Theor Appl Genet (2014) 127:1805–1816
DOI 10.1007/s00122-014-2342-7

ORIGINAL PAPER

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

Erik den Boer · Koen T. B. Pelgrom · Ningwen W. Zhang · Richard G. F. Visser · Rients E. Niks · Marieke J. W. Jeuken

Received: 3 January 2014 / Accepted: 29 May 2014 / Published online: 14 June 2014

Abstract

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

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

Abbreviations

BIL	Backcross inbred line (*L. saligna* introgression segment, 20–80 cM long, in a lettuce, *L. sativa*, genetic background)
sub-BIL	Line with a smaller introgression segment than the BIL of which it is derived
QTL	Quantitative trait locus
YDT	Young plant disease test
ADT_F	Adult plant disease test in the field
ISL	Infection severity level
RIS	Relative infection severity level

Introduction

Improving plant genotypes by breeding requires crossing and selection of the most desirable plants with the best combination of genes. The plants with the preferred genotype contain genes for desirable qualitative and quantitative traits from several parents stacked together. Stacking is often recommended when quantitative trait loci (QTLs)

Communicated by Emilio A. Carbonell.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-014-2342-7) contains supplementary material, which is available to authorized users.

E. den Boer · K. T. B. Pelgrom · N. W. Zhang · R. G. F. Visser · R. E. Niks · M. J. W. Jeuken (✉)
Laboratory of Plant Breeding, Wageningen University, 6700 AJ Wageningen, The Netherlands
e-mail: marieke.jeuken@wur.nl

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

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

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

In previous research, we detected in an *L. saligna* CGN05721 × *L. sativa* cv. Olof F_2 population no race-specific *R*-genes, but instead three QTLs and a resistance caused by a digenic interallelic interaction that leads to hybrid necrosis (Jeuken and Lindhout 2002; