesis de kans gaf om ook een promotie onderzoek te doen in de sla. Je hebt me altijd gesteund en geholpen met soms bijna dagelijkse discussies over het opzetten van experimenten, het interpreteren van resultaten en het opschrijven van de resultaten. Deze zeer goede begeleiding heeft mij zeer veel geholpen en zonder deze begeleiding was het voor mij niet mogelijk geweest om te promoveren en drie van de hoofdstukken te publiceren. Soms moet het wel moeilijk voor je geweest zijn, vooral in het stadium dat ik moest schrijven omdat mijn talent hier niet lag. Daarnaast was het ook persoonlijk heel prettig om met je samen te werken. Ik wens je veel succes met het nieuwe, opvolgende, project en voor het verkrijgen van andere projecten.

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very required coffee breaks, drinks after work, weddings, and many baby visits. Thanks for the good time you gave me in Plant Breeding and in Wageningen.

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Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Erik den Boer
Date: 19 December 2014
Group: Plant Breeding
University: Wageningen University & Research Centre

1) Start-up phase	date
► **First presentation of your project**	
Novel approaches for resistance breeding using phathogen effectors and their host plant targets: towards durable resistance to Bremia in lettuce	Sep 16, 2008
► **Writing or rewriting a project proposal**	
Novel approaches for resistance breeding using phathogen effectors and their host plant targets: towards durable resistance to Bremia in lettuce	Aug-Oct 2008
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*4.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
1st European Retreat of PhD Students in Experimental Plant Sciences (Wageningen)	Oct 02-03, 2008
PhD student day (Leiden)	Feb 26, 2009
PhD student day (Utrecht)	Jun 01, 2010
► **EPS theme symposia**	
Theme 4 'Genome plasticity', Wageningen University	Dec 10, 2010
Theme 2 'Interactions between plants and biotic agents (& Willie Commelin Scholten Day), University of Amsterdam	Feb 03, 2011
► **NWO Lunteren days and other National Platforms**	
NWO-ALW meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 06-07, 2009
NWO-ALW meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 19-20, 2010
NWO-ALW meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 04-05, 2011
► **Seminars (series), workshops and symposia**	
Plant breeding research day	Jun 17, 2008
Plant breeding research day	Mar 03, 2009
Plant breeding research day	Feb 08, 2010
Richard Michelmore, seminar at Keygene	Jun 03, 2010
Invited seminars Brigitte Mauch-Mani and Felix Mauch	May 31, 2010
TTI-GG symposium 2010	Sep 22, 2010
Rob Goldbach's memorial lecture David Baulcombe	Sep 27, 2010
Invited seminar, entitled 'How to write a world-class paper'	Oct 26, 2010
Invited seminar Kirsten Bomblies	Nov 18, 2010
Invited seminars Jose Jimenz-Gomez and Ales Pecinka	Nov 29, 2010
Plant sciences seminar 'Towards malaria elimination: what works?', Niels Verhulst, Sander Koenraadt , Willem Takken	Apr 26, 2011
Plant science seminar 'High throughput plant phenotyping'	May 10, 2011
Invited seminar Angus Dawe	May 13, 2011
Invited seminar Rosie Bradshaw, 'The genome of Dothistroma septosporum, a close relative of Cladosporium fulvum; what have we learnt so far?'	Aug 04, 2011
► **Seminar plus**	
► **International symposia and congresses**	
Plant & Animal genome XIX , San Diego, USA	Jan 15-19, 2011
Eucarpia Leafy Vegetables, Villeneuve d' Ascq, Lille, France	Aug 24-26, 2011
► **Presentations**	
Poster TTI-GG symposium	Sep 22, 2010
Presentation Eucarpia Leafy Vegetables congress	Aug 25, 2011
Meeting breeding companies & oral presentation	Oct 30, 2008
Meeting breeding companies & oral presentation	Apr 23, 2009
Meeting breeding companies & oral presentation	Oct 28, 2009
Meeting breeding companies & oral presentation	Apr 27, 2010
Meeting breeding companies & oral presentation	Oct 27, 2010
Meeting breeding companies & oral presentation	Apr 13, 2011
► **IAB interview**	
Meeting with a member of the International Advisory Board of EPS	Feb 18, 2011
► **Excursions**	
Subtotal Scientific Exposure	*16.9 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
Summer School ' On the Evolution of Plant Pathogen Interactions'	Jun 18-20, 2008
Phostgraduate course 'Natural Variation in Plants'	Aug 26-29, 2008
► **Journal club**	
Participation in a literature discussion group at Plant Breeding	2008-2012
► **Individual research training**	
Subtotal In-Depth Studies	*5.2 credits**

4) Personal development	date
► **Skill training courses**	
Scientific Writing	Oct 07-Nov 25, 2010
Academic Writing 1	Mar 04-Jun 24, 2008
Academic Writing 2	Sep 2009-Jan 2010
Interpersonal Communication for PhD students	Oct 26-27, 2009
PHD competence assessment	Nov 20 & Dec 09, 2008
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*7.7 credits**

TOTAL NUMBER OF CREDIT POINTS*	**34,3**

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 ECTS credits

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

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