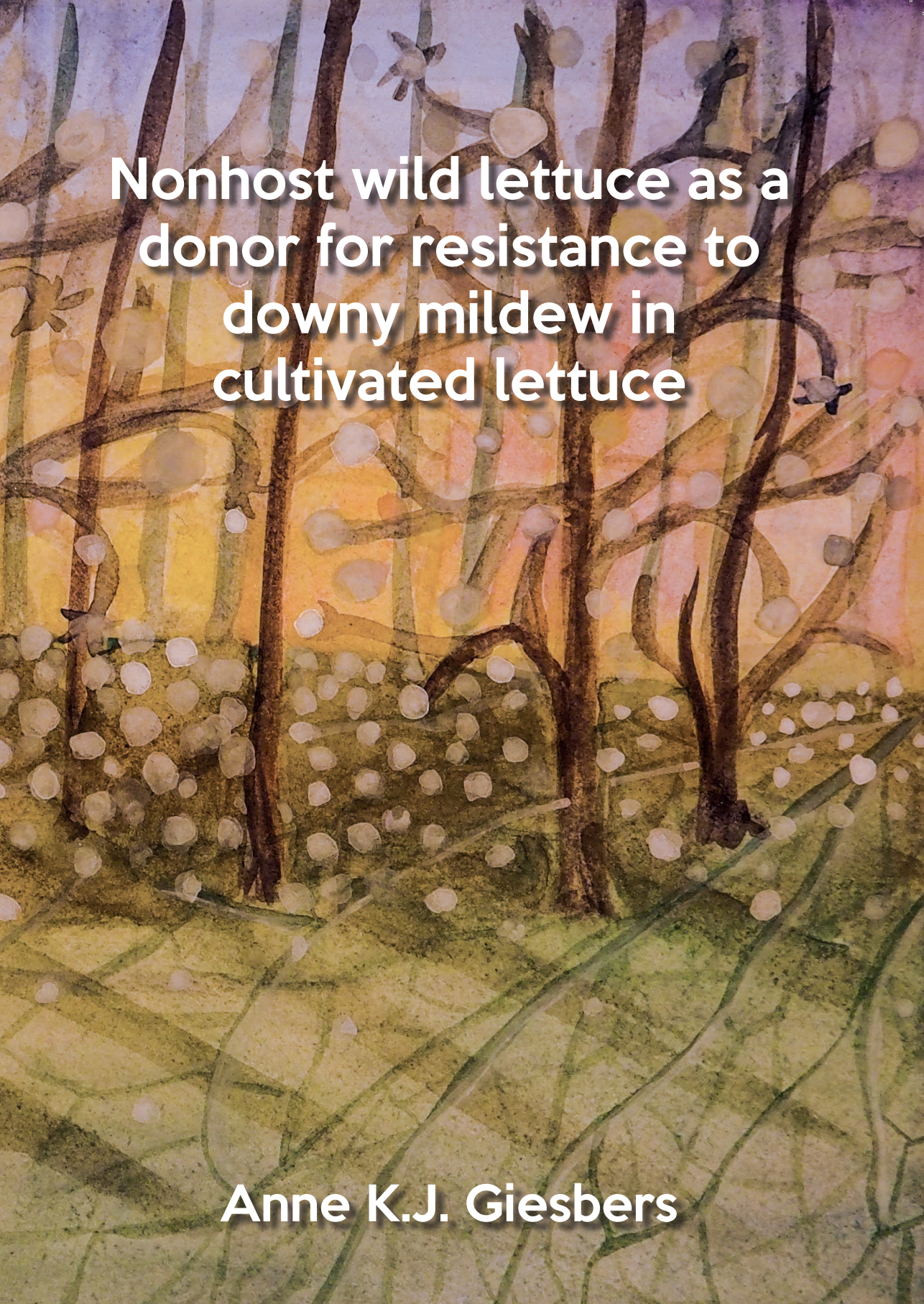

Nonhost wild lettuce as a donor for resistance to downy mildew in cultivated lettuce

Anne K.J. Giesbers

Propositions

1. For lettuce with durable downy mildew resistance, cultivar traits should be introgressed in *Lactuca saligna* instead of the reverse.
   (this thesis)

2. Not all quantitative trait loci for downy mildew resistance of *L. saligna* are essential for its nonhost resistance.
   (this thesis)

3. Eternal life will probably be accomplished through combining neuroscience and computer science.

4. Organs on a chip will soon replace all medicine research on animals.

5. Vegetables should be tax free.

6. PhD propositions incorrectly suggest that PhD candidates gain a very broad knowledge by four years of highly specialised study.

Propositions belonging to the thesis entitled

"Nonhost wild lettuce as a donor for resistance to downy mildew in cultivated lettuce"

Anne K.J. Giesbers

Wageningen, 18 April 2018

Nonhost wild lettuce as a donor for resistance to downy mildew in cultivated lettuce

Anne K.J. Giesbers

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This research was conducted under the auspices of the Graduate School Experimental Plant Sciences

Nonhost wild lettuce as a donor for resistance to downy mildew in cultivated lettuce

Anne K.J. Giesbers

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 18 April 2018
at 1.30 p.m. in the Aula.

Anne K.J. Giesbers
Nonhost wild lettuce as a donor for resistance to downy mildew in cultivated lettuce
150 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2018)
With references, with summaries in English and Dutch

ISBN: 978-94-6343-879-7
DOI: 10.18174/432584

Contents

Abbreviations and glossary

***Avr* gene**: avirulence gene from a pathogen that triggers host resistance when recognized by an *R* gene.
BC1cult: backcross (BC1) population from the cross F1 (*L. saligna* x *L. sativa*) x *L. sativa.*
BC1wild: backcross (BC1) population from the cross F1 (*L. saligna* x *L. sativa*) x *L. saligna.*
BIL: backcross inbred line, here: line containing a single or few chromosome segments from *L. saligna* in a *L. sativa* background.
cv: cultivar.
***Dm* gene**: dominant resistance gene (*R* gene) against lettuce downy mildew.
BDM incompatibility: genetic incompatibility in hybrids that can be explained by the Bateson-Dobzhansky-Muller model.
Conspecific: belonging to the same species.
Effector: secreted pathogen protein that contributes to virulence and confers avirulence in plant genotypes that carry the cognate *R* gene.
ETI: effector-triggered immunity caused by recognition of an avirulent pathogen effector mediated by a plant NLR protein.
Heterospecific: belonging to a different species.
Hybrid incompatibility (HI): reduced hybrid fitness due to heterospecific gene interactions.
HR: hypersensitive response.
MRC: major resistance cluster.
LG: linkage group.
NB-LRR (NLR): nucleotide-binding leucine-rich repeat receptor. Plant intracellular receptors, usually encoded by *R* genes, that recognize effectors .
NHR: nonhost resistance.
PAMP (or **MAMP**): pathogen (or microbe) associated molecular pattern that can be recognized by PRRs.
PTI: PAMP-triggered immunity, mediated by recognition of PAMPs by plant PRRs.
PRR: pattern recognition receptor. Plant receptor protein at the cell surface that detects a PAMP leading to PTI.
QTL: quantitative trait locus.
Qualitative resistance: complete absence of disease, often mediated by an *R* gene.
Quantitative resistance: reduced but not complete absence of disease, conferred by one or multiple genes.
***R* gene:** monogenic dominant resistance gene conferring resistance to a pathogen, often via ETI.
RIS: relative infection severity level, here: % sporulation compared to the susceptible parent *L. sativa* cv Olof.
***S* gene:** monogenic dominant susceptibility gene that is required for susceptibility to a pathogen.
TRD: transmission ratio distortion, a deviation from Mendelian segregation of alleles.
TRDL: transmission ratio distortion locus.

Allele codes
c: *L. sativa* (**c**ultivated species) allele
w: *L. saligna* (**w**ild species) allele

Chapter 1

General Introduction

One of the main challenges in crop production is combating infection by pathogens. Development and implementation of disease resistant crops is an effective and environmentally friendly method of control. Therefore, one of the priorities of plant breeding is to obtain plant genotypes with improved disease resistance. Classical resistance genes are often rendered ineffective by the occurrence of pathogenic strains with new virulence characteristics. A plant species that is never infected with a particular pathogen species is called a nonhost for that pathogen. Nonhost resistance (NHR) is often assumed to be the most durable form of resistance which would be useful to exploit for disease resistance in crop plants. However, the genetics of NHR are poorly understood (Heath 2000; Thordal-Christensen 2003; Mysore and Ryu 2004). Inheritance studies of NHR are difficult, because these require interspecific crossings between host and nonhost species. Most nonhost species cannot be crossed to host species, as they are genetically diverged. Lettuce-downy mildew is one of the few plant-pathosystems in which the inheritance of NHR can be studied. The nonhost lettuce species *Lactuca saligna* is cross-compatible with cultivated lettuce, *Lactuca sativa*, which is a host for downy mildew (*Bremia lactucae*). Downy mildew is the most devastating disease in lettuce cultivation, leading to high yield losses (Michelmore and Wong 2008). The lettuce-downy mildew plant-pathosystem is an interesting subject of study:
1) from a fundamental point of view: how does NHR work in a nonhost that is closely related to a host?
2) from a more practical point of view: can we identify novel, potentially durable resistance genes against downy mildew from the nonhost *L. saligna* and introgress these in the cultivated host species, *L. sativa?*

Pathogen resistance in plants

Plants have developed multiple layers of preformed and inducible defences against pathogens. Preformed physical barriers such as wax layers, rigid cell walls, anti-microbial enzymes or secondary metabolites form the first line of plant defence against pathogens (Nürnberger *et al.* 2004). If a pathogen manages to overcome these constitutive defensive layers, active defence responses may prevent further spread of the pathogen. Plant pathogens are often divided into biotrophs (feeding on living host tissue) and necrotrophs (feeding on dead host material). Mechanisms underlying active defence responses differ between biotrophs and necrotrophs (Zhang *et al.* 2013). Here, we will focus on defence responses against biotrophs, as the oomycete *B. lactucae* falls into this category of pathogens.

Two main strategies of pathogen recognition have evolved in plants (Fig. 1) (Jones and Dangl 2006; Dodds and Rathjen 2010):
1) extracellular detection of pathogen-associated molecular patterns (PAMPs)
2) intracellular detection of pathogen virulence molecules called effectors

PAMPs, previously known as general elicitors, are conserved pathogenic molecular structures that are often indispensable for the pathogen. Recognition of PAMPs by pattern recognition receptors (PRRs) on the cell surface of a plant results in PAMP-triggered immunity (PTI) and is part of a plant's innate immunity. Successful pathogens can circumvent recognition of their PAMPs through effector proteins that suppress PTI. These pathogens penetrate the host cell wall and invaginate the host cell membrane by forming structures called haustoria, that are used both for nutrient uptake from the host and delivery of effector proteins to the host (Jones and Dangl 2006; Stassen and Van den Ackerveken 2011). Pathogen effectors enhance host infection and colonization. Oomycete effectors can be divided into two main classes: RXLRs (containing amino acid motifs of

arginine, any amino acid, leucine, arginine) and crinklers (Rouxel and Balesdent 2010; Stassen and Van den Ackerveken 2011; Anderson *et al.* 2015).

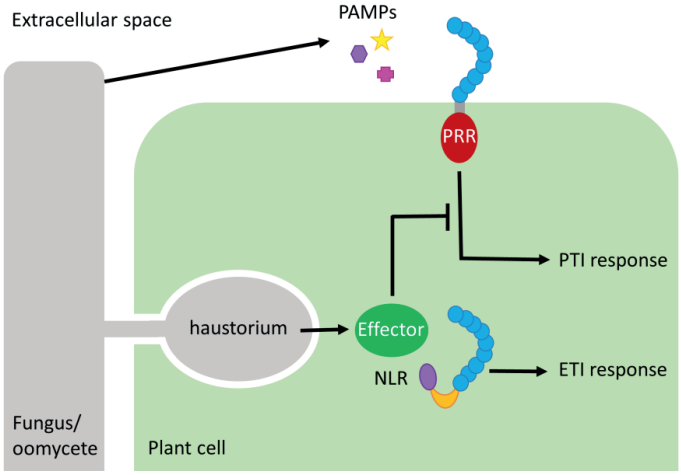

Figure 1. Two strategies of induced resistance against fungi or oomycetes. Detection of PAMPs on the plant surface through PRRs leads to a resistance response called PTI. Effectors that are secreted inside the plant cell may suppress the PTI response, but if recognized by NLR receptors lead to a resistance response called ETI (adapted from Dodds and Rathjen (2010)).

Plants can evolve to detect effectors through resistance (R) receptors, leading to effector-triggered immunity (ETI). When recognized by R proteins, effectors are called avirulence (Avr) proteins. The majority of R proteins are intracellular receptors that contain nucleotide binding (NB) and leucine-rich repeat (LRRs) domains (NLRs). NLRs are known to confer resistance to diverse pathogens including fungi, oomycetes, bacteria, viruses and insects (Dodds and Rathjen 2010). Recognition of effectors may either be direct by physical association or indirect through a plant target protein. The guard model explains the indirect detection of effectors: R proteins may "guard" plant targets, for instance PRRs, manipulated by effectors. Modification of the plant target results in activation of the R protein (Van Der Biezen and Jones 1998; Dangl and Jones 2001; van der Hoorn and Kamoun 2008). Direct or indirect recognition of effectors leads to ETI and is often associated with a localized cell death response at the site of infection, also called a hypersensitive response (HR).

The co-evolution between plants and pathogens is a continuous process as explained by the zigzag model of plant immunity (Jones and Dangl 2006). For instance, loss of function mutants in *Avr* genes will not be recognized by their cognate *R* gene anymore. Additionally, novel effectors may evolve that suppress ETI triggered by a certain effector (King *et al.* 2014; Teper *et al.* 2014). From the host side, mutations in *R* genes or plant targets may lead to altered resistance specificities. Some cases of PTI and ETI share much of the same downstream signalling machinery (Tsuda and Katagiri 2010). However, ETI induced responses are qualitatively stronger, faster (Dodds and Rathjen 2010), and prolonged compared to PTI (Tsuda and Katagiri 2010). Preformed defences and PTI typically function against non-adapted pathogens, while ETI is effective against adapted pathogens. However, these relationships are not exclusive (Dodds and Rathjen 2010) and there may

be a continuum between PTI and ETI rather than a strict distinction (Thomma *et al.* 2011), explained by the Invasion Model (Cook *et al.* 2015).

Qualitative versus quantitative resistance

Pathogen resistance in plants can broadly be divided into qualitative resistance and quantitative resistance (Fig. 2). Qualitative resistance is generally controlled by one or few major dominant *R* gene(s) and causes absence of disease, while quantitative resistance is typically mediated by multiple minor genes and causes reduction of disease (Niks *et al.* 2015). NHR however, is phenotypically qualitative (complete), mostly with a quantitative, polygenic inheritance (Niks and Marcel 2009; Niks *et al.* 2015). Quantitative resistance can also be mediated by a "defeated" or "weak" *R* gene (Brodny *et al.* 1986; Li *et al.* 1999; Fukuoka *et al.* 2014), for instance resulting from incomplete breakdown of resistance by mutation of its cognate *Avr* gene in the pathogen (Li *et al.* 1999).

The complete, qualitative resistance commonly conferred by *R-Avr* gene-for-gene interactions is infamous for its lack of resistance durability. Just one loss-of-function mutation in an effector (*Avr* gene) could prevent recognition by its cognate *R* gene, resulting in a virulent pathogen. The now virulent pathogen has a huge reproductive advantage, especially in present-day agriculture in which cultivars with the same *R* gene are grown on a large scale (Niks *et al.* 2015).

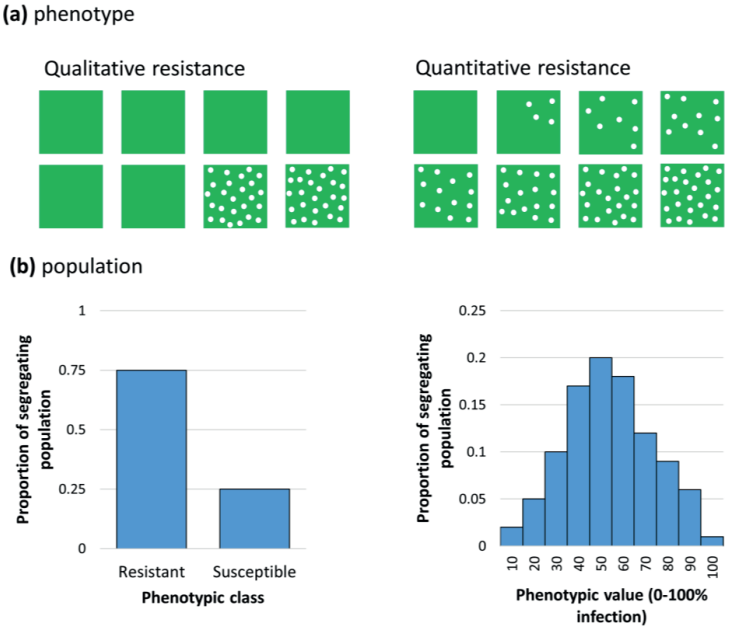

Figure 2. Qualitative versus quantitative resistance in segregating populations (a) phenotypically, two discrete classes of infection can be distinguished for qualitative resistance, while a continuous distribution of infection levels is observed for quantitative resistance. **(b)** a major dominant *R* gene results in a 3:1 ratio for resistance versus susceptibility, while multiple genes with small effect result in a continuous segregation of infection levels (adapted from Nelson *et al.* (2017)).

Another example of resistance that is usually assumed to be qualitative, but may also be quantitative, is due to a recessive loss-of-function mutation of a susceptibility (*S*) gene. *S* genes support plant-pathogen compatibility, and therefore a mutation in such a gene may hamper infection. To re-establish compatibility, the pathogen would have to acquire a new

way to infect the host through a gain-of-function mutation. Such a gain-of-function mutation is not very likely to occur, consequently resistance through *S* gene mutation is potentially durable. One well-known example is recessive mutation of the *Mlo* gene in many plant species, which was originally detected as a natural mutation in barley (Kusch and Panstruga 2017). Mutation of the *Mlo* gene prevents haustorial formation and confers durable broad-spectrum powdery mildew resistance. However, *S* genes also have an intrinsic function (pleiotropy) with a potentially essential function in the plant and consequently, loss-of-function mutation may lead to decreased plant fitness (Pavan *et al.* 2010; van Schie and Takken 2014).

Quantitative resistance based on multiple genes with a minor effect is not likely to be overcome rapidly. Multiple gain-of-function mutations in the pathogen would be required (Niks *et al.* 2015). Overcoming one minor-effect gene will provide only a small fitness gain, which is unlikely to spread rapidly and remain in the pathogen population. Still, pathogen populations probably also evolve to overcome quantitative resistance. Quantitative resistance may "erode" over time, characterized by a gradual increase in pathogenicity of the pathogen population (McDonald and Linde 2002; Brown 2015).

Nonhost (versus host) resistance

A plant species of which all germplasm is resistant to all genetic variants of a pathogen is known as a nonhost (Niks 1987; Heath 2000). In a host species that coevolved with its pathogen, certain accessions may display resistance to specific pathogenic strains. Host resistance is often governed by single gene-for-gene interactions in which *R* genes recognize pathogenic *Avr* genes. NHR more likely results from a continuum of layered defences (Heath 1997; da Cunha *et al.* 2006; Ham *et al.* 2007). Preformed, constitutive barriers and PAMP triggered immunity typically function against non-adapted pathogens and are therefore associated with NHR. Preformed barriers are likely to play a larger role in NHR to pathogens of plant species that are distantly related to the nonhost. Induced defences like PTI are probably most relevant in NHR to pathogens of a plant species closely related to the nonhost (Niks and Marcel 2009; Schulze-Lefert and Panstruga 2011). Successful pathogens can suppress host PTI through delivery of appropriate effectors within hours (Li *et al.* 2005; Caldo *et al.* 2006; Truman *et al.* 2006). In nonhosts, effectors apparently do not manage to suppress PTI. Likely the effectors cannot modify the plant targets in a nonhost, which is necessary for pathogen lifecycle completion. Little is known about the plant target genes that determine this failure of effector-triggered susceptibility (Niks *et al.* 2015). In 2011, Schulze-Lefert and Panstruga hypothesized that besides PTI, ETI might also contribute to NHR. The relative contribution of PTI might be more important in nonhosts that are distantly related to the host, whereas ETI might play a larger role in nonhosts that are closely related to the host. However, as far as we know there is no functional proof for the prevalence or essential involvement of ETI in NHR.

Like many biological phenomena, the division between host and nonhost status is not black and white, due to the quantitative nature of phenotypes in the transition between these two states (Niks 1987, 1988; Bettgenhaeuser *et al.* 2014). The evolutionary trajectory in which pathogen species try to establish a compatible interaction with a plant species is an ongoing process with transitions from compatibility to incompatibility and vice versa. Therefore, some distinguish subdivisions between host and nonhost status, like intermediate host and intermediate nonhost (Niks 1987; Bettgenhaeuser *et al.* 2014; Dawson *et al.* 2015).

Two evolutionary scenarios for NHR have been postulated by Antonovics *et al.* (2012):

"one-sided evolved" and "nonevolved". NHR may be "one-sided evolved", if the pathogen did not counter-evolve to genetic changes in the plant that directly or indirectly (e.g. adaptation to a different climate) led to decreased infection levels. Alternatively, NHR could be "nonevolved", if the inability to infect the plant is a property of the pathogen and not an evolved trait of the plant, for instance if the pathogen specialized on another host. Generally, genetic variation for resistance in a nonhost is not expected (but may be incidentally present), whereas polymorphism for resistance is expected for hosts that coevolved with a pathogen. More genes are expected to contribute to NHR with increasing phylogenetic distance from the source host (Antonovics *et al.* 2012).

What is known about the genetics of NHR?
The inheritance of NHR is difficult to study, as interspecific crosses between host and nonhost species are usually not possible due to reproductive barriers. One way to circumvent this problem was shown by Atienza *et al.* (2004), who developed barley, an intermediate nonhost (also called near nonhost) to certain rust fungi (*Puccinia*), into a fully susceptible barley line (SusPtrit) that can be crossed with regular resistant lines. Phenotypic and genotypic analyses in populations of SusPtrit crossed with resistant lines led to the identification of multiple QTLs that may play a role in NHR (Jafary *et al.* 2006, 2008). Also, some inheritance studies were attempted based on crossings within a nonhost species that shows natural variation in its degree of resistance to a non-pathogen (Shafiei *et al.* 2007; Ayliffe *et al.* 2011). Often such studies are hampered by the relatively small differences in the levels of components of the infection process and by the need for tedious microscopic observations. Only Shafiei *et al.* (2007) were able to map QTLs: three minor ones for substomatal vesicle formation in *Arabidopsis* against a wheat leaf rust pathogen.

Other approaches to study the genetics of NHR include gene-expression studies (Delventhal *et al.* 2017) and mutagenesis approaches. For instance, knock-out screenings have been used to identify nonhost genes that are necessary for the retention of resistance (An *et al.* 2016; Zhao *et al.* 2016). In *Arabidopsis*, mutant screenings and microscopic observation identified three genes (PEN1, PEN2 and PEN3) that, when mutated, lead to enhanced haustorial formation of non-adapted powdery mildew pathogens (Collins *et al.* 2003; Lipka *et al.* 2005; Stein *et al.* 2006). Additional mutation of components of a layer of posthaustorial defense permitted development of more microcolonies and occasional conidiophores by *Blumeria graminis* f. sp. *hordei* (*Bgh*) and even dense sporulation by *Erisiphe pisi* (Lipka *et al.* 2005).

The plant-pathosystem of lettuce and downy mildew provides a rare opportunity to study the inheritance of NHR directly, because the nonhost wild lettuce species *Lactuca saligna* is sufficiently cross-compatible with the cultivated host *Lactuca sativa* to allow inheritance studies.

Lettuce
Cultivated lettuce (*Lactuca sativa*) belongs to the genus *Lactuca* L. and the family of Compositae, also known as Asteraceae. Lettuce is most often grown as a leafy vegetable, but also for its stem (mainly in China) or in the past for its oil-containing seeds in ancient Egypt (Harlan 1986). Popular types of lettuce grown nowadays are butterhead, crisphead, looseleaf (cutting), romaine (cos), latin, stalk (stem lettuce) and Batavia.

Lettuce cultivation has probably started in South West Asia (Boukema *et al.* 1990). The highest number of wild lettuce species are found between the Euphrates and the Tigris rivers (De Vries 1997). This region is probably the most ancient origin of agriculture, and among other crops, cultivated lettuce may have its origin there. Wall-paintings and reliefs

of some Egyptian tombs from 2500 B.C. indicate its ancient cultivation in Egypt (De Vries 1997). The wild lettuce species *L. serriola* is probably the direct ancestor of cultivated lettuce, though one or two other species might have been involved (Lindqvist 1960; Kesseli *et al.* 1991; De Vries 1997). Domestication of lettuce into a leafy vegetable has resulted in a decrease in latex content and bitter taste, more tender and soft leaves, head formation, loss of prickles on stem and leaf, absence of early bolting, increase in seed size, non-shattering of the seeds (De Vries 1997).

Objectives of modern lettuce breeding include resistance to pests and diseases, particularly downy mildew and lettuce mosaic virus. Other modern lettuce breeding focus points include morphological traits, bolting resistance, a short growing period, low energy need and long shelf life (De Vries 1997; Ryder 2001; Hunter *et al.* 2017). The primary gene pool for cultivated lettuce improvement comprises *L. dregeana, L. altaica, L. serriola and L. aculeata*, as these can easily be intercrossed with *L. sativa* (Lebeda *et al.* 2009). The downy mildew nonhost *L. saligna* belongs to the secondary gene pool, whereas *L. virosa* can be considered as a secondary or tertiary gene pool species (Lebeda *et al.* 2014). *L. georgica* was previously often classified as *L. virosa*, but is now recognized as a separate species (http://www.cng.wur.nl). Based on our own phenogram (Fig. 3) *L. georgica* indeed forms a separate clade from *L. virosa*.

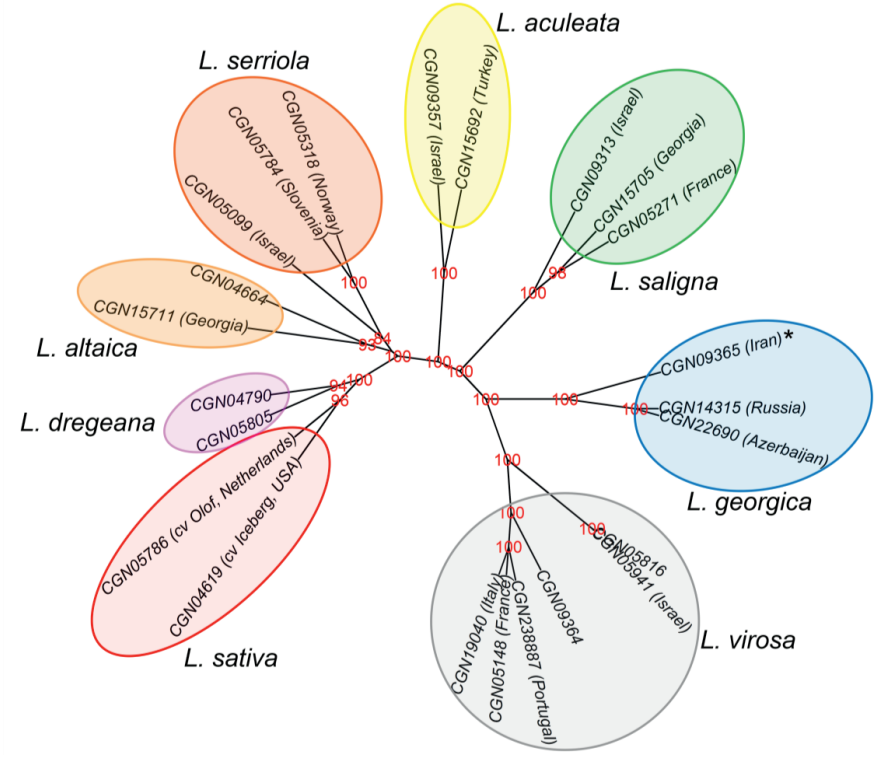

Figure 3. Phenogram based on 387 AFLP markers of *Lactuca* species cross-compatible with *L. sativa*. Distances were calculated using the 'DIST' function in the R package 'STATS' (R Core Team, 2016). A tree was obtained using the neighbourjoining method in the R package 'APE' (Paradis *et al.* 2004). Bootstrap values >60% (based on 1000 replicates) are indicated in red at the nodes. *classified as *L. virosa* by the Dutch Center for Genetic Resources (CGN), but based on AFLP patterns probably a *L. georgia*.

L. sativa is an autogamous and diploid species, with nine chromosomes (*2n=2x*=18) and an estimated genome size of 2.7 Gb. The relatively large size of this genome is probably partly due to an ancient genome triplication and the presence of many repetitive sequences (Reyes-Chin-Wo *et al.* 2017). A total number of 38,919 protein coding genes have been predicted for *L. sativa* cv. Salinas (Reyes-Chin-Wo *et al.* 2017). Phenotypic resistance genes and candidate disease resistance genes are clustered in major resistance clusters (MRCs) in the lettuce genome (Meyers *et al.* 1998; Christopoulou *et al.* 2015b).

The first *Lactuca* linkage map was constructed from the *L. sativa* cv Calmar x *L. sativa* cv Kordaat cross with 53 genetic markers, mostly RFLP (Landry *et al.* 1987). In a more detailed follow-up linkage map, the marker number was increased to 319 RLFP and RAPD markers (Kesseli *et al.* 1994). A first linkage map was based on two interspecific F2 from *L. saligna* x *L. sativa* with nearly 500 AFLP markers and resulted in nine linkage groups (Jeuken *et al.* 2001). A high-density genetic map based on RILs generated by crossing *L. sativa* cv Salinas x *L. serriola* was constructed by Truco *et al.* (2013). These RILs were recently also used to validate a *de novo* genome assembly of *L. sativa* cv. Salinas and generate chromosomal pseudomolecules for *L. sativa* (Reyes-Chin-Wo *et al.* 2017).

Downy mildew

Lettuce downy mildew is caused by *Bremia lactucae* Regel, which belongs to the order of *Peronosporales*, phylum of oomycetes and kingdom of Stramenopiles. Although oomycetes physically resemble fungi, they are actually more closely related to brown algae and diatoms (Coates and Beynon 2010). *B. lactucae* has an obligate biotrophic lifestyle, meaning it obtains its nutrients from living host tissue. Downy mildew is regarded as the most important disease affecting lettuce worldwide (Michelmore and Wong 2008). It is presently not possible to grow *B. lactucae* in axenic cultures away from host plants.

Figure 4. Symptoms of lettuce downy mildew infection (a) healthy, green lettuce plant **(b)** severely infected lettuce plant with brown leaves **(c)** white sporulation on a leaf segment from an adult plant **(d)** white sporulation on lettuce seedlings.

B. lactucae spores are naturally spread by wind and rain. Pathogen development is most successful under cool (15°C) and moist conditions. In compatible interactions of *B. lactucae* with a lettuce host, spores germinate on the leaf followed by the formation of appressoria that directly penetrate the plant epidermal cells. Subsequently, primary vesicles, secondary vesicles, hyphae and haustoria (feeding structures which invaginate the host plasma membrane) are formed. Symptoms of infection first include pale, yellow (chlorotic) areas on the lettuce leaves mostly delimited by the main veins, after which white and woolly sporulation develops on the abaxial (lower) leaf surface (Fig. 4). Infected leaf areas expand with time and ultimately turn brown (Sargent *et al.* 1973; Lebeda and Reinink 1994; Lebeda *et al.* 2008).

B. lactucae is diploid for the majority of its lifecycle and harbours a minimum of seven or eight pairs of chromosome plus a set of linear polymorphic molecules that are inherited in a non-Mendelian manner (Michelmore and Wong 2008). Its total genome size is estimated to be around 50 Mb, but this is probably an underestimate (Michelmore and Wong 2008). *B. lactucae* is predominantly heterothallic, with two sexual compatibility types (Michelmore and Sansome 1982). It has a short asexual life-cycle of a few weeks and a sexual cycle of several months(Michelmore and Wong 2008). In field conditions, downy mildew is primarily spread by asexual spores (Lebeda *et al.* 2008). *B. lactucae* adapts rapidly to host plant resistance or fungicides due to a successful combination of large population size, both sexual and asexual reproduction and high gene flow (McDonald and Linde 2002).

Transcriptome sequencing of *B. lactucae* race Bl:24 resulted in the prediction of 77 potential RXLR(-like) effectors that might contribute to pathogen virulence (Stassen *et al.* 2012). Transient expression of candidate effectors in a plant may result in a hypersensitive response (HR) resulting in localized cell death, which indicates the presence of an *R* gene against the effector. Transient expression assays may be a rapid and effective method for the identification of new *R* genes (Vleeshouwers *et al.* 2011; Vleeshouwers and Oliver 2014). Therefore, 34 of the 77 *B. lactucae* effector candidates were cloned and expressed in a *Lactuca* germplasm set (Stassen *et al.* 2013). Two effectors were found to trigger an HR, one in *L. saligna* and in *L. sativa*. However, these responses were not associated with resistance against the *B. lactucae* race from which the effectors originated (Stassen *et al.* 2013).

Downy mildew control

Strategies for control of downy mildew include the use of fungicides and genetic resistance. *B. lactucae* frequently evolves insensitivity to fungicides (Brown *et al.* 2004). Furthermore, the use of fungicides can be costly, is potentially harmful to consumers and is becoming increasingly limited by restrictive regulations. The deployment of resistant cultivars is an effective and economically sustainable method of control (Michelmore *et al.* 2017).

So far, 28 downy mildew resistance (*Dm*) genes (*R* genes), 23 resistance (*R*) factors and 15 quantitative trait loci for resistance to downy mildew have been identified (Parra *et al.* 2016). Resistance genes are denominated as *Dm* genes when they have been mapped to a single locus, whereas *R* factors have not (yet) been mapped. Most of these resistances have been identified from *L. sativa* and *L. serriola*, but some originated from *L. saligna* and *L. virosa*. All 28 *Dm* genes that have been described are located in major resistance clusters, mainly on linkage group 1, 2 and 4 (Parra *et al.* 2016). Until now, the sequence of only two of these *Dm* genes has been identified: *Dm*3 and *Dm*7, both in *L. sativa* (Shen *et al.* 2002; Christopoulou *et al.* 2015a). *Dm* genes are commonly used in lettuce cultivars and provide high levels of resistance, but are continually rendered ineffective by the occurrence of pathogenic strains with new virulence characteristics (Lebeda and Zinkernagel 2003). New strategies are needed to provide more durable forms of resistance (Michelmore and Wong 2008).

***L. saligna*: a source of NHR genes against downy mildew**

The wild lettuce species *L. saligna* is considered as a nonhost to downy mildew. Its broad resistance spectrum has been described since 1976 (Netzer *et al.* 1976; Norwood *et al.* 1981; Bonnier *et al.* 1992). Sparse sporulation of *L. saligna* has been observed at seedling stage for some accessions, but this may be a plant-stage dependent effect as the accessions showed full resistance at adult plant stage (Bonnier *et al.* 1992; Petrželová *et al.* 2011). Very sparse sporulation (maximally 5% of the leaf area) has been observed on

the edges of leaf segments at adult plant stage, but only under laboratory conditions (Jeuken and Lindhout 2002). *L. saligna* has never been reported as a host in natural habitats (Lebeda *et al.* 2002; Beharav *et al.* 2008). A large dataset of 33 *L. saligna* accessions tested with 27 downy mildew races confirmed the nonhost status of *L. saligna* (van Treuren *et al.* 2011).

What is known about NHR in *L. saligna*?

Microscopic observations on *L. saligna* inoculated with *B. lactucae* indicated that NHR is mainly based on prehyphal or prehaustorial resistance. Only malformed hypha-like structures and no haustoria were observed (Lebeda and Reinink 1994; Zhang *et al.* 2009b). The genetics of NHR in *L. saligna* has been studied previously for *L. saligna* CGN05271. A wide and continuous range of infection severity levels was observed in an F2 population generated from a cross between the nonhost *L. saligna* and the host *L. sativa* cv Olof, which is susceptible to all *B. lactucae* races (Fig. 5).

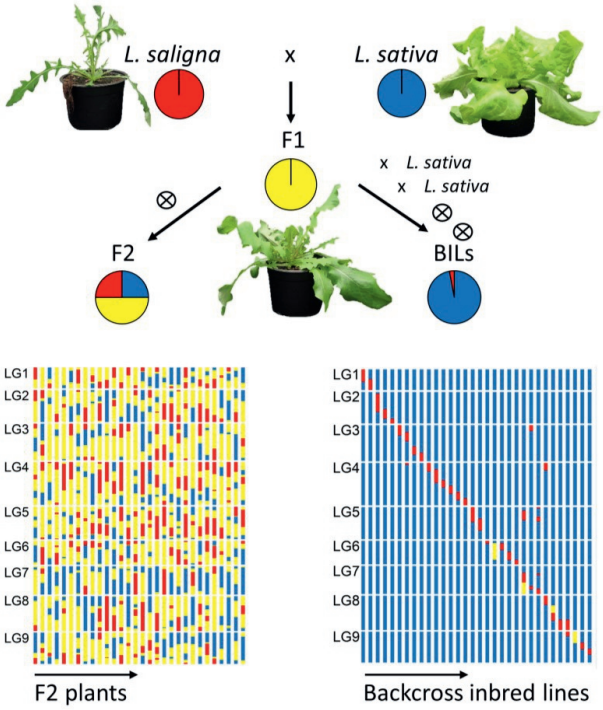

Figure 5. Crossing scheme and genetic composition of *L. saligna, L. sativa*, the interspecific F1, F2 and backcross inbred lines (BILs). Red: homozygous *L. saligna*, blue: homozygous *L. sativa,* yellow: heterozygous.

Few F2 plants were fully resistant like *L. saligna* or susceptible like *L. sativa*, but most plants showed infection severity levels in the continuum between *L. saligna* and *L. sativa* (Fig. 6a). Three minor QTLs for downy mildew resistance at adult plant stage were identified in this F2 population (Jeuken and Lindhout 2002). Furthermore, a set of 29 backcross inbred lines (BILs) that generally each contain one nonhost (*L. saligna*) introgression in a host (*L. sativa*) background has been developed (Jeuken and Lindhout 2004) (Fig. 5). Some BILs showed relatively low levels of infection severity compared to *L. sativa*, but none as low as *L. saligna* itself (Fig. 6b). In tests at seedling, young plant and

adult plant stage fifteen, probably race-nonspecific, QTLs from *L. saligna* were detected in the set of BILs (Jeuken *et al.* 2008; Zhang *et al.* 2009a). Of these 15 QTLs, only two were found to be effective at every plant developmental stage (Zhang *et al.* 2009a). Fine mapping three resistance QTLs was not possible, due to disintegration into sub-QTLs that reduced or even promoted infection (den Boer *et al.* 2013). Furthermore, stacking eight combinations of two race-nonspecific QTLs by intercrossing of BILs did not result in greatly elevated levels of resistance (Den Boer *et al.* 2014).

Some *L. saligna* accessions also contain race-specific *R* genes (Parra *et al.* 2016). However, *R* genes seem not essential for NHR, as the studies on the F2 and set of BILs from *L. saligna* CGN05271 indicated presence of quantitative resistance genes only (Fig. 6) (Jeuken and Lindhout 2002; Jeuken *et al.* 2008; Zhang *et al.* 2009a).

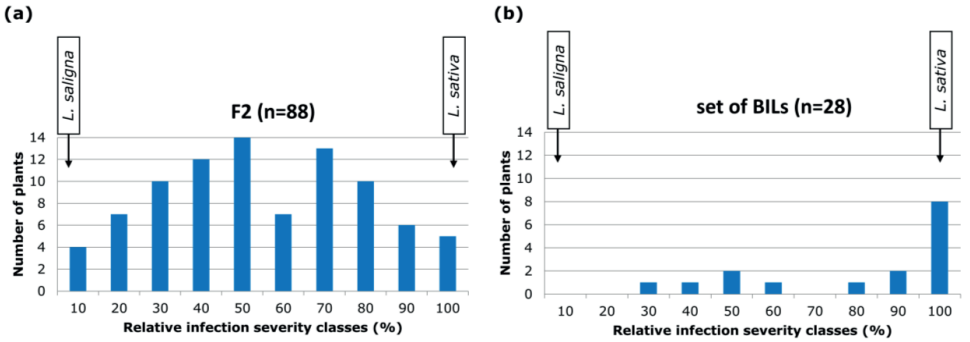

Figure 6. Infection severity at adult plant stage relative to *L. sativa* cv Olof in (a) an F2 population from a cross between *L. saligna* CGN05271 and *L. sativa* cv Olof, tested with *B. lactucae* Bl:16 (adapted from Jeuken and Lindhout (2002)) **(b)** a set of backcross inbred lines (BILs) each containing one or a few *L. saligna* CGN05271 introgressions in a *L. sativa* cv Olof background, tested with *B. lactucae* Bl:14 and Bl:16 (adapted from Zhang *et al.* (2009a)).

Apparently, the genetics of NHR in *L. saligna* is complex. NHR of *L. saligna* is probably based on interactive, possibly epistatic, loci that have little or no effect individually but in combination lead to high levels of resistance.

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

Disease testing and genetic analysis of recombinant inbred lines would be a useful strategy to identify epistatic interactions. However, the F2 generation showed hybrid breakdown with severely reduced fertility, probably due to interspecific genetic incompatibilities, which limited further inbreeding and hampered the development of recombinant inbred lines (Jeuken *et al.* 2008). Interspecific reproductive barriers are a common phenomenon, and are due to genetic incompatibilities between two different genomes. Deleterious interactions between the genomes of two species, also referred to as hybrid incompatibilities, can severely limit the exchange of genetic variants between species. Hybrid incompatibilities may lead to distortion of Mendelian segregation of alleles and genotypes in hybrids. Such transmission ratio distortion (TRD) is frequently observed in interspecific crosses and may indicate hybrid incompatibilities (Rieseberg *et al.* 2000; Koide *et al.* 2008). TRD has been observed in interspecific F2 populations of *L. saligna* and *L. sativa* (Jeuken *et al.* 2001) and in BILs that remained heterozygous even after several generations of selfing (Jeuken and Lindhout 2004).

The most common genetic mechanism leading to the evolution of hybrid dysfunction is the incidental accumulation of incompatible gene interactions (Presgraves 2010). A model

explaining how hybrid incompatibility (HI) can evolve without species themselves experiencing reduced fitness has independently been formulated by Bateson, Dobzhansky and Muller and is commonly referred to as the Bateson-Dozhansky-Muller (BDM) model (Bateson 1909; Dobzhansky 1937; Muller 1942; Orr 1996). The BDM model states that each pair of interacting genes evolves independently in separate lineages. Deleterious interactions between heterospecific alleles can occur in hybrids, when independently derived alleles meet (Maheshwari and Barbash 2011). Genetic changes that are adaptive or nearly neutral in their own (conspecific) genomic background can be functionally incompatible with alleles from a foreign (heterospecific) genomic background (Presgraves 2010). Deleterious heterospecific allele interactions can cause sporophytic, gametophytic or zygotic reproductive barriers (Fig. 7).

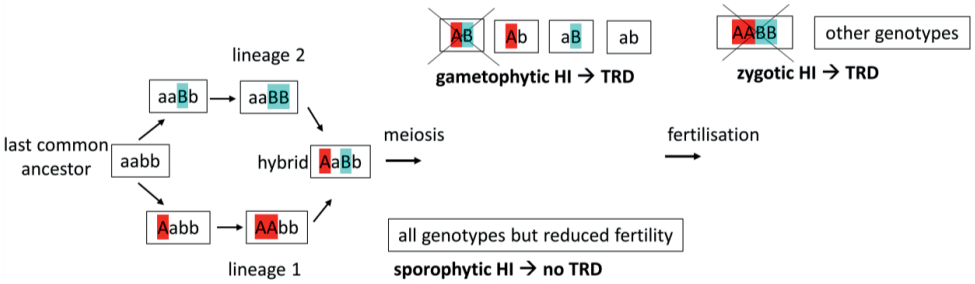

Figure 7. The Bateson-Dobzhansky-Muller (BDM) model for hybrid incompatibilities. New alleles (highlighted, capitalized letters) at one or more loci may arise in independent lineages that shared the same ancestor. These derived alleles have no negative fitness effect in their native, conspecific genomic context, but when combined in a hybrid they can cause genetic incompatibilities due to heterospecific gene interaction. Examples of sporophytic, gametophytic, and zygotic hybrid incompatibility (HI) are shown. Before fertilisation, HI can be caused by reduced fitness of the sporophyte (sporophytic HI), leading to formation of all genotypes (no TRD) but reduced fertility. Non-transmission of a heterospecific allele combination (e.g. AB) in the gametophyte (gametophytic HI), leads to transmission ratio distortion (TRD) in the offspring. After fertilisation, HI can be caused by reduced fitness of a heterospecific allele combination (e.g. AABB) in the zygote (zygotic HI), leading to TRD in the offspring.

One specific form of HI, hybrid necrosis, has been observed and characterized in hybrids between *L. saligna* CGN05271 and *L. sativa* cv Olof. This zygotic barrier was due to a heterospecific interaction of two loci. It caused lethal to mild necrotic lesions on the plants and was associated with resistance to downy mildew (Jeuken *et al.* 2009).

Hybrid incompatibilities can cause non-Mendelian segregation of alleles and hamper the introgression of traits of interest from *L. saligna* into *L. sativa*. Therefore, knowledge on heterospecific gene interactions leading to non-transmission of certain heterospecific allele combinations may be important for breeding of *L. sativa* cultivars with *L. saligna* introgressions.

Scope and structure of this thesis

The main aims of this thesis were to contribute to a better understanding of the genetics of NHR to lettuce downy mildew, and to provide new leads for breeding downy mildew resistant lettuce. Additionally, hybrid incompatibilities between the two lettuce species were studied, as these may complicate the introgression of resistance traits from *L. saligna* into *L. sativa*.

Chapter 2 describes the use of computationally predicted effector genes from *B. lactucae* as a tool to preselect *L. saligna* accessions with an HR response and consequently potential *R* genes. Due to the rapid defeat of the latest introduced *R* genes by *B. lactucae*, there is a constant need for new resistance genes in lettuce. Few *L. saligna* accessions are known to harbour *R* genes against downy mildew, but the general frequency of *R* genes in *L. saligna* is unknown. *L. saligna* is relatively uncharacterized and unexploited in lettuce breeding and may be a source of novel *R* genes and other types of resistance genes.

Previous studies on the NHR of *L. saligna* were all conducted on accession CGN05271. In **Chapter 3**, we extended this study to a total of nine accessions from a diverse geographic origin. To identify combinations of epistatically-acting loci resulting in NHR, we used two complementary approaches: (1) nonhost into host introgression: identification of *L. saligna* derived chromosome regions that were overrepresented in highly resistant BC1 plants, (2) host into nonhost introgression: identification of *L. sativa* derived chromosome regions that were overrepresented in BC1 inbred lines with relatively high infection levels.

In **Chapter 4**, postzygotic reproductive barriers between *L. saligna* and *L. sativa* were mapped using an F2 population, backcross populations and backcross inbred lines (BILs). We identified loci with TRD (TRDL) in the F2 and backcross populations. The BILs indicated which TRDL were associated with an absolute non-transmission of the homozygous *L. saligna* genotype. Three loci were associated with a heterospecific digenic hybrid incompatibility. One of these digenic interactions was fine mapped and characterized in more detail.

Finally, in **Chapter 5** the results of all previous chapters are integrated and discussed together with perspectives for future research on the genetics of NHR, hybrid incompatibilities and the application of our findings for downy mildew resistance breeding in lettuce.

Chapter 2

Effector-mediated discovery of a novel resistance gene against *Bremia lactucae* in a nonhost lettuce species

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Published in *New Phytologist* 2017, 216: 915–926

Abstract

- Candidate effectors from lettuce downy mildew (*Bremia lactucae*) enable high-throughput germplasm screening for the presence of resistance genes. The nonhost species *Lactuca saligna* comprises a source of *B. lactucae* resistance genes that has hardly been exploited in lettuce breeding. Its cross-compatibility with the host species *L. sativa* enables the study of inheritance of nonhost resistance (NHR).
- We performed transient expression of candidate RXLR effector genes from *B. lactucae* in a diverse *Lactuca* germplasm set. Responses to two candidate effectors (BLR31 and BLN08) were genetically mapped and tested for co-segregation with disease resistance.
- BLN08 induced a hypersensitive response (HR) in 55% of the *L. saligna* accessions, but responsiveness did not co-segregate with resistance to Bl:24. BLR31 triggered an HR in 5% of the *L. saligna* accessions, and revealed a novel *R* gene providing complete *B. lactucae* race Bl:24 resistance. Resistant hybrid plants that were BLR31 non-responsive indicated other unlinked *R* genes and/or nonhost QTLs.
- We have identified a candidate avirulence effector of *B. lactucae* (BLR31) and its cognate *R* gene in *L. saligna.* Concurrently, our results suggest that *R* genes are not required for NHR of *L. saligna*.

Introduction

Nonhost resistance (NHR) is defined as immunity occurring in all genotypes of a plant species against all genotypes of a specific pathogen (Heath, 1981; Niks, 1987; Niks & Marcel, 2009). Understanding the mechanisms of NHR may lead to the development of durable and broad-spectrum disease resistance in crop plants.

If a pathogen breaks through a plant's preformed defenses, it can be recognized by that plant through two overlapping layers of immunity (Jones & Dangl, 2006). The first layer depends on recognition of pathogen-derived molecules, also called pathogen-associated molecular patterns (PAMPs). PAMP recognition, commonly through pattern recognition receptors, can lead to resistance and is referred to as PAMP-triggered immunity (PTI). To counteract PTI, pathogens secrete effector molecules targeting host intracellular compartments that enhance infection through manipulation of host cellular processes, leading to effector-triggered susceptibility (ETS).

The second layer of immunity is triggered when host cells recognize avirulence effectors through resistance (R) proteins. In oomycetes, like *Phytophthora* and downy mildew, effectors are often characterized by RXLR and EER amino acids motifs (Rouxel & Balesdent, 2010; Anderson *et al.*, 2015). These conserved amino acid motifs are thought to mediate entry of oomycete effectors into host cells (Whisson *et al.*, 2007). R proteins are typically nucleotide-binding leucine-rich repeat (NLR) proteins that act on their own or in pairs to recognize effectors directly, or indirectly by detecting manipulation of a plant target (Van Der Biezen & Jones, 1998; Wu *et al.*, 2016). Recognition of effectors leads to effector-triggered immunity (ETI) and is usually associated with a hypersensitive response (HR) resulting in localized cell death.

Application of (candidate) effectors to host plant leaf tissue through transient expression triggered HR responses that were associated with novel and already known *R* genes (Vleeshouwers *et al.*, 2008; Rietman *et al.*, 2012; Gascuel *et al.*, 2016; Lenman *et al.*, 2016). Recognition of effectors in nonhost species is often assumed to contribute to NHR (Wroblewski *et al.*, 2009; Schulze-Lefert & Panstruga, 2011; Lee *et al.*, 2014; Adlung *et al.*, 2016). As NHR implies by definition that a complete species is resistant, it should be studied at the species level.

Just a few screenings of transiently expressed effectors in multiple accessions of nonhost species are described, viz.: *Pseudomonas* and *Ralstonia* effectors in 19 *Lactuca sativa* accessions (Wroblewski *et al.*, 2009), *B. lactucae* effectors in 52 *Lactuca saligna* accessions (Stassen *et al.*, 2013), *Phytophthora infestans* effectors in 42 *Capsicum annuum* accessions (Lee *et al.*, 2014), and *Xanthomonas campestris* pv. *vesicatoria* effectors in 46 *Nicotiana tabacum* accessions (Adlung *et al.*, 2016). Effector induced response patterns in these studies can be divided into: 1) responses induced in a broad range of accessions, and 2) responses induced in a narrow range of accessions. In all of these studies, some effectors triggered an HR in a broad range of accessions from the nonhost species, ranging from 52% (Lee *et al.*, 2014)) to 100% responsive accessions (Wroblewski *et al.*, 2009; Adlung *et al.*, 2016).

Yet, it is still unclear to what extent nonhost resistance is due to ETI. A way out could be to cross the resistant nonhost species to a susceptible host, and to find co-segregation between effector induced HR and resistance in the progeny. However, host and nonhost plants are usually sexually incompatible, which hampers classical genetic studies of NHR. The plant pathosystem of lettuce (*Lactuca* spp.) and downy mildew (caused by the

oomycete *Bremia lactucae* (Michelmore & Wong, 2008)) provides a rare opportunity to study the inheritance of NHR. The cultivated host species *L. sativa* is cross-compatible with the nonhost species *L. saligna.* Since 1976, several studies indicate that *L. saligna* accessions are totally disease-free (Netzer *et al.*, 1976; Norwood *et al.*, 1981; Lebeda, 1986; Gustafsson, 1989; Bonnier *et al.*, 1992; Petrželová *et al.*, 2011; Van Treuren *et al.*, 2011). Histology studies suggested that germinated conidia in *L. saligna* were arrested before normal hyphae formation (Lebeda *et al.,* 2008; Zhang *et al.,* 2009b).

Based on our previous genetic studies, the nonhost resistance of *L. saligna* CGN05271 seems to be explained by multiple race-non-specific quantitative effects (QTLs), though their QTL x QTL interactions have not been solved yet (Jeuken & Lindhout, 2002; Jeuken *et al.*, 2008; Zhang *et al.*, 2009a; den Boer *et al.*, 2014). In addition, a few *L. saligna* accessions are known to contain dominant monogenic race-specific *R* genes to *B. lactucae* (Parra *et al.*, 2016). The race-specific effect of these *R* genes makes it unlikely that they are the main factor to explain NHR.

Since the 1970s more than 50 *R* genes have been deployed in lettuce breeding (Parra *et al.*, 2016). Most of them originated from the primary genepool species *L. sativa* or *L. serriola* (Parra *et al.*, 2016). These genes do not provide broad spectrum resistance and are rapidly broken by *B. lactucae.* At least 25 of these *R* genes co-localize to one of the three major clusters of candidate resistance genes (NLRs) in the lettuce genome (Christopoulou *et al.*, 2015). The resistance of secondary genepool species, including the nonhost *L. saligna*, to *B. lactucae* is relatively uncharacterized and unexploited, because of a low success rate of crossings with cultivated lettuce and reduced fertility of the F1 generation. Testing computationally predicted effector genes (referred to as candidate effectors) in the nonhost *L. saligna* could be a helpful tool to preselect accessions with an HR and therefore potential *R* genes. By only using these preselected accessions for further mapping studies, a lot of time and effort involved in the development of wide crosses and segregating populations could be saved. In this manner, potential sources of *R* genes are expanded beyond the primary gene pool in an efficient way.

Compared to classical disease tests, screening *Lactuca* germplasm with effectors by transient expression assays could be faster and more effective to identify new *R* genes. Furthermore, *R* gene repertoires can be compared between accessions. In an effector transient expression assay the effect of a single effector is observed, while a classical disease test with spores reflects the sum of effects from a mix of effectors. In classical disease tests, resistance of a plant may be the result of activation of various *R* genes, triggered by separate effectors that are masking each other's effects. As a result, potential resistances may remain hidden (reviewed in Vleeshouwers *et al.*, 2011). Effectoromics has shown its potential in the potato-*Phytophthora* pathosystem by the identification of new *R* genes, accelerated *R* gene cloning, detection of resistance specificities and *R* gene deployment in agriculture (Vleeshouwers & Oliver, 2014). Possibly, this success can be transferred to the lettuce-downy mildew pathosystem. Lettuce is definitely in need for new *R* genes as *B. lactucae* variability rapidly defeats the latest introduced *R* genes.

Previously, responses to two *B. lactucae* Bl:24 effectors have been mapped in *Lactuca* germplasm (Stassen *et al.*, 2013). Effector BLG03 triggered a response specifically in *L. sativa* cultivars containing the *Dm2* resistance gene, whereas effector BLG01 induced a response in the majority (90%) of tested *L. saligna* accessions. Both responses were not associated with disease resistance against the *B. lactucae* race from which the effector was derived.

In the current study we tested some new candidate effectors with a specific focus on the nonhost *L. saligna*. The objectives were to: a) obtain a broader picture on the responsiveness of *L. saligna* to *B. lactucae* effectors, and b) verify if responsiveness to effectors in this nonhost contributes to disease resistance. We have identified two new candidate effectors that induce a response in the nonhost *L. saligna*. Responsiveness to only one candidate effector co-segregated with disease resistance.

Materials and Methods

Plant materials

Lactuca L. germplasm (n=150) was selected to include 1) a wide range of *B. lactucae* resistances (n=53) including a differential set of *L. sativa* cultivars with single dominant *R* genes and 2) a broad diversity of accessions from the primary (n=72: *L. sativa*, *L. serriola*, *L. aculeata, L. altaica*) and secondary (n=78: *L. saligna*, *L. virosa, L. georgica*) gene pool of lettuce (Table S1a).

L. saligna phenogram

A phenogram of 45 *L. saligna* accessions was based on 423 aflp-fragments, derived from eight primer combinations. Distances were calculated using the "dist" function in the R package "stats" (R Core Team 2016). A tree was obtained using the neighbour-joining method in R package "ape" (Paradis *et al.*, 2004). Two *L. sativa* cultivars represented the outgroup.

Mapping populations

Responsiveness to candidate effectors and resistance genes against *B. lactucae* were mapped in segregating populations like F2, backcross (BC1) or a previously developed set of backcross inbred lines (BILs). F1-plants from *L. saligna* accessions x *L. sativa* cv Olof were retrieved with difficulty, variable success rates, and a severely reduced (though still sufficient) fertility for further inbreeding (F2) and backcrossing (BC1). F1s were used as a mother in a backcross to *L. sativa* cv Olof to obtain three distinct BC1 populations, or selfed to obtain an F2 population (Fig. 2b). Populations were named after their generation (BC1 or F2) and the CGN number of the parental *L. saligna* accession, resulting in the following population names: BC1_CGN05947, BC1_CGN05304, BC1_CGN05318, and F2_CGN05310. The set of 29 BILs with *L. saligna* CGN05271 introgression segments in a *L. sativa* cv Olof background (Jeuken & Lindhout, 2004) will be referred to as BILs_CGN05271.

DNA isolation and genotyping

DNA was isolated from plant leaf tissue either by a high-throughput NaOH method (Wang *et al.*, 1993) or by a CTAB method (Jeuken *et al.,* 2001). Polymorphisms between PCR products of *L. saligna* and *L. sativa* alleles were visualized by high-resolution melting curve differences on a LightScanner System (den Boer *et al.*, 2014). Populations were genotyped to map their effector response and/or major resistance gene(s). Genetic markers were analysed at the major resistance cluster (MRC) regions in *L. sativa* (Christopoulou *et al.*, 2015), if necessary followed by markers in the rest of the genome (primer sequences, Table S2). Genetic map distances are derived from a reference F2 genetic linkage map of cross *L. saligna* CGN05271 x *L. sativa* cv Olof (Jeuken *et al.*, 2001). Physical map locations refer to the *L. sativa* cv Salinas reference lettuce genome V8 (Reyes-Chin-Wo *et al.,* 2017; https://lgr.genomecenter.ucdavis.edu/). Here, we will use the chromosome numbering and orientation of the reference *L. sativa* physical map, in contrast to our previous publications. In order to relate previously reported trait locations to the mapped loci in the current study, Table S3 presents the correspondence between the chromosome numbering

and orientation of the two maps. We use the following allele notation: w= wild lettuce (*L. saligna*) allele, c= cultivated lettuce (*L. sativa*) allele. Genotype notation: ww =homozygous *L. saligna*, wc= heterozygous, cc = homozygous *L. sativa.*

Candidate effector identification and cloning

RNA was isolated from *B. lactucae* race Bl:24 spores and infected lettuce as described by Stassen *et al.* (2012). Total RNA was further processed using the Illumina mRNA-Seq sample preparation kit and sequenced (Illumina HiSeq2000). Total raw yield was 36 Mb and 41Mb for infected lettuce and *B. lactucae* spores respectively, with ≥Q30 scores of 77.5% and 85.8%. The *B. lactucae* transcriptome was assembled using SOAPdenovo-Trans release 1.03 (Luo *et al.*, 2012) using default settings with the following adjustments: avg_ins = 120, map_len = 32, max_rd_len = 100 and K = 21. Candidate coding regions within the transcript sequences were identified using TransDecoder (Haas *et al.*, 2013). Resulting protein sequences were analysed with SignalP3.0 (Bendtsen *et al.*, 2004) to construct a secretome.To identify effector candidates, proteins were screened for RXLR-like, dEER and LXLFLAK motifs. *B. lactucae* effector candidates were cloned (primer sequences, Table S2), sequenced and electrotransformed into *Agrobacterium tumefaciens* C58C1 (pGV2260) as described by Stassen *et al.* 2013. Searches for best BLAST hits were performed using BlastP on non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects (https://blast.ncbi.nlm.nih.gov, accessed 16 March 2017).

qPCR

Time course qPCR experiments were performed on 3-4 days old *L. sativa* cv Olof seedlings in a similar way as described by Stassen *et al.* (2013). Cotyledons were collected at 3 hours after infection, 1 day post inoculation (dpi), 3 dpi and 6 dpi. Expression levels were determined as the number of qPCR cycles required for the abundance of each amplicon to reach the cycle threshold (Ct) level. We performed three independent experiments, each with three biological replicates (two technical replicates each). *B. lactucae* actin expression levels were calculated as ΔCt values relative to *Lactuca sativa* actin. Effector expression levels were calculated as ΔCt values relative to *B. lactucae* actin (primer sequences,Table S2).

***Agrobacterium*-mediated transient transformation**

A. tumefaciens strains with cloned candidate effectors were grown in selective media, resuspended to an OD_{600} of 0.5 and infiltrated in lettuce leaves according to Stassen *et al.* (2013). Strains containing a vector with yellow fluorescent protein (YFP) or a cell death inducing protein of *Phytophthora sojae* (PsojNIP) were included as a negative and positive control respectively.

Per plant genotype we infiltrated two leaves with Agrobacterium strains, but for the F2_CGN05310 per plant only one leaf was infiltrated. In the *Lactuca* germplasm screening, each leaf was infiltrated with candidate effectors, YFP and PsojNIP. In the mapping populations, each leaf was infiltrated with the candidate effector and YFP. PsojNIP was applied to all parental lines, and to one leaf in BC1_05947 and BC1_05318. For the set of 29 BILs on average two plants per BIL were infiltrated. In the germplasm screening, the majority of accessions were assessed in two or three independent experiments. Subsequently, responses in a subset of secondary genepool accessions were verified (Table S1b).

Plants were infiltrated at an age of 28 to 42 days. At 5-8 dpi we observed symptoms at the upper and lower leaf surface. Plant responses to candidate effectors, positive (PsojNIP) and negative (YFP) controls were scored on a scale from 0 to 3 in half unit steps according to the following classification: 0: no visible symptoms 1: bleaching or yellowing (chlorosis), 2: strong yellowing or cell death, or 3: strong cell death. The negative control YFP consistently elicited a score between 0 and 1, which was considered as the background response to *Agrobacterium*. We corrected the plant score to candidate effectors by subtracting the plant score to YFP. So, the 'plant response to the effector' per plant is defined as the difference between the score to the candidate effector and the score to YFP. An average plant response of ≥1 to candidate effectors in the germplasm screening was considered as a robust response. In the segregating populations also lower responses, as low as value 0.3, were qualified as a response to an effector on the basis of co-segregation with resistance and/or the responsible *L. saligna* allele.

***B. lactucae* disease test**

Per mapping population independent detached leaf assays were conducted on 5-8-wk-old adult plants as previously described in Jeuken & Lindhout (2002) with some modifications. Four leaf squares (2 by 2 cm) from four fully extended leaves were collected per plant and inoculated with 2-4 x 10^5 spores/ml of *B. lactucae* race Bl:24. The following control lines were included: parental *L. saligna* accessions (complete resistance), *L. sativa* cv Iceberg and combi-BIL[4.1+6.3]+8.2 (high levels of quantitative resistance; Zhang *et al.*, 2009b; den Boer *et al.*, 2014), and *L. sativa* cv Olof and *L. sativa* cv Cobham Green (susceptible). Infection severity level (ISL) was scored visually as the percentage of leaf area covered with sporangiophores between 10 and 14 days after inoculation. Relative infection severity (RIS) levels were calculated as percentage relative to the ISL on the susceptible parent *L. sativa* cv Olof. Plants with RIS levels ≤10% were considered as highly resistant.

Results

Two *B. lactucae* candidate effectors trigger an HR in *L. saligna* accessions

Sixteen candidate effectors with RXLR(-like) motifs were predicted from newly generated *B. lactucae* transcriptome data (described in M&M) and could be added to the 34 ones that were published previously (Stassen *et al.*, 2012). Responsiveness to these new candidate effectors was tested by *Agrobacterium*-mediated transient expression on a *Lactuca* germplasm set (n=150). The tested lines were selected from the primary and secondary genepool of cultivated lettuce (*L. sativa*), for a wide range of resistances to *B. lactucae,* and for a broad range of geographic origins. Plants showed a range of macroscopic responses to candidate effector application, from no reaction (0) to chlorosis (1) and to little or severe cell death (2 and 3) (described in Materials and Methods). Here, we report on two candidate effectors, BLR31 and BLN08 (GenBank accession numbers KY940276 and KY940275), that induced a robust response (average ≥1) in the secondary gene pool species *L. saligna* (Table S1).

BLR31 is 126 amino acids long with an N-terminal signal peptide followed by RLLR and EER motifs that are typical for host-translocated oomycete effectors (Fig. 1a, Fig. S1). It shows no homology to proteins in the NCBI database. BLN08 (463 amino acids) also has a signal peptide followed by an EER motif, but with an RSLR motif further away. It shows homology (BLASTp, 30% identity, E-value=1e-55) to a hypothetical protein (CEG42686.1) of the sunflower downy mildew pathogen (*Plasmopara halstedii*). Expression levels in planta were determined by qPCR. Expression of *BLR31* was relatively high, appeared stable over a time

course of 6 days, but peaked slightly at 1 dpi. *BLN08* expression was highest at 3 hours after infection and decreased markedly during the course of infection (Fig. 1b).

After the first survey, a subset of secondary gene pool accessions was re-examined for responsiveness to BLN08 and BLR31. Most of the HR responses could be confirmed. Only *L. saligna* CGN05310 showed an HR in response to BLN08 in two additional experiments, whereas it did not respond in the germplasm screening (Table S1b).

In the germplasm screening of 150 lines, BLR31 triggered a consistent HR in three *L. saligna* accessions (CGN05947, CGN05310, CGN05304), all of Israeli origin (Fig. 1c, Fig. 2a, Table S1b). BLN08 induced a response in 30 out of 55 *L. saligna* accessions, as well as in a few *L. sativa, L. georgica* and *L. virosa* accessions or lines (Table S1b). BLN08 responsive *L. saligna* accessions originated from the full geographical range of occurrence of this species and were not restricted to a particular region (Fig. 1c). We focused on responsiveness to BLN08 in *L. saligna* that is a nonhost to *B. lactucae* and is cross-fertile with *L. sativa*. The species *L. virosa* and *L. georgica* are also highly resistant to *B. lactucae*, but they are less cross-fertile with *L. sativa*, and most *L. virosa-L. sativa* F1 hybrids are completely sterile (Lebeda *et al.,* 2002; Maisonneuve, 2003).

To determine whether responsiveness to BLR31 and BLN08 co-segregates with resistance to *B. lactucae*, we tested five *L. saligna* x *L. sativa* cv Olof mapping populations (BC1, BIL or F2) for HR response and infection level (Fig. 2). For BLR31 the three Israeli accessions CGN05304, CGN05310 and CGN05947 were selected as responsive parental accessions. For BLN08 we selected as parental accessions: CGN05271, for which a set of BILs was available, CGN05318, and CGN05947. The latter was selected for its response to both BLN08 and BLR31 (Fig. 2a).

Candidate avirulence effector BLR31 reveals its cognate *R* gene

Three mapping populations were tested with BLR31 resulting in a range of plant responses, from no reaction to chlorosis and cell death (Table S4). Two groups were distinguished within mapping populations: plants without any response (value 0) and plants with a low to high BLR31 response (the minimum value differed per population). BLR31 responses ranged from 0.8 to 2.5 for BC1_CGN05947, from 0.3 to 1.8 for BC1_CGN05304, and from 0.5 to 2.0 for F2_CGN05310 (Table S4). Responsiveness to BLR31 in all three populations was closely associated with a dominant *L. saligna* allele at the top of chromosome 2 (C2) (Venn diagrams Fig. 3, Table S4). The map interval overlaps with the *Dm3* region within Major Resistance gene Cluster 2 (MRC2) in *L. sativa* cv Salinas (Christopoulou *et al.*, 2015). The smallest genetic interval ranged from 0-6.1 cM in *L. saligna* CGN05310 (Fig. 4).

In an adult plant disease test per population, we evaluated for co-segregation of BLR31 responsiveness with resistance to *B. lactucae* Bl:24, the race from which the candidate effectors were cloned. Control lines behaved as expected, with >70% relative infection severity (RIS) for all but one of the susceptible controls, 0-30% RIS for the quantitative resistant controls, and 0% RIS for the *L. saligna* accessions (Table S4).

All three populations showed a wide and continuous range of infection severities from 100% (susceptible) to 0% (resistant) RIS (Fig. 3a-c), instead of two discrete classes, viz. highly susceptible like the parental *L. sativa* line and completely resistant like the parental *L. saligna* accession. For each of the three populations more than half of the plants (73%, 55%, and 86% respectively) were highly resistant (0-10% RIS, Fig. 3a-c). These infection level distributions are consistent with the segregation of quantitative (QTLs) as well as qualitative (monogenic dominant *R*-genes) resistance loci.

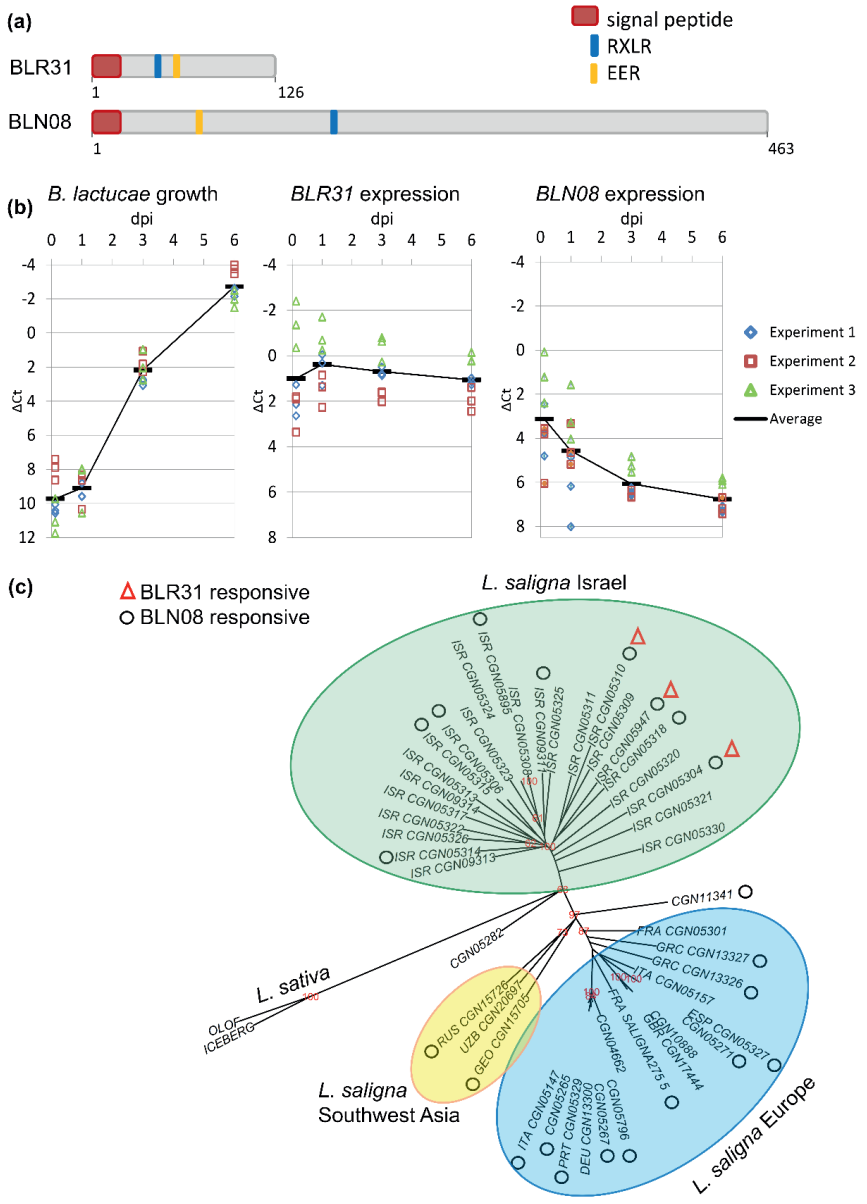
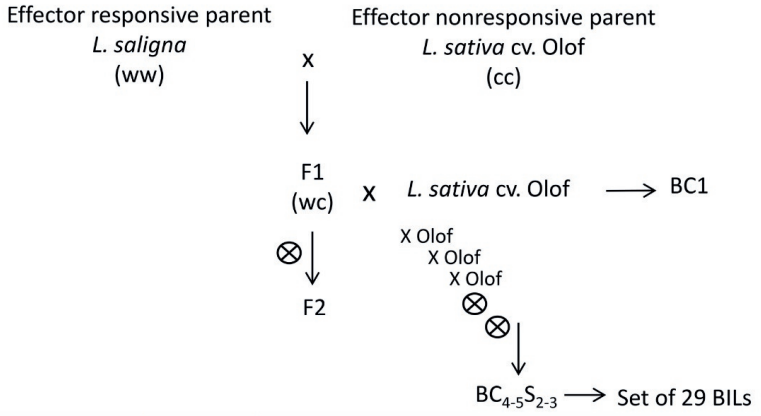


Figure 1. Characteristics of *Bremia lactucae* effector BLR31 and BLN08, and responsiveness of *Lactuca saligna* accessions. (a) Signal peptide, RXLR motif and EER motif within the amino acid sequences. **(b)** *Bremia lactucae* BI:24 growth and candidate effector gene expression during infection of *Lactuca sativa* cv Olof. Growth is inferred by the increase of *Bremia lactucae* actin relative to lettuce actin. Candidate effector gene expression is determined relative to *Bremia lactucae* actin. The y-axis has been reversed to ease interpretation, as lower ΔCt values indicate higher expression. **(c)** Unrooted neighbour joining tree constructed of 423 AFLP fragments from 45 *Lactuca saligna* and 2 *Lactuca sativa* accessions. Three main branches are distinguished: European, Israeli and Southwest Asian. Bootstrap values greater than 60% (based on 1000 replicates) are indicated in red at the nodes. Triangle: BLR31 responsive accession, circle: BLN08 responsive accession.

(a)

L. saligna	Response to 1. BLR31	2. BLN08
CGN05947	2.5	2
CGN05304	1.5	2.5
CGN05310	1	1.5
CGN05318	0	2.5
CGN05271	0	2.5

(b)



Population code	Generation	Original cross	Tested effectors
BC1_CGN05947	BC1	*L. saligna* CGN05947 x *L. sativa* cv. Olof	BLR31 & BLN08
BC1_CGN05304	BC1	*L. saligna* CGN05304 x *L. sativa* cv. Olof	BLR31
F2_CGN05310	F2	*L. saligna* CGN05310 x *L. sativa* cv. Olof	BLR31
BILs_CGN05271	BILs	*L. saligna* CGN05271 x *L. sativa* cv. Olof	BLN08
BC1_CGN05318	BC1	*L. saligna* CGN05318 x *L. sativa* cv. Olof	BLN08

Figure 2. Five BLR31 and BLN08 responsive *Lactuca saligna* accessions and their mapping populations. (a) Plant symptoms (left) after infiltration with BLR31 and BLN08 and their response values (right) in the five selected parental *Lactuca saligna* accessions. Numbers on leaves indicate infiltrations with: 1. BLR31, 2. BLN08, 3. YFP. **(b)** Crossing scheme to create backcross (BC1), backcross inbred lines (BILs) and F2 mapping populations. Allele notation: w= wild lettuce (*Lactuca saligna*) allele, c= cultivated lettuce (*Lactuca sativa*) allele.

Responsiveness to BLR31 co-segregated with complete resistance to Bl:24 in all three populations (Fig. 3, Table S4). All 126 (26+61+39) BLR31 responsive plants over three populations were completely resistant with 0% RIS, except for two plants (Fig. 3). One exception in BC1_CGN05304 might be explained by an overestimation of the BLR31 induced response, as the response to BLR31 was scored at the very low value of 0.3. In F2_CGN05310, the other exception had a relatively low RIS of 25%, suggesting incomplete expression of the BLR31-associated resistance. In conclusion, the response to BLR31 is mediated by a locus on C2 and associated with complete resistance to Bl:24. Hence, we identified the cognate *R* gene of BLR31 in three *L. saligna* accessions on C2.

Complexity of stacked resistances

The highly resistant (0-10% RIS) BC1 and F2 plants did not all show a response to BLR31 and/or had a *L. saligna* allele at the BLR31 response locus (C2). The resistance in these plants could be explained by: 1) other *R* genes, 2) nonhost QTLs, or (3) a combination of the two. The percentages of resistant BLR31 non-responsive plants in BC1_CGN05947 (52%, 17 out of 33 plants, Fig. 3d) and F2_CGN05310 (68%, 17 out of 25 plants, Fig. 3f) were close to the 50% and 75% as expected for resistant plants in case of a second *R* gene. Therefore, a dominant resistance gene unlinked to MRC2, was expected for BC1_CGN05947 and F2_CGN05310. To identify these potential additional *R* genes, populations were genotyped with additional genetic markers. All 17 resistant (RIS<10%) BLR31 non-responsive BC1_CGN05947 plants contained a *L. saligna* allele on chromosome 4 (C4) (Fig. S2). The map interval of this *R* gene on C4 in *L. saligna* CGN05947 (119.8-134.8 cM) partly overlaps with MRC4 in *L. sativa* cv Salinas (Fig. S3).

For F2_CGN05310, we could not assign a clear monogenic association to the additional resistance, despite genotyping with 73 markers distributed over the genetic map. Only a weak association with the MRC8(A-C) region on chromosome 8 (C8) was observed. The small number of susceptible (RIS>10%) plants (n=10), resulted in a too low resolution for accurate mapping.

In BC1_CGN05304 the percentage of resistant BLR31 non-responsive was 14% (10 out of 71 plants, Fig. 3e) which is not close to the 50% expected resistant plants in case of one *R* gene. In previous studies we have shown that the resistance of *L. saligna* CGN05271 to *B. lactucae* is probably based on QTLs and did not show evidence for monogenic dominant resistance loci (Jeuken & Lindhout, 2002; Jeuken *et al.*, 2008; Zhang *et al.*, 2009a). In 6 BC1 populations of *L. saligna* accessions x *L. sativa* cv Olof in which no *R* gene segregated, 4 to 12% of the plants were highly resistant and explained by nonhost QTLs (A.K.J. Giesbers & M.J.W Jeuken, unpublished). Therefore, the 14% resistant BLR31 non-responsive plants in BC1_CGN05304 are likely explained by nonhost QTLs. We did not further genotype BC1_CGN05304 as we have no concrete information about the number and locations of QTLs involved.

Summarizing, an additional dominant *R* gene was observed and mapped in *L. saligna* CGN05947 and suggested for *L. saligna* CGN05310. Our results indicate an additional high level resistance explained by a combination of nonhost resistance QTLs for *L. saligna* CGN05304. This combination of nonhost resistance QTLs may also be present in *L. saligna* CGN05947 and CGN05310. However, due to the presence of two *R* genes in relatively small populations, BC1_05947 (n=59) and F2_05310 (n=74), the expected number of plants that were resistant because of multiple QTLs only, was very low.

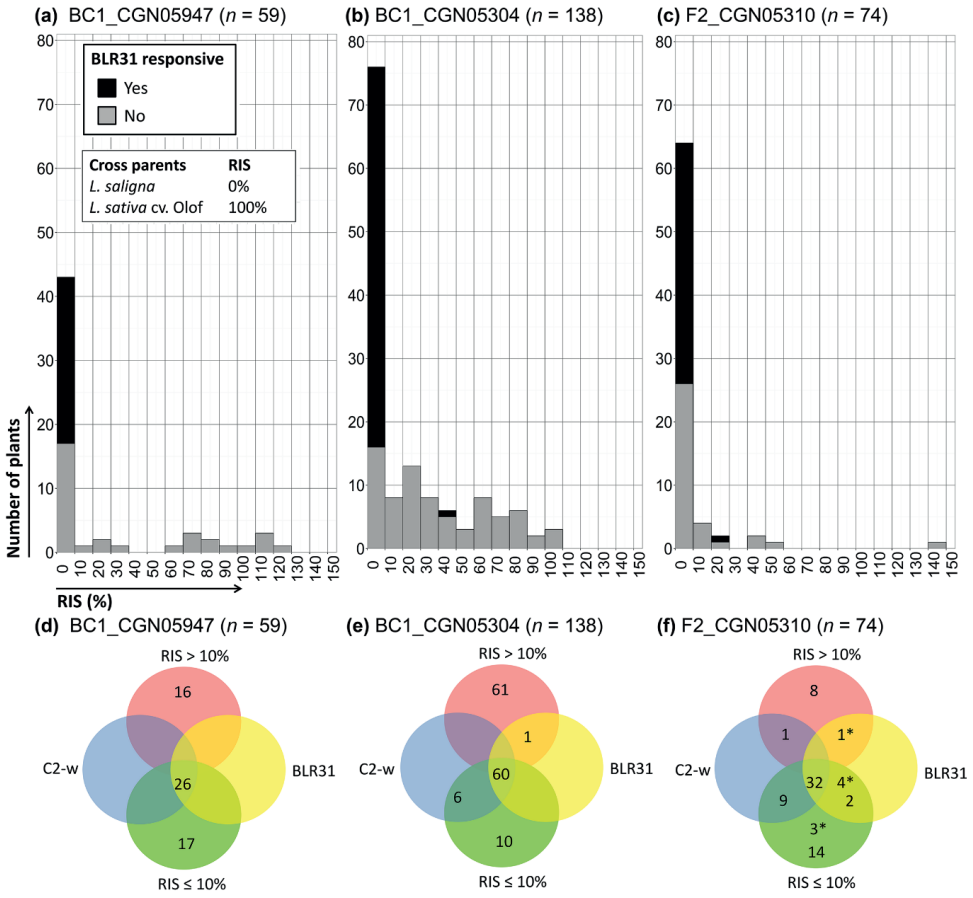


Figure 3. BLR31 responsiveness, Bl:24 infection severity and genotype on the chromosome 2 (C2) mapping locus for segregating populations of the three BLR31 responsive *Lactuca saligna* parents (a-c) BLR31 responsive plants and relative infection severity level (RIS) to race Bl:24 per segregating population. Black: plants with a response to BLR31, grey: plants without a response to BLR31. Note: all BLR31 responsive plants in RIS class ≤10% did not show any sporulation, so their exact RIS was 0% (completely resistant). **(d-f)** Venn diagrams depicting four conditions: BLR31 (yellow): BLR31 responsive plants; C2-w (blue) plants with at least one wild lettuce (*Lactuca saligna*) allele on the C2 mapping locus (most closely linked codominant marker depicted); RIS≤10% (green): plants highly resistant to Bl:24; RIS>10% (red): plants susceptible to Bl:24. * plants with missing genotype data.

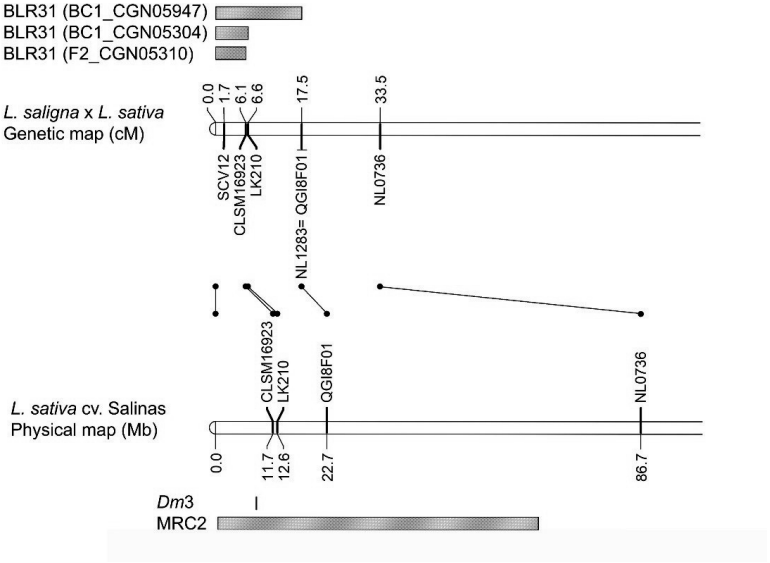


Figure 4. Map interval of responsiveness to BLR31 on chromosome 2 in *Lactuca saligna* CGN05947, CGN05304 and CGN05310. Alignment with the *Lactuca sativa* cv Salinas physical map and MRC2 and *Dm3* intervals is shown below.

Responsiveness to BLN08 is not associated with Bl:24 resistance

Responsiveness to BLN08 was tested in progenies of *L. saligna* CGN05947, CGN05271 and CGN05318. First, we screened F1 plants from *L. saligna* CGN05947 (n=4), CGN05271 (n=2) and CGN05318 (n=3). A response to BLN08 was absent or minimal in all plants of F1_CGN05947 (average response of 0.2) and present in all plants of F1_CGN05271 and F1_CGN05318 (average response of ≥1) (Table S4). The positive result of the latter F1s indicates dominance for the responsiveness gene. Second, we screened BC1 populations. BLN08 did not elicit a response in any of the BC1_CGN05947 plants (n=59).

For *L. saligna* CGN05271, candidate effector BLN08 was infiltrated in 29 BILs, containing one or a few *L. saligna* introgressions in a *L. sativa* cv Olof background, covering about 97% of the *L. saligna* genome. Four plants of a single BIL, preBIL6.2, responded to BLN08, whereas all other BILs were non-responsive. Genotyping revealed that preBIL6.2 contains a heterozygous *L. saligna* introgression from 20-59 cM on chromosome 8 (C8).

In BC1_CGN05318, a range of responses to BLN08 from 0.3 to 1.7 was observed in 32 (53%) out of 60 plants (Table S4). The response was mapped as a dominant gene on C8 from 20-33 cM. For both *L. saligna* accession CGN05271 and CGN05318, the gene causing responsiveness to BLN08 may overlap with resistance cluster MRC8A or B in *L. sativa* cv Salinas (Fig. 5).

BLN08 responsiveness did not co-segregate with *B. lactucae* Bl:24 resistance in adult disease tests in *L. saligna* CGN05271 and CGN05318 derived progeny. Control lines behaved as expected, with >70% RIS for the susceptible controls, 0-30% RIS for the quantitative resistant controls, and 0% RIS for the *L. saligna* accession (Table S4). BLN08 responsive preBIL6.2 plants (n=2) of *L. saligna* CGN05271 were as susceptible to Bl:24 as *L. sativa* cv Olof (susceptible parent). The response to BLN08 occurred in all infection

severity classes (0-100%) of BC1_CGN05318 (Fig. 6). However, 60% (36 out of 60) of the BC1_CGN05318 plants were highly resistant (RIS ≤10%, Fig. 6), indicating the presence of a dominant resistance gene (50% expected under Mendelian segregation), possibly with the remaining 10% resistant plants explained by QTLs for nonhost resistance. The other 40% of plants showed a wide range of infection severities (Fig. 6), indeed indicating segregation for QTLs. Genetic mapping showed that a dominant resistance gene explained 83% (30 out of 36, Table S4) of the resistant plants. This gene mapped on C1 between 32-73 cM, which overlaps with MRC1 in *L. sativa* cv Salinas (Fig. S4). Six (17%) resistant plants without a *L. saligna* allele at the C1 map interval are likely explained by a combination of nonhost QTLs.

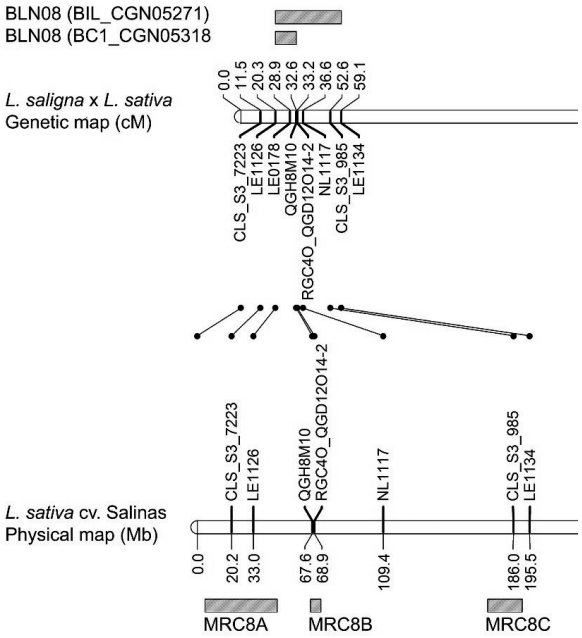


Fig. 5 Map interval of responsiveness to BLN08 on C8 in *Lactuca saligna* CGN05318 and CGN05271. Alignment with the *Lactuca sativa* cv Salinas physical map and MRC8(A-C) is shown below.

Taken together, the BLN08 induced response in *L. saligna* CGN05947 was for unknown reasons completely absent in its F1 and BC1 progeny, while it was mapped to C8 in *L. saligna* CGN05271 and CGN05318. In *L. saligna* CGN05271 and CGN05318 derived progeny the response to BLN08 was not associated with Bl:24 resistance. Instead, an *R* gene on C1 that was not associated with BLN08 responsiveness was identified in *L. saligna* CGN05318.

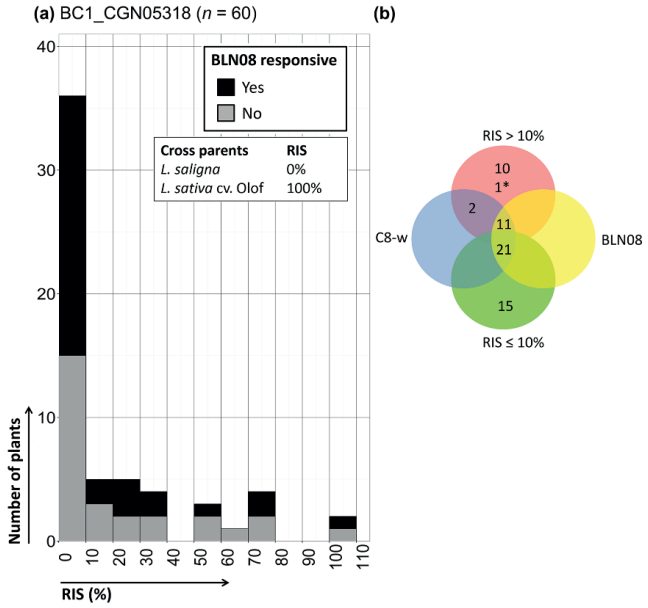


Figure 6. BLN08 responsiveness, Bl:24 infection severity and genotype on the chromosome 8 (C8) mapping locus for the BC1 population of *Lactuca saligna* CGN05318 (a) response to BLN08 and relative infection severity level (RIS) to race Bl:24. Black: plants with a response to BLN08, grey: plants without a response to BLN08. **(b)** Venn diagram depicting four conditions: 'BLN08': BLN08 responsive plants, 'C8-w': plants with one wild lettuce (*Lactuca saligna*) allele on the C8 mapping locus (most closely linked codominant marker depicted), 'RIS≤10%': plants highly resistant to Bl:24, and 'RIS>10%': plants susceptible to Bl:24. * plants with missing genotype data.

Intensity differences in plant responses to effectors

Lowest and highest responses of *L. saligna* and its derived progenies to BLR31 and BLN08 varied from light chlorosis (0.5) to strong cell death (2.5). We were interested whether these intensity differences of effector induced responses reflect *L. saligna* allele dose differences. Therefore, we compared the values of plants with one allele (F1 and responsive BC1/F2) and two alleles (responsive F2 and parental *L. saligna* accessions) per accession (Fig. S5). Average responses to effectors were significantly higher ($p<0.001$) for plants with two *L. saligna* alleles compared to plants with one *L. saligna* allele in all accessions, except for CGN05310 ($p>0.1$) (Fig. S5). However, response ranges of plants within both the one allele and the two allele dose group were wide and overlapping (except for CGN05318, which were wide but not overlapping). Therefore, individual plants cannot be classified for the responsive allele dose based on their effector response value alone (except for CGN05318, Fig. S5).

Overall, our BLR31 and BLN08 data revealed that resistance in the tested *L. saligna* accessions is composed of qualitative resistance by *R* genes and quantitative resistance by nonhost QTLs. The induced response to BLR31 in a narrow range of accessions was associated with Bl:24 resistance, while the response to BLN08 in a broad range of accessions was not.

Discussion

Our first objective was to obtain a broader picture of responsiveness to effectors in the nonhost *L. saligna*. Secondly, we wanted to verify if effector induced responses in a nonhost are associated with disease resistance to the effector producing pathogen strain. Unlike all other effector screenings in nonhost species, our study verified the co-segregation of effector induced responsiveness with resistance in segregating populations. This analysis of co-segregation was possible because of the exceptional situation in which the nonhost, *L. saligna*, is cross-fertile with the host species, cultivated lettuce. We detected a response to BLR31 in 5% (3 out of 55) of the *L. saligna* accessions, and a response to BLN08 in 55% (30 out of 55) of the *L. saligna* accessions.

BLR31 induced responsiveness co-segregated with complete resistance to *B. lactucae* Bl:24. Therefore,BLR31 is a candidate avirulence effector of lettuce downy mildew, and it probably interacts with an *R* gene on C2 (in *L. saligna* CGN05304, CGN05947, CGN05310). This resistance locus is new for the species *L. saligna.* All other *R* genes against *B. lactucae* that have been mapped in *L. saligna* are located on C1 (n=3) and C9 (n=1) (Parra *et al.*, 2016).The *R* gene on C2 co-localized with major resistance cluster MRC2 on C2 of *L. sativa*. We are unaware of the presence of MRCs in *L. saligna* due to the absence of a genome sequence, but similar MRCs in *L. sativa* and *L. saligna* are conceivable due to synteny. In *L. sativa*, MRC2 contains 61 NLR-encoding genes (Christopoulou *et al.*, 2015) and nine known *Dm* genes of which *Dm3* is cloned and explained by an NLR gene (Parra *et al.*, 2016). This indicates that the *L. saligna* C2 resistance gene may be a putative NLR gene. *R* gene cloning could functionally proof this hypothesis. Effector intensity differences of BLR31 responsive plants further showed that an HR can vary from chlorosis to cell death, as both phenotypes co-segregated with one allele of the *R* gene.

BLN08 induced a response in the majority of tested *L. saligna* accessions. Interestingly, the response to BLN08 did not co-segregate with Bl:24 resistance. However, the response to BLN08 was mapped to C8 and co-localized with MRC8(A-B) on C8 in *L. sativa.* MRC8A and MRC8B contain 42 NLR-encoding genes (Christopoulou *et al.*, 2015) without known *Dm* genes (Parra *et al.*, 2016). Previously, the induced response to BLG01 in a broad range of *L. saligna* accessions (in 47 out of 52 accessions; 90%) was also not associated with disease resistance. The response to BLG01 in *L. saligna* CGN05271 localized to the bottom of C9 (Stassen *et al.*, 2013), which is now known to co-localize with MRC9C in *L. sativa* (Christopoulou *et al.*, 2015). The fact that induced responses to two candidate effectors in a broad range of nonhost *L. saligna* accessions do not co-segregate with resistance, whereas the induced response to BLR31 in a narrow range of accessions does, raises the question of whether this is a pattern. Are responses to effectors induced in a narrow range of accessions directly associated with resistance, whereas responses induced in a broad range of accessions are not (directly) associated with resistance? Due to the currently limited number of *B. lactucae*-derived candidate effectors that induce an HR in *L. saligna* (n=3), we cannot answer this question yet.

A potential explanation for the lack of co-segregation between responsiveness to an effector and resistance is that the *R* gene is not functional against the *B. lactucae* isolate from which the effector has been cloned, because other effectors in this isolate suppress the resistance response (King *et al.*, 2014; Teper *et al.*, 2014). Maybe, the *R* gene is effective against older downy mildew isolates, in which the *R* gene suppressing effectors have not yet evolved. Another possibility is that a second unlinked gene is required for

resistance (Cooley *et al.*, 2000; Kachroo *et al.*, 2000). Or a functional version of a second tightly linked gene, like in NLR pairs (Cesari *et al.*, 2014; Williams *et al.*, 2014) is required as an inducer of disease resistance signalling. The *R* gene might also confer resistance against another pathogen and recognize the downy mildew effector as a side effect. Other explanations for the absence of co-segregation between responsiveness to an effector and disease resistance may be a lack of proper effector translocation in the host, mis-timed expression of the effector or the *R* gene, or incomplete resistance mediated by the *R* gene (Krasileva *et al.*, 2011; Goritschnig *et al.*, 2012). The latter explanation can be excluded, as the average resistance level of BLN08 responsive BC1 plants was not significantly lower than of BLN08 non-responsive BC1 plants.

Even though *L. saligna* CGN05947 showed a clear BLN08 induced cell death response, its derived F1 and BC1 plants did not respond to BLN08. Possibly, *L. sativa* alleles (50% per F1-plant and on average 75% per BC1-plant) interfere with BLN08 induced cell death in the hybrid plants. Variation at the BLN08 response locus, like unexpected heterozygosity, in the *L. saligna* parent can be excluded, because *L. saligna* CGN05947 (n=26) showed a consistent response to BLN08 in three independent experiments. These deviating responses between F1-hybrid plants of the three BLN08-tested *L. saligna* accessions, make us wonder how F1 plants of the other 27 responsive *L. saligna* accessions will respond. Testing more of these F1s (and their BC1s) gives a better reflection of the true character of BLN08 response and could be tested in future experiments, but requires very laborious and difficult crossing work.

Recognition of effectors in a nonhost is often hypothesized to contribute to NHR (Wroblewski *et al*. 2009; Schulze-Lefert & Panstruga, 2011; Lee *et al.*, 2014; Adlung *et al.*, 2016). However, HR responses are also histologically observed in susceptible lettuce and potato cultivars, and are associated with both host and nonhost resistance in potato (Vleeshouwers *et al.*, 2000; Zhang *et al.*, 2009b). Therefore, the presence of responsiveness to an effector in a nonhost does not necessarily mean that this contributes to NHR. Our current and previous results characterized nonhost responses to three candidate effectors. It showed that the responses to BLN08 and BLG01 in *L. saligna*, do not co-segregate with disease resistance, though they segregated as single dominant loci and co-localized with NLR loci, as has been found before for *Pseudomonas* effectors in lettuce (Wroblewski *et al.*, 2009).

Also in host-pathogen interactions, an effector induced cell death response does not necessarily imply a functional *R* gene mounting an HR to the pathogen delivering that effector. For instance: recognition of *Hyaloperonospora arabidopsidis* (*Hpa*) effectors in Arabidopsis did not inhibit growth of *Hpa* strains expressing the effector (Krasileva *et al.*, 2011; Goritschnig *et al.*, 2012).

A complexity of multiple and diverse resistances was identified in the *L. saligna* accessions. Besides the resistance explained by the BLR31 responsive *R* gene, additional highly resistant plants without an response to BLR31 were observed in all three mapping populations tested with BLR31. Also in BC1_CGN05318 tested with BLN08, highly resistant plants without a response to BLN08 were observed. Two dominant monogenic *R* genes (on C4 for CGN05947 and C1 for CGN05318) and some evidence for a putative *R* gene on C8 for CGN05310 explained all or a large part of the highly resistant plants in three populations. For the remaining highly resistant BC1 plants (6% of total) no evidence for dominant monogenic resistance was found. Based on our prior genetic studies in *L. saligna*, we assume that these highly resistant plants are explained by several quantitative effect

resistance genes (nonhost QTLs), as discovered in *L. saligna* CGN05271. (Jeuken & Lindhout, 2002; Jeuken *et al.*, 2008; Zhang *et al.*, 2009a). These resistant plants explained solely by nonhost QTLs suggest that NHR is independent from *R* genes.

The general frequency and distribution of *R* genes in the species *L. saligna* is unknown, though a few *R* genes have been found in *L. saligna* accessions and those with known genetic positions co-localize with *L. sativa* MRC1 and MRC9C (Parra *et al.*, 2016). Our results add three new *R* genes and one putative *R* gene, at one known (MRC1) and three new resistance loci for *L. saligna* (co-localization with MRC2, MRC4 and MRC8A-B in *L. sativa* respectively). These findings suggest that the presence of *R* genes in *L. saligna* accessions is not uncommon (at least two *R* genes out of five *L. saligna* accessions from a non-targeted approach). This may be surprising, since *R* genes are typically assumed to have evolved under selection pressure by a harmful pathogen. Since *L. saligna* is a nonhost to *B. lactucae* it remains an interesting question what selective forces have resulted in the evolution of so many *R* genes. Maybe, *L. saligna* has previously been a host for *B. lactucae* or for an extinct related pathogen and the *R* genes are remnants of this ancient host status.

We have shown that *R* gene discovery by effectors is successful if responsiveness of the plant is associated with resistance against the pathogen (like for BLR31). But it leads to the wrong track if there is no association (like for BLN08 and BLG01). Furthermore, *R* gene discovery in *L. saligna* is very well possible without the use of effectors, as *R* genes appear not to be uncommon in *L. saligna.*

In summary, we have identified a candidate avirulence effector of *B. lactucae* (BLR31) and its cognate *R* gene in *L. saligna*. *L. saligna* accessions contain QTLs for NHR and some also carry one or more *R* genes. The presence of resistant hybrid plants with solely nonhost QTLs and no *R* genes against Bl:24 suggests that *R* genes are not required for NHR, though they might still contribute. The broadly induced response to BLN08 was not associated with resistance, like our earlier finding with the broadly induced response to BLG01 (Stassen *et al.*, 2013). Therefore, effector induced responses in a nonhost may be independent of the NHR mechanism.

Acknowledgements

This work was part of the research programme "Open Technology" with project number 12683 which is (partly) financed by the Netherlands Organisation for Scientific Research (NWO). We thank Joël Klein and Marcel van Verk (Utrecht University, The Netherlands) for bioinformatic support.

Author contributions

A.K.J.G. and M.J.W.J. designed, performed and analysed experiments, with the contribution of A.J.E.P. and G.V.d.A. for candidate effector identification and expression analysis. A.K.J.G. and M.J.W.J. wrote the manuscript. R.G.F.V., R.E.N. and all other authors were involved in revising the manuscript critically.

Supplemental Figures

>BLR31

```
   1 ATGCTTCTTT CCCGTGCCAT CTCTGTACTC GCCCTACTCG CATGTATTCG TTGTGGGGTG
  61 CACGCACAAA ACACGGAACA AAATCTTAAG ACTCAACTAA CCACCGACAG CGCGATGATC
 121 ACTTCGCAGC GGCTTCTCAG AACCAGCGTG GACTTTAAAG ACAGTGAAGA ACGTTGGCCT
 181 ACTGAAAGTA GTAGGATCAG AAGTGCCATT AAGGACTATT TCCGTGAATT TCCAGAAAAA
 241 GTAAGTATTG CTATGGCGAT AAGACAGATA GATGCACACG GCGTTCGTCA TGTTGAGAAG
 301 GTGCTCTCAC AATACAAATT CCCTGCCGCA GACCAAGGAA ACATACGATT AGCGATAATT
 361 CATCACAAAG CTCCAAAATA A
```

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   1 MLLSRAISVL ALLACIRCGV HAQNTEQNLK TQLTTDSAMI TSQRLLRTSV DFKDSEERWP
  61 TESSRIRSAI KDYFREFPEK VSIAMAIRQI DAHGVRHVEK VLSQYKFPAA DQGNIRLAII
 121 HHKAPK
```

>BLN08

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   1 ATGAATTTGC ACTTCTTGCT ACTGTCCCTA CTCCTTACAA CAGCGAATGC CGCGCTTAAT
  61 AAGAATGCAA GCGAAAATAG CGCTCAACCC CCCTCAAGCC TCCCCGAACA TAGCCGTCCC
 121 TTCCTTCCTA AAAGTCCGAA CAAAGACCTC GCAATCCAAG GCCCTCGATT CAACGATGCA
 181 CATGACATGA CTGGCTCTCA AGCCACATCA ATTGAAGAAA GAAACCTTCT GGACTCGATT
 241 AAGCACACAA CGCTCGATGC CGTCTACAAA TTGGCCGCGA AACTCCGTGC GAGTCCGCGA
 301 TTTATGTTTT ATGCCATGGG AATTTTCATT TCGCGCCTTA CGAAAAAGTT GCACAAAAAA
 361 ATCAATCTCT ACCAGTGGCT TCTTTACGTA GACAAGCACA TGTTTCAACC GTGCAAGTCT
 421 CACGACGAGC TCTTGGCCGC AAGCAGCACT TTCTTTTCCT TCTTTCAACG AAAATTTACC
 481 GACGTACAGC TTGCGGGGTT CTTTCGATCG CTTCGAACAT ATCCGGGTCT GTCAAACTTG
 541 GGAGATTGGA TGCAAACGTA CATGGCTACA AACGTCGCTA CGAGCTCAGC GATGCGGGAG
 601 GCGTGGAGTT GGTATGGCGA TACGATCGAC GTTGTTTTCA AGACCCTGCG CGTTGAAAAC
 661 GAAGCAGATC TTGTCGGTAG CCGTGTGGTG ACTGCGTGGT TAGAATACTG TCATGCGCGC
 721 CGTCACGTGG CCACGCGAGA TCCAATGATT AATCTCATAG CGCTAGAAAA CATCGTGCGG
 781 TTGCTCAAGA CGACCAAGCC GGATCAAGAC CTAAAGACCG TATTTAAGAC GTTTAGTGGT
 841 GTAAATGGGA TGGAAGAATT TGCCAAGGAA TTGATTGAAG TCATTGAGCG GGAAGCACAA
 901 ATTGAAGCCT GGGCGGCGAA GAAGGTGCAT CCATCGAAAG TCTATGACGA GTTGGAGCTA
 961 GGTACGACGA ATTCGATTGA TATTACCCGA TTTATTCAGT GGCTTCGATA TTTGCAAAAG
1021 ATTCAAGTCG AAAACGATGT TTTTGTGCAT TTTTCTAAAA CGATTCCTAA AGGACAAGAG
1081 ATTGAGTATG CATCGATTTT AAAAGACATG ACACTCTTTC CGGATTTAGA AACGTTCTCG
1141 AAAGACCTAC GTAGCGTCTT GTATAAAAAC TGGGCCGCCG ACACCGACAT GACGCCGCTT
1201 ATGCTGATGA AGCGCATGAC TTCTTCCGTT GCTACTCTCT CGAGTATTGA TCCAAAACGC
1261 GTGGTGCTGC TTGAGTACAC CAAGTATTTT ATCATTCGAT ATAACGCGGC TTTATGGCCA
1321 CAATTCCAAA AAATAGTGGA AAAGAATGGT ATTGTTGCTG CGGTGAAATT TGCATCGAAT
1381 GTAAACCTTT GA
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   1 MNLHFLLLSL LLTTANAALN KNASENSAQP PSSLPEHSRP FLPKSPNKDL AIQGPRFNDA
  61 HDMTGSQATS IEERNLLDSI KHTTLDAVYK LAAKLRASPR FMFYAMGIFI SRLTKKLHKK
 121 INLYQWLLYV DKHMFQPCKS HDELLAASST FFSFFQRKFT DVQLAGFFRS LRTYPGLSNL
 181 GDWMQTYMAT NVATSSAMRE AWSWYGDTID VVFKTLRVEN EADLVGSRVV TAWLEYCHAR
 241 RHVATRDPMI NLIALENIVR LLKTTKPDQD LKTVFKTFSG VNGMEEFAKE LIEVIEREAQ
 301 IEAWAAKKVH PSKVYDELEL GTTNSIDITR FIQWLRYLQK IQVENDVFVH FSKTIPKGQE
 361 IEYASILKDM TLFPDLETFS KDLRSVLYKN WAADTDMTPL MLMKRMTSSV ATLSSIDPKR
 421 VVLLEYTKYF IIRYNAALWP QFQKIVEKNG IVAAVKFASN VNL
```

Figure S1. Nucleotide and amino acid sequence of candidate effector BLR31 and BLN08. Red: signal peptide, blue: RXLR motif, yellow: EER motif.

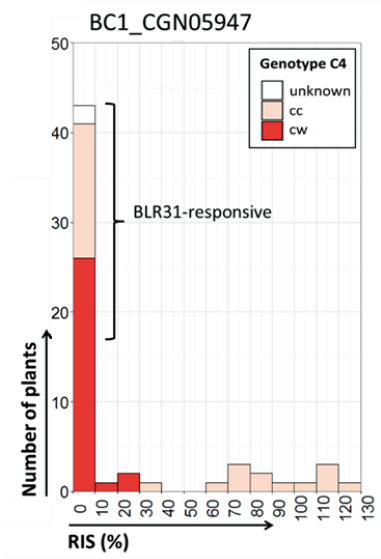


Figure S2. Distribution of segregating population BC1_CGN05947 for genotype on the chromosome 4 (C4) *R* gene locus, BLR31 responsiveness, and relative infection severity (RIS) to *Bremia lactucae* race BI:24. Red: plants with a *Lactuca saligna* allele on the C4 locus (cw), pink: plants without a *Lactuca saligna* allele on the C4 locus (cc), white: genotype unknown.

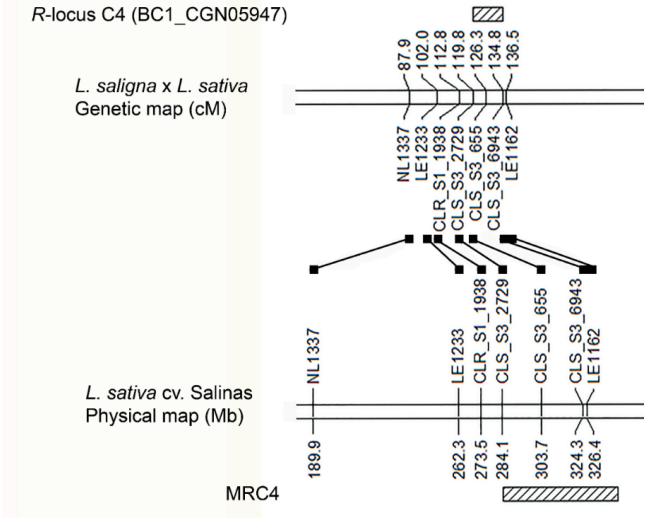


Figure S3. Map interval of the *R* gene on chromosome 4 (C4) in *Lactuca saligna* CGN05947. Major resistance cluster 4 (MRC4) in *Lactuca sativa* cv. Salinas is shown below the physical map. The genetic map is derived from an F2 population of *Lactuca saligna* CGN05271 x *Lactuca sativa* cv. Olof. The physical map is from *Lactuca sativa* cv. Salinas (http://lgr.genomecenter.ucdavis.edu/).

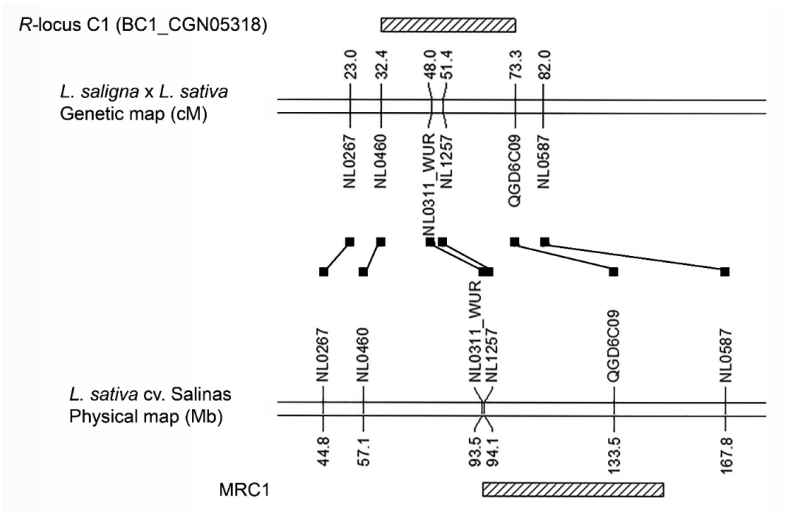
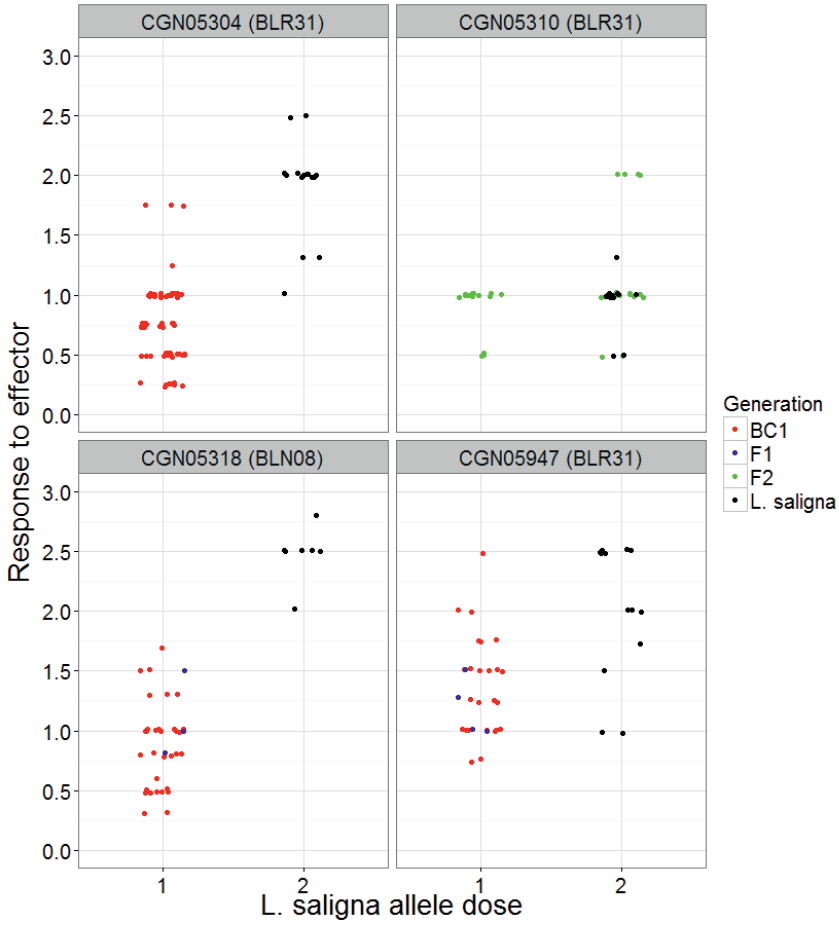


Figure S4. Map interval of the *R* gene on chromosome 1 (C1)) in *Lactuca saligna* CGN05318. Major resistance cluster 1 (MRC1) in *Lactuca sativa* cv. Salinas is shown below the physical map. The genetic map is derived from an F2 population of *Lactuca saligna* CGN05271 x *Lactuca sativa* cv. Olof. The physical map is from *Lactuca sativa* cv. Salinas (http://lgr.genomecenter.ucdavis.edu/).



	Allele dose 1		Allele dose 2		Allele dose 1 vs 2
Accession	**Mean**	**SD**	**Mean**	**SD**	**p-value (Anova)**
CGN05304	0.8	0.4	1.9	0.4	<0.001
CGN05310	0.9	0.2	1.1	0.4	0.123
CGN05318	0.9	0.4	2.5	0.2	<0.001
CGN05947	1.4	0.4	2.1	0.6	<0.001

Figure S5 Range of effector scores for responsive plants with one *Lactuca saligna* allele (BC1, F1, F2) and plants with two *Lactuca saligna* alleles (F2, *L. saligna*) per accession. A jitter function was used to add a small amount of noise, in order to make all data points visible.

Supplemental Tables

Table S1 Germplasm set and candidate effector screening results.

Available online:
https://doi.org/10.18174/430413

Table S2 List of primers used in this study.

Available online:
https://doi.org/10.18174/430413

Table S3 Chromosome numbering and orientation.

Map description + reference	Chromosome numbering								
L. sativa cv. Salinas physical map V8 [1]	1	2	3	4	5	6	7	8	9
F2 *L. saligna* x *L. sativa* genetic linkage map [2]	1^R	2	8^R	4	5^R	3	7	6^R	9

^{R} reversed chromosome orientation

[1] Reyes-Chin-Wo S, Wang Z, Yang X, Kozik A, Arikit S, Song C, Xia L, Froenicke L, Lavelle DO, Truco MJ et al. 2017. Genome assembly with in vitro proximity ligation data and whole-genome triplication in lettuce. *Nature Communications* 8: 14952.

[2] Jeuken M, Peleman J, Lindhout P. 2001. An integrated interspecific AFLP map of lettuce (*Lactuca*) based on two *L . sativa* × *L . saligna* F2 populations. *Theoretical and Applied Genetics* 103: 638–647.

Table S4 Effector responsiveness, RIS and genetic marker data per segregating population.

Available online:
https://doi.org/10.18174/430413

Chapter 3

Bidirectional backcrosses between wild and cultivated lettuce identify loci involved in nonhost resistance to downy mildew

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Submitted

Abstract

The commonplace observation that plants are immune to most potential pathogens is known as nonhost resistance (NHR). However, the genetic basis of NHR is poorly understood. Inheritance studies of NHR require crosses of nonhost species with a host, but these crosses are usually not successful. The plant-pathosystem of lettuce and downy mildew, *Bremia lactucae*, provides a rare opportunity to study the inheritance of NHR, because the nonhost wild lettuce species *Lactuca saligna* is sufficiently cross-compatible with the cultivated host *Lactuca sativa*. Our previous studies on NHR led to the hypothesis that multi-locus epistatic interactions might explain NHR and was focused on one *L. saligna* accession, CGN05271. Here, we studied NHR at the species level by segregating populations based on nine nonhost accessions. Besides the commonly used approach of studying a target trait from a wild donor species in a cultivar genetic background, we also explored the opposite, complementary approach of cultivar introgression in a wild species background. This bidirectional approach encompassed (1) nonhost into host introgression: identification of *L. saligna* derived chromosome regions that were overrepresented in highly resistant BC1 plants (F1 x *L. sativa*), (2) host into nonhost introgression: identification of *L. sativa* derived chromosome regions that were overrepresented in BC1 inbred lines (F1 x *L. saligna*) with relatively high infection levels. Here we demonstrated that the genetic dose for NHR differs between accessions. NHR seemed explained by combinations of epistatic genes on three or four chromosome segments, of which one was validated by the host into nonhost approach.

Introduction

Plants are generally resistant to most pathogenic organisms that they encounter. This is due to the narrow specialization of most pathogens: by far most pathogens have only a limited host range. The ability of a plant species to remain unaffected by all genotypes of a non-adapted pathogen is known as nonhost resistance (NHR) (Heath 2000; Thordal-Christensen 2003). When only a limited number of genotypes of a plant species remain unaffected by an adapted pathogen, this resistance is called host resistance. Considering host and nonhost status as distinct as black and white is a too simplistic representation. The phenomenon in which microbe species try to establish a compatible interaction with a plant species is an ongoing evolutionary process with transitions from compatibility to incompatibility and the reverse. Therefore, some authors distinguish intermediate cases between host and nonhost status that we could refer to as the grey area between host and nonhost (Niks 1987; Bettgenhaeuser *et al.* 2014; Dawson *et al.* 2015).

NHR results from a continuum of layered defences, constitutive and induced (Heath 1997; da Cunha *et al.* 2006; Ham *et al.* 2007). Current models of plant immunity state that NHR and host resistance involve the same components of the non-self-detection system: combined actions of basal immunity mediated by recognition of pathogen-associated molecular patterns (PAMPs) by cell surface receptors and NLR-triggered immunity by recognition of pathogen effectors (Chisholm *et al.* 2006; Jones and Dangl 2006; Niks and Marcel 2009; Schulze-Lefert and Panstruga 2011). For NHR, basal immunity is hypothesized to be relatively more important in interactions where the nonhost species is distantly related to the normal host, whereas NLR immunity would be more important in interactions involving a more closely related nonhost species (Schulze-Lefert and Panstruga 2011). This could be mechanistically explained by a failure of the microbe's effectors to effectively suppress basal immunity in nonhosts. In nonhosts, the host cellular targets for the effectors may have diverged to an extent that hampers manipulation by the effectors.

Several approaches have been used to unravel the genetics of NHR in different plant-pathosystems. Most commonly, knock-out screenings have been used to pinpoint genes. One approach is to identify genes in a nonhost that are necessary for the retention of resistance in a plant by microscopic examination. The mutant phenotype shows further developed infection (Collins *et al.* 2003; Lipka *et al.* 2005; Stein *et al.* 2006; An *et al.* 2016; Zhao *et al.* 2016). Other knock-out screenings on host plants have been used to identify host genes that are necessary for plant-pathogen compatibility and the retention of susceptibility (*S* genes) (Pavan *et al.* 2010; van Schie and Takken 2014). The mutant phenotype shows a decreased infection. Loss-of-function mutations in *S* genes, such as *Mlo* (Kusch and Panstruga 2017), can lead to a generally recessive resistance to the pathogen, reminiscent of NHR.

A more direct way to identify the responsible genes for natural variation of NHR would be genetic mapping in segregating populations (Niks and Marcel 2009). However, most host and nonhost species cannot be intercrossed, as most nonhost species are too much diverged from the host species to be cross-fertile. A creative solution to this problem was applied by Atienza *et al*. (2004): barley has an intermediate nonhost status to several species of *Puccinia* rusts, which means that some accessions show slight susceptibility. Such accessions were intercrossed to develop a fully susceptible experimental barley line (SusPtrit). Subsequently, SusPtrit was crossed with regular resistant barley lines to study

NHR. Mapping populations with SusPtrit have led to the identification of multiple individual QTLs that may contribute to NHR (Jafary *et al.* 2006, 2008).

One of the few plant-pathosystems in which host and nonhost species can be crossed is lettuce-downy mildew. Lettuce (*Lactuca*) is a self-fertilizing diploid species (2n=18). The nonhost *Lactuca saligna* is cross-compatible with the cultivated host species *L. sativa*, though severely reduced F1 fertility, reduced F2 fertility and hybrid breakdown are common and reflect their genetic distance (De Vries 1990; Jeuken *et al.* 2001). Lettuce downy mildew is caused by *Bremia lactucae*, an obligate biotrophic oomycete, and leads to high yield losses in lettuce cultivation.

An interesting review about NHR in the context of evolutionary ecology by Antonovics *et al*. (2012) postulates two scenarios for nonhost resistance: "one-sided evolved" and "nonevolved". The nonhost resistance of *L. saligna* may be "one-sided evolved", if *B. lactucae* did not counter-evolve to genetic changes in *L. saligna* that led to decreased infection levels. Alternatively, nonhost resistance in *L. saligna* could be "nonevolved", if the inability to infect *L. saligna* is a property of *B. lactucae* and not an evolved trait of *L. saligna,* for instance if *B. lactucae* specialized on another host like *L. serriola*. Generally, genetic variation for resistance in a nonhost is not expected, though may be incidentally present. Polymorphism for resistance is mainly expected for hosts that coevolved with a pathogen (Antonovics *et al.* 2012).

Histological analysis suggested that germinated conidia in *L. saligna* are arrested before normal hyphae formation (Lebeda *et al.* 2008; Zhang *et al.* 2009b). Previous genetic studies on progenies from *L. saligna* CGN05271 crossed with *L. sativa* cv Olof identified three minor QTLs in F2 populations at adult plant stage (Jeuken and Lindhout 2002). Fifteen race-nonspecific QTLs were identified at seedling, young and adult plant stage in a set of 29 backcross inbred lines (BILs) that each contain one nonhost (*L. saligna*) introgression in a host (*L. sativa*) background (Zhang *et al.* 2009a). Only one of the QTL intervals detected in the F2 clearly overlapped with the introgression segments of the intermediately resistant BILs. Only two QTLs of the BILs were effective during the entire lettuce life cycle and the remaining QTLs showed plant stage dependence to a greater or lesser extent (Jeuken and Lindhout 2004; Jeuken *et al.* 2008; Zhang *et al.* 2009a). Stacking eight combinations of two race-nonspecific QTLs did not result in greatly elevated levels of resistance (Den Boer *et al.* 2014). In conclusion, our earlier approach based on detection and stacking of individually effective QTLs did not lead to a combination of genes giving full resistance. Instead, NHR of *L. saligna* might be based on interactive, possibly epistatic, loci that have no individual effect but in combination lead to high levels of resistance. Such epistatic alleles should all be present in resistant segregants (F2 or backcross plants), whereas segregants with intermediate or susceptible phenotypes would not carry such a combination of epistatic alleles.

Some *L. saligna* accessions contain race-specific major gene resistances known as *R* genes (Parra *et al.* 2016; Giesbers *et al.* 2017). *R* genes typically encode nucleotide-binding leucine-rich repeat (NLR) proteins and are mostly identified in resistant host plants. The race-specific *R* genes in *L. saligna* seem not essential for NHR, as segregating interspecific populations (F2s and BC1s backcrossed to the host) showed that segregants can be resistant while lacking such a monogenic dominant nonhost allele (Jeuken and Lindhout 2002; Giesbers *et al.* 2017). These resistant segregants may be explained by a combination of epistatic NHR loci.

This paper aims to broaden our insight into the genetics of NHR in *L. saligna* as a species. Most of our previous studies on NHR were based on a single *L. saligna* accession and individual QTLs. Here, we test whether multiple *L. saligna* accessions indeed share the same NHR genes. We have developed bidirectional backcross populations with 1) *L. saligna* (nonhost) introgressions in a *L. sativa* (host) background (F1 x host: BC1cult) to find which nonhost chromosome regions confer resistance, and 2) host introgressions in a nonhost background (F1 x nonhost: BC1wild and three subsequent inbred generations) to find which host chromosome regions are required to confer susceptibility. Segregation for infection levels was studied in these backcross populations, originating from a diverse set of nine *L. saligna* accessions. Selective genotyping of highly resistant BC1cult plants and increased susceptible inbred lines of BC1wild enabled identification of four loci that in interaction with each other may play a major role in NHR.

Materials and Methods

1. Plant materials

Over a period of several years, we made many crossings with variable success rates between nine *L. saligna* accessions (nonhost) as a mother and *L. sativa* cv. Olof (host) as a father (Fig. S1). The latter is susceptible to all *B. lactucae* races and does not harbour any known *R* genes against *B. lactucae*. Eight *L. saligna* accessions were received from the Centre for Genetic Resources, The Netherlands (http://www.cgn.wur.nl): CGN05721, CGN05304, CGN05318, CGN05947, CGN11341, CGN15705, CGN15726, and CGN19047. One *L. saligna* accession from the island Corse, 275-5, was kindly provided by Dr. A. Beharav from the University of Haifa, Israel. The geographic origin of all accessions is listed in Table S1. F1 plants were all derived from a cross between a single mother and a single father plant. F1 plants as a mother were crossed to *L. sativa* cv. Olof as a father to develop for each of the nine *L. saligna* accessions a BC1 progeny, indicated as BC1cult (followed by an underscore and accession number to indicate the *L. saligna* parent) (Fig. 3b). Only the F1 from CGN05271 was also backcrossed to *L. saligna* CGN05271 as the father, indicated as BC1wild. This BC1wild population was further inbred for three generations until BC1wildS3 (Fig. 3c). In the generations BC1wildS1 and BC1wildS2, individuals were selected for enhanced infection severity compared to the previous generation (Relative Infection Severity>0% in BC1wildS1 and>10% in BC1wildS2) (see crossing scheme Fig. 3c). An F2 population of *L. saligna* CGN05271 x *L. sativa* cv Olof (n=126) was genotyped and used for linkage analysis to calculate genetic distances. The following control lines were included in most disease tests: *L. sativa* cv Iceberg and dBIL468 (high levels of quantitative resistance at all plant stages except seedling stage (Zhang *et al.* 2009b; Den Boer *et al.* 2014), BIL8.2 (low level of quantitative resistance at all plant stages except seedling stage) and *L. sativa* cv Olof, cv Cobham Green or occasionally cv Norden (susceptible). BIL268 and *L. sativa* cv Grand Rapids (high levels of quantitative resistance at young plant stage (Zhang *et al.* 2009b) were included in the histological analysis and in the young plant disease test.

2. Host-nonhost classification of *Lactuca* species

Data of seedling infection severity to multiple (n=36) *B. lactucae* isolates of *L. sativa* (1154 accessions), *L. serriola* (639 accessions), *L. virosa* (58 accessions) and *L. saligna* (55 accessions), were gathered from the Centre for Genetic Resources (http://cgn.websites.wur.nl/Website/downloads/DownloadCnr06.htm). *B. lactucae test* isolates included Bl:1–7, Bl:10–26, UPOV set S1, SF1, IL4, CS9 and TV, and seven races collected from *L. serriola* (Lebeda 1986). We additionally tested seedlings of *L. saligna* 275-

5, CGN11341 and CGN15726 with Bl:21, Bl:24 and Bl:29, as the CGN dataset lacked those infection severity data. Seedling infection severity was originally scored on a discrete 1 (resistant) to 9 (susceptible) scale. In a few cases, data were not scored on a 1 to 9 scale, but only on a 0, 3, 6, 9 scale (0=complete resistant. 3=incomplete resistant 6=incomplete susceptible. 9=susceptible). Score 0 was changed into score 1 for comparison between experiments. Details of the screening are described in Van Treuren et al. (2013). The numerical scores of each *B. lactucae* isolate were averaged per accession by taking the average of experiments scored on a 1 to 9 scale (experiments scored on a 0, 3, 6, 9 scale were only included if no data on a 1-9 scale was available), after which the resulting values ranging from 1.0 to 9.0 were transformed to a scale ranging from 0% to 100%, using the formula: infection severity level=(score-1)/8*100.

3. L. saligna phenogram

A phenogram of 73 *L. saligna* accessions was based on 423 aflp-fragments, derived from eight primer combinations. Distances were calculated using the "dist" function in the R package "stats" (R Core Team 2016). A tree was obtained using the neighbour-joining method in R package "ape" (Paradis *et al.* 2004). Three *L. sativa* cultivars represented the outgroup.

4. Disease tests with *B. lactucae*

Seedling disease test

Seven to ten days after seedling emergence 8-16 seedlings per lineage/genotype were inoculated with 2-4 x 10^5 spores/ml of *B. lactucae* Bl:21, and for some also Bl:24 and Bl:29. Infection severity level (ISL) was scored at 7-12 dpi as the percentage of cotyledon area covered with sporulation, per cotyledon.

Histology and young plant disease test (YDT)

Nine 19-day-old plants per genotype (*L. saligna* 275-5, CGN05271 and CGN11341) were inoculated with 1.1 x 10^5 spores/ml of *B. lactucae* Bl:21, as described by Zhang et al. (2009a). Six leaf samples per genotype were stained 48 hours post inoculation as described by Zhang et al. (2009b) and Van Damme et al. (2005). Developmental stages of the pathogen were counted per leaf sample. Five different types of infection units (IUs) were discerned: IU with only a primary vesicle (PV), IU that formed a secondary vesicle (SV), IU that formed intercellular hyphae (IH), IU with malformed hyphae (MAL-HY) and IU with haustoria (HA). Pictures showing these different developmental stages of *B. lactucae* are shown by Zhang et al. (2009b). Macroscopic ISL was scored visually as the percentage of leaf area covered with sporangiophores 10 days after inoculation on the two youngest fully expanded leaves at the moment of inoculation.

Adult plant disease test (ADTg)

Plant with hybrid necrosis symptoms (HN), observed as necrotic spots on leaves and associated with resistance, were excluded from disease phenotyping. Mostly HN symptoms can be observed easily at a macroscopic level. Incidentally, plants with less clear HN symptoms were classified and excluded based on genotypic data (markers at two loci, LG8 and LG9 (Jeuken *et al.* 2009)). Independent detached leaf assays were conducted on 5-7-wk-old adult plants as described by Jeuken & Lindhout (2002). Leaf squares (2 by 2 cm, four per genotype) from fully extended leaves were inoculated with 2-4 x 10^5 spores/ml of *B. lactucae*. Infection severity level (ISL) was scored visually as the percentage of leaf area covered with sporangiophores between 10 and 14 days after inoculation.

Relative infection severity
Relative infection severity (RIS) levels were calculated as percentage relative to the absolute infection severity level (ISL) of the susceptible parent *L. sativa* cv. Olof. Plants with RIS≤10% were considered as highly resistant.

Statistical analysis
To improve data normality the percentage data of the F1 adult plant disease test were arcsine root transformed. General Analysis of Variance (ANOVA) was performed in GenStat 18, with genotype*plant as treatment structure. Predicted mean RIS values per line were compared in a Bonferroni test (p=0.05).

5. Genotyping

DNA isolation, DNA markers and genotyping
DNA was isolated from plant leaf tissue either by a high-throughput NaOH method (Wang *et al.* 1993) or by a modified CTAB method (Jeuken *et al.* 2001). For genotyping we used EST-based markers and KASPar markers based on SNPs between *L. sativa* and *L. saligna*. The SNPs were obtained by mapping Illumina paired-end reads from *L. sativa* cv. Olof and a pool of five *L. saligna* accessions (CGN05304, CGN05318, CGN15705, CGN15726 and 275-5) against *the L. sativa* cv. Salinas genome version 8 (Reyes-Chin-Wo *et al.* 2017) using BWA-mem, version 0.6.3 (Li and Durbin 2009), with default settings. The Illumina raw read file for *L. sativa* cv. Olof and for pooled *L. saligna* accessions is available through NCBI Short Read Archive (BioProject ID PRJNA434185). SNP calling was performed using Freebayes, version v1.0.2-29 (Garrison and Marth 2012) with default parameters. Subsequently the SNPs were filtered with SNPsift version 4.3 (Cingolani *et al.* 2012), with parameters: RPL&RPR >1, SAF&SAR>1, PAIRED&PAIREDR>0.8, 6<DP>20, isHom&isRef for the *L. sativa* cv. Olof reads and isHom&isVariant for the *L. saligna* pooled reads. Flanking sequences were checked against the reference genome (*L. sativa* v8) using BLASTn (Altschul *et al.* 1990) to select for unique sites. The criterion for a SNP was: a same base for cv Salinas and cv Olof and the same alternative base in all reads of *L. saligna* accessions. From a collection of 9000 identified SNPs, we selected 293 genome-wide SNPs (with an average distance of 3.7 cM between markers) and seven chloroplastic SNPs for KASPar assays.

We distinguish three genotyping procedures:

1. All individuals of BC1cult_CGN05271, BC1wild_CGN05271 and BC1cult_CGN15705 were genotyped with 79, 83 and 77 EST-based markers respectively, more or less evenly distributed across the genome (Table SH2). Polymorphisms between PCR products of *L. saligna* and *L. sativa* alleles were visualized by high-resolution melting curve differences on a LightScanner System (Den Boer *et al.* 2014).

2. We observed BC1cult populations with extremely skewed but not bimodal distributions for infection levels to *B. lactucae*. They contained a high proportion (>20%) of highly resistant plants (RIS≤10%). These distributions resembled the segregation of a dominant monogenic *R* gene (50% complete resistant under normal segregation) in combination with segregation for quantitative resistance. To test whether and how many of these resistant plants were explained by dominant monogenic *R* genes, we genotyped these resistant plants with EST-markers until a high percentage of co-segregation between linked EST markers and the resistance phenotype was observed. Co-segregation of full resistance and a *L. saligna* allele at one locus, indicating an *R* gene, was observed and described previously

for three BC1 populations (CGN05304, CGN05318, CGN05947) in Giesbers *et al*. (2017). Plants for which the very low infection level was explained by an *R* gene were excluded from further NHR genotyping experiments.

3. The remaining highly resistant BC1cult plants (n=32) from six *L. saligna* accessions were genotyped with 300 SNP based genome-wide KASPar markers by Dr. Van Haeringen Laboratorium B.V., Wageningen, the Netherlands. KASPar SNP positions with 150 surrounding base pairs are listed in Table S3. The 300 SNP based genome-wide genotyping further included the following plants: cross parents and their F1s (as controls), BC1wildS3 plants and their ancestors (back to three generations), an F2 population (n=126 (Jeuken *et al.* 2001), and a set of backcross inbred lines (Jeuken and Lindhout 2004) from reference cross *L. saligna* CGN05271 x L. sativa cv. Olof.

Detection of NHR regions

The observed genotypic ratio (heterozygous : homozygous host) of highly resistant BC1cult plants was compared to the expected Mendelian ratio of 1:1 for each marker using Chi-square tests. At least 18 independent genomic regions are expected, assuming that each of the nine linkage groups contains at least two independent regions due to one crossover per chromosome arm. To correct for multiple-testing, a genome-wide significance threshold of 0.05/18=0.003 was applied to obtain a genome-wide error rate of p=0.05. Resistant BC1cult plants were tested together and per geographic subset (Israel, Southwest Asia, Europe). To exclude the possibility that one accession dominated the outcome of all tested plants, we also tested subsets: all BC1cult plants minus all plants of one accession (with more than five individuals). Subsequently, the identified loci with an overrepresentation of *L. saligna* alleles were compared with distorted segregation loci in genotyped BC1cult and BC1wild populations without phenotypic selection. In the BC1wildS3 lineages with enhanced susceptibility, introgression segments that were fixed for the homozygous *L. sativa* genotype were considered as regions nullified for NHR.

Genetic map

KASPar markers were added to our latest F2 genetic linkage map (based on EST and aflp-markers) from the cross *L. saligna* CGN05271 x *L. sativa* cv Olof (Jeuken *et al.* 2001). Linkage analyses were performed using JoinMap v5 software (Van Ooijen 2006). A new consensus genetic linkage map was calculated per linkage group using regression mapping and Kosambi's mapping function with default settings: linkages with a recombination frequency smaller than 0.40, LOD scores higher than 1, a jump threshold of 5 and a third round. Marker intervals for studied traits in all populations were based on this F2 consensus map. Physical map locations refer to the *L. sativa* cv. Salinas reference lettuce genome v8 (Reyes-Chin-Wo *et al.* 2017); https://lgr.genomecenter.ucdavis.edu/). Here, we use the linkage group numbering and orientation of that reference *L. sativa* physical map, which differs from the numbering used in our previous publications. In order to relate previously reported gene and marker locations to the mapped loci in the current study, we present a conversion table (Table S4).

Results

Host-nonhost classification

The broad resistance spectrum of *L. saligna* was described in a couple of studies since 1976 (Netzer *et al.* 1976; Norwood *et al.* 1981; Bonnier *et al.* 1992), but has not been graphically visualized. To illustrate the host and nonhost classification in *Lactuca* species, we have visualized a large dataset obtained from the Dutch Centre for Genetic Resources (CGN) of

four *Lactuca* species and their *B. lactucae* infection scores at seedling stage, supplemented with some seedling disease test data for three accessions not tested by CGN.

L. sativa (cultivated lettuce) and *L. serriola* (wild lettuce from the primary gene pool) have a high average infection severity level (ISL) of 62% and 70%, respectively, and are classified as a host species (Fig. 1). Most lines and accessions show either very low or very high ISL to individual *B. lactucae* races (heat map, Fig. S2ab). Resistances are explained mainly by the presence of one or more race-specific monogenic dominant *R* genes, and occasionally by some additional QTLs (Parra *et al.* 2016). *L. serriola* accessions show more intermediate interactions than *L. sativa* lines, possibly due to a higher frequency of minor genes for resistance.

L. virosa (wild lettuce from the secondary gene pool) has a low average ISL (18%, Fig. 1). Most accessions are highly resistant to all *B. lactucae* races but several accessions show high levels of ISL (Fig. S2c). Therefore, at the species level *L. virosa* seems neither a true host nor a true nonhost. Not much is known about the genetics of resistance in *L. virosa*, except for the presence of a few *R* genes (Parra *et al.* 2016).

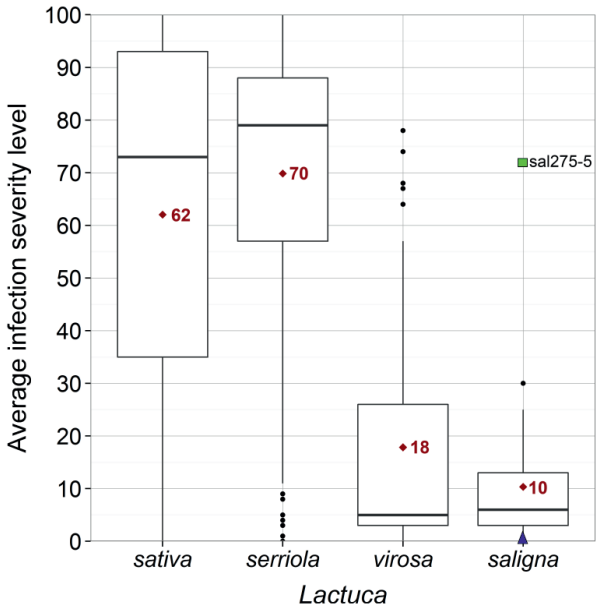


Figure 1. Boxplot of average infection severity level (%, at seedling stage) per *Lactuca* species based on public data of the Centre for Genetic Resources, the Netherlands (CGN) supplemented with our own data on three *L. saligna* accessions. The band inside each box depicts the median. Red numbers and diamonds indicate the species average. Averages are based on all tested interactions per accession tested with 10-23 Bl: races and for *L. saligna* also include the three additional accessions tested with only three races. Number of accessions included per species: *L. sativa* (n=780), *L. serriola* (n=490), *L. virosa* (n=45), and *L. saligna* (n=54). Green square (our data): *L. saligna* 275-5 average infection severity level to three Bl: races (21, 24 and 29). Blue triangle (our data): *L. saligna* CGN11341 and CGN15726 average infection severity level to three Bl: races (21, 24 and 29).

L. saligna (wild lettuce from the secondary gene pool) has on average a very low ISL (10%, Fig. 1). One exception is *L. saligna* 275-5 tested with three *B. lactucae* races (average ISL

72% at seedling stage, relative infection severity (RIS) 17% at adult plant stage). This *L. saligna* accession with unusually high infection at seedling stage was first reported by Petrželová *et al*. (2011) and we included it in our NHR study. Based on phenetic analysis (Fig. 3a), accession 275-5 fits in the *L. saligna* clade. We consider *L. saligna* as a nonhost, with the exception of this one accession.

Multiple single-dose *L. saligna* alleles already result in low levels of infection severity

To get an impression of required gene and allele dosages for NHR, we compared infection levels of the F1 generation and bidirectional backcross populations of the reference accession *L. saligna* CGN05271, crossed to the susceptible *L. sativa* cv Olof (Fig. 2). The F1, designated as F1_CGN05271, was backcrossed bidirectionally, to its resistant *L. saligna* parent and to its susceptible *L. sativa parent*, resulting in two distinct BC1 populations that we designated as BC1wild and a BC1cult respectively (Fig. 3).

F1_CGN05271 showed a low infection severity level (average RIS 12%), similar to or lower than our quantitative resistant controls Iceberg and dBIL468 (Fig. 2b). All BC1wild plants (n=28) showed a very low infection severity level (average RIS 1%, Fig. 2a). The low infection level of the F1 and BC1cult show that a single dosage of host (*L. sativa*) alleles on multiple loci does not lead to infection levels as high as the susceptible parent. The BC1cult population showed a continuous distribution from no infection to an infection severity similar to or higher than the *L. sativa* host parent (mean RIS 59%, Fig. 2c). A small proportion (7%) of BC1cult plants showed infection severity levels similar to or lower than the F1. This BC1cult result indicates that a combination of nonhost (*L. saligna)* alleles, each in a single dose (heterozygous state), can already result in low infection severity.

These results indicate that NHR is based on resistance genes from *L. saligna* and not on absence of dominant susceptibility alleles from *L. sativa* (*S* genes). In the latter case, we would have expected the F1 and BC1cult to show a high infection severity and a proportion of BC1wild plants with high infection severity levels. Instead, all BC1wild plants showed very low infection severity levels. Infection levels in F1 and bidirectional BC1 populations suggest that identification of NHR loci can be done by selective genotyping of resistant plants (RIS≤10%) in a BC1cult population, and by selective genotyping in inbred generations of BC1wild if increased infection phenotypes are observed.

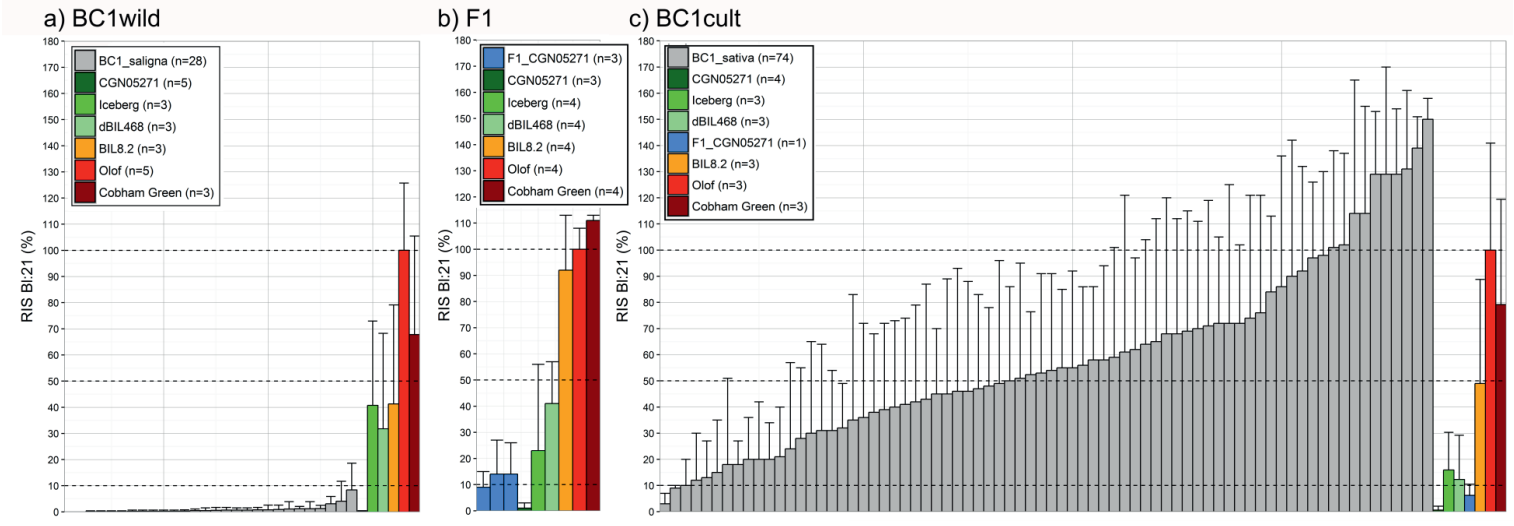
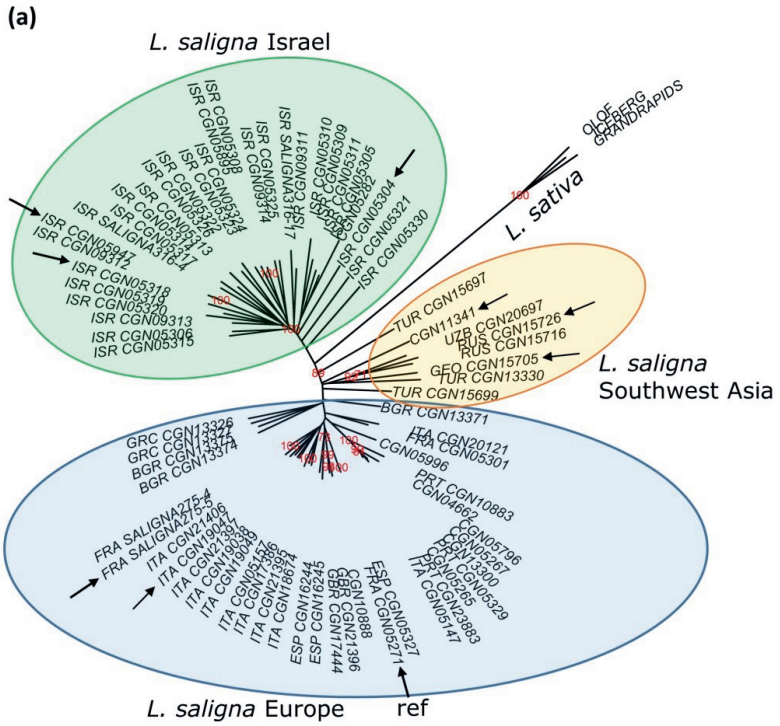


Figure 2. **Relative infection severity levels to BI:21 at adult plant stage in three independent experiments, including disease test controls (see insert boxes). (a)** BC1_saligna (n=28) **(b)** F1 (n=3); **(c)** BC1_sativa (n=74). For the test plants (BC1wild, F1 and BC1cult), averages are shown per individual plant. For the control lines, averages from multiple plants are shown.

Strategies to dissect NHR of *L. saligna*

On the basis of these findings, we denominated two strategies of selective genotyping: 1) nonhost (*L. saligna*) into host (susceptible *L. sativa*) introgression and selection for resistant phenotypes (Fig. 3b), and the other way around: 2) host into nonhost introgression and selection for enhanced infection (Fig. 3c). Strategy 1 was executed at the species level by using nine different *L. saligna* accessions (pictures in Fig. S2) from a broad range of geographic regions, including reference accession CGN05271 (Fig. 3a), in order to capture possible genetic variation in genes underlying NHR. Strategy 2 was executed for the reference *L. saligna* CGN05271 accession only.



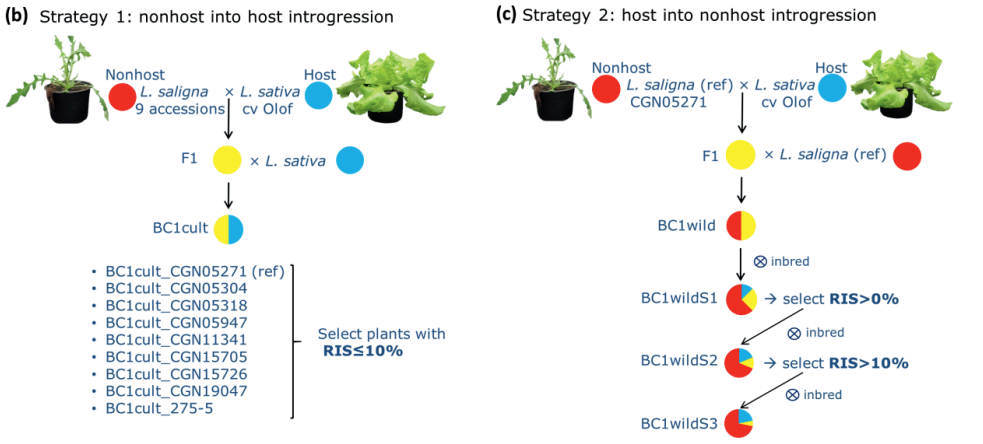


Figure 3. Selected *L. saligna* accessions and crossing scheme to obtain F1 plants and BC1 populations. Pie charts depict the average genotypic composition of each generation. Red: homozygous *L. saligna*, blue: homozygous *L. sativa*, yellow: heterozygous. **(a)** Unrooted neighbour joining tree constructed of 423 aflp fragments from 73 *L. saligna* and 3 *L. sativa* accessions. Three main branches are distinguished: European, Israeli and Southwest Asian. Bootstrap values greater than 60% (based on 1000 replicates) are indicated in red at the nodes. Arrows point to the nine selected accessions. **(b)** Each selected *L. saligna* accession was crossed with susceptible *L. sativa* cv. Olof (*L. sativa* ref), F1 plants were backcrossed to *L. sativa* cv. Olof resulting in BC1cult populations. **(c)** An F1 from *L. saligna* CGN05271 (*L. saligna* ref) was backcrossed to its *L. saligna* parent to obtain a BC1wild population and an inbred BC1wildS1 population. Subsequently, additional inbreeding was performed on plants which showed sporulation in disease tests (BC1wildS1: RIS>0%, BC1wildS2: RIS>10%).

Strategy 1: nonhost into host introgression

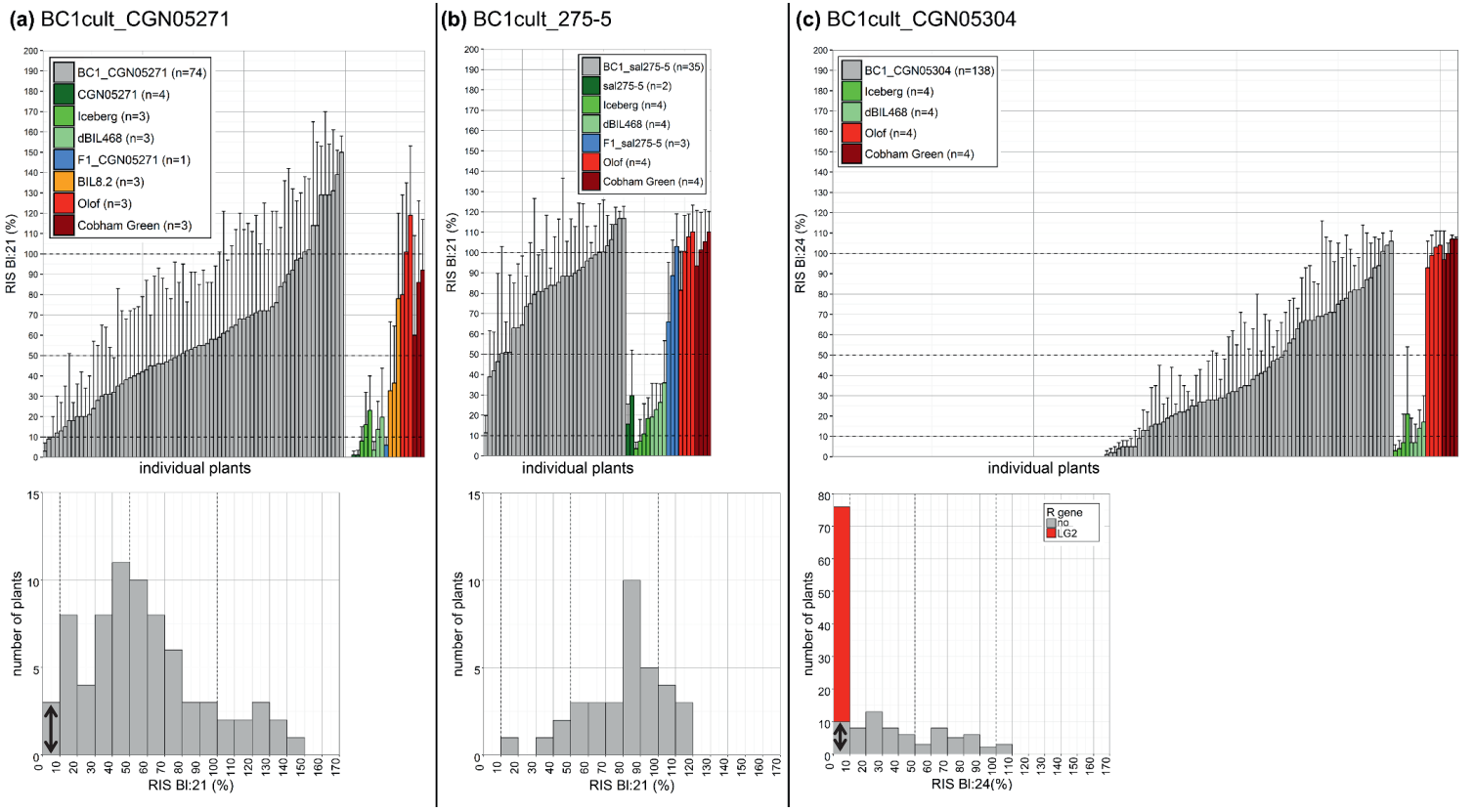
L. saligna accessions differ in their degree of NHR

All nine BC1cult populations were tested with Bl:21 or Bl:24. Bl:24 was used for three accessions that were tested for responsiveness to an effector from this isolate (Giesbers *et al.* 2017). Four BC1cult populations contained plants with hybrid necrosis (HN) symptoms or HN associated allele combinations. These HN plants were discarded (Table S1). All BC1cult populations showed a wide range of infection severity scores. Based on differences in skewness of the distributions between BC1cult populations we distinguished three types, each represented by one example in Figure 4 (lower panels with histograms). Bar charts and histograms for all nine BC1cult populations are depicted in Fig. S3-5.

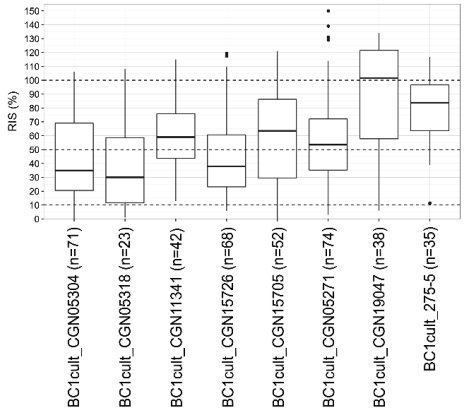
- Distribution type 1 (three BC1cult populations (Fig S3), example: BC1cult_CGN05271 shown in Fig. 4a): Normally or right skewed distributions with a few percent of highly resistant plants (RIS≤10%), indicating segregation of minor genes for quantitative resistance only.
- Distribution type 2 (two BC1cult populations (Fig S4), example BC1cult_275-5 shown in Fig. 4b): Left skewed distributions with no highly resistant plants (RIS≤10%) (except one BC1cult_19047 plant) and a low proportion of plants with RIS<50% compared to type 1 distributions. This distribution indicates segregation of minor genes for quantitative resistance only, with higher average infection scores than distribution type 1 plants.

- Distribution type 3 (four BC1cult populations (Fig S5), example BC1cult_CGN05304 shown in Fig. Dc): Extremely right skewed distributions with a very high proportion of highly resistant plants (RIS≤10%). This distribution indicates the presence of qualitative resistance (due to *R* genes) while the remaining plants without *R* genes show a wide and continuous range of infection severity scores, indicating segregation for quantitative resistance. To distinguish the *R* gene based resistant plants from those with quantitative resistance, each resistant plant of the four BC1 populations was checked for co-segregation with markers. In each BC1 population we mapped one (and once two) loci for a major dominant resistance gene (Table S1). Furthermore, in three of the four populations a proportion of resistant plants did not have *L. saligna* alleles for the markers indicative of the *R* gene. This is illustrated by ten of the 76 resistant plants in BC1cult_CGN05304 (histogram Fig. 4c).

Figure 4 (next pages). Relative infection severity (RIS) to Bl:21 or Bl:24 of BC1cult populations at adult plant stage (ADTg method) with distinct infection ranges (bar charts) and distribution patterns (histograms). Bar charts include disease test control lines (see legend per population; number of plants tested per line or population indicated in brackets). Bar chart (upper) and corresponding histogram (lower) are shown for **(a)** BC1cult_CGN05271 **(b)** BC1cult_CGN19047, and **(c)** BC1cult_CGN05304; plants in red had a particular marker allele indicating an *R* gene on LG2 against the *B. lactucae* test isolate. Double-headed arrow: plants with RIS ≤10% selected for NHR genotyping. **(d)** RIS boxplots of BC1cult populations. Plants that were highly resistant and explained by qualitative resistance (*R* gene) were excluded. BC1cult_CGN05947 was not included as it had only 13 plants without qualitative resistance. Dashed lines at 10, 50 and 100% RIS are included to ease comparison between figures; 100% RIS: average RIS of susceptible control cv Olof; plants with RIS ≤ 10% were considered as highly resistant.


(a) BC1cult_CGN05271
BC1_CGN05271 (n=74)
CGN05271 (n=4)
Iceberg (n=3)
dBIL468 (n=3)
F1_CGN05271 (n=1)
BIL8.2 (n=3)
Olof (n=3)
Cobham Green (n=3)
RIS BI:21 (%)
individual plants
number of plants
RIS BI:21 (%)
(b) BC1cult_275-5
BC1_sal275-5 (n=35)
sal275-5 (n=2)
Iceberg (n=4)
dBIL468 (n=4)
F1_sal275-5 (n=3)
Olof (n=4)
Cobham Green (n=4)
RIS BI:21 (%)
individual plants
number of plants
RIS BI:21 (%)
(c) BC1cult_CGN05304
BC1_CGN05304 (n=138)
Iceberg (n=4)
dBIL468 (n=4)
Olof (n=4)
Cobham Green (n=4)
RIS BI:24 (%)
individual plants
R gene
no
LG2
number of plants
RIS BI:24(%)

(d) RIS distribution per BC1cult population (plants with *R* genes excluded)



R genes in *L. saligna* are accession-specific and race specific ((Parra *et al.* 2016) and our own unpublished findings). However, *R* genes seem not essential for NHR as we also observed resistant plants not co-segregating with a wild parent allele at an *R* gene locus. Therefore, BC1cult plants with qualitative resistance due to *R* genes were filtered out to compare population distributions of the remaining plants between *L. saligna* parental accessions (Fig. 4d). It is apparent from this figure that BC1cult_CGN19047 and BC1cult_275-5 have higher average infection severity level compared to other BC1cult populations. Their nonhost parents were the only *L. saligna* accessions showing low levels of infection severity at adult plant stage: RIS averaged 5% (CGN19047) or 17% (275-5) in a test with three *B. lactucae* races. Furthermore, their F1s showed infection severity levels to Bl:21 (RIS 73% and 87%) similar to their susceptible *L. sativa* parent, while all F1s from the other *L. saligna* parents showed low levels of infection severity to Bl:21 (RIS 0%, 0%, 3%, 12%, 15%, 33% and 47%, Fig. S7). Histological analysis of three *L. saligna* accessions, 275-5, CGN05271 and CGN11341, at young plant stage also confirmed that development of *B. lactucae* differs among *L. saligna* accessions (Fig. S8). *L. saligna* 275-5 showed a similar percentage of haustoria as quantitatively resistant control lines with low infection severities, viz. *L. sativa* lines cv Grand Rapids, cv Iceberg and tripleBIL (described in Materials and Methods). *L. saligna* CGN05271 allowed formation of a low proportion of hyphae and haustoria. *L. saligna* CGN11341 only contained a large proportion of malformed hyphae, few normal hyphae and no haustoria.

In three distribution type 1 (right skewed) populations small proportions (2, 8, and 9%, Table S1) of BC1cult plants were highly resistant. For distribution type 3 (extremely right skewed) populations, a large proportion (17, 48, 62 and 78%, Table S1) of the BC1cult plants was explained by one or two monogenic dominant loci (potential *R* genes). Next to these, fair proportions (13, 14 and 17%, Table S1) of highly resistant plants were present in three BC1cult populations that did not contain the *R* locus. On average, BC1cult populations with distribution type 1 or 3 harboured 9% highly resistant plants without HN and *R* gene(s) against the test isolate. These highly resistant plants may contain the epistatic alleles underlying NHR.

BC1cult selective genotyping

To find the possibly interactive loci underlying high levels of resistance in BC1cult populations, we selected the highly resistant BC1cult plants without HN and *R* gene(s) against the test isolate (criterion: RIS≤10% or occasionally <15%). This resulted in 32 highly resistant BC1cult plants derived from six *L. saligna* accessions, one to nine plants per population (Table S1). For the progenies of three *L. saligna* accessions the selection criterion was not met (Table S1). We genotyped the selected plants by 300 genome-wide SNP markers. For these 32 resistant BC1cult plants, we compared the observed genotypic ratio, 'heterozygous : homozygous host', with the expected genotypic ratio assuming random inheritance in an unselected population being '1:1'. Individual BC1cult subpopulations were too small for statistical analysis to identify significant differences.

Chi-square tests of actual versus Mendelian DNA-marker segregation for all 32 plants revealed a preference for *L. saligna* alleles ($p<0.003$) at five genomic regions (Fig. 5a, Table S5a). Subanalysis of the 32 plants in three subsets of geographic origin (Israel, Southwest Asia, Europe Fig. 3a) or in plants from only five accessions (Table S5a) did not reveal other regions. The five regions were identified on LG4 (two regions), LG7 (two regions), and LG8. However, the overrepresentation of *L. saligna* alleles on the top of LG4 is likely due to distorted segregation, because such preference for the *L. saligna* allele was observed earlier in four genome-wide genotyped interspecific populations without phenotypic selection: BC1cult_CGN05271 and BC1cult_CGN15705 (Table S5b), F2_CGN05271 and F2_CGN05310 (data not shown). We found proof that this distortion in hybrids of CGN05271 is due to a digenic hybrid incompatibility (unpublished data). Therefore, we exclude the top of LG4 as a NHR locus. The four remaining regions that may carry NHR genes (Table S5) carried the saligna allele in 70-80% of the plants (Table S6a). Six combinations of heterozygous introgressions at two, three or four loci were present. On average between three and four of the four loci were heterozygous in the 32 resistant BC1cult plants (Table 1). Only two plants were detected with just two, and two plants with just one heterozygous introgression(s) out of the four loci. These four plants had *L. saligna* alleles at other loci in the genome, which, in combination, may explain their resistance. Our results suggest that NHR in all accessions is explained by combinations of several genes, in which in particular three or more of the four regions on LG4, LG7 (two regions) and LG8 participate.

Table 1 (next page). Genotype within the four NHR regions for each individual resistant BC1cult plant. RIS: relative infection severity. Genotypes for each interval are represented by the marker(s) with the highest chi-square value. Genotype codes: a (blue): homozygous *L. sativa*, h (yellow): heterozygous. Numbers are colour-formatted with intensities from green (low) to high (red) to facilitate interpretation.

			LG4	LG7	LG7	LG8	NHR regions	Total genome
Origin	Plant	RIS (%)	131-157 cM	21-37 cM	54-64 cM	17-33 cM	# heterozygous introgressions	heterozygous frequency
Europe	BC1cult_CGN05271.31	13	h	h	h	h	4	0.56
Europe	BC1cult_CGN05271.49	9	h	h	h	h	4	0.48
Europe	BC1cult_CGN05271.51	12	h	h	h	h	4	0.54
Europe	BC1cult_CGN05271.63	15	h	h	h	h	4	0.55
Europe	BC1cult_CGN05271.66	10	a	a	a	h	1	0.49
Europe	BC1cult_CGN05271.79	3	h	a	a	h	2	0.49
Israel	BC1cult_CGN05304.105	5	h	h	h	h	4	0.66
Israel	BC1cult_CGN05304.109	4	h	h	h	a	3	0.62
Israel	BC1cult_CGN05304.18	5	h	h	a	h	3	0.33
Israel	BC1cult_CGN05304.19	5	h	h	h	h	4	0.68
Israel	BC1cult_CGN05304.53	5	h	a	h	h	3	0.31
Israel	BC1cult_CGN05304.68	2	h	h	h	h	4	0.74
Israel	BC1cult_CGN05304.72	9	a	h	h	h	3	0.66
Israel	BC1cult_CGN05304.75	2	h	h	h	h	4	0.49
Israel	BC1cult_CGN05304.76	1	h	h	a	h	3	0.54
Israel	BC1cult_CGN05318.19	7	h	h	h	h	4	0.70
Israel	BC1cult_CGN05318.37	9	a	h	h	h	3	0.46
Israel	BC1cult_CGN05318.38	8	h	h	a	h	3	0.49
SW Asia	BC1cult_CGN11341.11	13	a	h	h	h	3	0.69
SW Asia	BC1cult_CGN15705.23	6	h	h	h	h	4	0.60
SW Asia	BC1cult_CGN15705.33	8	h	h	h	h	4	0.77
SW Asia	BC1cult_CGN15705.35	0	h	h	h	a	3	0.48
SW Asia	BC1cult_CGN15705.41	0	h	h	h	a	3	0.52
SW Asia	BC1cult_CGN15705.48	9	h	h	h	h	4	0.50
SW Asia	BC1cult_CGN15705.51	0	h	h	h	a	3	0.52
SW Asia	BC1cult_CGN15705.61	2	h	h	h	a	3	0.67
SW Asia	BC1cult_CGN15726.08	8	a	h	h	h	3	0.56
SW Asia	BC1cult_CGN15726.18	10	h	a	a	a	1	0.36
SW Asia	BC1cult_CGN15726.39	8	h	h	h	h	4	0.47
SW Asia	BC1cult_CGN15726.41	8	a	h	h	h	3	0.53
SW Asia	BC1cult_CGN15726.49	8	h	h	h	h	4	0.62
SW Asia	BC1cult_CGN15726.53	6	h	a	a	h	2	0.45
Average							3.3	0.55

Strategy 2: host into nonhost introgression

As a complementary approach that could reveal evidence for NHR loci, we selected for enhanced infection severity in inbred generations of BC1wild of *L. saligna* CGN05271 (Fig 3c). The genotypes of. BC1wild plants are on average half 'homozygous nonhost' and half heterozygous. BC1wild plants were inbred for three generations. The frequency of the 'homozyous host (*L. sativa*)'-genotype is increasing in every next inbred generation. In the third inbred generation (BC1wildS3) the expected genotype frequencies will be, in the absence of selection: 0.72 'homozygous nonhost' (*L. saligna*), 0.06 heterozygous and 0.22 'homozygous host' (*L. sativa*) (Fig. 3c). Repeated inbreeding increases the rate of homozygous host regions, some of which may be associated with enhanced levels of susceptibility (absence of certain NHR alleles). In each generation, we therefore selected plants with sporulation (BC1wildS1: RIS>0%, BC1wildS2: RIS>10%) as the ancestors of the subsequent inbred population. Hundreds of plants were disease phenotyped over the three inbred generations (Fig. S9).

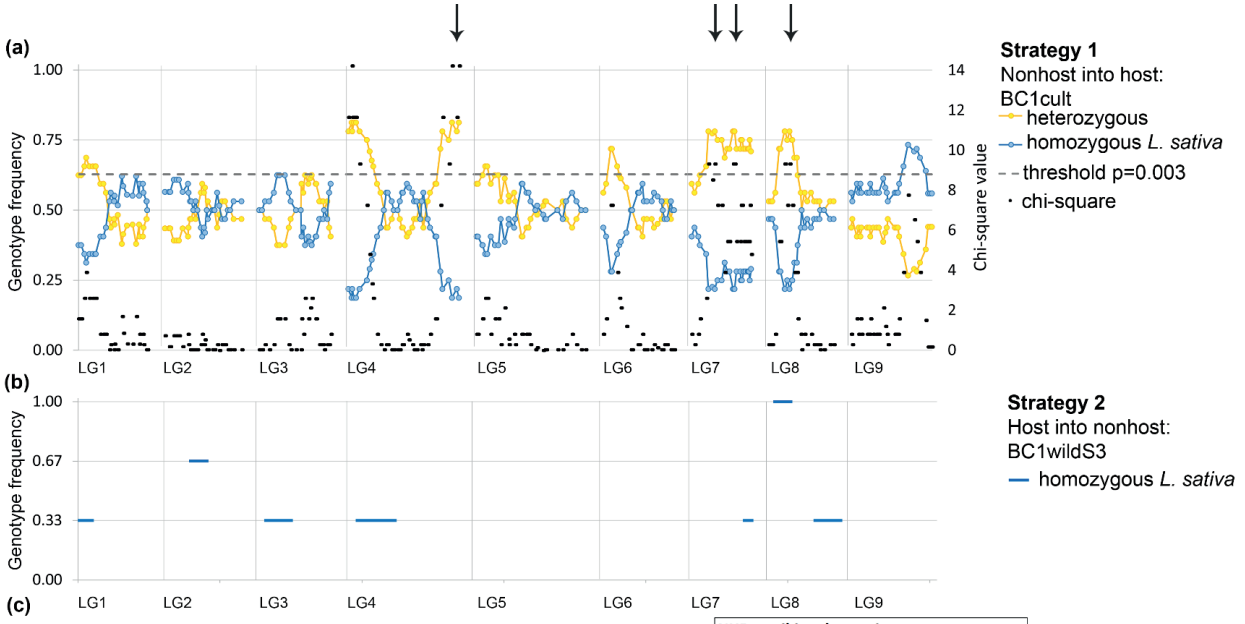
As shown in Fig. 2a all BC1wild plants displayed a very low infection severity level (mean RIS 1%) which indicated that increased infection can only be achieved by homozygous *L. sativa* (host) introgressions. Twenty-eight BC1wild plants were inbred resulting in 26 BC1wildS1 families (two BC1wild plants did not produce offspring), with theoretically expected genotype frequencies of 0.63 homozygous *L. saligna*, 0.25 heterozygous and 0.13 homozygous *L. sativa*. The complete genome of the host parent *L. sativa* cv Olof was covered by the population of 26 BC1wild plants. Ten BC1wild plants were enough to cover

each locus at least once (heterozygous introgressions in ten BC1wild plants, Table S6b). We tested 26 BC1wildS1 families (average: 20 plants/family) at young plant stage (method YDT), for enhanced susceptibility, observed as sparse sporulation symptoms. In total, 18 plants from seven families showed slight levels of sporulation (1-9% RIS). These 18 plants were retested in an ADTg and showed RIS levels of 0-10% (Fig. S10).

Sixteen BC1wildS1 plants showing sporulation in the YDT and/or ADTg were selected for further inbreeding and resulted in only five BC1wildS2 families from three different BC1wild ancestors. Eleven BC1wildS1 plants did not produce offspring due to low vitality and/or low fertility. The germination frequency of BC1wildS2 families was severely reduced and ranged from 0.12 to 0.60, with an average of 0.36 for all families together. On average six plants per BC1S2 family were disease phenotyped (Fig. S11). Eight BC1wildS2 plants with RIS>10% were selected for further inbreeding, resulting in only five BC1wildS3 families from three different BC1wild ancestors. Three BC1wildS2 plants did not produce offspring due to low vitality and/or fertility.

The five resulting BC1wildS3 families (17-20 plants/family) were genotyped and phenotyped. They contained five to nine regions with host introgressions and had average genotype frequencies for the 'homozygous host' genotype below or close to the expected 0.22 (range 0.05-0.21, Table S6). The five BC1wildS3 families (17-20 plants/family) showed increased levels of infection severity compared to the original *L. saligna* CGN05271 parent (Fig. S12). Their infection severity levels ranged from similar to control lines with 'partial resistance' (cv Iceberg and dBIL468) to intermediate levels (between Iceberg and BIL8.2). Disease test validation at seedling stage in subsets of BC1S4 lines per BC1S3 family showed similar infection levels as at adult plant stage (Fig. S13). These five BC1wildS3 families originated from three BC1wild individuals, in which 90% of the genome from the susceptible host parent *L. sativa* cv. Olof was covered.

All BC1wildS3 plants (from five families) contained the same introgressed region with a homozygous *L. sativa* introgression on LG8 (3.4–26.4 cM); this region overlapped with the locus on LG8 identified in resistant BC1cult plants (Fig. 5). Other commonly found homozygous *L. sativa* introgressions were present in maximally three out of five families and did not overlap with NHR regions identified in the resistant BC1cult plants (Fig. 5). We noticed that 38% (5/13) of the segregating introgression segments showed distorted genotype frequencies with a lack of the homozygous host genotype. Possibly this affected the introgression and detection of host segments that nullify NHR.



				NHR candidate intervals			
	***L. saligna* accessions**	**phenotype**	**criterion**	**LG4**	**LG7**	**LG7**	**LG8**
nonhost into host	six	RIS ≤ 10%	p<0.003	131-157 cM	21-37 cM	54-64 cM	17-33 cM
host into nonhost	one (CGN05271)	average RIS > 20%	homozygous *L. sativa* in all families	n/a	n/a	n/a	3-26 cM
overlap both strategies				n/a	n/a	n/a	17-26 cM

Figure 5. Overview of NHR intervals identified from the "nonhost into host" approach and from the "host into nonhost" approach. (a) Segregation distortion of 300 SNP markers in 32 highly resistant BC1cult plants. Yellow: heterozygous, blue: homozygous *L. sativa*. X-axis: linkage map with white space between linkage groups. Left y-axis: genotype frequency, right y-axis: chi-square values, dashed line: threshold of p=0.003. Arrows indicate the four loci with significant preference for the heterozygous genotype that were identified as NHR loci (the top of LG4 was excluded because this segregation distortion is likely due to a digenic hybrid incompatibility between *L. saligna* and *L. sativa*) **(b)** Homozygous *L. sativa* introgressions in BC1wildS3 plants selected for enhanced susceptibility. X-axis: linkage map with white space between linkage groups, left y-axis: genotype frequency. Blue horizontal lines indicate the frequency of the presence of a homozygous *L. sativa* introgression in all BC1wildS3 plants originating from one (out of three) BC1wild ancestors. A homozygous *L. sativa* introgression with frequency one is considered as a region that nullifies NHR **(c)** Overview of map intervals underlying NHR based on heterozygous introgressions in BC1cult (p<0.003) and homozygous *L. saligna* introgressions in all BC1wildS3 plants. Centimorgans are based on the F2 consensus map (see Materials and Methods)

Three BC1wildS1 plants with sporulation levels of 3-30% RIS were also backcrossed to the host parent *L. sativa* and the 24 cross-products were included in adult plant disease tests along BC1wildS2 and F1-plants (code for cross product: BC1wildS1BC1cult, expected genotypic composition is 0.75 heterozygous and 0.25 homozygous *L. sativa*). It resulted in 3 out of 24 plants with much higher RIS levels than in its F1 and BC1wildS1 ancestor(RIS>80%, Fig. S14) which were subsequently genotyped. As the F1 with only heterozygous introgressions was highly resistant (low RIS), only homozygous *L. sativa* introgressions are expected to lead to high RIS levels. Therefore, loci with homozygous *L. sativa* introgressions are promising loci for NHR genes. Apart from a homozygous *L. sativa* introgression on LG8 (which was already fixed in the two BC1wildS1-ancestors), two of the three genotyped BC1wildS1BC1cult plants harboured a homozygous *L. sativa* segment on LG5 (26-40 cM) and LG9 (22-51 cM), that was heterozygous in their BC1wildS1 ancestor. One BC1wildS1BC1cult plant with RIS 100% had a relatively low percentage of homozygous host genome, genotypic composition: 0.08 homozygous *L. sativa* (host), 0.92 heterozygous. This plant harboured a homozygous *L. sativa* introgression on LG3 (21-63 cM) and LG4 (133-157 cM) that was heterozygous in its BC1wildS1 ancestor (Table S6c). The latter region on LG4 falls within the region on LG4 identified from BC1cult plants (strategy 1: nonhost into host introgression).

Summarizing all results, four NHR regions with an overrepresentation of *L. saligna* (nonhost) alleles were identified in highly resistant BC1cult plants from six *L. saligna* accessions. One of these loci, on LG8, overlapped with a locus identified as a homozygous *L. sativa* (host) introgression in all BC1wildS3 lineages from CGN05271 selected for enhanced infection severity. Based on the BC1wildS3 lineages, the position of the putative gene for NHR on LG8 was narrowed down to an interval of 17-26 cM (Fig. 5c).

Discussion

Our goal was to identify the genetic basis of nonhost resistance (NHR) in *L. saligna* at the species level. We used a bidirectional backcross approach, in which we developed and screened backcross populations with nonhost introgressions in a host background (BC1cult, from nine *L. saligna* accessions) and host introgressions in a nonhost background (BC1wild, from one *L. saligna* accession). We identified four components of a putative set of epistatic genes for NHR in *L. saligna* through genotyping of thoseBC1cult plants that were highly resistant and the BC1wild inbred plants that showed enhanced susceptibility.

NHR status of *L. saligna*

Our analysis of seedling disease test data from the Dutch Centre for Genetic Resources (CGN) demonstrated that *L. saligna* as a species is highly resistant to *B. lactucae* and confirms the results of a less elaborate dataset (van Treuren *et al.* 2013). The observation of some not fully resistant *L. saligna B. lactucae* interactions at seedling stage (Fig. S2) may be a plant stage dependent effect. Bonnier *et al*. (1992) and Petrželová *et al*. (2011) reported occasional sparse sporulation on *L. saligna* at seedling stage, but full resistance at adult plant stage. Petrželová *et al*. (2011) identified accession 275-5 as one of the lines with the highest level of infection severity, with 24% sporulation at seedling stage but no sporulation at adult plant stage. We validated the taxonomic classification of this accession. In our experiments, its observed infection severity to *B. lactucae* was 72% RIS at seedling stage and even 17% RIS at adult plant stage. In conclusion, we confirm the nonhost status of *L. saligna*, with the exception of this one accession.

NHR is based on resistance factors from *L. saligna*

Comparing F1, BC1wild and BC1cult of CGN05271 showed, respectively, a low infection, overall low infection and a continuous range of infection severity levels. If dominant susceptibility genes of host *L. sativa* were involved, all BC1cult plants should have been susceptible, as well as the F1 and part of the BC1wild. The low percentage of highly resistant BC1cult plants and the high resistance levels of the F1 plants indicated that multiple heterozygous introgressions, each with one copy of the nonhost allele, can lead to high levels of resistance. The few highly resistant BC1cult plants most probably harbour a combination of particular heterozygous introgressions leading to resistance.

The degree of NHR differs between accessions

To study NHR at the species level, we extended our study to eight additional *L. saligna* accessions from a wide range of geographic origins. Variation for resistance genes is not expected in a nonhost, but may be incidentally present (Antonovics *et al.* 2012), therefore we expected to identify NHR loci in common within the nine accessions. Infection severity levels in F1 plants and BC1cult populations derived from different *L. saligna* accessions showed that the genetic dose of NHR differs between *L. saligna* accessions. *L. saligna* CGN19047 and 275-5 had a higher RIS average for F1 and BC1cult populations than the other *L. saligna* accessions. Furthermore, *L. saligna* 275-5 itself showed an average RIS of 17% at adult plant stage and in histological analysis it had a similar proportion of haustoria as in quantitatively resistant control lines, whereas the other tested *L. saligna* accessions showed very few or no haustoria. Accession 19047 and 275-5 may lack the same NHR locus allele(s) by descent as they are genetically most similar and collected from geographically close regions among the nine accessions (Fig. 3a). If the remaining NHR genes of these accessions would lose function due to incidental mutations, *L. saligna* might eventually evolve into a novel host species for *B. lactucae*.

Confounding factors

Two factors complicated our study of NHR, in certain crosses of *L. saligna* accessions with *L. sativa*: 1) a hybrid incompatibility symptom, hybrid necrosis (HN), visible as necrotic flecks on leaves in four of the nine BC1cult populations. Jeuken *et al.* (2009) demonstrated that such an HN reaction is associated with resistance to *B. lactucae.* 2) The segregation of monogenic dominant resistances (qualitative resistance, *R* genes) in four other BC1cult populations.

The presence of *R* genes as well as genes for quantitative resistance in *L. saligna* seems in line with the presumed combined action of *R* genes and pattern recognition receptors (PRRs) in NHR as proposed by the model of Schulze-Lefert and Panstruga (2011). However, we showed that *R* genes are not essential for NHR, as five out of nine *L. saligna* accessions did not contain an *R* gene against the test isolate, but were completely resistant. Furthermore, in all accessions QTL mediated resistance was present that led to high or intermediate resistance levels in BC1cult populations.

Possibly, the occasional *R* genes are remnants of an ancient host status of *L. saligna*. *B. lactucae* may later have specialized on *L. serriola,* the probable ancestor of *L. sativa* (De Vries 1997)*(De Vries 1997)(De Vries 1997)*. Alternatively or additionally, accession-specific *R* genes may be an accidental by-product of gene duplications, recombination, unequal crossing-over, point mutations and diversifying selection that are common within *R* gene clusters (Meyers *et al.* 2005). Sequence exchange between *R* genes that are effective against a specific pathogen can even result in the generation of novel *R* genes with resistance specificities to other, phylogenetically unrelated pathogens (Slootweg *et al.*

2017). This flexibility of *R* genes to generate novel resistance specificities may have resulted in coincidental presence of *R* genes in the *L. saligna* nonhost. Another point to be addressed here is that the presence of *R* genes to a nonadapted pathogen in a nonhost remains unnoticed as long as nonhosts are not interfertile with hosts, which is commonplace with the exception of our interspecific cross. Possibly the frequency of *R*-genes against nonpathogens in nonhosts is higher than we are aware of and we generally expect.

Identification of four potential components for NHR

Two to 17% of the BC1cult plants per population were highly resistant (≤10% RIS; after exclusion of HN and *R* gene explained plants), resulting in 32 BC1cult plants of six *L. saligna* accessions. If the high level of resistance would be explained by a particular combination of a certain number of loci, it would imply a set of three to five underlying genes ($½^3$=0.125, $½^4$=0.0625, $½^5$=0.0313). In NHR studies of *Arabidopsis* mutants, minimal two (*pen*3+*eds*1) and three (*pen*2+*pad*4+*sag*101) mutated genes were needed to lead to dense sporulation of pea powdery mildew and occasional sporulation of barley powdery mildew (*Bgh*) (Lipka *et al.* 2005; Stein *et al.* 2006).

Our strict phenotypic selection of highly resistant BC1cult plants (RIS≤10%) and subsequent strict genotypic selection (overrepresentation of *L. saligna* alleles with $p<0.003$) resulted in the identification of four NHR loci. These loci were located on LG4, LG7 (two regions) and LG8. In former NHR genetic studies on accession CGN05271 we detected, among others, individual QTLs at three similar locations, but these were effective only in certain plant developmental stages: *rbq15* (LG4), *rbq6* (LG8) and *rbq1* (LG7) (Jeuken and Lindhout 2002; Zhang *et al.* 2009a). The fact that the remaining 12 QTLs that were identified earlier were not detected this time may suggest that these QTLs are not essential for NHR, or not in all accessions.

The high resistance level of the selected BC1cult plants may be explained by five combinations of heterozygous segments at three or four of the identified NHR loci. Therefore, resistance alleles at minimal three loci seem necessary for NHR of *L. saligna*. If all five possible combinations of at least three loci (combinations in binary code: 1110, 1101, 1011, 0111, 1111) would lead to high resistance, the probability of resistant plants in a BC1cult would be 31% (5 out of 16 possible combinations of 4 loci=0.315, if under Mendelian segregation). However, we found on average 9% highly resistant BC1 plants. We have indications that the NHR loci are closely linked to regions with distorted segregation with a preference for host (*L. sativa*) alleles (data not shown). This could explain our lower proportion of resistant plants. In these 32 BC1 genotypes, we could not identify a particularly common combination out of these five combinations. Possibly, few other loci together with three of the four detected loci might explain NHR. Other possible NHR loci were identified in enhanced susceptible (RIS>80%) BC1wildS1 plants backcrossed to *L. sativa* on LG3, LG5 and LG9.

Individual BC1cult populations were too small to study whether there was significant genetic variance for NHR between accessions, as 32% of all 615 tested BC1cult plants had to be discarded due to the presence of confounding HN or *R* genes. To obtain a larger dataset, genotyping resistant BC1cult plants of *L. saligna* CGN15726 with Bl:21 would be most efficient, as these plants did not show HN and no *R* genes against Bl:21. The previously identified QTLs in BILs were race-nonspecific (Zhang *et al.* 2009a). Testing with multiple isolates would be necessary to prove race-nonspecificity of the identified NHR loci.

Furthermore, field tests should be conducted to exclude plant-stage dependent effects of NHR loci, as previously found for some partially resistant BILs (Zhang *et al.* 2009a).

Additional evidence for a NHR component on LG8
Through introgression of host segments into a nonhost genetic background (CGN05271), we identified one host (*L. sativa*) derived introgression region leading to enhanced infection in all five BC1wildS3 lineages (with low to intermediate sporulation). This region overlapped with the NHR region on LG8 identified in resistant BC1cult plants with an interval from 17 to 26 cM (spanning 15 Mb). The host into nonhost approach did not convincingly confirm the other detected NHR loci on LG4 and LG7 nor did it identify a new locus. This might partly be explained by chance, due to relatively low numbers (average n=6 per ancestor) in the second inbred generation, BC1wildS2. On the other hand it might be explained by the distorted segregations at five of the thirteen segregating introgression segments, which showed distorted genotype frequencies with a lack of the homozygous host genotype.

Conclusion and recommendations for further research
Our findings indicate that the genetic dose of NHR differs between *L. saligna* accessions. Furthermore, NHR in *L. saligna* seems explained by combinations of epistatic genes on three or four chromosome segments. The first finding can be followed up by studying the *L. saligna* accessions 275-5 and CGN19047 with the lowest dosage of NHR. Crossings with completely resistant *L. saligna* accessions could lead to identification of the gene(s) that lowered the dosage of NHR. To validate the four identified loci for NHR and characterize their interactions, we could study the next inbred generations of the resistant BC1cult plants, or intercross BILs with *L. saligna* segments that overlap with the four loci. Our unusual but successful approach of bidirectional backcrossing in combination with dense genotyping of selected offspring might be an effective strategy to also study the inheritance of other complex traits.

Acknowledgements

This work was part of the research programme "Open Technology" with project number 12683 which is (partly) financed by the Netherlands Organisation for Scientific Research (NWO). We thank M.J.M. Smulders for critically reading the manuscript.

Supplemental Figures

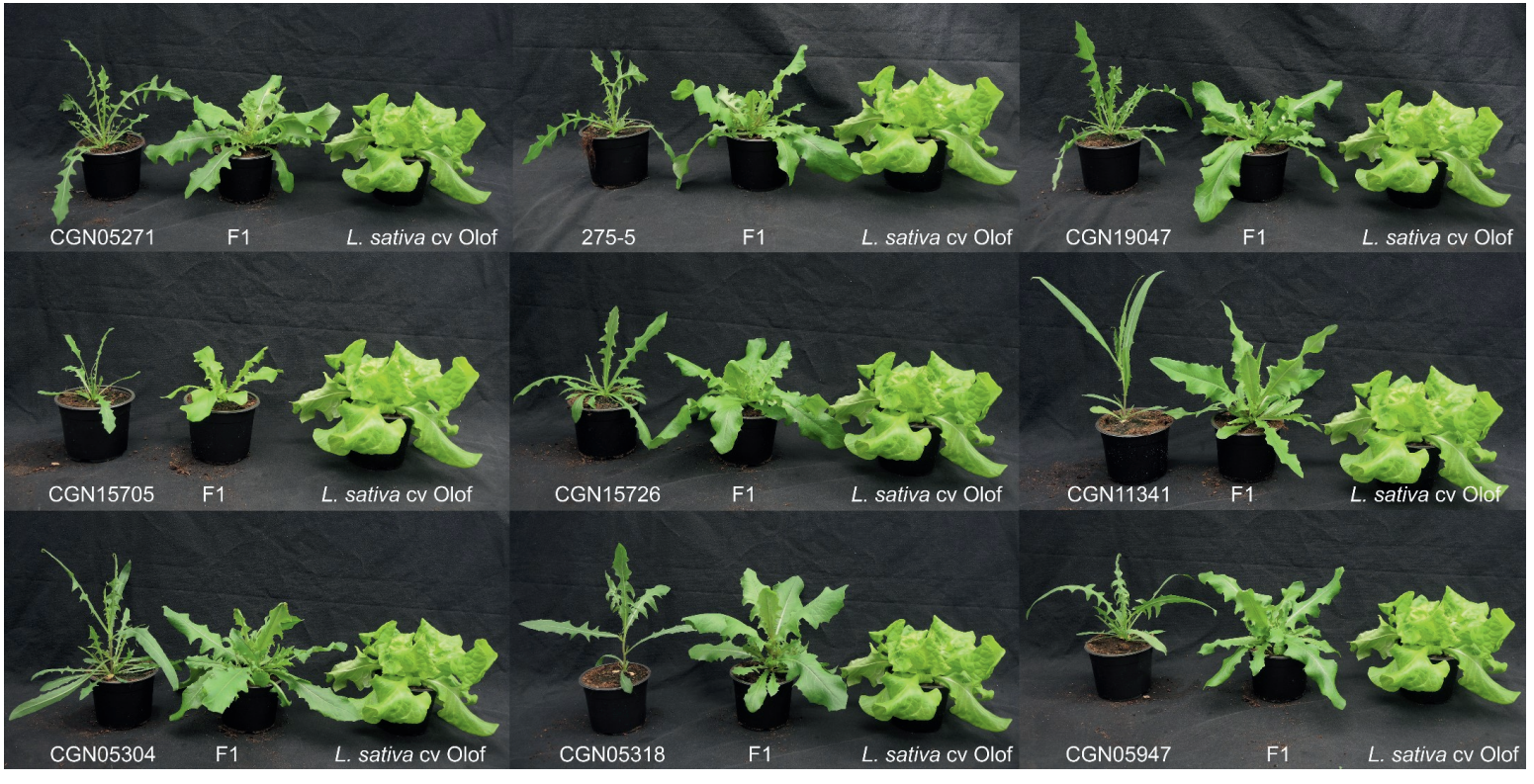


Figure S1. Phenotypes of all tested *L. saligna* accessions, the F1 hybrid and *L. sativa* cv Olof at five weeks of age.

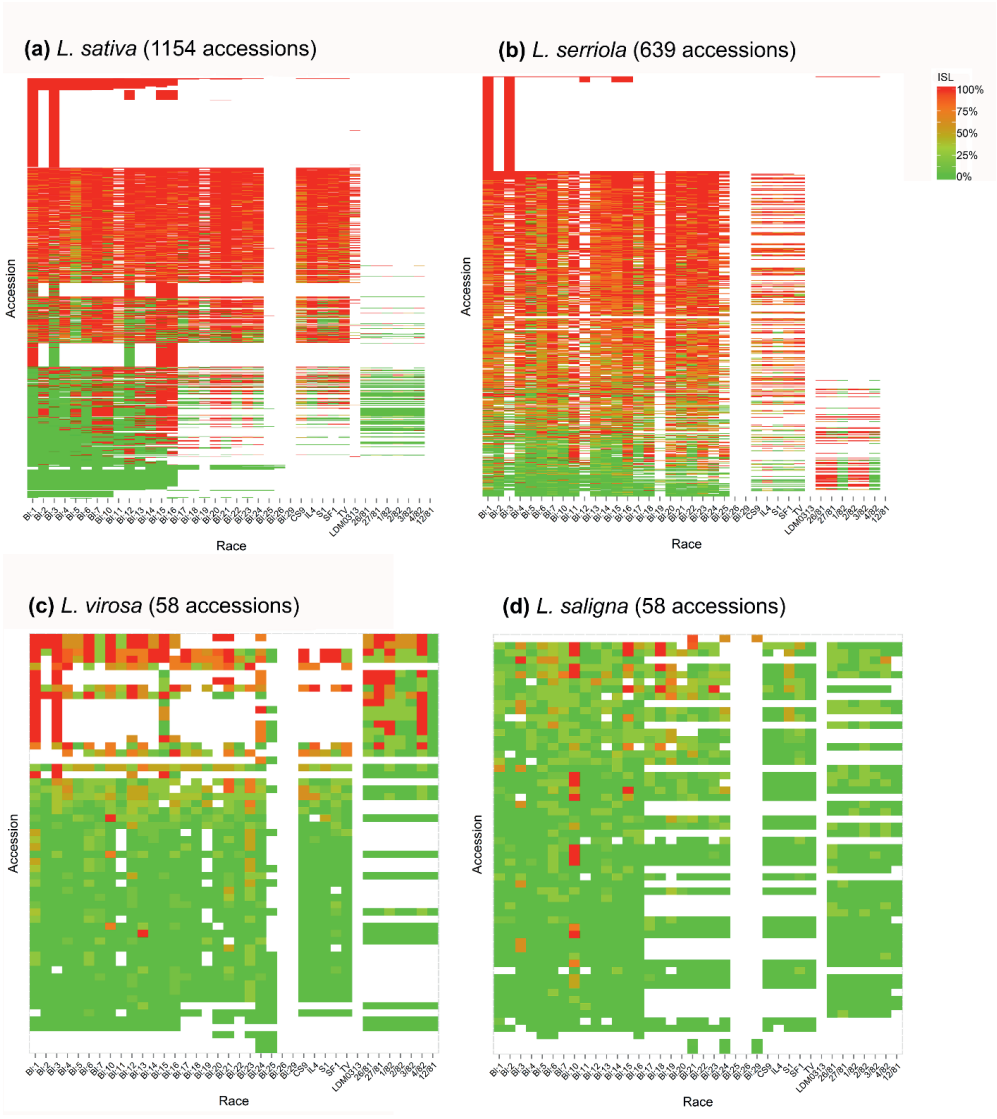


Figure S2. Heatmap of seedling infection severity level (ISL) of all individual interactions between each *Lactuca* accession and *B. lactucae* race. (a) *L. sativa* **(b)** *L. serriola* **(c)** *L. virosa* **(d)** *L. saligna*. Resistant (0%: green) to susceptible (100%: red) of all tested accessions to each race, per Lactuca species. No colour (white) means that a certain accession x *B. lactucae* race combination has not been tested.

Data are derived from http://cgn.websites.wur.nl/Website/downloads/DownloadCnr06.htm and supplemented with our own data on three *L. saligna* accessions.

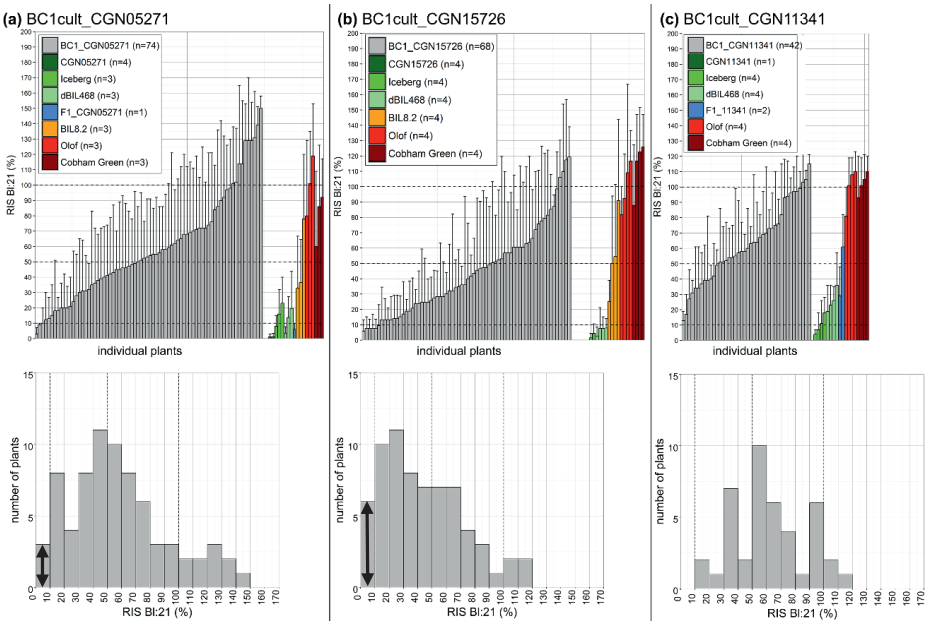


Figure S3. Bar charts including disease test controls (see legend) and corresponding histograms with normally or right skewed distributions (type 1) of relative infection severity levels (RIS) of three BC1cult populations at adult plant stage without a major resistance (*R*) gene against the test isolate (a) BC1cult_CGN05271 **(b)** BC1cult_CGN15726 **(c)** BC1cult_CGN11341. Double-headed arrow: plants with RIS ≤10% selected for NHR genotyping.

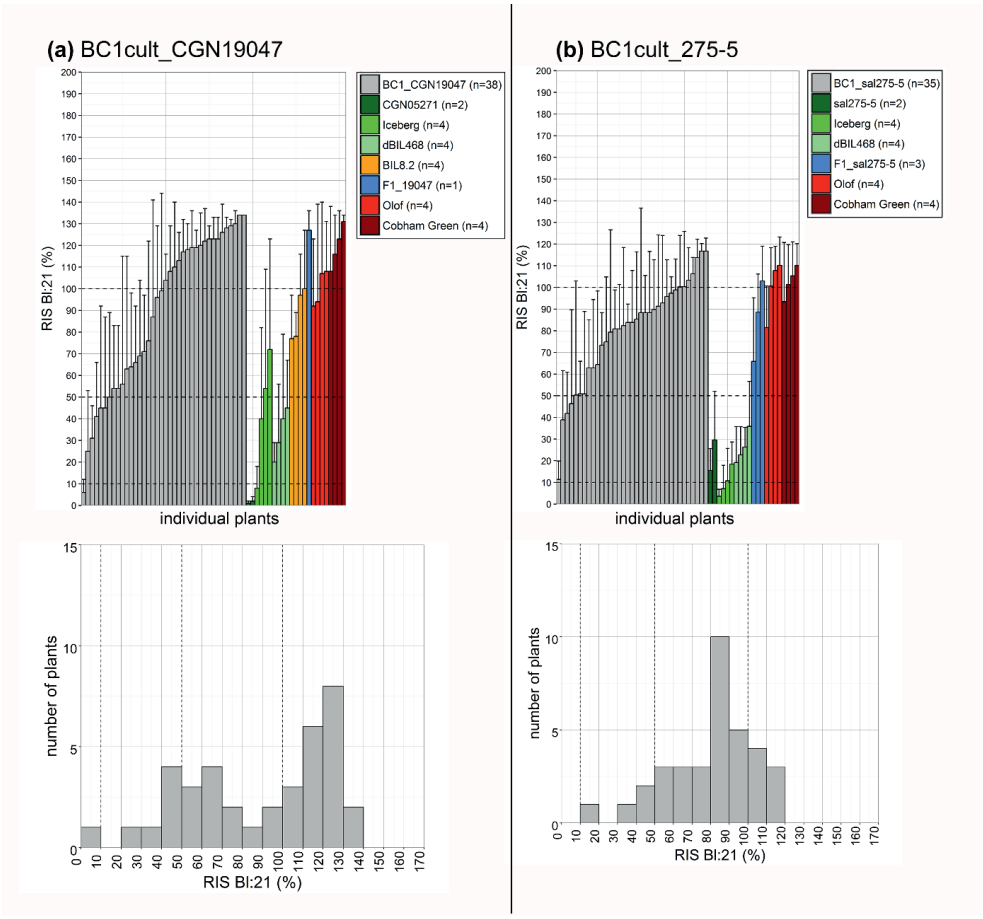


Figure S4. Bar charts including disease test controls (see legend) and corresponding histograms with left skewed distributions (type 2) of relative infection severity levels (RIS) of two BC1cult populations at adult plant stage without a major resistance (*R*) gene against the test isolate (a) BC1cult_CGN19047 **(b)** BC1cult_275-5.

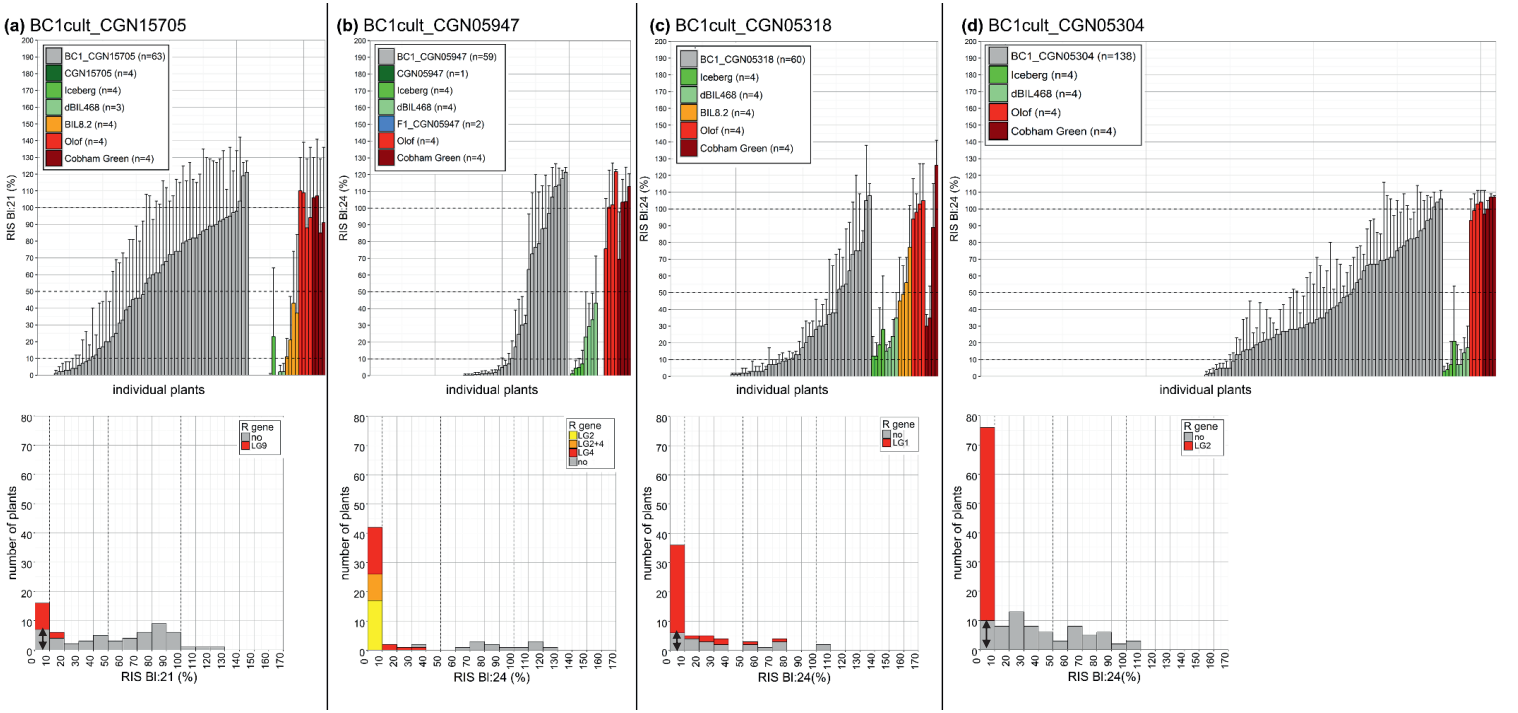


Figure S5. Bar charts including disease test controls (see legend) and corresponding histograms with extremely right skewed distributions (type 3) of relative infection severity levels (RIS) of four BC1cult populations at adult plant stage with a major resistance (*R*) gene against the test isolate (a) BC1cult_CGN15705 **(b)** BC1cult_CGN05947 **(c)** BC1cult_CGN05318 **(d)** BC1cult_CGN05304. In the histograms, plants in red and yellow showed co-segregation between a single dominant *L. saligna* allele (*R* gene) and resistance against the *B. lactucae* test isolate on LG1, LG2, LG4 or LG9 as indicated in the legend. Double-headed arrow: plants with RIS ≤10% selected for NHR genotyping

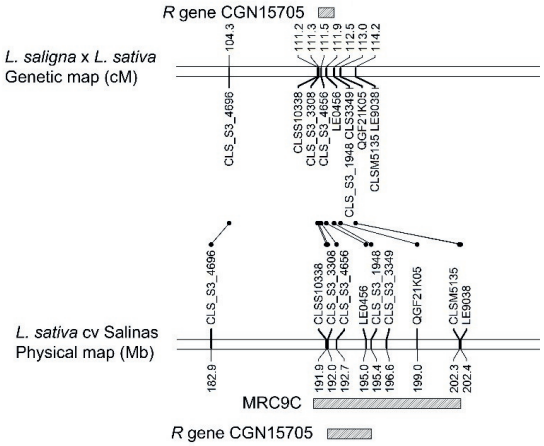


Figure S6. Map interval of the *R* gene on linkage group 9 in *Lactuca saligna* CGN15705. Major resistance cluster 9C (MRC9C) in *Lactuca sativa* cv. Salinas is shown below the physical map. The genetic map distances are based on an F2 population of *L. saligna* CGN05271 x *L. sativa* cv. Olof. The physical map distances are from *Lactuca sativa* cv Salinas genome assembly v8 (http://lgr.genomecenter.ucdavis.edu/, Reyes-Chin-Wo *et al.* 2017).

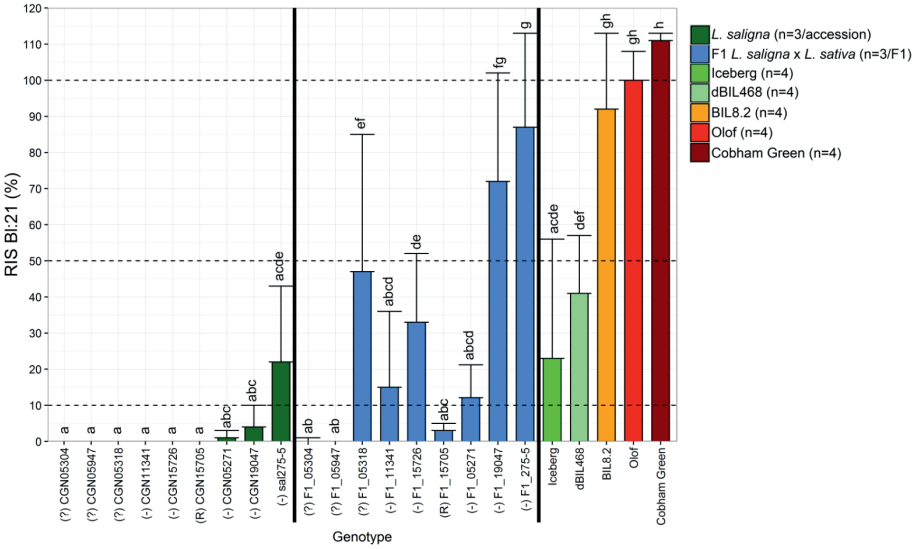


Figure S7. Relative infection severity (RIS) to *B. lactucae* Bl:21of *L. saligna* parental accessions, F1-genotypes and disease test control lines at adult plant stage. For ease of interpretation, the three groups of plants are separated by black bars. Letters in common indicate no significant difference (α =0.05, Bonferroni test). Per genotype three plants were evaluated with 2-4 leaf parts per plant. Prefix to test lines (-) no *R* gene identified against the test isolate, (R) *R* gene identified against the test isolate (by RIS segregation in BC1cult population and molecular marker analysis), (?) presence of *R* gene against test isolate unknown, because the corresponding BC1cult population was not tested with this test isolate.

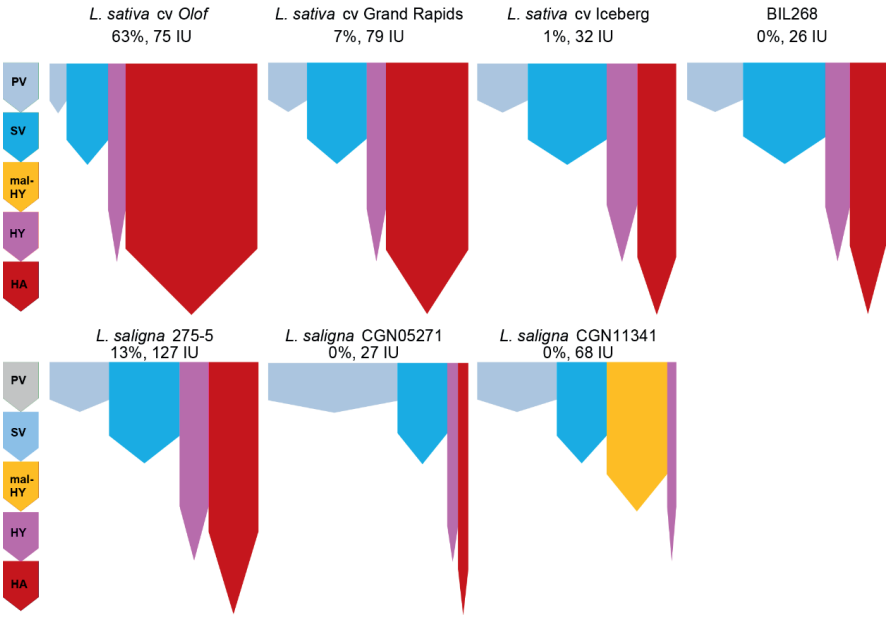
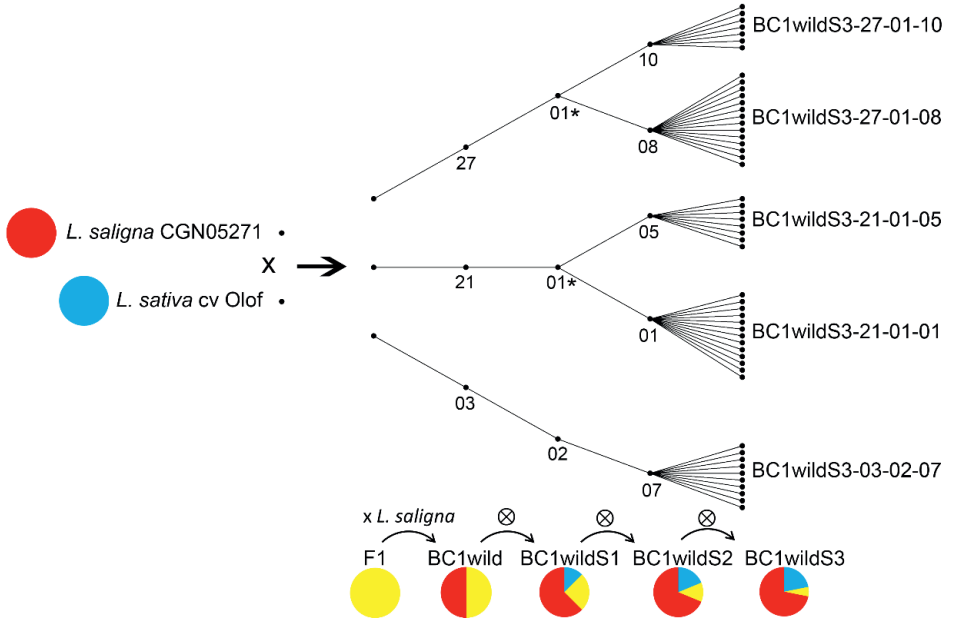


Figure S8. Graphical representation of the infection process of *B. lactucae* race Bl:21 in tested *Lactuca* genotypes 48 hours post inoculation. The infection structures, which *B. lactucae* may develop while invading lettuce plants, are represented on the left side with differently coloured arrows: PV= primary vesicle, SV= secondary vesicle, MAL-HY= malformed hypha, HY= hypha and HA= complete infection unit with haustoria. The width of an arrow indicates relatively how many infection units reached a developmental stage. The width of the arrows together represents per genotype 100% of the infection units. Values mentioned below the name of each accession/line represent respectively the infection severity level (%) of the macroscopic controls at 10 dpi and the total number of observed infection units (IU).



	# phenotyped	selection criterion	# fulfilling selection criterion	# selected (1 or 2 plants/family)	# succesful next generation	# genotyped	# visualized in pedigree
BC1sal	28	NA	NA	NA	26	10	3
BC1salS1	531	RIS>0%	23	16	5	3	3
BC1salS1BC1sat	24	RIS>80%	3	3	NA	3	n/a
BC1salS2	41	RIS>10%	11	8	5	5	5
BC1salS3	97	n/a	n/a	n/a	n/a	52	52

Figure S9. Pedigree of genotyped BC1wildS3 lineages and overview of phenotyped and genotyped plants. * Backcrossed to *L. sativa*, resulting in BC1wildS1BC1cult families (Fig. S14). Pie charts depict the average genotypic composition of each generation. Red: homozygous *L. saligna*, blue: homozygous *L. sativa*, yellow: heterozygous. The table lists details on plant numbers that were phenotyped and genotyped. Not all selected plants resulted in a successful next inbred generation, due to reduced vitality and fertility. RIS: relative infection severity, n/a: not applicable.

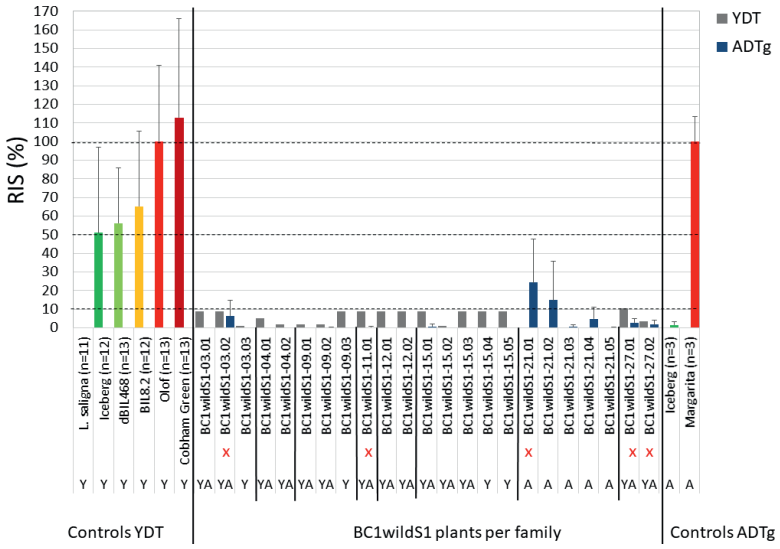


Figure S10. Relative infection severity (RIS) to Bl:21 of BC1wildS1 plants in a young plant disease test (YDT) and adult plant disease test (ADTg). Plants from lineage BC1wildS1-21 were included in the ADTg as they escaped proper disease test conditions due to local inferior humidity conditions in the YDT. YDT controls are depicted on the left, ADTg controls on the right. Results of BC1wildS1 plants are depicted in the middle, with different families separated by vertical lines. Y: tested in YDT, A: tested in ADTg. Red crosses indicate plants selected as mothers for BC1wildS2 families.

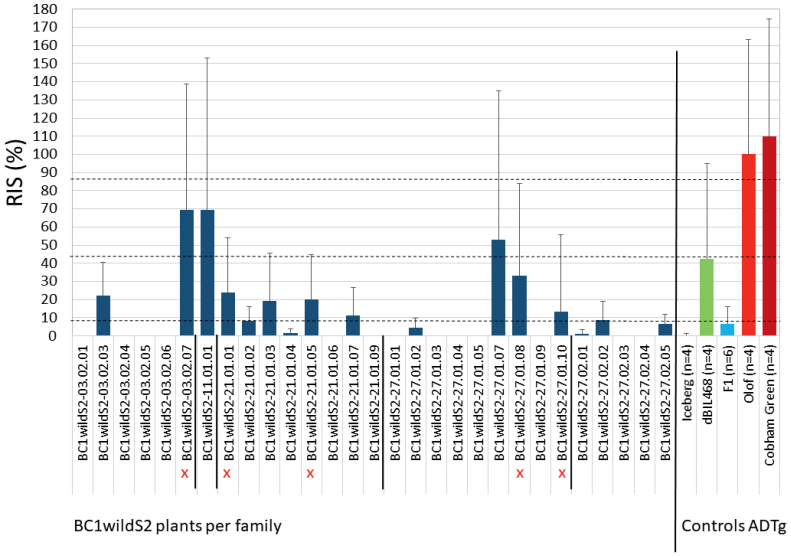


Figure S11. Relative infection severity (RIS) to Bl:21 of BC1wildS2 families with BC1wildS1 mothers with enhanced susceptibility and control lines in an adult plant disease test (ADTg). Relative infection severity levels of BC1wildS2 families are separated by vertical lines. Red crosses indicate plants serving as mothers for BC1wildS3 families.

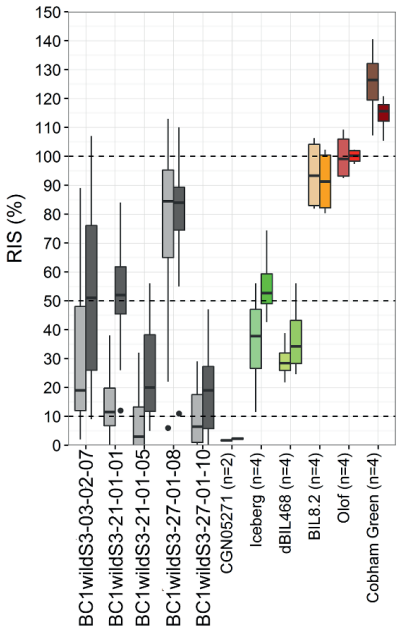


Figure S12. Relative infection severity (RIS) to BI:21 of BC1wildS3 families selected for enhanced susceptibility and controls. Per population type, RIS at 9 dpi is plotted in a light colour followed by RIS at 12 dpi in a darker colour.

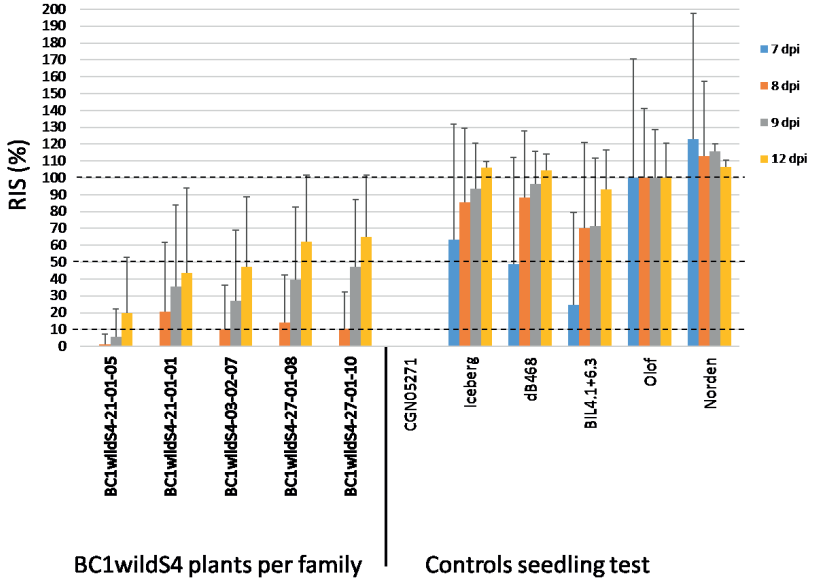


Figure S13. Relative infection severity (RIS) at seedling stage in inbred offspring of five BC1wildS3 lineages selected for enhanced infection severity. Remark: cv Iceberg and dB468 are control lines for reduced infection (partial resistant) at adult plant stage, but not at seedling stage.

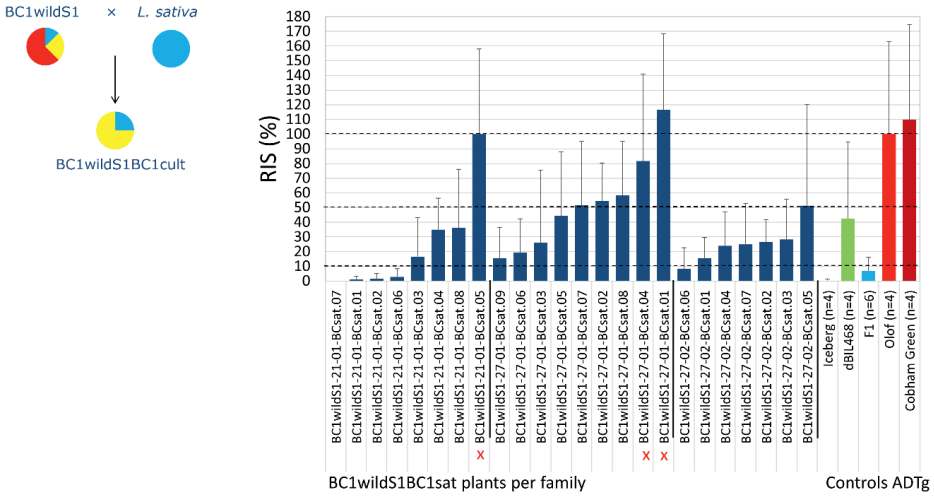


Figure S14. Crossing scheme to obtain BC1wildS1BC1cult plants from three BC1wildS1 founders and relative infection severity levels to Bl:21 of BC1wildS1BC1cult families in an adult plant disease test (ADTg). Pie charts depict the average genotypic composition of each generation. Red: homozygous *L. saligna*, blue: homozygous *L. sativa*, yellow: heterozygous. Different families are separated by vertical lines. Red crosses indicate plants selected for genotyping.

Supplemental Tables

Table S1. Details of BC1cult plants per *L. saligna* accession. (a) geographic origin of each accession, the exact geographic location can be found at http://www.cgn.wur.nl **(b)** *B. lactucae* test race used. **(c)** total number of BC1cult plants **(d)** number of discarded plants with hybrid necrosis (HN) **(e)** number of plants tested for relative infection severity (RIS) to BI:21 or BI:24 **(f)** number of plants with qualitative resistance by *R* gene(s) against the test isolate **(g)** percentage of plants with an *R* gene against the test isolate **(h)** *R* gene locus, n/a: not applicable [a] detailed map interval in Fig. S6 [b] detailed map interval in Giesbers et al (2017) **(i)** number of plants without HN and *R* genes **(j)** number of highly resistant plants (RIS≤10%) without *R* genes against the test isolate and HN **(k)** percentage of highly resistant plants (RIS≤10%) without *R* genes against the test isolate and without HN **(l)** number of highly resistant plants genotyped per population [c] three plants with RIS 10-15% [d] one plant died before sampling [e] one plant with RIS 10-15%.

	(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)	(j)	(k)	(l)
L. saligna	Origin	test race	# plants (total)	# plants with HN (discarded)	# tested plants (total - HN)	# plants with *R* gene(s)	% plants with R gene(s)	*R* gene locus	# plants without HN and *R* genes	# highly resistant plants	% highly resistant plants	# highly resistant plants genotyped
CGN05271	France	BI:21	94	20	74	0	0	n/a	74	6[c]	8[c]	6[c]
275-5	Corse	BI:21	46	11	35	0	0	n/a	35	0	0	0
CGN19047	Italy	BI:21	42	4	38	0	0	n/a	38	1[d]	3	0
CGN15726	Russia	BI:21	68	0	68	0	0	n/a	68	6	9	6
CGN11341	SW Asia	BI:21	45	3	42	0	0	n/a	42	1[e]	2[e]	1[e]
CGN15705	Georgia	BI:21	63	0	63	11	17	LG9: 111.3-112.5 cM[a]	52	7	13	7
CGN05304	Israel	BI:24	138	0	138	67	48	LG2: 0.0-6.1 cM[b]	71	10[d]	14	9
CGN05947	Israel	BI:24	59	0	59	46	78	LG2: 0.0-6.1 cM & LG4: 119.8-134.8 cM[b]	13	0	0	0
CGN05318	Israel	BI:24	60	0	60	37	62	LG1: 32.4-73.3 cM[b]	23	4[d]	17	3

Table S2. Primer sequences of EST-based markers.
Data available online:
https://doi.org/10.18174/430413

Table S3. SNPs and their surrounding sequence used for design of KASPar markers (marker design by Dr. van Haeringen Laboratorium B.V. (VHL), Wageningen, the Netherlands)
Data available online:
https://doi.org/10.18174/430413

Table S4. Correspondence between the linkage group (chromosome) numbering and orientation of the original F2 genetic linkage map (used in our previous publications) and the reference physical map of *L. sativa* cv. Salinas (used here).

Map description + reference	Linkage group								
L. sativa cv. Salinas physical map V8 [1]	1	2	3	4	5	6	7	8	9
F2 *L. saligna* x *L. sativa* genetic linkage map [2]	1[R]	2	8[R]	4	5[R]	3	7	6[R]	9

[R] reversed chromosome orientation [1] Reyes-Chin-Wo S, Wang Z, Yang X, Kozik A, Arikit S, Song C, Xia L, Froenicke L, Lavelle DO, Truco MJ et al. 2017. Genome assembly with in vitro proximity ligation data and whole-genome triplication in lettuce. *Nature Communications* 8: 14952. [2] Jeuken M, Peleman J, Lindhout P. 2001. An integrated interspecific AFLP map of lettuce (*Lactuca*) based on two *L . sativa* × *L . saligna* F2 populations. *Theoretical and Applied Genetics* 103: 638–647.

Table S5. Overview of candidate NHR loci, identified in resistant BC1cult plants and BC1wild inbred families that were selected for enhanced infection severity.
Data available online:
https://doi.org/10.18174/430413

Table S6. Genotype data of individual plants of BC1cult, BC1wild and BC1wildS1-S3 (1 to 3 generations of inbreeding).
Data available online:
https://doi.org/10.18174/430413

Chapter 4

Non-transmission of male and female heterospecific gametophytes is one of the reproductive barriers between wild and cultivated lettuce

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Submitted

Abstract

Interspecific crosses can be hampered by reproductive barriers, resulting in progeny with reduced vitality and fertility due to genetic incompatibilities between species, a phenomenon known as hybrid incompatibility (HI). Understanding the genetic architecture of HI can provide novel insights into the nature and evolution of postzygotic reproductive barriers between species. HI often leads to a distortion of Mendelian segregation of alleles in hybrid-derived progenies. Transmission ratio distortion loci (TRDL) in segregating populations can give an indication of loci for HI. We determined the genome-wide distribution of HI between wild lettuce, *Lactuca saligna,* and cultivated lettuce, *L. sativa*, through identification of TRDL in an F2 and in a set of backcross inbred lines (BILs) containing single introgression segments of wild lettuce in a cultivated lettuce background. Here we show that ten loci of severe TRD were detected in the F2, of which eight with a *L. sativa* allele preference. Half of these TRDL were associated with complete non-transmission of a heterospecific allele combination (absolute HI) in the BILs. The F2, which segregates for all loci, was used to identify candidates of conspecific interacting partners with the HI loci. Crosses between BILs with absolute HI and BILs with the conspecific interacting allele demonstrated three cases of HI by deleterious heterospecific two-locus interactions. One of the digenic interactions leading to absolute HI pointed to a sex-independent two-locus gametophytic barrier, as it was caused by complete non-transmission of one heterospecific allele combination, surprisingly in both male and female gametophytes.

Introduction

Understanding the genetic basis of speciation is an important aspect in evolutionary biology. Speciation often starts with a reduction of gene flow between lineages due to reproductive barriers. These barriers can arise by divergent ecological or sexual selection or by the evolution of genetic incompatibilities (Seehausen *et al.* 2014). Ecological adaptation may play only a minor role in the divergence between species, whereas purely mutational mechanisms or internal genetic conflicts play a larger role (Maheshwari and Barbash 2011). When diverging lineages meet in hybrids, heterospecific genetic incompatibilities can hamper proper development of the organism, resulting in hybrid inviability, sterility, weakness or necrosis, collectively known as hybrid incompatibility (HI). In general, the genetic and molecular mechanisms behind HI are not well characterized. The underlying genetics of these postzygotic hybrid incompatibilities may provide clues to the evolutionary forces and molecular mechanisms that lead to the formation of different species (Coyne and Orr 2004).

Postzygotic isolation can be caused by diverse genetic mechanisms, such as ploidy levels, chromosomal rearrangements or mutational processes (Coyne and Orr 2004; Rieseberg and Willis 2007; Hoffmann and Rieseberg 2008). The latter is the most common mechanism that leads to the evolution of HI through the accumulation of dysfunctional genic interactions (Presgraves 2010; Maheshwari and Barbash 2011). A model that explains how HI can evolve without species themselves having a reduced fitness has been formulated by Bateson, Dobzhansky and Muller and is commonly referred to as the BDM model (Orr 1996; Bomblies 2013). The BDM model states that each pair of interacting genes evolves independently in separate lineages, and deleterious interactions between them only occur in hybrids and their derived progeny as a secondary consequence of intraspecific divergence (Bateson 1909; Dobzhansky 1937; Muller 1942). Single locus BDM interactions also occur (Todesco *et al.* 2014). Genetic changes that are adaptive or nearly neutral in their own genomic background can be functionally incompatible with alleles from a foreign genomic background (Presgraves 2010).

BDM incompatibilities can evolve by various processes. One of these processes is gene duplication, followed by loss of function in one of the redundant gene copies (Bikard *et al.* 2009). When functional genes end up in different genomic locations in related species, interspecific hybrids may inherit only non-functional copies. Another process is internal genetic conflict, often caused by selfish genes (Presgraves 2010). Selfish genes can cause segregation distortion in their own favour but with deleterious effects on their host, for instance through meiotic drive and gamete-killing. Hosts can evolve suppressor genes to compensate for these negative effects. In interspecific hybrids, recombination may uncover the effects of a selfish gene when it is present without its suppressor (Presgraves 2010).

Many studies on the genetics of speciation have focused on *Drosophila* (Castillo and Barbash 2017), but studies have also been conducted on other organisms including yeast, nematodes, salamander, African clawed frog and plant species such as *Arabidopsis*, rice, wheat and *Mimulus* (Hermsen 1963; Harushima *et al.* 2001; Leppälä *et al.* 2013; Snoek *et al.* 2014; Hou *et al.* 2015; Case *et al.* 2016; Niedzicka *et al.* 2017; Gibeaux *et al.* 2018). BDM genes that cause hybrid lethality or sterility have been reviewed by Presgraves (2010), Rieseberg and Blackman (2010) and Maheshwari and Barbash (2011).

Apart from the fundamental interest in reproductive barriers because of their impact on the evolution of species, reproductive barriers have a practical impact on the improvement

of crops with genes from wild relatives. The narrow genetic base of many crops has become a major constraint in crop improvement. Introgression of genetic material from wild relatives or exotic accessions of the same species is an attractive natural means to broaden crop genetic resources (Chandnani *et al.* 2017). However, intra- or interspecific hybrid incompatibilities (HI) can result in a complete or incomplete non-transmission of certain genotypes. This often leads to distortion of Mendelian segregation of alleles and genotypes in hybrid-derived progeny. Such transmission ratio distortion (TRD) is frequently observed in interspecific segregating populations (Rieseberg *et al.* 2000; Koide *et al.* 2008). TRD can severely hamper the exchange of genetic variants between and within species. Therefore, understanding the mechanisms responsible for TRD is important for the introgression of agriculturally interesting alleles (Truco *et al.* 2007).

Transmission ratio-distorted loci (TRDL) co-locate with HI loci more frequently than is expected by chance (Moyle and Graham 2006). Therefore, TRDL have been used as indicators of genetic incompatibilities in several plant species. However, TRD is not always a sign of HI, but may also occur due to various other reasons before or after fertilization like meiotic drive (Lyttle 1991), competition between gametes (Howard 1999) or zygotes (Korbecka *et al.* 2002). Genome scan approaches are also used to identify the candidate barrier loci involved in the reduction of gene flow between species. Regions of high genomic differentiation between species are often assumed to be related to reproductive barriers. However, in order to conclusively identify a HI additional experimental evidence, such as transgenic approaches or experimental crosses, is necessary (Ravinet *et al.* 2017).

Genetic mapping (e.g. QTL analysis) in segregating populations can provide additional evidence of an association between the genotype and the HI phenotype, like sterility or inviability. Besides mapping in segregating populations, HI genes can also be mapped in advanced backcross inbred lines (BILs), which contain a chromosomal segment of one species in the genetic background of another species. BILs can be very useful for validation and fine-mapping of HI genes, but cannot identify inter-locus interactions in contrast to multi-locus segregating populations (Maheshwari and Barbash 2011).

Here, we studied a wide interspecific cross between two autogamous, diploid *Lactuca* species: wild lettuce *L. saligna* and cultivated lettuce *L. sativa*. Apart from fundamental insights into the genetic basis of *Lactuca* divergence, knowledge on cases of HI between *L. saligna* and *L. sativa* might be useful for the introgression of horticulturally interesting wild lettuce alleles into cultivated lettuce. An easily recognizable form of HI, hybrid necrosis, has previously been observed in an F2 population and a backcross inbred line (BIL) of an interspecific cross between *L. saligna* x *L. sativa*. This hybrid necrosis was caused by a heterospecifc interaction of two loci and acted as a zygotic barrier (Jeuken *et al.* 2009).

Many studies start with a multi-locus segregating population to identify TRD loci and/or hybrid sterility loci and their colocations. Our starting point was a set of BILs, each containing one or few chromosomal segment(s) from the wild parent in the genomic background of the cultivated parent. While monitoring the introgression of wild parent DNA into the cultivated parent we came across regions that were associated with distorted segregation and prevented the occurrence of homozygous wild parent segments in a homozygous cultivated parent background. This showed that there was a complete non-transmission of heterospecific allele combinations, which can be considered as HI. We then used an interspecific F2 to identify TRD, and compared the detected TRDL with HI loci in BILs. Secondly, the F2 was used to find potential conspecific genetic loci or 'dance partners' that can nullify HI (Moyle and Graham 2006). Validation studies by intercrossing BILs

containing HI loci with BILs containing candidate dance partners, demonstrated the existence of two new digenic interactions involved in HI. We analysed one of these digenic interactions in detail, and found evidence for non-transmission of male and female gametophytes with a particular heterospecific genotype.

Materials and Methods

Plant materials

An F2 population of 126 plants was derived from a cross between wild parent *L. saligna* CGN05271 (mother) and cultivated parent *L. sativa* cv Olof (Jeuken *et al.* 2001). F1 plants of the same cross were backcrossed as a mother to the cultivated parent resulting in a BC1 population of 88 plants designated as BC1cult, and to the wild parent resulting in a BC1 population of 33 plants designated as BC1wild.

Backcross inbred lines (BILs) with wild parent chromosome segments introgressed into cultivated lettuce were developed by several generations of backcrossing to the cultivated parent followed by minimal one generation of selfing (Jeuken and Lindhout 2004). In this paper, a BIL number reflects the linkage group (LG) number where the wild lettuce introgression segment resides, according to the *L. sativa* cv Salinas genome map (Reyes-Chin-Wo *et al.* 2017) and letters discriminate among segments on the same LG (Fig. S1).

DNA isolation and genotyping

DNA was isolated by either a high-throughput NaOH method (Wang *et al.* 1993) or a CTAB method (van der Beek *et al.* 1992). Genetic markers were based on *L. sativa* cv Salinas ESTs or on SNPs between *L. sativa* cv Salinas and a pool of five *L. saligna* accessions (Chapter 3, this thesis). For EST-based markers (Table S1), polymorphisms between PCR products of *L. saligna* and *L. sativa* alleles were visualized by high-resolution melting curve differences on a LightScanner System (Den Boer *et al.* 2014) or by gel electrophoresis. For SNPs, 300 KASPar markers were designed and used for genotyping by Dr. Van Haeringen Laboratorium B.V., Wageningen, the Netherlands. KASPar SNP positions with 150 surrounding base pairs are listed in Table S2.

Genetic map

KASPar markers were added to our latest F2 genetic linkage map (based on EST and AFLP-markers) from the cross *L. saligna* CGN05271 x *L. sativa* cv Olof (Jeuken *et al.* 2001). Linkage analyses were performed using JoinMap v5 software (Van Ooijen 2006). A new consensus genetic linkage map was calculated per linkage group using regression mapping and Kosambi's mapping function with default settings: linkages with a recombination frequency smaller than 0.40, LOD scores higher than 1, a jump threshold of 5 and a third round. Marker intervals for studied traits in all populations were based on this F2 consensus map. Physical map locations refer to the *L. sativa* cv. Salinas reference lettuce genome v8 (Reyes-Chin-Wo *et al.* 2017); https://lgr.genomecenter.ucdavis.edu/). Here, we use the linkage group numbering and orientation of that reference *L. sativa* physical map, which differs from the numbering used in our previous publications. In order to relate previously reported gene and marker locations to the mapped loci in the current study, we present a conversion table (Table S3).

Genetic nomenclature

Alleles of wild lettuce, *L. saligna*, are referred to as 'w' and alleles of cultivated lettuce, *L.*

sativa, as 'c'. Consequently, genotypes are 'cc': homozygous *L. sativa*, 'cw' or 'wc': heterozygous, and 'ww': homozygous *L. saligna*.

Detection of TRD
Observed genotype frequencies were compared to the expected Mendelian ratio of 1:2:1 in the F2, or to the Mendelian 1:1 ratio in BC1 populations. Assuming that the nine homologous chromosomes of lettuce contain at least two independent regions, at least 18 independent genomic regions were expected. To correct for genome-wide testing, we applied a threshold of $\alpha=0.05/18=0.003$. Chi-square tests were performed per marker and in the F2 distorted regions with three or more distorted consecutive markers were considered as regions with TRD. As BC1 populations were genotyped with only ~80 markers, our criterion of at least three distorted markers may be too strict, therefore regions with at least one distorted marker were considered as TRD regions.

Zygotic or gametophytic barriers
Reproductive barriers may be zygotic or gametophytic. As explained in Fig. S2, distortion of heterozygote frequency may indicate a zygotic barrier (or possibly two gametophytic barriers, one affecting the male and the other affecting the female gametophyte), whereas non-distortion of heterozygote frequency may indicate a gametophytic barrier (Fig. S2). For each representative marker in a distorted region in the F2, the observed heterozygote frequency and the sum of the two homozygote frequencies were compared to the expected Mendelian ratio of 1:1 in a chi-square test at $\alpha=0.05$.

Identification of digenic interactions
For each HI region that was identified in the set of BILs, F2 genotypes with a homozygous *L. saligna* introgression along the HI region were selected. Loci with at least one conspecific (*L. saligna*) allele in all of these plants were considered as candidate interacting partners of the HI gene (i.e. 'dance partners', (Moyle and Graham 2006)). Crosses were made between BILs with a heterozygous segment and the BILs containing their candidate dance partners in a homozygous wild parent segment. A limited number of candidates were tested per HI. After selfing the F1 of each cross, segregation of the HI region was assessed in all plants homozygous *L. saligna* for the candidate dance partner. If segregation was normal (no TRD), we considered this as proof of a conspecific digenic interaction.

HI on linkage group segments 4A+8A

Segregation analysis
Inbred progeny (n=691) of an F1 (double heterozygote '4cw8cw') from cross BIL4A+8A x *L. sativa* cv Olof segregated for segment 4A and 8A. Individuals were genotyped with two markers per segment to determine segregation ratios of the nine expected genotypes: markers NL1151 and NL0897 on LG4 and markers M7120 and LE1211 on LG8. Individuals with crossovers between either pair of markers (n=56) were excluded. Segregation of individual heterozygous segments 4A and 8A in the *L. sativa* background was also determined. Segregation of segment 4A was assessed in inbred progeny (n=118) of a plant with genotype 4cw8cc. A heterozygous segment 8A was only available in a line that also contained a homozygous *L. saligna* introgression at 4A. Therefore, segregation of segment 8A was assessed in inbred progeny (n=545) of a plant with genotype 4ww8cw.

Hypothesis testing
Hypotheses for the HI based on gametophytic and/or zygotic barriers were tested by chi-square tests at $\alpha=0.05$. To validate a non-rejected hypothesis of distorted segregation in

the F2, we tested again for TRD in different population types. We developed two backcross populations: the double heterozygote 4cw8cw was backcrossed reciprocally to BIL4A (4ww8cc). Only the gametophytes of the double heterozygote segregate and therefore their maternal or paternal effects on the segregation ratios can be observed separately in the reciprocal backcross populations. Observed segregation ratios of these two BC1 populations (n=117 and n=140 plants) were tested against the hypothesized segregation by a chi-square test (α=0.05).

Pollen vitality and seed set were assessed, as a reduction is a functional validation of the non-rejected hypothesis. To test pollen vitality, capitula that just starting flowering were collected from the double heterozygote 4cw8cw and from the control genotype *L. sativa* cv Olof. From the same genotypes, developing capitula (flower buds) of 2-3 mm in length were collected to observe tetrads. Capitula were individually dissected and examined microscopically after treatment with Alexander stain, which differentially stains aborted and non-aborted pollen (Peterson et al 2010, Alexander 1980). Seed set was assessed in *L. saligna* CGN05271, *L. sativa* cv Olof, the double heterozygote 4cw8cw and recombinants of the double heterozygote. Per plant, at least five capitula that flowered on the same day were labelled. For unique recombinant genotypes, at least ten capitula that flowered on the same day were labelled. The number of seeds (achenes) per labelled capitulum were counted to determine the percentage of aborted and non-aborted seeds. An aborted seed was distinguished as a thin, empty seed coat. Statistical differences were tested by ANOVA followed by a Tukey HSD test in Genstat 18th Edition.

Mapping

The HI conferred by a deleterious heterospecific combination of genes on segment 4A and 8A was first identified in the BILs and delineated by the borders of the introgression segments. Map intervals were further reduced by mapping the TRD in the F2 with additional markers. Genotyping the recombinant offspring of the 4cw8cw double heterozygote further reduced the map interval by two approaches described in the results section.

Results

F1 and F2 hybrid phenotypes

L. saligna CGN05271 (female) was unidirectionally crossed to *L. sativa* cv Olof (male), as reciprocal crosses with *L. sativa* as a female and *L. saligna* as a male are less successful (De Vries 1990). The resulting F1 seeds showed normal germination. F1 plants displayed a vital phenotype intermediate between its parents (Fig. 1a), but with severely decreased fertility (Fig. 1b). F1s had a seed set of only 2% of the seed set of each of its parents. The 1.4 times higher seed number of *L. saligna* compared to *L. sativa* can be explained by the 1.5 times as many capitula on *L. saligna* (Table S3). F1 selfing resulted in an F2 population of 162 seeds yielding 126 adult plants (Table S4). Twenty-two per cent of F2 seeds did not result in adult plants due to hybrid inviability (no germination or early plant death). Of the adult plants, 11% showed hybrid necrosis and about 10% showed malformed growth (hybrid weakness). Variation in hybrid fertility was observed: from 36% of F2 we did not harvest seeds either due to complete sterility or loss before flowering, 55% of F2 had severely reduced seed set up to 2% of the parental seed set and 9% of F2 had a severely reduced seed set of 2-13% of the parental seed set. These aberrant F2 phenotypes are likely associated with genetic incompatibilities between the two species.

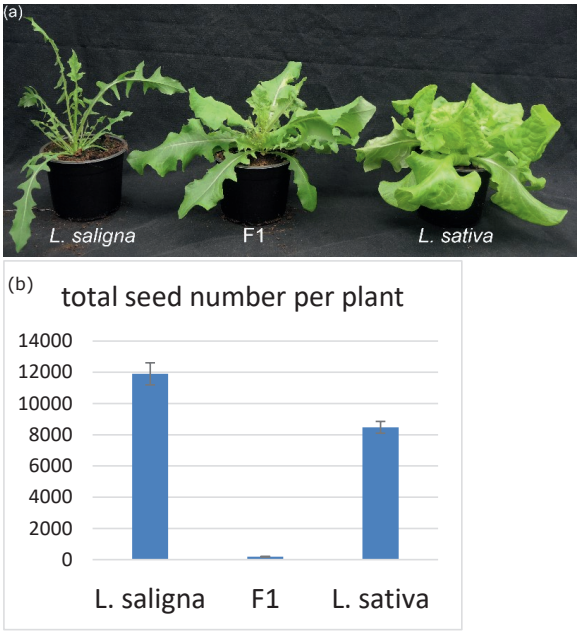


Figure 1. Phenotype and seed number of *L. saligna* CGN05271, *L. sativa* cv Olof and the interspecific F1 (a) phenotype at five weeks of age **(b)** average estimated total seed number per plant, based on three plants per genotype (Table S3).

TRDL in F2 and BC1

Marker TRDs in segregating populations are usually a consequence of deleterious allelic combinations that cause HI. Ten transmission ratio distortion loci (TRDL) were indicated by chi-square tests (p-value<0.003) after genome-wide genotyping of the F2 (n=126) with 492 markers (Fig. 2, Table 1). They were distributed over six chromosomes, leaving three chromosomes free of TRD (LG1, LG2 and LG6). Eight TRDL had a preference for *L. sativa* alleles and two TRDL had a preference for *L. saligna* alleles. For some loci the frequency of one of the homozygous genotypes was decreased from the expected 0.25 down to almost 0, or increased from 0.25 up to 0.65. Some heterozygous genotype frequencies were distorted from the expected frequency of 0.5 down to 0.35 or up to 0.7. Non-distortion of heterozygote frequency within a TRDL indicates a gametophytic reproductive barrier with altered allele transmission through one of the gametophytes, male or female. Distortion of the heterozygote frequency within a TRDL can be explained by a zygotic reproductive barrier with altered viability of the zygote via its genotype or theoretically by a sex-independent gametophytic barrier affecting the male as well as female gametophytes (examples in Fig. S2). Heterozygote frequency analysis indicated that five TRDL may be due to a zygotic (or possibly sex-independent gametophytic) barrier, and five TRDL may be due to a male or female gametophytic barrier (Table 1).

We compared these TRDL in the F2 with indications for TRDL in two genome-wide genotyped bidirectional backcross (BC1) populations from the same original *L. saligna* CGN05271 x *L. sativa* cv Olof cross (genotyped with 79 and 83 markers respectively) (Fig. S3). Fewer TRDL, with less severe segregation distortion were detected in the BC1 populations compared to the F2 (Table 1, Table S5). In the BC1cult (F1 x *L. sativa*) five TRDL overlapped with TRD intervals in the F2 and one additional TRDL was identified. In

the BC1wild (F1 x *L. saligna*) three TRDL overlapped with TRD intervals in the F2 and one additional TRDL was identified (Table S5).

Overlap of TRD in F2 with HI in BILs

Previously, backcross inbred lines (BILs) with wild (*L. saligna*) segments in cultivar (*L. sativa*) background were obtained by several generations of backcrossing with *L. sativa*, starting from the F1 generation and ending with at least one generation of selfing (Jeuken and Lindhout 2004). Recently, one newly developed BIL was added (BIL6D). Each BIL contains one or a few homozygous *L. saligna* introgression segments in a *L. sativa* background and four lines harbour only a heterozygous introgression segment at one region. Genotyping with 300 new KASPar markers gave a more detailed picture of the introgressions present in the BILs (Fig. S1). Few missing *L. saligna* segments were revealed at distal chromosomal ends that were unnoticed during earlier BIL development: the top of LG2 and the bottom of LG5. Absence of an introgression on the top of LG7 was already observed in the earlier genotyping (Jeuken and Lindhout 2004). Probably it was already lost in one of the early backcross generations (BC2 or BC3). We did not further study this region.

For five genomic regions, we were only able to obtain a line with a single heterozygous introgression in a *L. sativa* background. Their inbred progeny showed TRD with a complete lack of individuals with a homozygous wild parent segment (Table S5), which indicated complete non-transmission of a heterospecific allele combination that we consider as absolute hybrid incompatibility (HI). HI regions were based on the borders of the heterozygous segment. For four of these five HI loci, we also observed TRD in the F2. One absolute HI in BIL8B showed no TRD in the F2. For three remaining TRDL with a *L. sativa* preference in the F2, we did obtain BILs with single homozygous *L. saligna* introgressions from relatively small segregating populations (n=14). Therefore, these three TRDL were not due to absolute HI.

The wild parent introgression segments (homozygous or heterozygous) in our set of BILs cover 90% of the *L. saligna* genome. Consequently in 10% of the genome HI could not be determined, due to absence of a *L. saligna* introgression caused by a lack of genetic markers in this region at the time of selection and/or due to loss of the introgression segment in an early backcross generation.

Digenic interactions

According to the BDM model, TRD in hybrid-derived progeny may be due to deleterious interactions between heterospecific genes. Hybrid incompatibility caused by such a heterospecific gene pair can therefore be nullified by the presence of conspecific genes at each interacting locus. Genotypes with homozygous *L. saligna* introgressions at HI regions can only be present in plants that also carry the *L. saligna* allele for the interacting locus. As the F2 segregates for all loci, some plants obtain *L. saligna* alleles at both interacting loci, in contrast to BILs with a single *L. saligna* introgression in a purely *L. sativa* background.

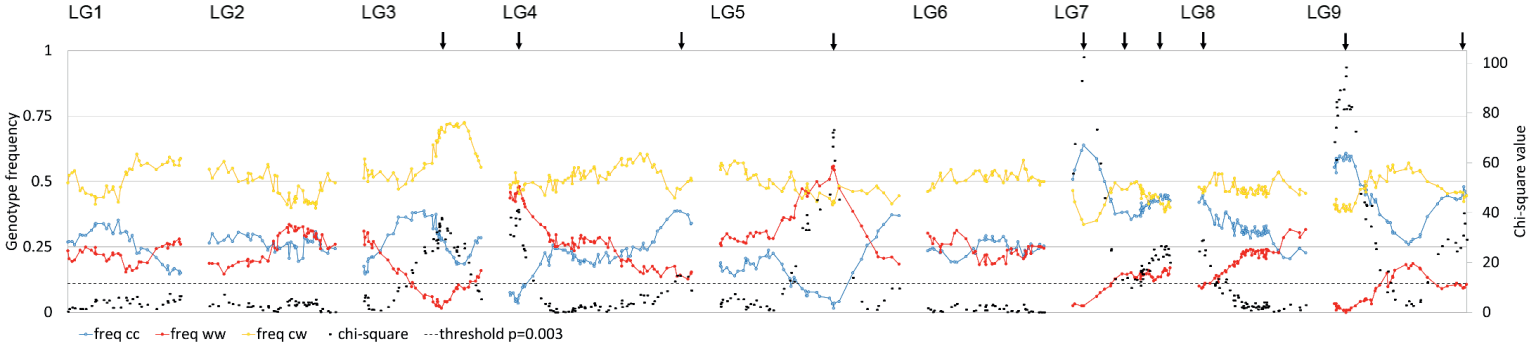


Figure 2. Frequency of wild parent *L. saligna* (red), heterozygous (yellow), cultivated parent *L. sativa* (blue) genotypes and chi-square value per marker in an F2 population of *L. saligna* CGN05271 x *L. sativa* cv Olof. Black dots above the black dashed line indicate deviation from a 1:2:1 Mendelian segregation ($p<0.003$). X-axis: nine linkage groups in cM. Arrows indicate loci with significant transmission ratio distortion.

Table 1. Overview and characteristics of transmission ratio distortion loci (TRDL) identified with p<0.003 in the F2 and validated in BC1cult, BC1wild and backcross inbred lines (BILs) and absolute hybrid incompatibility loci identified in BILs. Details can be found in Table S5. HI: absolute hybrid incompatibility (complete non-transmission of homozygous segment of the wild parent in a homozygous cultivated parent background). In the F2, TRDL are designated as potentially zygotic or gametophytic barriers based on distortion or non-distortion of heterozygote frequency respectively, *p<0.05, **p<0.01, ***p<0.0005, ****p<0.00001. Three TRDL peaks were identified on LG7 based on a switch of the most prominent genotype. Genotype and allele frequencies (freq) are colour-shaded from low (green) to high (red) values to facilitate interpretation. c: allele from cultivated parent *L. sativa* cv Olof, w: allele from wild parent *L. saligna* CGN05271. ✓: TRDL validated ✗: TRDL not validated. ns: not significant, n/a: not applicable. [a] no TRD in F2, but absolute HI in BIL. [b] TRDL on LG5 in BC1cult with a slightly less strict p-value of 0.006 (instead of 0.003). [c] TRD with a wild parent allele preference cannot be validated in BILs with wild parent introgressions in cultivated parent background. [d] segment could not be studied due to loss in an early backcross generation.

F2 (n=126)						genotype freq			allele freq			BC1cult	BC1wild	BILs	Conclusion
LG	interval TRD (cM)	representative marker	cM	Mb	Chi^2	cc	cw/wc	ww	c	w	barrier	(n=88)	(n=33)		HI wild parent into cultivated parent
				Mendelian segregation		**0.25**	**0.50**	**0.25**	**0.50**	**0.50**					
3	36-92	NL1187	66.9	134	38	0.28	0.71	0.02	0.63	0.37	zygotic ****	✓	✓	✗	no HI
4	0-26	Ls_v8_lg_4_020626270	5.6	21	41	0.05	0.50	0.46	0.30	0.70	gametophytic	✓	✗	n/a[c]	n/a
4	136-157	Ls_v8_lg_4_348066968	147	348	15	0.39	0.47	0.14	0.62	0.38	gametophytic	✗	✗	✗	no HI
5	55-122	Ls_v8_lg_5_255102715	97.2	255	74	0.03	0.40	0.57	0.23	0.77	zygotic *	✓[b]	✓	n/a[c]	n/a
7	0-33	Ls_v8_lg_7_021291267	9.3	21	102	0.64	0.34	0.03	0.81	0.19	zygotic ***	✗	✗	n/a[d]	n/a
7	33-69	NL1001	55.2	131	13	0.37	0.50	0.13	0.62	0.38	gametophytic	✗	✗	✓	**HI**
7	69-84	LE9018	80.5	183	27	0.45	0.39	0.16	0.64	0.36	zygotic ****	✗	✗	✗	no HI
8	0-17	Ls_v8_lg_8_000481920	2.3	0.5	29	0.44	0.47	0.09	0.67	0.33	gametophytic	✗	✗	✓	**HI**
8[a]	26-47	NL1117	38.4	109	ns	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	**HI**
9	0-50	RIN4	8	15	89	0.59	0.40	0.01	0.79	0.21	zygotic *	✓	✗	✓	**HI**
9	74-114	Ls_v8_lg_9_195009606	111.7	195	40	0.48	0.41	0.10	0.69	0.31	gametophytic	✓	✓	✓	**HI**

We used our F2 genotype data set to identify candidates of conspecific interacting partners – genetic "dance partners" (Moyle and Graham 2006) for HI loci identified in the set of BILs. F2 genotypes with a homozygous *L. saligna* introgression in the distorted region (defined as HI in BILs) were selected. Loci with at least one conspecific (*L. saligna*) allele in all of these plants were identified as candidate dance partners (Table S6). The number of candidate dance partners per HI locus varied from one to more than ten. Crosses between heterozygous BILs and BILs containing candidate dance partners resulted in three BILs with a homozygous *L. saligna* introgression in the TRD region in combination with a conspecific introgression at another locus. The presence of the conspecific allele elsewhere on the genome nullified the previously observed TRD at the HI locus. (Table S6). This indicated a two-locus hybrid incompatibility between heterospecific alleles, most probably caused by a digenic interaction.

The digenic interaction of (BIL)9A-1+8C has been characterized previously (Jeuken *et al.* 2009). Additionally, we have now identified a two-locus interaction for BIL7C with BIL3A, and for BIL8A with BIL4A. In this paper, we describe the functional characterization of the hybrid incompatibility between heterospecific alleles at introgression segments 8A and 4A.

TRDL with a wild parent allele preference

In the F2, two TRDL with a preference for *L. saligna* alleles were identified on LG5 and LG4 (Table 1). The TRD on LG5 was observed in all three segregating populations (F2, BC1cult, and BC1wild). Interestingly, this region of TRD overlapped with a recombination coldspot between *L. saligna* and *L. sativa* (Fig. S4). The TRD on the top of LG4 (Table 1) can be explained by its interaction with the top of LG8 and will be described in the following paragraphs.

Non-transmission of male and female heterospecific gametophytes (4c8w)

The F2 derived TRD region on LG8 with cultivated parent (*L. sativa*) allele preference overlaps with the interval of segment 8A in BIL4A+8A. The F2 derived TRD region on LG4 with wild parent (*L. saligna*) allele preference overlaps with segment 4A in BIL4A. Segment 4A was present in BIL4A as a single homozygous *L. saligna* segment, whereas 8A was only present in combination with 4A (but we could not trace back if any effort had been taken to retrieve it singly). We wondered whether it was coincidence or not, that segment 8A and 4A for which opposite strong allele preferences were observed in the F2, appear together in a backcross line.

To answer this question, we studied the segregation of a population that only segregated for the introgression segments 4A and 8A in further purely cultivated parent background (inbreds of F1 from cross BIL4A+8A x *L. sativa* cv Olof). Plants (n=691) were genotyped with a pair of markers per introgression segment (see Materials and Methods). Individuals with crossovers between either pair of markers (n=56) were excluded. The seed germination rate of the population segregating for both loci was normal (>95%) and no seedling lethality was observed.

The segregation ratio of 635 plants (Fig. 3a) was significantly different from a Mendelian segregation of two independent genes (Hypothesis 1, Fig. 3b). In a Mendelian segregation of two loci, four gametophyte genotypes are produced in equal frequencies (two conspecific ones 4c8c and 4w8w, and two heterospecific ones 4c8w and 4w8c), leading to 16 gametophyte combinations (4 male x 4 female) representing nine genotype classes. These occur in frequencies of 1/16, 1/8 or 1/4. We observed only six genotypes instead of nine (Fig. 3a). Three out of nine expected genotypes were absent. The remaining six genotyped

showed nearly doubled frequencies (1/8 and 1/4) compared to Mendelian segregation, except for the double heterozygote (4cw8cw) which remained close to 1/4. The non-observed genotypes were 4cc8cw, 4cc8ww and 4cw8ww. Numbers refer to chromosome numbers of the introgressions, 'c' is a cultivated parent (*L. sativa*) allele, 'w' is a wild parent (*L. saligna*) allele. Consequently, plants that were homozygous *L. saligna* at the 8A introgression were always homozygous *L. saligna* at the 4A introgression, genotype 4ww, 8ww. So, we conclude that it was no coincidence that the two segments appeared together in one BIL (4A+8A). Segregation of 8A was not distorted when 4A was homozygous *L. saligna* (4ww8cw) (Fig. S5 panel b). Segregation of a single 4A segment in cultivated background was almost Mendelian with a slight preference, not for *L. saligna* but for *L. sativa* alleles (allele freq 0.59) (Fig. S5 panel c).

The three absent genotypes have in common that they are a product of at least one gametophyte with the heterospecific genotype '4c8w', whereas half of the expected double heterozygotes are expected to be a product of this gametophyte. Therefore, we postulated a second hypothesis stating that the heterospecific gametophytic genotype '4c8w' is not transmitted for both male and female gametophytes (Fig. 3c). Segregation according to this hypothesis, in which maternal and paternal 4c8w gametophytes are absent, resulted in expected genotype numbers close to the observed numbers (Fig 3c). This hypothesis was not rejected ($p=0.8$).

To validate the maternal and paternal effects, segregation ratios of a reciprocal cross between a double heterozygote 4cw8cw (gametophytes 4c8c, 4w8w, 4c8w, 4w8c) and a 4ww8cc plant (gametophytes: 4w8c only) were analysed. Indeed, in both reciprocal progenies the 4cw8cw genotype resulting from gametophyte 4c8w was absent or observed in a very low frequency, probably due to some selfings. These results confirmed that the 4c8w gametophytes were not transmitted (Fig. S5 panel j-k).

To exclude the possibility of an alternative explanation for the distorted segregation, we tested four alternative hypotheses, regarding genotype lethality, non-transmission of 4c8w gametophytes by male or female gametophytes only, combined hypotheses and genotype frequencies based on observed allele frequencies (Fig. S5 panel f-i). However, these hypotheses were all rejected as expected genotype numbers were very different from the observed numbers ($p<0.001$).

Overall, our results suggested that the observed TRD between 4A and 8A is explained by the male and female 4c8w gametophytes not participating in the reproduction, due to a deleterious digenic interaction (HI). In the F2 population (n=126) we observed a similar two-locus TRD by absence of the three non-transmitted genotypes. This population had *L. saligna* cytoplasm, whereas the double heterozygote 4cw8cw and its selfed progeny had *L. sativa* cytoplasm, suggesting that cyto-nuclear interactions do not play a role.

(a) Observed segregation inbred progeny of genotype 4cw8cw

observed plant numbers

	LG8					
LG4	**cc**	**cw/wc**	**ww**	sum	geno freq	allele freq
cc	75	0	0	75	0.12	c=0.32
cw/wc	126	130	0	256	0.40	
ww	80	151	73	304	0.48	w=0.68
sum	281	281	73	**635**		
geno freq	0.44	0.44	0.11			
allele freq	c=0.66		w= 0.34			

genotype frequencies

	LG8		
LG4	**cc**	**cw/wc**	**ww**
cc	0.12	0.00	0.00
cw/wc	0.20	0.20	0.00
ww	0.13	0.24	0.11

(b) Hypothesis 1: Mendelian segregation
Chi2= 395, p=0, rejected
expected plant numbers

	LG8					
LG4	**cc**	**cw/wc**	**ww**	sum	geno freq	allele freq
cc	40	79	40	159	0.25	c=0.50
cw/wc	79	159	79	317	0.50	
ww	40	79	40	159	0.25	w=0.50
sum	159	317	159	**635**		
geno freq	0.25	0.50	0.25			
allele freq	c=0.50		w=0.50			

genotype frequencies

	LG8		
LG4	**cc**	**cw/wc**	**ww**
cc	0.06	0.13	0.06
cw/wc	0.13	0.25	0.13
ww	0.06	0.13	0.06

(c) Hypothesis 2: non-tranmission of male and female 4c8w gametophytes
Chi2= 4.6, p=0.8, not rejected
expected plant numbers

	LG8					
LG4	**cc**	**cw/wc**	**ww**	sum	geno freq	allele freq
cc	71	0	0	71	0.11	c=0.33
cw/wc	141	141	0	282	0.44	
ww	71	141	71	282	0.44	w=0.67
sum	282	282	71	**635**		
geno freq	0.44	0.44	0.11			
allele freq	c=0.67		w=0.33			

expected gamete segregation (bold underlined: non-transmitted)

	male			
female	4c8c	**4c8w**	4w8c	4w8w
4c8c	4cc8cc	**4cc8cw**	4cw8cc	4cw8cw
4c8w	**4cc8wc**	**4cc8ww**	**4cw8wc**	**4cw8ww**
4w8c	4wc8cc	**4wc8cw**	4ww8cc	4ww8cw
4w8w	4wc8wc	**4wc8ww**	4ww8wc	4ww8ww

Figure 3. Segregation of inbred progeny (n=635) of double heterozygote 4cw8cw (segregating for loci on LG4 (4A segment) and LG8 (8A segment)) compared to segregation according to two hypotheses. Genotype plant numbers and genotype frequencies are colour-shaded from low (red) to high (green) numbers. **(a)** Observed segregation of inbred progeny of the doubly heterozygous genotype, 4cw8cw **(b)** Expected segregation according to hypothesis 1: Mendelian 1:2:1 segregation (both loci allele frequencies of 0.5). **(c)** Expected segregation according to hypothesis 2: non-transmission of male and female 4c8w gametophytes, lower panel: expected gamete segregation, bold underlined: non-transmitted gametes. Cells with the same colour indicate gamete combinations that result in the same genotype. Genetic nomenclature: c= *L. sativa* allele, w= *L. saligna* allele; cc= homozygous *L. sativa* genotype, cw/wc= heterozygous genotype, ww= homozygous *L. saligna* genotype. geno freq = genotype frequency, allele freq = allele frequency.

Functional proof for non-transmission of female heterospecific gametophytes
Non-transmission of the heterospecific (4c8w) gametophytes may be due to disturbance of different processes in reproduction. This heterospecific gametophyte may not be formed in meiosis, or formed but dysfunctional anywhere in process to double fertilisation. From the male side, non-transmission of one out of four gametophytes may result in 25% non-vital pollen. However, no phenotypic abnormality was observed at the tetrad stage and pollen vitality in the double heterozygote was similar to *L. sativa* cv Olof (>95%). These results indicate that the heterospecific male gametophyte was formed but dysfunctional.

The ratio of aborted and non-aborted achenes (seeds) per capitulum could indicate if reproduction is disturbed before or after fertilisation. If 4c8w gametophyte transmission is disturbed before fertilization, we would expect only the female gametophytes to influence seed set, as the defect 4c8w pollen can be compensated by other pollen genotypes. For the double heterozygote, this would result in 75% viable seeds and 25% aborted seeds (one out of four female gametophytes is not transmitted). If disturbed by a process after fertilization (e.g. zygote lethality), 4c8w pollen are not compensated by other pollen and 75% (transmitted female gametophytes) * 75% (transmitted male gametophytes)=56% viable seeds and 44% aborted seeds would be expected for the double heterozygote.

In the double heterozygote 4cw8cw, 29% of the seeds were aborted, compared to 4 to 7% for *L. saligna* and *L. sativa. L. saligna* and *L. sativa* both had a similar number of around 12 achenes per capitulum (Table S3.) The 71% remaining seeds indeed only consisted of six genotypes (and three genotypes 4cc8ww, 4cw8ww and 4cc8cw were absent). These results are consistent with a disturbance of gametophyte transmission before fertilization, in which 4c8w pollen are compensated by other pollen.

Mapping the hybrid incompatibility between 4A and 8A
In the F2 we observed the same two-locus TRD with only six out of nine expected genotypes within the marker intervals on the segment 4A and 8A. The three non-transmitted genotypes by HI, 4cc8ww, 4cw8ww and 4cc8cw, were only present in recombinants. Presence of these genotypes is directly informative for the HI map interval, as it indicates absence of HI. We analysed the TRD between 4A and 8A in F2 recombinants within those regions to more precisely locate the genes responsible for this TDR. This resulted in an HI interval from 2.9-7.1 cM on LG4 and 0.0-9.5 cM on LG8.

Recombinant offspring of the 4A8A double heterozygote (with introgressions of about 25 cM on both LG4 and LG8) were used to further fine map the HI loci. Two HI phenotyping approaches were used to narrow down the HI map intervals (Fig. S6). The first approach (Fig. S6a) was also used in the F2 and is based on the fact that plants with three specific genotypes (4cc8ww, 4cc8cw and 4cw8ww) are not formed in the HI with the typical distorted segregation. Formation of these three genotypes within the current map intervals therefore indicates absence of HI phenotype. Two recombinants with a 4cc8ww and 4cc8cw genotype within the 4A segment narrowed down the HI locus on LG4 to an interval of 1.2 cM, from 4.4 to 5.6 cM (Fig S7).

The second HI phenotyping approach (Fig. S6b) is based on two types of recombinant plants of which the genotype is not directly informative, but only in the next inbred generation after determination of the presence or absence of HI based on seed set and TRD in inbred progeny. These two recombinants have a heterozygous genotype at one locus and a recombinant genotype at the other locus (at LG4 switching from heterozygous to homozygous *L. saligna* and at LG8 switching from heterozygous to homozygous *L.*

sativa). If the HI phenotype is present (determined by 25% reduced seed set and TRD in inbred progeny), the HI must map within the heterozygous interval. If the HI loci phenotype is absent (normal seed set and no TRD in inbred progeny), the HI must map outside of the heterozygous interval. One plant with a recombination in the 8A segment showed normal seed set and presence of all expected nine genotypes in its offspring, indicating a non-HI phenotype. Therefore the HI phenotype on LG8 should be situated outside of the 8A heterozygous segment of this recombinant plant. This narrowed down the HI locus on LG8 to an interval of 4.7 cM, from 0 to 4.7 cM (Fig S7).

In summary, segregation ratios in inbred and reciprocal backcross progenies indicated that the distorted segregation between 4A and 8A segments can be explained by non-transmission of male and female gametophytes with the heterospecific allele combination, 4c8w. Functional proof for this specific non-transmission was obtained from the female gametophyte side. The 4A8A hybrid incompatibility was fine mapped to 1.2 and 4.7 cM intervals on LG4 and LG8.

Discussion

The genome-wide analysis of TRD and HI in interspecific crosses may shed light on postzygotic reproductive barriers, i.e. barriers after formation of an F1 hybrid. Domesticated lettuce is closely related and cross-fertile to its primary gene pool species (*L. serriola* and *L. aculeata* amongst others) and far-related and less crossable to species of the secondary gene pool (*L. saligna* and *L. virosa*). Among the *Lactuca* species, we have no estimations for the divergence time between the primary gene pool of lettuce and the secondary gene pool species *L. saligna* based on genomic comparisons. However, the divergence between *L. saligna* and *L. sativa* is clearly demonstrated by the observation that they show nearly complete reproductive isolation. From a fundamental point of view, we were interested in the amount, mechanism and genetic basis of reproductive barriers in this wide interspecific cross. From a practical perspective, knowledge on HI loci that prevent introgression of wild parent DNA into the cultivated parent may be useful for breeding.

Types of observed postzygotic reproductive isolation

F1s from the cross between *L. saligna* CGN05271 and *L. sativa* cv Olof were as vital as the parents, but showed severely reduced hybrid fertility with only 2% of the normal seed set of both species. F1 seed set of other wide interspecific crosses is generally also low. Seed set by selfing on diploid *Arabidopsis thaliana* x *A. arenosa* hybrids was only 0 to at most 30% (Burkart-Waco *et al.* 2012). In our F2, around 40% of the plants suffered from hybrid inviability or weakness but all F2 plants suffered from severely reduced fertility. Most F2 individuals were even less fertile than the F1. Hybrid inviability and hybrid weakness were only observed in the F2, not in the F1, and indicate hybrid breakdown.

Identification of TRDL

Marker TRD in genetic mapping populations is regularly ascribed to selection against heterospecific allelic combinations that cause hybrid incompatibility. Accordingly, genomic regions of TRD could be predictive for locations of genes underlying HI. To directly test this hypothesis, we compared the genomic location of TRDL in an F2 population with location of regions associated with HI in a set of backcross inbred lines (BILs).

Genome-wide TRD analysis in the F2 resulted in ten loci with severe TRD, in most cases with an almost complete lack of one homozygous genotype. Eight of the ten TRDL showed

a preference for *L. sativa* alleles, which was not significantly different from an equal distribution of wild and cultivated allele preferences over TRDL (8:2 is not significantly different from 5:5). In recombinant inbred lines from the narrow cross, *L. sativa* x *L. serriola,* five regions were distorted ($p<0.01$) (Truco *et al.* 2013). The number of TRDL was higher in our wide than in Truco *et al*'s narrow cross. This suggests that the number of TRDL reflects the genetic distance between populations. Two regions with a *L. sativa* allele preference in the narrow cross overlap with the *L. sativa* allele preferences observed in our *L. saligna* x *L. sativa* F2 on LG3 and the middle of LG7. Possibly, heterospecific digenic interactions with wild parent alleles on LG3 and LG7 are highly incompatible or a *L. sativa* allele gets transmission advantage by a selfish genetic element. The genes underlying these two TRDL may be involved in processes specific to *L. sativa*. Furthermore, these two TRDL colocate with distant expression QTL (eQTL) hot spots that were identified in a transcriptomics study on 200 *L. sativa* accessions (Zhang *et al.* 2018). The locus on LG3 even corresponds with the most significant distant eQTL hotspot containing 58 distant eQTLs. This indicates that the genes at these eQTL loci are highly interactive with genes at other loci and potentially vulnerable for hybrid incompatibilities.

The number of observed TRDL in the bidirectional BC1 populations was in both cases about half of the number observed in the F2. The more introgressed regions from one species that are present in the other species' genetic background, the more deleterious interactions that may occur (Moyle and Graham 2006).

Genome-wide studies of TRD in intra and interspecific crosses of other organisms show variation in numbers of TRDL that can give a first indication of potential candidate HI regions (Gadau *et al.* 1999; Fishman *et al.* 2001; Harushima *et al.* 2001; Myburg *et al.* 2004; Hall and Willis 2005; Nakazato *et al.* 2007; Wu *et al.* 2010; Leppälä *et al.* 2013; Reflinur *et al.* 2014). However, comparison of numbers of TRDL between studies seems inappropriate/meaningless, as different detection criteria and a diverse width of crosses were used.

Non-distortion of heterozygote frequency at a TRDL is an indication for a gametophytic barrier. In our F2 population, half of the TRDL may be due to single gametophytic barriers and the other half may be due to zygotic reproductive barriers. In rice and *Arabidopsis*, gametophytic incompatibilities appear more common than zygotic incompatibilities (Koide *et al.* 2008; Leppälä *et al.* 2013; Ouyang and Zhang 2013). Some indications for zygotic barriers may actually be indications of double gametophytic barriers, in which both male and female allele transmission are distorted which also leads to a distortion of heterozygote frequency. Reciprocal backcross experiments can validate whether the TRD is due to reduced fitness of zygotes, of male gametophytic or of female gametophytic barriers (Harushima *et al.* 2001).

Hybrid incompatibilities

TRD analysis in the F2 provided a first indication of potential hybrid incompatibilities. Eight out of ten TRDL in F2 showed distortion towards the cultivated allele, which may limit the introgression of wild alleles in these regions in a cultivated parent background. The top of LG7 with a TRD towards the cultivated allele is a candidate HI, as a *L. saligna* introgression was lost in an early backcross generation during the introgression process towards development of a set of BILs. Lack of backcross plants with wild parent alleles at this locus prevented further in-depth studies on this region, but are needed to ascertain a true HI.

While developing the set of BILs, we observed that some introgression regions segregated abnormally, showing complete absence of the homozygous wild parent genotype class, indicating absolute HI. Such genomic regions were only represented by wild parent alleles in a heterozygous state. The BILs showed that four out of seven of the TRDL with cultivated allele preference (identified in the F2) were related to an absolute HI. One additional HI region, on LG8, was detected in the BILs that did not show TRD in the F2 (or BC1). The two other TRDL with cultivated parent allele preference were not associated with absolute HI, as we did obtain BILs with these homozygous wild regions in cultivated background. Possibly these two TRDL may be due to incomplete non-transmission of a heterospecific genotype. In the F2, TRDL associated with absolute HI showed Chi^2 values of 13, 29, 40 and 89, whereas TRDL not associated with absolute HI showed Chi^2 values of 15, 27 and 38 (Table 1). Therefore, the degree of segregation distortion in the F2 was not predictive for the degree of non-transmission of heterospecific allele combinations.

Summarized, about half of the TRDL with cultivated allele preference were predictive for a HI that prevented introgression of wild alleles in cultivated background. Knowledge on TRDL in F2 that are not associated with absolute HI (fitness of zero), but with a strong selection against certain alleles or allele combinations (reduced fitness) will also be useful for both research and breeding. Anticipation of TRD can prevent loss of certain desired introgressions by adjustment of population size.

Dance partners

Intrinsic postzygotic reproductive barriers in diploids are generally thought to be caused by two mechanisms (Myburg *et al.* 2004): 1) large chromosomal rearrangements between parental species may result in abnormal meiotic products and reduced fertility in hybrids 2) deleterious interactions between heterospecific genes may cause hybrid sterility and inviability. There are no indications of large chromosomal rearrangements between *L. saligna* and *L. sativa*, as our interspecific F2 linkage map was collinear with the *L. sativa* de novo genome (Reyes-Chin-Wo *et al.* 2017). Therefore, we expected interaction between heterospecific genes to lead to HI between wild and cultivated lettuce.

For the five HI regions that were not obtained as single homozygous wild introgressions in cultivated background, few plants with homozygous wild introgressions were present in the F2, probably due to the presence of other conspecific regions present in these plants. HI might be nullified when two conspecific genetic components or genetic "dance partners'' are present (Moyle and Graham 2006). We identified candidate dance partner regions in the F2. Crosses between BILs that each carry an appropriate genetic dance partner required for a fully functional interaction, proved three digenic HI interactions. We presume the other two regions carrying genes causing HI to be due to a digenic interaction as well, but more candidate dance partners should be tested to verify this.

In conclusion, TRD in segregating populations can give a first indication of potential candidate HI regions. Our study showed that BILs are ideal for validation and fine mapping of genes causing HI, as they exclude effects of other loci. Furthermore, crosses between BILs can provide the ultimate evidence of a rescued digenic HI, if the presence of two conspecific genetic dance partners nullifies TRD.

Sex independent non-transmission of heterospecific 4c8w gametophytes

We genetically analysed the striking observation that homozygous introgression segments 8A and 4A, for which TRDLs with opposite strong allele preferences were observed in the F2, appear together in a BIL. Inbreds from the double heterozygote (cross product of

BIL4A+8A x *L. sativa*) showed deviant segregation with absence of three genotypes out of the nine expected genotypes. The observed segregation fitted the hypothesis that heterospecific 4c8w male and female (sex-independent) gametophytes were not transmitted. This explanation was further validated by segregation analysis in reciprocal backcrosses. Functional proof was given from the female side by seed set analysis. Double heterozygotes (4cw8cw) showed 25% aborted seeds which was expected if one out of four female gametophytes is non-transmitted. Male 4c8w gametophytes seemed compensated by other pollen genotypes that fertilize the egg cells. The HI loci were fine mapped to intervals of 1.2 and 4.7 cM. F1 plants of *L.saligna* x *L. sativa* showed 98% reduced seed set compared to the parental lines. A quarter of this reduced seed set in the F1 may be explained by the HI caused by a digenic interaction between genes on segments 4A and 8A. Future analysis of seed set in BILs with heterozygous introgression segments may reveal HI loci that cause the remaining percentage of reduced seed set.

Most reported segregation distortion systems act in the male gametes only (Taylor and Ingvarsson 2003). Sex-independent TRD due to preferential allele transmission in the heterozygote through both sexes appears to be much less common (Koide *et al.* 2008). Still, several of the mutations that affect female gametogenesis in *Arabidopsis* also affect male gametogenesis (Drews *et al.* 1998; Drews and Yadegari 2002; Wang *et al.* 2012). Apparently, some processes of gametogenesis are identical in gametophytes of both sexes (Christensen *et al.* 1998; Ding *et al.* 2012) and disruption of such a similar process may explain our case of sex-independent gametophytic HI.

An explanatory model for the digenic HI between cultivated and wild lettuce alleles on LG4 and LG8 might be an internal genetic conflict in one of the two species. Selfish genes may negatively affect their own species, which can lead to the evolution of suppressor genes (Maheshwari and Barbash 2011). If the selfish gene is uncoupled from its suppressor in certain individuals by the selfing of the hybrid, HI can arise. For *L. sativa* a selfish allele on LG4 that is suppressed by an allele on LG8 could explain the HI, or for *L. saligna* a selfish allele on LG8 that is suppressed by an allele on LG4. The observed digenic HI also fits the duplicate gametophytic lethal model proposed by Oka (1957), in which genetic incompatibility is caused by reciprocal loss of duplicated genes. The HI genes on LG4 and LG8 may be ancient duplicates, after which the gene on LG8 lost its functionality in *L. saligna* and the gene on LG4 lost functionality in *L. sativa.* This would explain the non-transmission of 4c8w gametophytes, as both genes in this gametophyte would be non-functional. Lettuce has undergone an ancient whole-genome triplication (Reyes-Chin-Wo *et al.* 2017), which could make this type of incompatibility plausible. However, the regions on 4A and 8A that are involved in HI have not been found to be each other's syntelogs (Reyes-Chin-Wo *et al.* 2017). Three cases of the gametophytic lethal model have been demonstrated molecularly for male gametophytes in rice (Mizuta *et al.* 2010; Yamagata *et al.* 2010; Nguyen *et al.* 2017). Until now, only one case of gametophytic hybrid lethality that acts in both male and female gametophytes has been reported, viz. in *Mimulus* (Kerwin and Sweigart 2017). However, in that case the heterospecific gametophytes were only undertransmitted and this was not the sole explanation of the observed TRD. Our case of non-transmission of a heterospecific gametophyte in both males and females completely explains the TRD and may be the first identified two-locus sex-independent gametophytic HI.

Our data on TRD and HI provide new insight into postzygotic reproductive barriers of *Lactuca* species. We identified ten loci with severe to extreme TRD in an F2 of the wide

cross between *L. saligna* and *L. sativa*. Through the combination of this F2 genetic data of with BIL genetic data, we identified three two-locus allelic interactions resulting in absolute HI. Through in-detail analysis of one specific digenic HI we found proof for non-transmission of one heterospecific allele combination in both male and female gametophytes. This HI may be due to ancient gene duplication and subsequent reciprocal loss of function or internal genetic conflict that is suppressed in one species but shows up (again) in hybrids. To our knowledge, such a sex-independent two-locus gametophytic HI has not been described earlier. Functional analysis of the other cases of HI could further expand our knowledge of the mechanisms of divergence between species. Here, we have laid the foundation for experiments that can reveal the identity of these HI genes and ultimately the selective forces acting upon them.

Acknowledgements

This work was part of the research programme 'Open Technology' with project number 12683, which is (partly) financed by the Netherlands Organization for Scientific Research (NWO). We thank M.J.M. Smulders for critically reading the manuscript.

Supplemental Figures

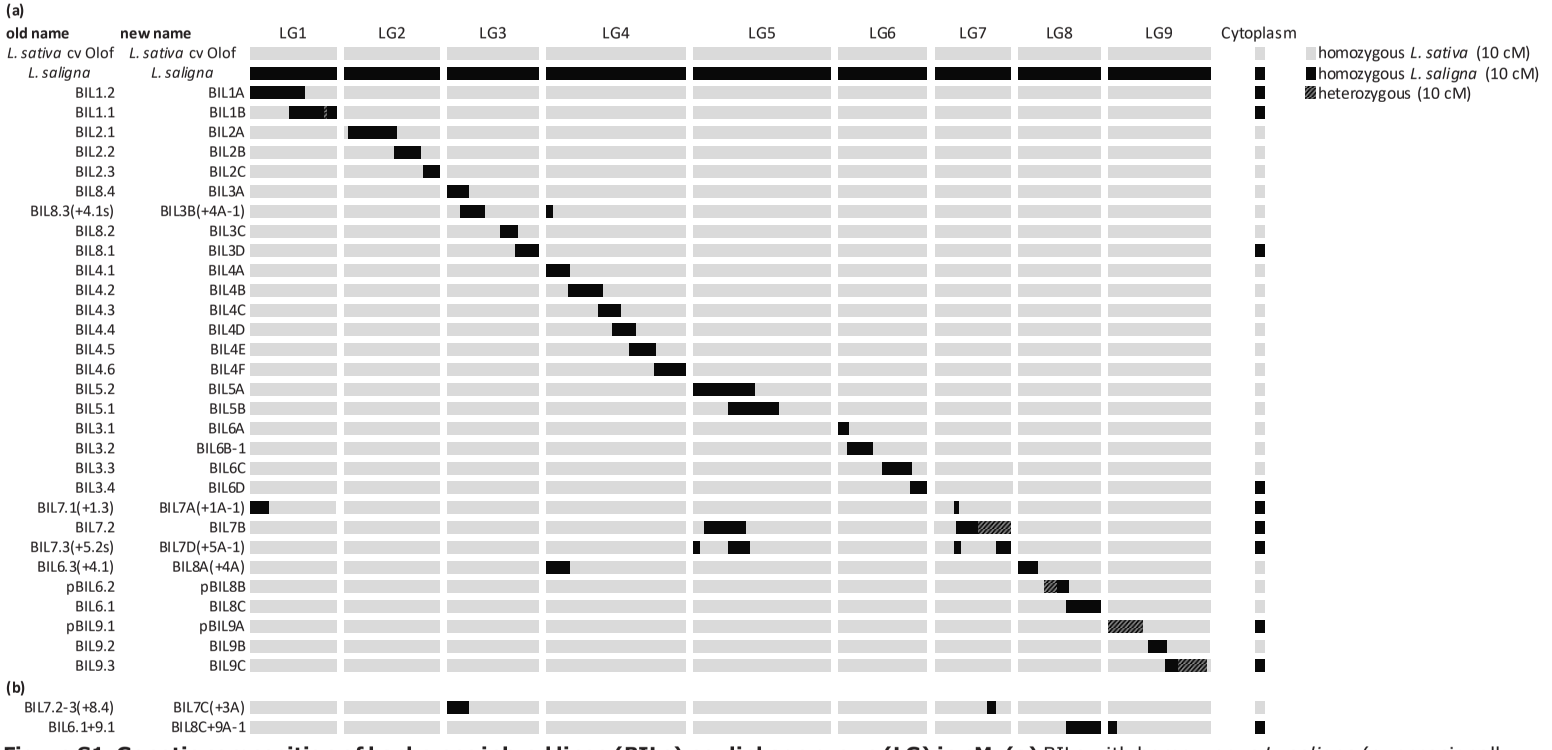


Figure S1. Genetic composition of backcross inbred lines (BILs) per linkage group (LG) in cM. (a) BILs with homozygous *L. saligna* (or occasionally heterozygous) introgressions in *L. sativa* background. **(b)** BILs with homozygous *L. saligna* introgression retrieved only after combination with the conspecific (dance partner) locus.

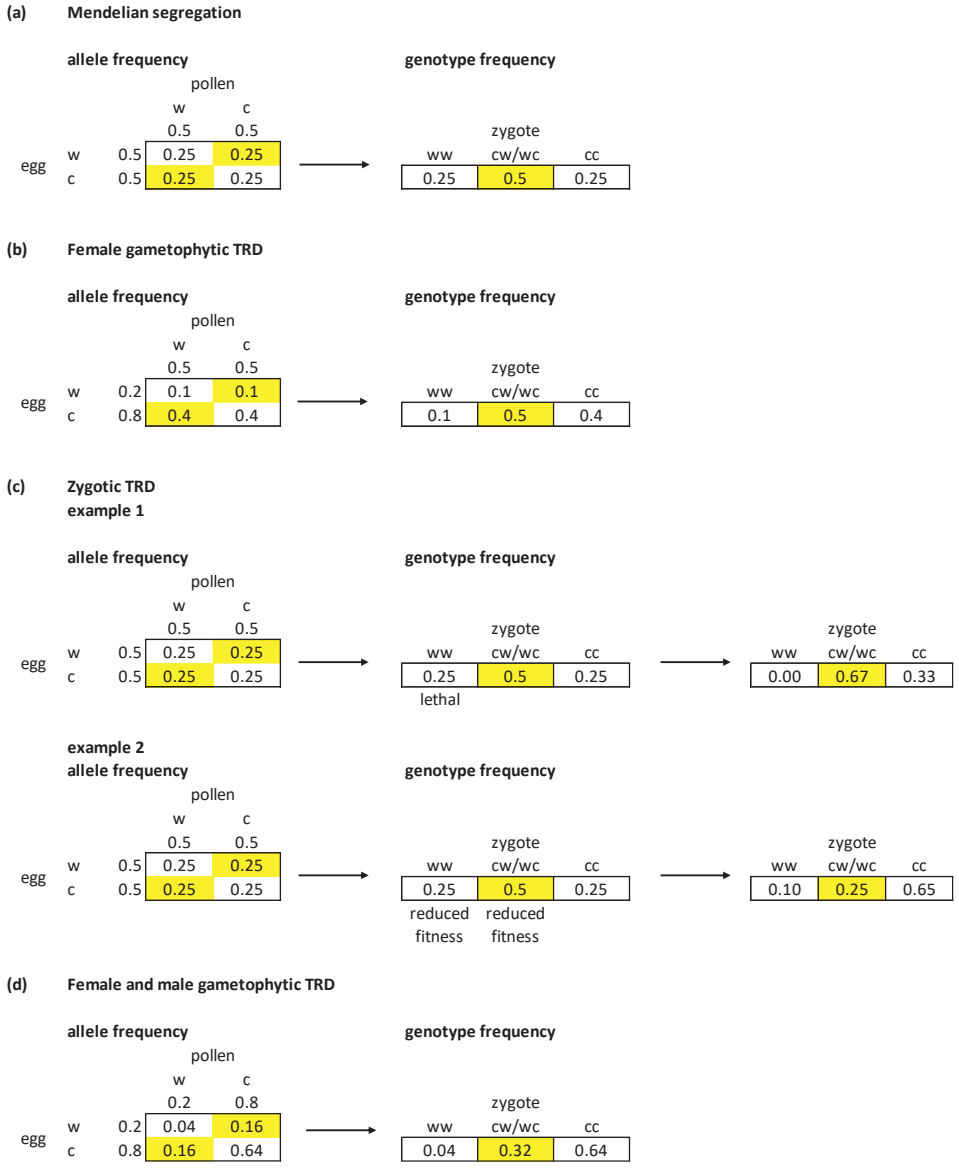


Figure S2. Examples of the effect of gametophytic and zygotic transmission ratio distortion (TRD) on the allele frequency of gametes and the genotype frequency in zygotes. (a) Expected allele and genotype frequency under Mendelian segregation. **(b)** Theoretical example of a female gametophytic TRD with preference for *L. sativa* alleles. The frequency of the heterozygous genotype is not distorted; only the frequencies of the homozygous genotypes are distorted. **(c)** Theoretical examples of a zygotic TRD. Example 1: lethality of the homozygous *L. saligna* (ww) genotype, resulting in distorted segregation of all genotypes. Example 2: reduced fitness of the homozygous *L. saligna* (ww) genotype and the heterozygous (cw/wc) genotype, resulting in distorted segregation of all genotypes. **(d)** Theoretical example of a female and male gametophytic TRD with equal preference for *L. sativa* alleles, resulting in distorted segregation of all genotypes. w: *L. saligna* allele, c: *L. sativa* allele, yellow: heterozygous genotype.

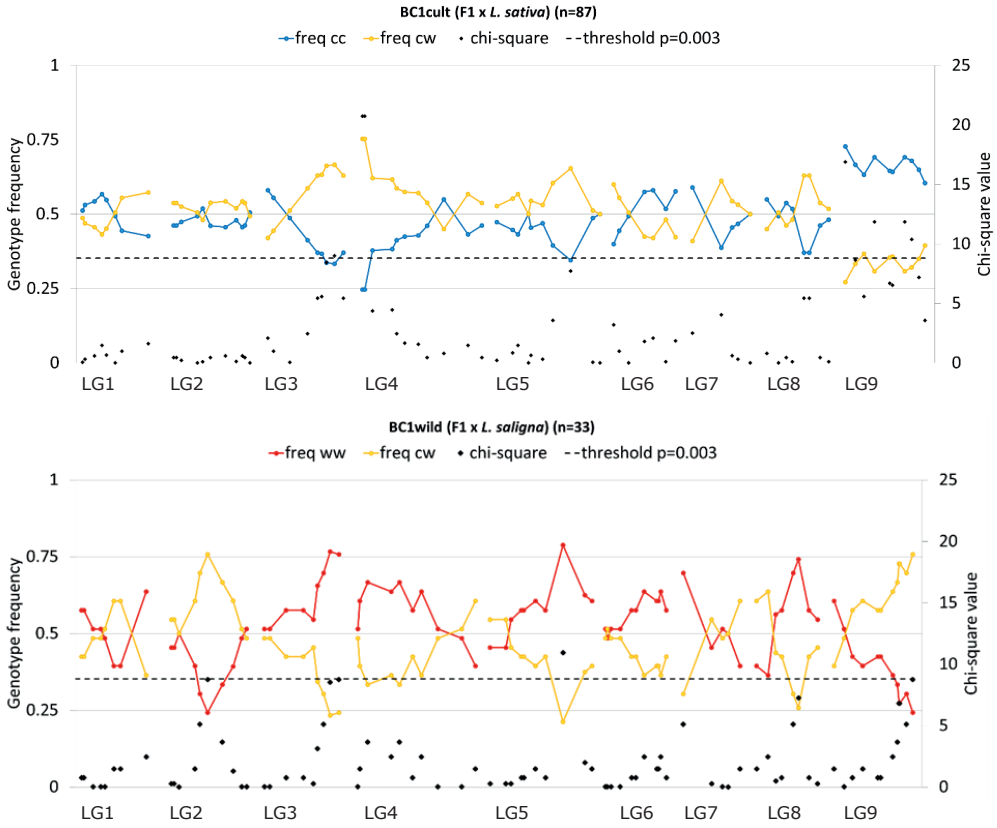


Figure S3. Genotype frequency and chi-square value per marker in two backcross populations of *L. saligna* CGN05271 x *L. sativa* cv Olof. (a) BC1cult: F1 backcrossed to *L. sativa* **(b)** BC1wild: F1 backcrossed to *L. saligna*. Black dots above the black dashed line indicate deviation from Mendelian segregation (p<0.003). X-axis: nine linkage groups in cM separated by white space. freq = frequency. Genetic nomenclature: cc= homozygous *L. sativa* (blue), cw= heterozygous (yellow), ww= homozygous *L. saligna* (red).

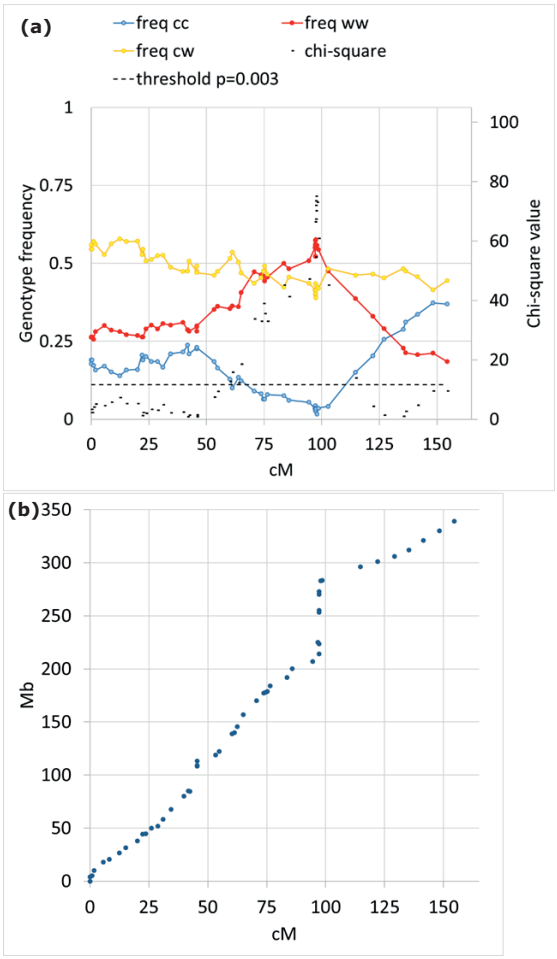


Figure S4. Transmission ratio distortion (TRD) and recombination on LG5. (a) Frequency of *L. saligna* (red), heterozygous (yellow), *L. sativa* (blue) genotypes and chi-square value per marker in an F2 population of *L. saligna* CGN05271 x *L. sativa* cv Olof. Black dots above the black dashed line indicate deviation from Mendelian segregation ($p<0.003$) **(b)** An Mb versus cM plot indicates a lack of recombination around 98 cM, the same region that showed the largest TRD.

Observed segregation from progeny that segregates only for loci on LG4 (4A segment) and/or LG8 (8A segment).

(a) inbred progeny of genotype 4cw8cw

	LG8					
LG4	**cc**	**cw/wc**	**ww**	sum	geno freq	allele freq
cc	75	0	0	75	0.12	c=0.32
cw/wc	126	130	0	256	0.40	
ww	80	151	73	304	0.48	w=0.68
sum	281	281	73	**635**		
geno freq	0.44	0.44	0.11			
allele freq	c=0.66		w= 0.34			

(b) inbred progeny of genotype 4ww8cw Chi^2= 0.02, p=0.99, concl: Mendelian segregation

	LG8					
LG4	**cc**	**cw/wc**	**ww**	sum	geno freq	allele freq
cc	n/a	n/a	n/a	0	0.00	c=0.0
cw/wc	n/a	n/a	n/a	0	0.00	
ww	137	271	137	545	1.00	w=1.0
sum	137	271	137	**545**		
geno freq	0.25	0.50	0.25			
allele freq	c=0.50		w=0.50			

(c) inbred progeny of genotype 4cw8cc Chi^2=8.2 ,p=0.02, concl: distorted segregation

	LG8					
LG4	**cc**	**cw/wc**	**ww**	sum	geno freq	allele freq
cc	41	n/a	n/a	41	0.35	c=0.59
cw/wc	58	n/a	n/a	58	0.49	
ww	19	n/a	n/a	19	0.16	w=0.41
sum	118	0	0	**118**		
geno freq	1.00	0.00	0.00			
allele freq	c=1.0		w=0.0			

Expected segregation ratios according to hypotheses

(d) H1: Chi^2= 395, p=0, rejected

	LG8					
LG4	**cc**	**cw/wc**	**ww**	sum	geno freq	allele freq
cc	40	79	40	159	0.25	c=0.50
cw/wc	79	159	79	317	0.50	
ww	40	79	40	159	0.25	w=0.50
sum	159	317	159	**635**		
geno freq	0.25	0.50	0.25			
allele freq	c=0.50		w=0.50			

(e) H2: Chi^2= 4.6, p=0.8, not rejected

	LG8					
LG4	**cc**	**cw/wc**	**ww**	sum	geno freq	allele freq
cc	71	0	0	71	0.11	c=0.33
cw/wc	141	141	0	282	0.44	
ww	71	141	71	282	0.44	w=0.67
sum	282	282	71	**635**		
geno freq	0.44	0.44	0.11			
allele freq	c=0.67		w=0.33			

(f) H3: Chi^2= 74, p=0, rejected

	LG8					
LG4	**cc**	**cw/wc**	**ww**	sum	geno freq	allele freq
cc	58	0	0	58	0.09	a=0.36
cw/wc	115	231	0	346	0.55	
ww	58	115	58	231	0.36	b=0.64
sum	231	346	58	**635**		
geno freq	0.36	0.55	0.09			
allele freq	a=0.64		b=0.36			

(g) H4: Chi^2= 165, p=0, rejected

	LG8					
LG4	**cc**	**cw/wc**	**ww**	sum	geno freq	allele freq
cc	53	53	0	106	0.17	c=0.42
cw/wc	106	159	53	318	0.50	
ww	53	106	53	212	0.33	w=0.58
sum	212	318	106	**635**		
geno freq	0.33	0.50	0.17			
allele freq	c=0.58		w=0.42			

(h) H5: Chi^2= 31, p=0.0001, rejected

	LG8					
LG4	**cc**	**cw/wc**	**ww**	sum	geno freq	allele freq
cc	64	0	0	64	0.10	c=0.35
cw/wc	127	191	0	318	0.50	
ww	64	127	64	254	0.40	w=0.65
sum	254	318	64	**635**		
geno freq	0.40	0.50	0.10			
allele freq	c=0.65		w=0.35			

(i) H6: Chi^2=193, p=0, rejected

	LG8					
LG4	**cc**	**cw/wc**	**ww**	sum	geno freq	allele freq
cc	33	33	9	75	0.12	c=0.32
cw/wc	113	113	29	256	0.40	
ww	134	135	35	304	0.48	w=0.68
sum	281	281	73	**635**		
geno freq:	0.44	0.44	0.11			
allele freq	c=0.66		w= 0.34			

(j) Progeny cross (4cw8cw) x (4ww8cc)
Informative for female gamete transmission

female	male 4w8c	Obs abs	Obs freq	H1 Exp abs	H2 Exp abs
4c8c	4cw8cc	31	0.26	29	39
4c8w	4cw8wc[a]	2	0.02	29	0
4w8c	4ww8cc	47	0.40	29	39
4w8w	4ww8wc	37	0.32	29	39
	total	117		116	117
			Chi^2	39	3.4
			p-value	0	0.34
				rejected	not rejected

(k) Progeny reciproke cross (4ww8cc) x (4cw8cw)
Informative for male gamete transmission

male	female 4w8c	Obs abs	Obs freq	H1 Exp abs	H2 Exp abs
4c8c	4cw8cc	46	0.33	26	39
4c8w	4cw8wc	0	0.00	26	0
4w8c	4ww8cc	32	0.23	26	39
4w8w	4ww8wc[b]	62[b]	0.44[b]	n/a	n/a
	total	140		78	78
			Chi^2	43	2.5
			p-value	0	0.28
				rejected	not rejected

Figure S5 (previous page). Hypothesis testing for transmission ratio distortion (TRD) of inbred progeny (n=635) from double heterozygote 4cw8cw (segregating for loci on LG4 (4A segment) and LG8 (8A segment)).
Genetic nomenclature: c= *L. sativa* allele, w= *L. saligna* allele; cc= homozygous *L. sativa* genotype, cw/wc= heterozygous genotype, ww= homozygous *L. saligna* genotype. Genotype plant numbers are colour-shaded from low (red) to high (green) numbers. geno freq = genotype frequency, allele freq = allele frequency, concl = conclusion, n/a = not applicable.
Observed segregation of inbred progeny segregating for LG4 and/or LG8:
(a) Inbred progeny of double heterozygote 4cw8cw.
(b) Inbred progeny of 4ww8cw (segregating only for LG8). The segregation ratio is compared with Mendelian segregation (1:2:1) in a chi-square (Chi^2) test.
(c) Inbred progeny of 4cw8cc (segregating only for LG4). The segregation ratio is compared with Mendelian segregation (1:2:1) in a chi-square test.
Expected segregation ratios based on six hypotheses for TRD were compared with the observed segregation presented in panel (a) by chi-square tests.
(d) H1 = Hypothesis 1: Mendelian 1:2:1 segregation (both loci allele frequencies of 0.5).
(e) H2 = Hypothesis 2: non-tranmission of male and female gametophytes with genotype 4c8w.
(f) H3 = Hypothesis 3: lethality of genotypes 4cc8cw, 4cc8ww and 4cw8ww.
(g) H4 = Hypothesis 4: non-transmission of male or female gametophytes with genotype 4c8w.
(h) H5 = Hypothesis 5: Hypothesis 3 and 4 combined.
(i) H6 = Hypothesis 6: Mendelian 1:2:1 segregation with the observed allele frequencies from panel (a).
(j,k) To validate the accepted H2 (non-transmission of male and female 4c8w gametophytes) per gametophyte type, we analysed segregation ration in backcross populations of the reciprocal cross 4cw8cw x 4ww8cc (and also tested it for H1, Mendelian segregation 1:1:1:1). The presumed non-transmitted gametophyte is bold and underlined. obs = observed, exp = expected
[a] in **(j)** two plants with 4cw8wc genotype were observed, that are probably due to a selfing of the mother plant instead of a backcross. Four plants with 4cc8cc genoype were detected as well, which can only be derived from a selfing of the mother plant. The other three observed genotypes might also contain some selfings besides backcross plants.
[b] in **(k)** the plant number of the 4ww8cc genotype is much higher than expected for both hypotheses. This observation can be explained by the fact that 4ww8cc is the only genotype in which the number of backcross plants can be overestimated due to occurrence of selfing of the mother plant. Because the actual number of backcross plants cannot be distinguished from plants generated by selfing, this genotype class is excluded from the chi-square test.

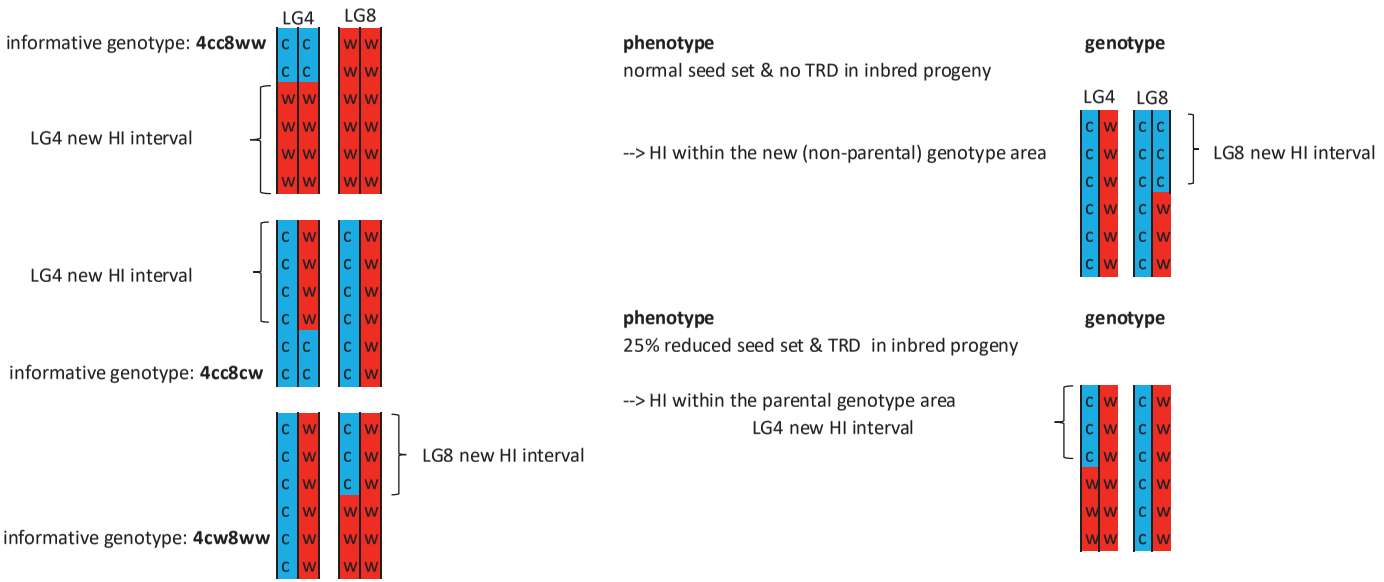
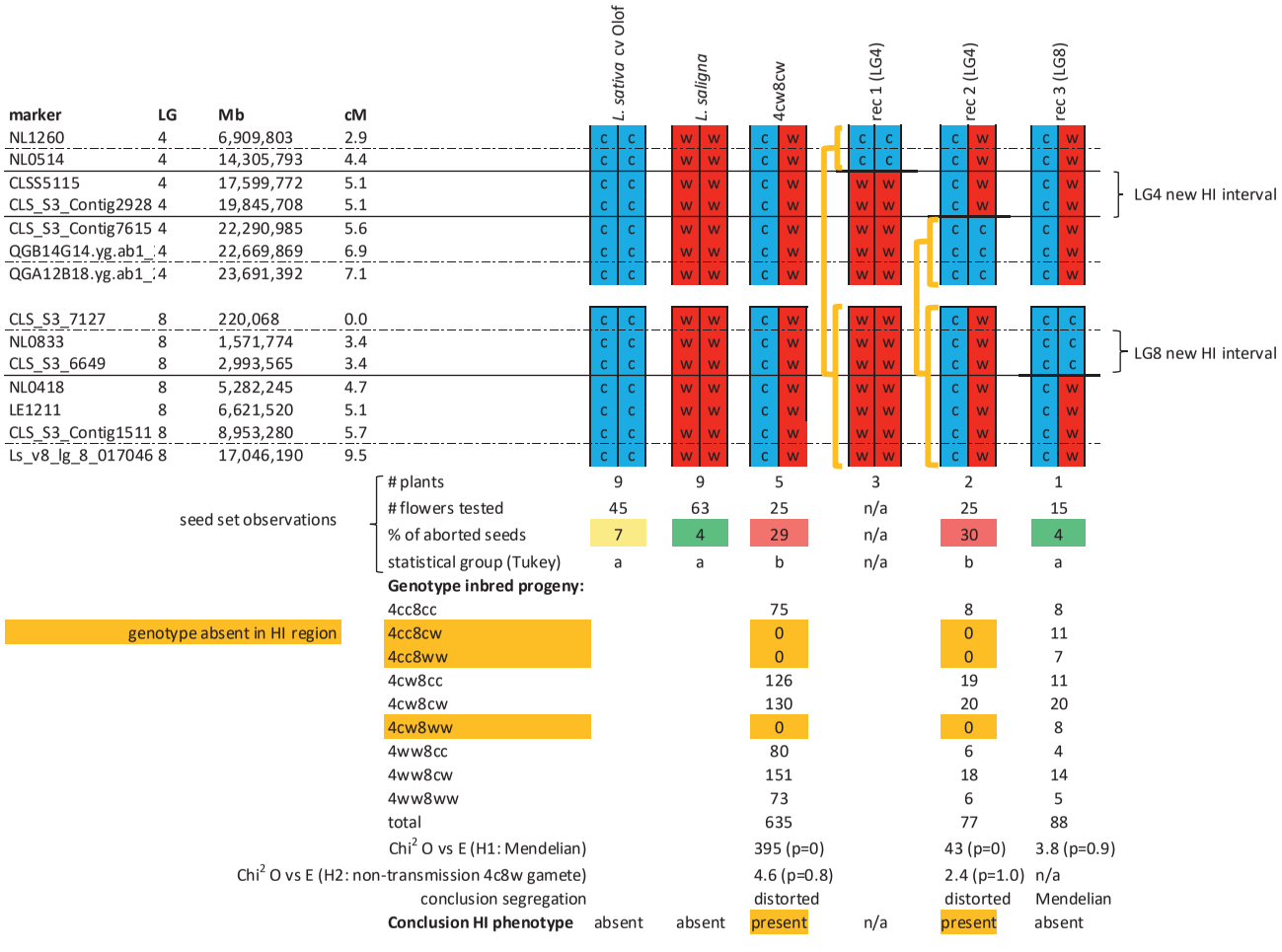


Figure S6. HI mapping approaches. Inbred progeny (1st and 2nd generation) of double heterozygotes (genotype 4cw8cw) were genotyped to find recombinants within the HI interval. Genetic nomenclature: c= *L. sativa* allele, w= *L. saligna* allele; cc= homozygous *L. sativa* genotype, cw/wc= heterozygous genotype, ww= homozygous *L. saligna* genotype. Numbers (4 and 8) indicate the original HI regions on linkage group (LG) 4 and LG8. Recombinants within the original HI map interval can reduce the map interval: **(a)** directly by genotype. Three genotypes are not formed due to HI: 4cc8ww, 4cc8cw, 4cw8ww. Presence of one of these three genotypes indicates that the HI locus is outside of this region. **(b)** after determination of presence/absence of HI through seed set and/or transmission ratio distortion (TRD) analysis in inbred offspring. 25% reduced seed set & TRD with absence of 4cc8ww, 4cc8cw, 4cw8ww genotypes indicate presence of HI. normal seed set & no TRD indicate absence of HI. note: segregation in inbred progeny is based on genetic markers within the double heterozygous genotype region (4cw8cw).



marker	LG	Mb	cM
NL1260	4	6,909,803	2.9
NL0514	4	14,305,793	4.4
CLSS5115	4	17,599,772	5.1
CLS_S3_Contig2928	4	19,845,708	5.1
CLS_S3_Contig7615	4	22,290,985	5.6
QGB14G14.yg.ab1_	4	22,669,869	6.9
QGA12B18.yg.ab1_	4	23,691,392	7.1
CLS_S3_7127	8	220,068	0.0
NL0833	8	1,571,774	3.4
CLS_S3_6649	8	2,993,565	3.4
NL0418	8	5,282,245	4.7
LE1211	8	6,621,520	5.1
CLS_S3_Contig1511	8	8,953,280	5.7
Ls_v8_lg_8_017046	8	17,046,190	9.5

seed set observations	# plants	9	9	5	3	2	1
	# flowers tested	45	63	25	n/a	25	15
	% of aborted seeds	7	4	29	n/a	30	4
	statistical group (Tukey)	a	a	b	n/a	b	a
	Genotype inbred progeny:						
	4cc8cc			75		8	8
genotype absent in HI region	4cc8cw			0		0	11
genotype absent in HI region	4cc8ww			0		0	7
	4cw8cc			126		19	11
	4cw8cw			130		20	20
genotype absent in HI region	4cw8ww			0		0	8
	4ww8cc			80		6	4
	4ww8cw			151		18	14
	4ww8ww			73		6	5
	total			635		77	88
	Chi^2 O vs E (H1: Mendelian)			395 (p=0)		43 (p=0)	3.8 (p=0.9)
	Chi^2 O vs E (H2: non-transmission 4c8w gamete)			4.6 (p=0.8)		2.4 (p=1.0)	n/a
	conclusion segregation			distorted		distorted	Mendelian
	Conclusion HI phenotype	absent	absent	present	n/a	present	absent

Figure S7 (previous page). New HI map interval (LG4: 4.4-5.6 cM, LG8: 0.0-4.7 cM) based on the three most informative recombinants. Dashed lines: HI interval identified from recombinants in the F2 population (*L. saligna* CGN05271 *x L. sativa* cv Olof*).* Black lines: new HI interval (thick black lines indicate informative cross-overs). Genetic nomenclature: c= *L. sativa* allele, w= *L. saligna* allele; cc= homozygous *L. sativa* genotype, cw/wc= heterozygous genotype, ww= homozygous *L. saligna* genotype. Prefix numbers (4 and 8) indicate the linkage group (LG)4 and LG8.
Recombinants (rec) that reduced the map interval on LG4 directly by genotype: rec1 by 4cc8ww genotype and rec2 by 4cc8cw genotype (indicated by orange lines). Recombinant 3 reduced the map interval on LG8 by the combination of genotype and absence of HI phenotype (no reduced seed set, no TRD). Percentages of aborted seeds are colour-shaded from low (green) to high (red) numbers to facilitate interpretation. O: observed, E: expected, Chi^2: chi-square test value, n/a: not applicable, orange shading: genotypes absent in the HI region. note: segregation in inbred progeny is based on genetic markers within the double heterozygous genotype region (4cw8cw).

Supplemental Tables

Table S1. Contigs and primer sequences of EST-based markers

Available online:
https://doi.org/10.18174/430413

Table S2. SNPs and their surrounding sequence used for design of KASPar markers (marker design by Dr. van Haeringen Laboratorium B.V. (VHL), Wageningen, the Netherlands)

Available online:
https://doi.org/10.18174/430413

Table S3. Seed weight, total seed number, and number of capitula for *Lactuca saligna*, F1 (*L. saligna* x *L. sativa*) and *Lactuca sativa*. Total seed number is calculated by the formula: seed weight of all seeds/seed weight of 100 seeds * 100. Number (#) of capitula/plant is calculated by the formula: average total seed number/(average # seeds/capitula).
* Bonferroni test, SD: Standard Deviation, n/a: not available.

		All seeds	100 seeds				Total seed number				# Seeds/capitulum	# Capitula/plant
Genotype	Plant nr	Seed weight (g)	Seed weight (g)	Average	SD	Statistical group*	Calculated	Average	SD	Statistical group*	Average	Calculated
L. saligna	1	7.1	0.056	0.057	0.003	a	12703	11900	706	a	11.84	1005
L. saligna	2	6.6	0.055				12015					
L. saligna	3	6.7	0.061				10984					
F1	1	0.2	0.125	0.125	0.015	b	174	190	21	b	n/a	n/a
F1	2	0.3	0.144				220					
F1	3	0.2	0.107				176					
L. sativa	1	8.3	0.103	0.105	0.007	b	8035	8475	378	c	12.52	677
L. sativa	2	8.3	0.098				8432					
L. sativa	3	10.2	0.114				8958					

Table S4. Number of plants with phenotypes indicating hybrid incompatibility, observed in the F2 from the cross *L. saligna* CGN05271 x *L. sativa* cv Olof.

		hybrid necrosis	malformation	no seed set or died before flowering	severely reduced seedset	less severely reduced seed set
Total F2 seeds	162					
no germination or died early	36					
adult plants	126	14	13	46	69	11

Table S5. Overview and characteristics of transmission ratio distortion loci (TRDL) identified with p<0.003 in the F2, BC1cult, BC1wild and backcross inbred lines (BILs) of *L. saligna* segments in *L. sativa* background.

Available online:
https://doi.org/10.18174/430413

Table S6. Overview of candidate and validated conspecific segments (dance partners) that can nullify transmission ratio distortion (TRD) of backcross inbred lines (BILs) with *L. saligna* segments in *L. sativa* background.

Available online:
https://doi.org/10.18174/430413

Chapter 5

General Discussion

In this thesis, the nonhost *L. saligna* was studied as a donor for resistance to downy mildew, a devastating disease in lettuce cultivation that can result in high yield losses. Through genetic analyses we identified effector responsiveness genes and major monogenic *R* genes **(chapter 2)**, loci for nonhost resistance (NHR) genes **(chapter 3)** and postzygotic reproductive barriers between *L. saligna* and cultivated lettuce **(chapter 4).** An overview of the genetic position of these traits is presented in Figure 1.

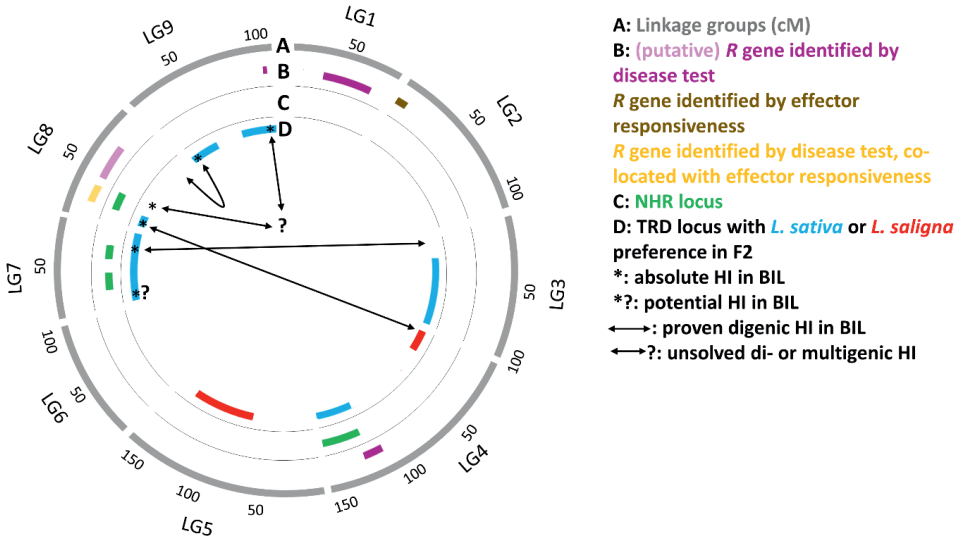


Figure 1. Overview of *Lactuca saligna* traits mapped in this thesis: *R* genes and nonhost resistance (NHR) loci against *Bremia lactucae*, transmission ratio distortion (TRD) loci identified in an F2 from the cross *L. saligna* x *L. sativa* and hybrid incompatibility (HI) loci identified in backcross inbred lines (BILs), each with a single *L. saligna* introgression segment in a *L. sativa* background. For three HI loci we identified the genetic interacting partner resulting in hybrid dysgenesis. One of these digenic cases of HI was described previously (Jeuken et al 2009).

R genes and effectors

Pathogen-associated molecular patterns (PAMPs) are an important trigger of a plant's host defence response, resulting in PAMP-triggered immunity (PTI). Successful pathogens can circumvent PTI through effector proteins that favour host infection. However, host plants may recognize effectors through *R* genes, which leads to effector-triggered immunity (ETI) and is often associated with a hypersensitive response (HR) at the site of infection (Jones and Dangl 2006). While ETI, and PTI to a lesser extent, are effective against adapted pathogens in host plants, preformed defences and PTI typically function against non-adapted pathogens in nonhost plants.

R genes against downy mildew (*Dm* genes) are commonly deployed in lettuce cultivars as they are genetically simple (single genes) and provide high levels of resistance. Even though *R* genes are continually rendered ineffective by the occurrence of pathogenic strains with new virulence characteristics (Lebeda and Zinkernagel 2003; Michelmore and Wong 2008), they can provide effective short-term protection against downy mildew. Most of the *Dm* genes have been derived from the primary gene pool species *L. sativa* and *L. serriola* (Parra *et al.* 2016). *L. saligna*, a nonhost for downy mildew, has hardly been exploited for lettuce breeding. Three *Dm* genes have been found in some *L. saligna* accessions (Parra *et al.* 2016), but the general frequency and distribution of *Dm* genes in this nonhost species is unknown. *L. saligna* may contain *Dm* genes with other

characteristics, for instance in terms of resistance spectrum, genomic locations, or pathogen recognition strategy, than the commonly exploited primary gene pool species. As all *L. saligna* accessions are completely resistant, segregating populations derived from crosses with a host are needed to identify *R* genes by disease testing. Besides disease testing of segregating populations, transient expression of effectors in *L. saligna* itself can be used as a tool for *R* gene discovery (Vleeshouwers *et al.* 2011; Vleeshouwers and Oliver 2014). If the frequency of *R* genes in *L. saligna* is low, effectors as a screening tool to identify *R* genes could be more efficient than disease testing random segregating populations. Effector responsiveness screening could also indicate which *L. saligna* accessions harbour novel *R* genes compared to the primary gene pool.

Previously, 34 candidate effectors were transiently expressed in a *Lactuca* germplasm set (Stassen *et al.* 2013). One effector, BLG01, induced a hypersensitive response (HR) in 90% of the *L. saligna* accessions, but not in primary gene pool species (mainly *L. sativa* and *L. serriola*) and was mapped to LG9. An HR response to effector BLG03 was induced only in *L. sativa* lines containing the resistance gene *Dm2* on LG2. However, both HR responses were not associated with resistance to Bl:24, the *B. lactucae* isolate from which the effectors were cloned.

In **chapter 2**, 16 new candidate effectors were screened on *Lactuca* germplasm. Two effectors, BLR31 and BLN08, induced an HR response in respectively 5% and 55% of the tested *L. saligna* accessions. BLR31 revealed a novel *R* gene providing complete resistance to isolate Bl:24. Consequently, we identified a candidate avirulence effector of *B. lactucae* and its cognate *R* gene in *L. saligna.* BLN08 responsiveness did not cosegregate with resistance to Bl:24. In a follow-up experiment, inbred offspring of backcross plants that displayed BLR31 or BLN08 responsiveness were tested with five *B. lactucae* isolates (data not shown). The results indicated that the cognate *R* gene of BLR31 probably provides race-specific resistance, similar to other *R* genes (Parra *et al.* 2016). Surprisingly, the BLN08 responsiveness locus on LG8 did cosegregate with resistance against other isolates than Bl:24 and therefore also indicates an *R* gene (follow-up experiment, data not shown). An explanation may be that Bl:24 secretes another effector that prevents ETI triggered by BLN08, whereas other isolates lack this additional effector(King *et al.* 2014; Teper *et al.* 2014).

In total **(chapter 2)**, we studied segregating populations of five *L. saligna* accessions crossed with *L. sativa* and mapped five *R* genes, of which one was putative (Fig. 1). For the putative *R* gene we had less evidence due to a low number of susceptible individuals. One *R* gene was identified by transient expression of a candidate effector. Another *R* gene was initially identified by effector responsiveness, but only cosegregated with resistance to other *B. lactucae* isolates than Bl:24, from which the effectors were cloned. The other three *R* genes, of which one putative, were accidentally identified by disease testing the segregating populations. All five *R* genes co-located with major resistance clusters in *L. sativa*: MRC1, MRC2, MRC4 and MRC8A-B (Christopoulou *et al.* 2015). Furthermore, in the genetic analysis for nonhost resistance described in **chapter 3**, we mapped an *R* gene in *L. saligna* CGN15705 on the bottom of LG9, co-locating with MRC9C. This is currently a new location for an *R* gene against *B. lactucae* and a patent application has been filed (patent WO2015136085).

Based on our results, the *R* gene frequency in *L. saligna* is not low. Identification of *R* genes by *Lactuca* germplasm pre-screening with effectors or by disease testing random interspecific segregating populations both require significant investments of time and effort. Candidate effectors need to be identified, cloned and transiently expressed, whereas the *L. saligna* x *L. sativa* crosses have a low success rate and severely reduced F1 fertility. Both approaches have different disadvantages: effector responsiveness may

not cosegregate with disease resistance, whereas a random *L. saligna* accession may not harbour *R* genes against *B. lactucae* (see **chapter 2**). Therefore, effector screening to preselect *L. saligna* accessions with potential *R* genes is probably not more efficient than disease testing random segregating populations of *L. saligna* x *L. sativa* to identify *R* genes.

Effector screenings may be especially useful to identify resistance against pathogens for which it is difficult or takes much more effort to perform disease tests, like *Fusarium oxysporum*, which causes lettuce wilt and root rot and affects lettuce production world-wide (Gordon and Koike 2015). For downy mildew, effectors can be useful to indicate the frequency of *R* genes in a germplasm set. Furthermore, effectors that do not induce an HR in the primary gene pool, but only in *L. saligna*–indicate novel *R* genes that are probably not yet used for breeding. *R* genes from an unexploited species like *L. saligna* potentially have a higher chance to map at unique genomic positions compared to *R* genes from the primary gene pool, which has already been extensively screened for *R* genes. This was already demonstrated once by the new *R* gene locus on LG9 that we mapped in *L. saligna* CGN15705. Development of an *R*-*Avr* catalogue listing all known *R* genes with their cognate effector (*Avr*) gene would be useful for breeders, as it could allow easy detection of known *R* genes in new sources for resistance and the discrimination of multiple *R* genes, even closely linked ones. Some scientists assume that effector-directed breeding may also allow identification of *R* genes that recognize broadly conserved (core) effectors, which play an important role in virulence (Bart *et al.* 2012). Loss of AVR effector genes is a common mechanism to evade ETI (Yoshida *et al.* 2009; Huang *et al.* 2014), but core effectors are unlikely to mutate. Therefore, *R* genes that recognize these core effectors are potentially more durable (Dangl *et al.* 2013).

Pathogen effectors do not only provide tools for discovering *R* genes but may also enable identification of the host plant target proteins that can be manipulated by effectors to suppress PTI and/or enhance infection. Host plant targets subsequently may be good candidates for susceptibility (*S*) genes, which are defined as infection promoting genes that when mutated can cause resistance (Pavan *et al.* 2010). Allelic variants of *S* genes that are insensitive to manipulation by the pathogen could be used for breeding disease resistant plants (van Schie and Takken 2014).

Effector responsiveness in a nonhost is often assumed to contribute to NHR (Wroblewski *et al.* 2009; Schulze-Lefert and Panstruga 2011; Adlung *et al.* 2016). For instance, HR responses to multiple *Phytophthora infestans* effectors in pepper accessions were suggested to underpin NHR of pepper to *P. infestans* (Lee *et al.* 2014). However, cosegregation between effector responsiveness and resistance is needed to prove a causal and complete association between *Avr* and *R* genes. Our study and several others have shown that the effector responsiveness does not always cosegregate with resistance (Vleeshouwers *et al.* 2008; Krasileva *et al.* 2011; Goritschnig *et al.* 2012). Furthermore, 6% of our tested *L. saligna* x *L. sativa* BC1 plants were fully resistant with no evidence for dominant monogenic resistance and/or effector responsiveness. Apparently, *R* genes are not essential for NHR of *L. saligna*.

Novel insights into nonhost resistance

One of the goals of this thesis was to elucidate the genetic basis of NHR in *L. saligna*. Inheritance studies based on crosses between nonhost and host species are rare and have only been performed in lettuce and pear. In pear, an indication for a nonhost QTL against scab caused by *Venturia pirina* was found (Won *et al.* 2014). Previous NHR studies in *L. saligna* were performed on F2 populations and a set of backcross inbred lines (BILs), each with a single *L. saligna* introgression segment in a *L. sativa* background. Individual QTLs were identified from *L. saligna* that each cause some reduction in infection severity, but only a minority were effective at each plant stage of the lettuce life cycle. Eight QTLs

identified in BILs were race-nonspecific under field conditions (Jeuken and Lindhout 2002, 2004; Jeuken *et al.* 2008; Zhang *et al.* 2009). All previous studies were based on only one *L. saligna* accession (CGN05271). In **chapter 3**, we studied the inheritance of downy mildew resistance in nine *L. saligna* accessions from a diverse geographic origin, to identify genes underlying NHR in *L. saligna* as a species.

Previously, stacking eight combinations of two race-nonspecific QTLs did not result in greatly elevated levels of resistance (Den Boer *et al.* 2014). We hypothesized that a combination of genes, which individually show no or only small effects, may be responsible for NHR in *L. saligna.* To identify such a combination of epistatic genes **(chapter 3)**, we developed bidirectional backcross populations between the nonhost species, *L. saligna* and the host species, *L. sativa*. Highly resistant BC1cult plants (F1 x cultivated parent *L. sativa*) with *L. saligna* introgressions into a *L. sativa* background were genotyped to identify which combination of nonhost chromosome regions confer resistance. As a complementary approach, increased susceptible inbred lines of BC1wild plants (F1 x wild parent *L. saligna*) were genotyped to find which (combination of) host chromosome regions confer susceptibility. The selective genotyping of highly resistant BC1cult plants identified four loci for NHR on LG4, LG7 (two loci) and LG8. Selective genotyping of increased susceptible inbred lines of BC1wild plants identified the same locus on LG8. For the latter, we hypothesize that a homozygous *L. sativa* introgression on LG8 may have replaced *L. saligna* alleles of genes involved in PTI, therefore resulting in enhanced susceptibility. The highly resistant BC1cult plants showed that combinations of at least three of the four identified loci may lead to complete resistance. Likewise, broad spectrum resistance has been achieved by pyramiding four QTLs each controlling a different aspect of rice resistance to blast disease (Fukuoka *et al.* 2015).

We noticed that the four NHR loci were located at or near regions with a strong preference for *L. sativa* alleles (Fig. 1). Still, we might have missed few other loci for NHR due to an underrepresentation of *L. saligna* chromosome regions caused by linkage to a gene involved in hybrid incompatibility (HI) or genes we selected against (*R* genes or hybrid necrosis genes). One HI locus in unselected BC1cult resulted in a reduced frequency of *L. saligna* alleles on the bottom of LG9. Additionally, our selection against plants with hybrid necrosis (HN), caused by a *L. saligna* allele on the top of LG9 likely decreased the detection power for NHR loci on the top of LG9. Furthermore, we selected against *R* genes, as we observed that these are not essential for high resistance levels. Three accessions harboured an *R* gene on the top of LG2, which likely resulted in a decreased detection power for NHR loci there. To prevent such reduction of detection power as much as possible, future selective genotyping should focus on accessions without HN and *R* genes against the test isolate. Of the *L. saligna* accessions that we tested, only CGN15726 did not show HN symptoms and revealed no *R* genes against the test isolate (Bl:21). Therefore, NHR research in CGN15726 would be most efficient.

By a bidirectional and multi-accession approach, we showed that a combination of three out of four NHR loci may explain the complete resistance of the species *L. saligna*. Therefore, I propose that these loci harbour a core set of four epistatic genes that are essential for NHR. We did find indications for variation in genes underlying NHR. For instance, LG8 was overrepresented in resistant backcross plants from five *L. saligna* accessions, but not in backcrosses from CGN15705. However, due to confounding factors like additional *R* genes and hybrid necrosis, plant numbers were too low for statistical analysis between accessions. Still the four detected NHR loci in *L. saligna* are probably important in the majority of accessions, as they were also detected when a single accession was excluded from the total analysis of all accessions.

Another indication for variation in genes underlying NHR is that the genetic dosage of NHR may differ between *L. saligna* accessions. *L. saligna* 275-5 and CGN19047 are the only accessions with slight levels of sporulation. They may lack the same NHR alleles by descent, as they cluster very closely together in a phenogram and their geographic origins (the island Corse and Italy, Siena) are near. *L. saligna* 275-5 may have an even lower dosage of NHR than CGN19047, as its infection level is higher than CGN19047. Possibly, *L. saligna* 275-5 does not harbour the full core set of NHR genes that together lead to effective PTI. Variation in NHR response has been observed for other interactions between nonhosts and microbes (Tosa and Shishyama 1984; Mellersh and Heath 2003; Shafiei *et al.* 2007; Aghnoum and Niks 2010; Ayliffe *et al.* 2011).

Based on our results, I propose a genetic model for NHR in *L. saligna* (Table 1). Combinations of at least three genes from a core set of four NHR genes are essential for NHR. Previously identified individual QTLs that conferred some reduction of infection severity (Zhang *et al.* 2009) and *R* genes form an additional layer of resistance that contributes to, but is not essential for NHR (Table 1). For CGN11341 and CGN05947 I hypothesize that the same four NHR genes were present as in their closest related accessions from the selection of nine accessions (Fig. 2). For CNG19047 and 275-5, which showed 5% and 20% RIS, I hypothesize that respectively one and two of the four core NHR genes are missing. The four essential loci for NHR could be validated in next inbred generations, or the loci could be stacked using our set of backcross inbred lines (BILs). Field studies would be needed to verify that the combined genes also protect lettuce under field conditions and to determine race non-specificity in the field.

Table 1. Proposed genetic model for NHR of *Lactuca saligna* to *Bremia lactucae* (based on disease tests at adult plant stage in the greenhouse): a core set of four epistatic NHR genes are essential for NHR in combinations of three out of four genes, whereas additional layers of resistance by individually effective QTLs and *R* genes are non-essential for NHR.

			Essential for NHR				**Non-essential for NHR**				
			Three out of four epistatic NHR genes (chapter 3)				Minor genes with individual quantitative effect (Screening set of BILs by Zhang et al 2009)				Major genes with qualitative effect (chapter 3) # *R* genes
L. saligna	Origin	RIS (%)	[c]								
CGN05271	Europe	0	✓	✓	✓	✓	✓[d]	✓[d]	✓	✓	0
CGN19047[a]	Europe	5	✓	✗	✓	✓					0
275-5[a]	Europe	20	✓	✗	✗	✓					0
CGN05318	Israel	0	✓	✓	✓	✓					1
CGN05947[b]	Israel	0	✓	✓	✓	✓					2
CGN05304	Israel	0	✓	✓	✓	✓					1
CGN11341[b]	SW Asia	0	✓	✓	✓	✓					0
CGN15726	SW Asia	0	✓	✓	✓	✓					0
CGN15705	SW Asia	0	✓	✓	✓	✗					1

✓: gene present, ✗: gene absent, grey shading: hypothetical presence/absence. [a]: hypothesized data as no genetic analysis for NHR was performed. [b]: hypothesized data as no genetic analysis for NHR was feasible. [c]: also identified as a QTL at adult plant stage in the field, but not in the greenhouse (Zhang *et al.* 2009). [d]: effective at all plant stages (seedling, young plant, adult plant in the greenhouse and field). RIS: relative infection severity level compared to susceptible control *L. sativa* cv Olof.

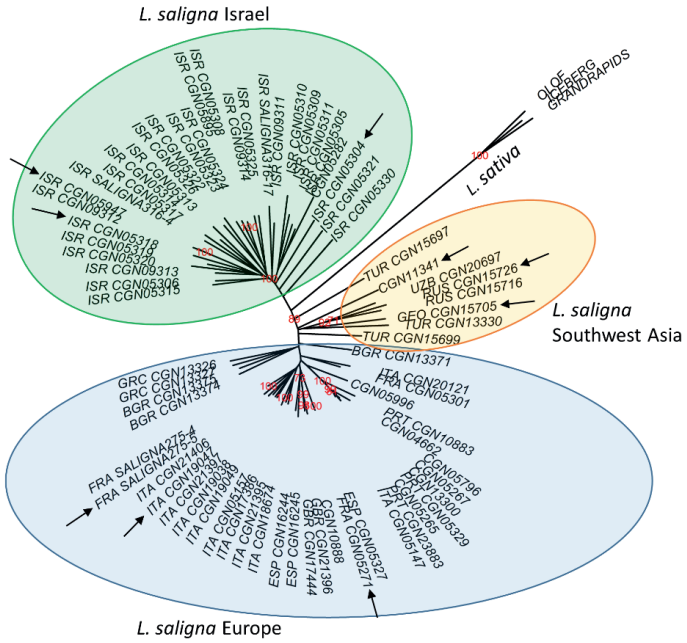


Figure 2. Phenogram of *Lactuca saligna*, based on 423 AFLP fragments from 73 *L. saligna* and 3 *L. sativa* accessions (chapter 3). Three main branches are distinguished: European, Israeli and Southwest Asian

A high level of specificity of nonhost responses has been found both for different pathosystems of wheat and barley, based on gene expression studies (Delventhal *et al.* 2017). Likely, the genes for NHR against downy mildew in *L. saligna* are specific for lettuce-downy mildew, but their applicability to other pathosystems remains to be investigated. However, enhancing the fundamental understanding of NHR genes will expand our knowledge of the mechanisms controlling both immunity and susceptibility. Furthermore, our unconventional approach of selective genotyping in bidirectional backcrosses could be applied to identify NHR loci in other systems, or to genetically map other complex traits.

Speciation of wild and cultivated lettuce

L. saligna and *L. sativa* share a common ancestor. Speciation likely occurred as a result of the accumulation of multiple reproductive barriers that reduced or prevented gene flow between diverging lineages. Barriers to gene flow between plant species can occur at the prepollination or postpollination stage (Coyne and Orr 2004; Chen *et al.* 2016). Prepollination barriers include geographic isolation, temporal isolation and mating system divergence. Postpollination barriers can act before or after fertilization, prezygotically or postzygotically (Rieseberg and Willis 2007). Postzygotic genetic incompatibilities occur in hybrids and/or their derived progenies of *L. saligna* x *L. sativa*, leading to i.a. hybrid sterility and inviability, known as hybrid incompatibility (HI). Another easily recognizable form of HI, hybrid necrosis (HN), has previously been observed in hybrids of *L. sativa* cv Olof with *L. saligna* CGN05271 and CGN11341 (Jeuken *et al.* 2009). This HN was due to a digenic interaction between a *L. sativa* allele of a potential *R* gene on LG8 and a *L. saligna Rin4* allele on LG9 leading to autoimmunity. In **chapter 3**, we observed similar HN symptoms in hybrids of *L. sativa* cv Olof with two out of seven other *L. saligna* accessions, namely 275-5 and CGN19047, that were associated with the same digenic interaction at the same loci. This postzygotic barrier appeared in interspecific progenies of four out of

nine *L. saligna* accessions, from two phenetic branches (Europe and Southwest Asia, Fig. 2).

In **chapter 4**, we performed a whole-genome analysis of postzygotic reproductive barriers in segregating populations and in a set of BILs of the cross *L. saligna* CGN05271 (mother) X *L. sativa* cv Olof (father). A first indication of HI was observed by severely reduced fertility in the F1 of only 2% of the parental fertility. A second indication of HI was observed in the F2, by non-Mendelian segregation of alleles at certain loci, i.e. transmission ratio distortion loci (TRDL), and by symptoms of hybrid breakdown, like hybrid inviability, weakness, necrosis and sterility.

We detected ten TRDL with severe to extreme distortions in the F2 mainly due to a preference for the *L. sativa* allele (paternal cross parent). About half of these TRDL were also detected in bidirectional BC1 populations (*L. saligna* and *L. sativa* respectively as the paternal cross parent). Four out of eight TRDL with a *L. sativa* allele preference in the F2 were associated with complete non-transmission of a heterospecific allele combination in certain BILs, defined as absolute HI. As a consequence, the introgression segments of these specific BILs were obtained only with the wild parent introgression segment in heterozygous state and their inbred progeny showed TRD with a complete lack of individuals with a homozygous wild parent segment. Future analysis of seed set in the BILs may reveal which TRDL and/or HIs are associated with reduced seed set. Seed set analysis of BILs with heterozygous introgressions, followed by analysis of TRD in the inbred offspring may provide more clues on the cause of the severely reduced fertility of the F1 (*L. saligna* x *L. sativa*) and its derived progeny. For instance, reduced seed set but no TRD would indicate a (parental) sporophytic effect, while reduced seed set and TRD would indicate a gametophytic or zygotic cause. LG7, LG8 and LG9 all harbour at least two loci for absolute HI Therefore, introgression of *L. saligna* segments from these chromosomes could be more of a challenge, for instance for the four NHR loci identified in **chapter 3** (see Fig. 1).

It would be interesting to examine to which extent the identified genetic incompatibilities for hybrids of *L. saligna* CGN05271 x *L. sativa* cv Olof are present in a broad or narrow range of *L. saligna* accessions. If the majority of *L. saligna* accessions display the same cases of HI with the majority of *L. sativa* accessions, these cases may be ancient and could have played a role in early divergence of the two species. Pairs of genes that confer HI only in a narrow subset of accessions may have evolved later. As mentioned earlier, hybrid necrosis caused by the same digenic interaction was present in interspecific progenies of four out of nine *L. saligna* accessions, from two phenetic branches. Another TRD/HI on the bottom of LG9 was observed in two interspecific *L. saligna* x *L. sativa* populations with *L. saligna* parents from different phenetic branches, CGN05271 and CGN15705 (Europe and Israel Fig. 2; supplemental data chapter 3). These two TRD/HI on the top and bottom of LG9 may be present as reproductive barriers in a broad range of accessions and could therefore have been involved in early divergence of the two species. All TRD discussed here were detected in F2 and BC1 populations with a *L. saligna* cytoplasm. Segregating populations with *L. sativa* cytoplasm could show whether certain TRD depend upon cytonuclear interactions. Cytonuclear incompatibility contributed to early stages of speciation in *Campanulastrum americanum* (Barnard-Kubow *et al.* 2016). However, our study showed an allele preference for *L. sativa* (the paternal cross parent) for eight out of ten TRDL, which suggests that genetic incompatibilities between *L. saligna* cytoplasmic genes and *L. sativa* nuclear genes do not play a major role.

Although no single homozygous *L. saligna* introgression segments were obtained in *L. sativa* background for five HI regions, a few F2 plants with these homozygous *L. saligna* introgressions were observed in the F2 population, probably due to the presence of other

conspecific regions. HI may be nullified or rescued when two conspecific genetic components or genetic "dance partners" are present (Moyle and Graham 2006), proving a compatible and non-deleterious digenic interaction. We identified three digenic cases of HI with complete non-transmission of a heterospecific allele combination. For the digenic HI between loci on LG4 and LG8, we found evidence for non-transmission of one heterospecific allele combination in both male and female gametophytes (with a *L. sativa* allele on LG4 and a *L. saligna* allele on LG8). This may be the first identified two-locus sex-independent gametophytic HI.

The three digenic hybrid incompatibilities that we identified can be explained by the Bateson-Dobzhansky-Muller (BDM) model (Bateson 1909; Dobzhansky 1937; Muller 1942), which states that each pair of interacting conspecific genes evolves independently in separate lineages. Subsequently, deleterious interactions between heterospecific alleles occur in hybrids or in later inbred stages. The extent to which the detected TRDL and/or hybrid incompatibilities have contributed to the initial branching of *Lactuca* lineages remains unclear. Possibly, they just reflect incompatibilities that developed after the speciation process had been completed. The fact that we identified two cases of HI in multiple accessions (on the top and bottom of LG9, explained above) from different phenetic branches indicates that at least some cases of HI have an ancient origin.

Evolution of NHR in *L. saligna*

An intriguing question is how *L. serriola/sativa* evolved into a host for downy mildew, while *L. saligna* ended up as a nonhost. As *L. serriola* is (one of) the direct ancestors of *L. sativa* (Lindqvist 1960; De Vries 1990; Kesseli *et al.* 1991; de Vries and van Raamsdonk 1994) I refer to *L. serriola* in the next paragraphs.

In one possible evolutionary scenario (Fig. 3, scenario 1), *L. saligna* was an ancient host for *B. lactucae*. A host range expansion to *L. serriola,* followed by a host shift occurred later. For grapevine downy mildew, a host range expansion from wild to cultivated grapevines was reconstructed by Rouxel *et al.* (2013). A host range expansion of *B. lactucae* to *L. serriola* could have been possible due to commonalities in the basal defence system between *L. saligna* and *L. serriola* and to continuous effector repertoire diversification of the pathogen. *L. saligna's* basal resistance by PTI had already been suppressed by effectors of *B. lactucae*. *L. saligna* counter-evolved effector triggered immunity (ETI), while an initial lack of ETI in *L. serriola* may have provided an advantage to *B. lactucae* that favoured a complete host shift to *L. serriola*. Choi and Thines (2015) showed that host-shift driven speciation of the pathogen likely played a large role in the evolution of the downy mildew genus *Bremia*. *L. serriola* is often considered as an invasive species (Hooftman *et al.* 2006) and has advanced northward during the last 250 years (D'Andrea *et al.* 2009). An increased *L. serriola* population size compared to *L. saligna* may have favoured a host shift to *L. serriola.*

Another evolutionary scenario (Fig. 3, scenario 2), starts with *B. lactucae* being able to infect the last common ancestor of *L. saligna* and *L. serriola*, so, before the divergence of *L. saligna* and *L. serriola*. Initial co-speciation of plant and pathogen may have resulted in two pathogen subspecies, each on their own host. Later, a loss of the ability to infect *L. saligna* appeared (pathogen extinction), as currently no *Bremia* pathogen that infects *L. saligna* is known. Continuous co-speciation would be expected to have resulted in parallel speciation patterns between pathogen and host phylogenies, which have not been observed for *Bremia* species (Choi and Thines 2015). In these first two scenarios, the occasional presence of *R* genes would be remnants of an ancient co-evolution between *L. saligna* and ancestral *B. lactucae*. NHR of *L. saligna* may be one-sided evolved or non-evolved (Antonovics *et al.* 2012). One-sided evolved NHR may be applicable to the first

two scenarios. *B. lactucae* may not have counter-evolved to genetic changes in *L. saligna* that caused decreased infection levels, but instead shifted to *L. serriola*.

In a third evolutionary explanation (Fig. 3, scenario 3), *L. serriola* became a host after divergence from *L. saligna*. *B. lactucae* specialized on *L. serriola* and never adapted to *L. saligna.* In this case, NHR of *L. saligna* would be non-evolved (Antonovics *et al.* 2012) as the incompatibility between *B. lactucae* and *L. saligna* is a property of the pathogen and not an evolved trait of *L. saligna*. In this scenario, the occasional presence of accession-specific *R* genes against *B. lactucae* may seem counter-intuitive, because *R* genes are commonly believed to evolve through co-evolution of a host and a pathogen. However, the *R* genes, effective to *B. lactucae*, may also be accidental by-products of other evolutionary or mutational processes in *R* genes effective to other pathogens and pests. Genes within *R* gene clusters are prone to duplications, recombination, unequal crossing-over, point mutations and diversifying selection (Meyers *et al.* 2005). Sequence exchange between *R* genes that are effective against a specific pathogen can even result in the generation of novel *R* genes with resistance specificities to other, phylogenetically unrelated pathogens (Slootweg *et al.* 2017). In these ways, *R* genes with specificity against certain *B. lactucae* races may have evolved by chance instead of by a host-pathogen interaction.

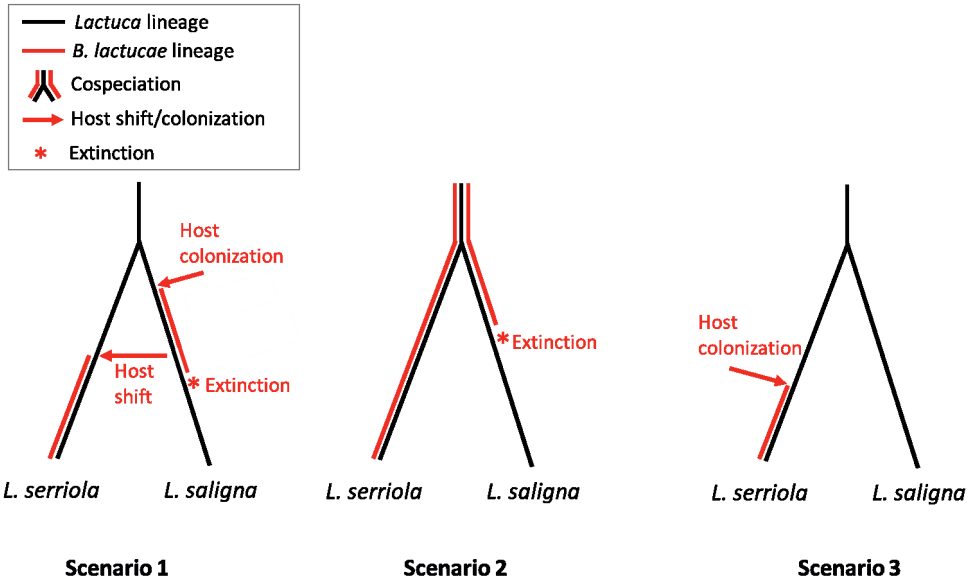


Figure 3. Evolutionary scenarios for NHR in *Lactuca saligna.*

Future perspectives

Rapid technological advances can tremendously impact genetic research. This is exemplified by the last 30 years, during which we went from the first *Lactuca* genetic linkage map with 50 markers (Landry *et al.* 1987), to a complete, high quality genome of *L. sativa* (Reyes-Chin-Wo *et al.* 2017). Here, I provide a glimpse into the future of lettuce and downy mildew genetic research. Recent and future sequencing of *Lactuca* germplasm and *B. lactucae* will provide important genomic resources for research and breeding. The sequencing of different *Lactuca* genomes has started in several projects:

In 2015, a two-year collaboration between public and private parties called the International *Lactuca* Genomics Consortium (ILGC) was started (http://topsectortu.nl/nl/international-lactuca-genomics-consortium-iglc). The goal of this project was to sequence, assemble and annotate the genomes of *L. saligna* and *L. virosa*. High quality *de novo* genomes have been assembled, but are not yet publicly available (Marieke Jeuken, personal communication, December 2017). In another project that was initiated recently, the Chinese genomics institute BGI will resequence, with a depth of 10x, the complete collection of 2500 accessions of several wild lettuce species and cultivated lettuce from the Dutch Centre for Genetic Resources (CGN). The digital genomes are expected to become publicly available in summer 2019.

The upcoming reference *L. saligna* genome will facilitate the genetic mapping and cloning of NHR genes and of genes conferring HI with *L. sativa*. The combination of *Lactuca* genomic resources with genome-wide association studies (GWAS) with field validation of the effect of new alleles could be used to predict which accessions harbour certain traits, and to identify genetic variants encoding interesting traits. Genomic prediction of phenotypic traits is a novel method that has the potential to improve selection and reduce costs (Hickey *et al.* 2017). Furthermore, the comparison of *Lactuca* genomes could provide a better understanding of the genetic diversity and speciation of lettuce. For instance, a pan-genome could be constructed, which is composed of a "core genome'' containing genes present in all species or accessions, and a "dispensable genome'' representing genes present only in a subset (Tettelin *et al.* 2005). Genome scans that interpret the genomic landscape by extracting effects of gene flow, divergent selection and reproductive isolation, could contribute to a better understanding of differentiation and divergence between species (Barrett and Hoekstra 2011).

Access to large-scale sequence and phenotypic information at unprecedented levels will catalyse trait and/or gene discovery. Combined with the advent of targeted genome modification this could have far-reaching implications for fundamental research and breeding of downy mildew resistant lettuce. The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system from *Streptococcus pyogenes* has been developed as a technology for genome editing in a precise and predictable manner (Brouns *et al.* 2008). Genome editing by CRISPR/Cas9 is more efficient and versatile than older genome editing tools like zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (Bortesi and Fischer 2015). Woo *et al.* (2015) have demonstrated the feasibility of CRISPR/Cas9 genome-editing in lettuce.

Conventional breeding depends on existing genetic variation within the crop species and its wild relatives, or on induced genetic variation by random mutagenesis. To use alleles from wild relatives, intercrossing an elite cultivar with the allele donor followed by extensive backcrossing to get rid of other, undesired, alleles is necessary. Creation of new genetic variation induced by random mutagenesis mainly delivers loss-of-function mutations and is also time-consuming, as it requires screening of large populations to identify mutants with interesting traits (Bortesi and Fischer 2015). CRISPR/Cas9 enables the creation of novel genetic variation directly in a cultivar background. This could result in alleles with altered or novel functions, like *R* genes with new resistance specificities, other classes of potential resistance genes (e.g. PRRs), or host plant targets (susceptibility factors) that are insensitive to pathogen manipulation by effector proteins. CRISPR/Cas9 also enables targeted loss-of-function screening of annotated or predicted genes, which can accelerate the identification of existing interesting alleles. Multiple genes could even be modified simultaneously, which would be useful to validate the combined action of for instance the NHR genes that we identified in **chapter 3**. Another often mentioned option is the creation of *R* gene "cassettes'' (by CRISPR/Cas9) that harbour multiple *R* genes so

close together that they behave as a single Mendelian locus that does not segregate in future generations or crosses (Ainley *et al.* 2013).This strategy could also be used to create stacks of NHR genes, optionally supplemented with *R* genes. New *R* genes from the untapped *L. saligna* species will be a welcome addition to the current *R* gene arsenal. However, until legal laws consider plants modified by CRISPR/Cas9 as non-GMO, CRISPR/Cas9 will mainly be used for research, and to identify and validate the function of naturally occurring genes.

A reference genome of *B. lactucae* is almost finished (Richard Michelmore, personal communication, September 2017), which opens the door for targeted genome editing from the pathogen side as well. For instance, systematic knock-out screening of *B. lactucae* genes could identify (effector) genes that are critical for the survival or virulence of the pathogen. If subsequently resistance genes are identified or artificially created that recognize such indispensable *B. lactucae* genes, this could result in durable disease resistance. Increased knowledge of the evolutionary capacity of the pathogen and mechanisms of plant immunity will lay the foundation for durable interventions.

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Summary

Downy mildew, caused by the oomycete *Bremia lactucae*, is a devastating disease in lettuce (*Lactuca sativa*) cultivation, leading to high yield losses. An effective method of downy mildew control is the deployment of resistant lettuce cultivars. Over 50 monogenic, dominant resistance (*R*) genes have been deployed in lettuce breeding. *R* genes provide high levels of resistance, but are continually rendered ineffective by the occurrence of pathogenic strains with new virulence characteristics. This demonstrates the presence of an arms race between plant and pathogen. *R* genes may be an effective but short-term solution to control downy mildew in lettuce, as long as sufficient resistance sources can continuously provide novel *R* genes. However, the continuous introgression of new *R* genes in elite breeding material demands much time of breeders and is not a durable solution. Until now, most resistance genes have been derived from the primary gene pool of cultivated lettuce. The wild lettuce species *L. saligna* is a member of the secondary gene pool, completely resistant to all *B. lactucae* isolates and considered as a nonhost for downy mildew. Despite its potential attractiveness as a resistance source, *L. saligna* has hardly been exploited for lettuce breeding.

In **chapter 2**, we explored the potential of screening for *R* genes in *L. saligna* by use of pathogen candidate effectors. Effectors are pathogenic proteins to aid infection of specific plant species. We transiently expressed 16 candidate effectors from *B. lactucae* in a diverse *Lactuca* germplasm set (n=150). Accessions that react with a hypersensitive response (HR) are supposed to carry an *R* gene that recognizes the effector as an avirulence factor. Two candidate effectors (BLN08 and BLR31) induced an HR in *L. saligna* accessions. BLN08 triggered an HR in 55% of the accessions, but in segregating populations responsiveness did not co-segregate with resistance to Bl:24, the *B. lactucae* race from which the effectors were cloned. BLR31 induced an HR in 5% of the accessions, and revealed a novel *R* gene providing resistance to isolate Bl:24. Consequently, we identified a candidate avirulence effector of *B. lactucae* and its cognate *R* gene in *L. saligna.* Additionally, resistant backcross plants that were BLR31 nonresponsive indicated other unlinked *R* genes and/or nonhost QTLs. Our results suggested that *R* genes against *B. lactucae* seem common in *L. saligna*, but they are not essential for nonhost resistance (NHR).

As a nonhost for downy mildew, *L. saligna* harbours potentially durable resistance genes. The genetics of NHR are poorly understood. Inheritance studies of NHR are uncommon, because in general host and nonhost species are so much diverged that they are not cross-fertile anymore. The plant-pathosystem of lettuce and downy mildew provides a rare opportunity to study the inheritance of NHR, as the nonhost species *L. saligna* is cross-compatible with the host species *L. sativa.* In previous genetic studies on backcross inbred lines (BILs) and F2 populations, multiple individual, often plant stage dependent QTLs for resistance were identified in *L. saligna* CGN05271. However, stacking eight combinations of two race-nonspecific QTLs by intercrossing of BILs did not result in greatly elevated levels of resistance. These findings indicated that NHR in *L. saligna* may be due to a combination of epistatic genes, which individually show no or only small effects.

To identify such a combination of epistatic genes that together confer NHR, we used a bi-directional backcross approach in **chapter 3**. All previous NHR studies in *L. saligna* were based on one accession, CGN05271. Here, we selected nine *L. saligna* accessions from a wide geographic origin, to study NHR in *L. saligna* as a species. Variation in infection severity levels among F1 and backcross (F1 x *L. sativa*) populations of these nine accessions, suggested genetic variation for gene dose of NHR. Two accessions probably harbour a lower genetic dose for NHR than the other accessions. Selective genotyping of highly resistant backcross (F1 x *L. sativa*) plants of the multiple accessions, showed four loci with an overrepresentation of *L. saligna* alleles. Confirmation of one of these four loci (LG8) was found in inbred offspring of F1 plants backcrossed to the *L. saligna* parent with a homozygous *L. sativa* introgression on LG8 and relatively high infection levels.

Combinations of three out of these four *L. saligna* loci seem to lead to complete resistance and could represent a core set of essential NHR genes.

Interspecific crosses are often hampered by reproductive barriers, which can lead to inviability, infertility and non-Mendelian skewed segregation of alleles (transmission ratio distortion, TRD) in hybrids and their derived progeny. This is due to genetic incompatibilities between species, a phenomenon known as hybrid incompatibility (HI). In **chapter 4**, we studied postzygotic reproductive barriers between *L. saligna* and *L. sativa*. Genome-wide analysis of TRD in F2 and two BC1 populations gave an indication of HI genes between wild and cultivated lettuce. Ten TRD loci (TRDL) and subsets of these were detected in the F2, and in the BC1 respectively. Four out of eight TRDL with a *L. sativa* allele preference in the F2 were associated with an absolute HI in the BILs, meaning that no single homozygous *L. saligna* introgression could be retrieved in a *L. sativa* background. Three HI loci were due to a heterospecific two-locus interaction. One of the deleterious digenic interactions leading to HI was characterized in detail and was caused by non-transmission of one heterospecific allele combination, in both the male and female gametophyte.

In **chapter 5** the main results of this thesis are evaluated in a broader perspective. The contribution of these results to our understanding of NHR in *L. saligna* are addressed. A genetic model for NHR is proposed in which a core set of epistatic loci is possibly supplemented with *R* genes or individually effective QTLs. Three scenarios for the evolution of NHR in *L. saligna* are suggested and some future perspectives for lettuce and downy mildew research are discussed.

Samenvatting

Valse meeldauw is het grootste ziekteprobleem in de teelt van sla (*Lactuca* sativa) en leidt tot grote opbrengstverliezen. Deze ziekte wordt veroorzaakt door de oomyceet *Bremia lactucae*. Een effectieve aanpak van valse meeldauw is het gebruik van resistente slarassen. Tot nu toe zijn er meer dan 50 monogene, dominante resistentiegenen (*R*-genen) ingezet in de slaveredeling. Deze klassieke *R*-genen geven een hoog niveau van resistentie, maar dit effect wordt regelmatig doorbroken/ondermijnd door het ontstaan van pathogene varianten (fysio's) met nieuwe virulentie-eigenschappen. Dit toont de aanwezigheid van een wapenwedloop tussen de plant en de ziekteverwekker. *R*-genen kunnen een effectieve kortetermijnoplossing zijn tegen valse meeldauw in sla, zolang er voldoende aanvoer is van resistentiebronnen met nieuwe *R*-genen. Maar de continue introgressie van nieuwe *R*-genen in elite veredelingsmateriaal vergt veel tijd van veredelaars en is geen duurzame oplossing. Tot nu toe zijn de meeste resistentiegenen verkregen uit de primaire genenpool van cultuursla. De wilde slasoort *L. saligna* behoort tot de secundaire genenpool, is compleet resistent tegen alle *B. lactucae* fysio's en wordt beschouwd als een zogenaamde "niet-waard" voor valse meeldauw. Ondanks zijn potentieel als resistentiebron, is *L. saligna* tot dusverre nauwelijks gebruikt in de slaveredeling.

In **hoofdstuk 2** verkenden we de mogelijkheid van het screenen op klassieke *R*-genen in *L. saligna* door middel van kandidaat-effectoren van het pathogeen. Effectoren zijn pathogene eiwitten om infectie in specifieke plantensoorten te bevorderen. We hebben 16 kandidaat-effectoren van *B. lactucae* tijdelijk tot expressie gebracht in een diverse set van 150 *Lactuca* accessies. Accessies die vervolgens een overtrokken resistentie-symptoom als "necrose" of "celdood" lieten zien, bezitten waarschijnlijk een *R*-gen dat de effector herkent als een avirulentie-factor. Twee kandidaat-effectoren (BLN08 en BLR31) veroorzaakten een celdood-reactie in *L. saligna* accessies. De herkenning van BLN08 in 55% van de accessies was in splitsende populaties echter niet geassocieerd met resistentie tegen *B. lactucae* fysio Bl:24, het fysio waaruit de effectoren gekloneerd waren. BLR31 induceerde een celdood-reactie (herkenning) in 5% van de accessies, en verder onderzoek leidde tot de identificatie van een nieuw *R*-gen dat resistentie tegen fysio Bl:24 geeft. We hebben dus een kandidaat avirulentie-effector van *B. lactucae* en het bijbehorende *R*-gen in *L. saligna* geïdentificeerd. Bovendien lieten resistente terugkruisingsplanten, die BLR31 niet herkenden, zien dat er andere, genetisch ongekoppelde *R*-genen en/of "niet-waard" kwantitatieve resistentiegenen (Engelse term: "quantitative trait loci", afgekort als "QTLs") aanwezig waren. Onze resultaten suggereerden dat *R*-genen tegen *B. lactucae* regelmatig vookomen in *L. saligna*, maar dat deze niet essentieel zijn voor de niet-waard-resistentie.

Als niet-waard voor valse meeldauw heeft *L. saligna* potentieel duurzame resistentiegenen. Er is weinig bekend over de genetica van niet-waard-resistentie. Overervingsstudies van niet-waard-resistentie zijn zeldzaam, omdat waard- en niet-waard-soorten meestal teveel van elkaar verschillen om onderling kruisbaar te zijn. Het plant-pathogeen-systeem van sla en valse meeldauw biedt een uitzonderlijke mogelijkheid om de overerving van niet-waard-resistentie te bestuderen, aangezien de niet-waard-soort *L. saligna* wel kruisbaar is met de waard-soort *L. sativa*. In eerdere genetische studies met terugkruisingslijnen (Engelse term: "backcross inbred lines", afgekort als "BILs") en F2 populaties zijn meerdere individuele, vaak plant-stadium afhankelijke QTLs voor resistentie geïdentificeerd in *L. saligna* CGN05271. Echter, het stapelen van acht combinaties van twee niet fysio-specifieke QTLs door middel van het kruisen van BILs resulteerde niet in sterk verhoogde niveaus van resistentie. Deze bevindingen suggereren dat de niet-waard-resistentie van *L. saligna* gebaseerd zou kunnen zijn op een combinatie van epistatische genen, die individueel geen of slechts een klein effect hebben.

Om een combinatie van epistatische genen te vinden die samen de niet-waard-resistentie verklaren, hebben we een bidirectionele terugkruisingsmethode gebruikt in **hoofdstuk 3**. Alle voorgaande studies over niet-waard-resistentie in *L. saligna* waren gebaseerd op één accessie, CGN05271. In deze studie, hebben we negen *L. saligna* accessies van diverse

geografische herkomsten geselecteerd, om de niet-waard-resistentie in *L. saligna* als soort te bestuderen. Variatie in infectieniveaus binnen F1 planten en terugkruisingpopulaties (F1 x *L. sativa*) van deze negen accessies, duidden op variatie in de genetische dosis voor niet-waard-resistentie. Twee accessies hebben waarschijnlijk een lagere genetische dosis voor niet-waard-resistentie dan de andere accessies. Het selectief genotyperen van zeer resistente terugkruisingsplanten (F1 x *L. sativa*) van meerdere accessies leidde tot de identificatie van vier loci met een oververtegenwoordiging van *L. saligna* allelen. Eén van deze vier loci (op chromosoom 8) werd bevestigd in zelfbevruchte nakomelingen van F1 planten teruggekruisd met de *L. saligna* ouder, aangezien deze planten een homozygote *L. sativa* introgressie hadden op chromosoom 8 en relatief hoge infectieniveaus. Combinaties van drie van de vier *L. saligna* loci lijken te leiden tot complete resistentie en zouden een basisset van essentiële genen voor niet-waard-resistentie kunnen vormen.

Interspecifieke kruisingen worden vaak gehinderd door reproductive barrières, die kunnen leiden tot sterfte, infertiliteit en niet-Mendeliaanse scheve uitsplitsing van allelen (Engelse term: "transmission ratio distortion", afgekort als TRD) in hybriden en nakomelingen hiervan. Dit komt door genetische incompatibiliteit tussen de soorten, een fenomeen bekend als hybride incompatibiliteit (HI). In **hoofdstuk 4** hebben we postzygotische reproductieve barrières tussen *L. saligna* en *L. sativa* bestudeerd. Genoom-brede analyse van TRD in F2 en twee BC1-populaties gaven een indicatie van HI-genen tussen wilde sla en cultuursla. Tien TRD loci (TRDL) en subsets hiervan werden gedetecteerd in respectievelijk de F2 en de BC1-populaties. Vier van de acht TRDL met een voorkeur voor het *L. sativa* allel waren geassocieerd met een absolute HI in terugkruisingslijnen (BILs), wat inhield dat geen enkele homozygote *L. saligna*-introgressie kon worden verkregen in een *L. sativa*-achtergrond Drie HI loci werden verklaard door een heterospecifieke twee-locus-interactie. Eén van de fatale digene interacties leidend tot HI werd in detail gekarakteriseerd en bleek veroorzaakt doordat één van de twee heterospecifieke allel-combinaties niet doorgegeven werd door zowel de mannelijke als de vrouwelijke gametofyt.

In **hoofdstuk 5** worden de hoofdresultaten van dit proefschrift in een breder perspectief geplaatst. De bijdrage van deze resultaten aan ons begrip van niet-waard-resistentie in *L. saligna* wordt besproken. Ik stel een genetisch model voor, waarin de niet-waard-resistentie van *L. saligna* verklaard wordt door een basisset van epistatische loci, mogelijk aangevuld met klassieke *R*-genen of individuele QTLs. Ook introduceer ik drie scenarios voor de evolutie van niet-waard-resistentie in *L. saligna* en bespreek ik enkele toekomstperspectieven voor het onderzoek naar de interactie tussen sla en valse meeldauw.

Acknowledgements

My PhD has been an amazing period of intense learning in the exciting international atmosphere of Wageningen. It is a pleasure to thank the many people who have contributed to this thesis or supported me in another way.

First and foremost I would like to thank Marieke Jeuken. You always made time for discussion and helped me out wherever you could. You know how to ask the right questions which enabled me to solve many problems. Thank you for all your support, ideas, and good company. I have also come to know you as a very fun person, especially during our trips to international conferences in the US and Spain. I simply could not have imagined a better supervisor for my PhD.

Special thanks to my co-promotor Rients Niks, for your ever timely and constructive criticism, interest and enthusiasm. I enjoyed our discussions and learned a lot from your detailed feedback. I appreciate that you are very committed to everything that you do, from science and teaching to lab trip games and sport events. In our WeDay sports team you were the oldest, but definitely most fanatic member! I am also grateful to my promotor Richard Visser. You had an impressive overview of my project and the previous ones and gave valuable feedback and suggestions on our manuscripts.

Many thanks also to everyone from Utrecht University who has been involved in our project. Guido van den Ackerveken, thank you for setting up this interesting project together with Marieke, resulting in a great combination of expertise on downy mildew and lettuce genetics. Alexandra Pelgrom, thank you for your work on the downy mildew side of the story, your helpful attitude and your contribution to our publication in *New Phytologist*. Claudia Meisrimler, thanks for joining the last stage of our project and presenting your findings during our meetings.

I gratefully acknowledge the Netherlands Organisation for Scientific Research (NWO Domain Applied and Engineering Sciences, a continuation of Technology Foundation STW) and the breeding companies that were involved for their financial and practical support. Special thanks to everyone from the companies for their feedback during our meetings and for performing effector screenings, greenhouse and field tests.

Erik den Boer and Ningwen Zhang, thank you for all your previous work, which provided the basis for my research. Many thanks also to the MSc students that have contributed to this thesis during my PhD: Jacqueline Ulen, Jeroen Wolfkamp, Steven Kaandorp and Thijs Bouten, and even before I started: David Braspenning and Johan Specken.

Thanks also go to many people who have given their valuable time, expertise and advice, especially everyone from the "Nonhost and Insect Resistance" group and also Vivianne Vleeshouwers, Chris Maliepaard, Roeland Voorrips and Danny Esselink. Martijn van Kaauwen thank you for your bioinformatics support. René Smulders, many thanks for your elaborate feedback on two of my chapters.

A big "Thank you!" also goes out to everybody who supported the practical aspects of this thesis. Koen, thank you very much for your cheerful way of making every practical problem seem tiny. Thanks to all other technicians who have helped me in one way or another, especially Betty, Fien, Doret, Johan, Patrick, Irma, and Jos. I also want to thank everyone from Unifarm who took care of my lettuce plants and experiments over the last years, especially Cees, Gerda, Henk, Bert, Dirkjan, Casper, Erik, André and Bertus. Lots of thanks also to our secretaries Nicole, Letty, Janneke and Daniëlle for happily arranging many things whenever I requested.

Thanks to my fellow PhD and postdocs for all our shared breaks, dinners, parties, sports and other fun activities: Andres, Arwa, Atiyeh, Behzad, Carolina, Christos, Cynara, Ernest, Geert, Izan, Jarst, Jasper, Johan, Jordi, Kaile, Kim, Manos, Mathilde, Mengjing, Michela, Myluska, Pauline, Peter Dinh, Raana, Valentina, Viviana, Xiao, Xuan, Yan and Yajun. Marine, Narges, and Peter, thank you for being the best office mates. Peter, Tim and Jeroen, thanks for the amazing trip to China that we made together. Special thanks to Mas and Charlotte for being my paranymphs. And Mas, thanks for keeping me well-fed with all your delicious food and for cheering me up with your loud laughter!

I would like to thank my friends for all the great times that we have shared. My friends from Stick Together, for distracting my mind simply with a ball and a stick, and many other fun activities. Francine, Niek, René, thanks for your friendship and our countless game nights. Wieneke and Bas, I hope we will keep sharing many nice trips and dinners in the future. Many thanks to Eva for our shared adventures and for your creativity, resulting in the cover of this thesis.

Then finally, but not least, thanks goes to my whole family and family-in-law, for all the nice times that we shared, and for your interest in my work and life in general. Special thanks to my parents for giving me all the chances to get here and supporting me in every possible way.

Luc, you literally contributed to this thesis by assisting a few times in the lab and greenhouse and helping me think things through. But most importantly, you were always there for me and I look forward to our coming adventures.

Thank you very much, everyone!

Wageningen, March 2018

About the author

Anne K.J. Giesbers was born in Roermond, the Netherlands, on January 7, 1988. She obtained her BSc degree in Biology with a specialisation in Ecology, and her MSc degree in Plant Biology with a minor in Neurobiology from Wageningen University & Research. In 2013, Anne started her PhD at the Department of Plant Breeding under the supervision of Dr. Marieke Jeuken. The research presented in this PhD thesis was performed as part of a larger research project in collaboration with the Van den Ackerveken group at Utrecht University, funded by the Netherlands Organisation for Scientific Research (NWO Domain Applied and Engineering Sciences, a continuation of Technology Foundation STW) and five breeding companies. In May 2018, Anne will start as a Research Associate for Rijk Zwaan USA at the UC Davis Michelmore lab, to conduct molecular and genetics research on disease resistance in lettuce and virulence of its pathogens.

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Anne Giesbers
Date: 18 April 2018
Group: Plant Breeding, Wageningen University & Research

1) Start-up phase	date
► **First presentation of your project**	
Title: "Effectors and their plant targets as leads for downy mildew resistance breeding in lettuce"	24 Oct 2013
► **Writing or rewriting a project proposal**	
► **MSc courses**	
Plant Breeding: Basic Concepts and their Applications (PBR21803)	02 Sep 2013
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*4.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student day, Leiden, NL	29 Nov 2013
EPS PhD student day 'Get2Gether', Soest, NL	29-30 Jan 2015
► **EPS theme symposia**	
EPS theme 2 symposium "Interactions between Plants and Biotic Agents", Amsterdam, NL	25 Feb 2014
EPS theme 2 symposium "Interactions between Plants and Biotic Agents", Wageningen, NL	23 Jan 2017
► **National meetings (e.g. Lunteren days) and other national platforms**	
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	14 Apr 2014
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	11 Apr 2016
► **Seminars (series), workshops and symposia**	
Flying & Invited Seminars	2013-2017
Symposium: "All-Inclusive Breeding: Integrating High-Throughput Science"	16 Oct 2014
Symposium: "Omics Advances for Academia and Industry - Towards True Molecular Pant Breeding"	11 Dec 2014
Symposium: "Crop Pathology and Plant-Microbe Interactions"	08 May 2015
Symposium: "Plant Breeding Research Day"	29 Sep 2015
Workshop: "WGS PhD Workshop Carousel"	02 Jun 2014
Workshop: "Pathogen-Informed Crop Improvement"	08-10 Apr 2015
Workshop: "WGS PhD Workshop Carousel"	08 Apr 2016
► **Seminar plus**	
► **International symposia and congresses**	
"International Plant & Animal Genome XXIII" (San Diego, USA)	10-14 Jan 2015
"EUCARPIA Leafy Vegetables" (Murcia, Spain)	10-13 Apr 2015
"Second International Academic Conference for Graduate Students" (Nanjing, China)	27-30 Oct 2015
"Pathogen-informed Strategies for Sustainable Broad-spectrum Crop Resistance" (Bled, Slovenia)	01-03 Mar 2017
► **Presentations**	
Talk: Meetings STW and breeding companies	2013-2017
Talk: Wageningen PhD Council symposium (Wageningen, NL)	10 Dec 2013
Talk: EUCARPIA Leafy Vegetables 2015 (Murcia, Spain)	17 Apr 2015
Talk: Second International Academic Conference for Graduate Students (Nanjing, China)	30 Oct 2015
Talk: EPS theme 2 symposium "Interactions between plants and biotic agents" (Wageningen, NL)	23 Jan 2017
Poster: Pathogen-informed strategies for sustainable broad-spectrum crop resistance" (Bled, Slovenia)	02 Mar 2017
► **IAB interview**	
► **Excursions**	
Visit to Rijk Zwaan	27 Sep 2013
Visit to Key Gene	12 Oct 2017
Subtotal Scientific Exposure	*21.1 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
Basic Statistics	20-22, 26 & 27 May 2015
Linear Models	08-10 Jun 2015
Introduction to R (www.datacamp.com)	Jul 2016
Intermediate R (www.datacamp.com)	Jul 2016
Cleaning data in R (www.datacamp.com)	Jul 2016
Data manipulation in R with dplyr (www.datacamp.com)	Jul 2016
Writing functions in R (www.datacamp.com)	Jul 2016
Data visualisation with ggplot2 (www.datacamp.com)	Aug 2016
► **Journal club**	
► **Individual research training**	
Subtotal In-Depth Studies	*3.6 credits**

4) Personal development	date
► **Skill training courses**	
Competence Assessment	05 & 26 Nov 2013
Effective behaviour in your professional surroundings	15 Apr & 13 May 2014
Data Management	27 May 2014
Efficient writing strategies	05, 12, 26 Apr, 10 & 24 May 2016
Career Orientation	04, 11, 18 & 24 Mar 2016
Bridging across cultural differences	24 Nov, 01 & 15 Dec 2016
► **Organisation of PhD students day, course or conference**	
Organisation of bi-weekly group meetings "Nonhost and Insect Resistance", Plant Breeding Department	Sep 2014-2015
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*5.7 credits**

TOTAL NUMBER OF CREDIT POINTS*	**34.9**

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

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

The work described in this thesis is part of the research programme “Open Technology” with project number STW12683 (Title: 'Effectors and their plant targets as leads for downy mildew resistance breeding in lettuce'), which is (partly) financed by the Netherlands Organisation for Scientific Research (NWO) and five breeding companies: Bayer CropScience Vegetable Seeds, Enza Zaden, Rijk Zwaan, Syngenta Seeds and Vilmorin & Co.

Cover design: Eva van Rijsingen

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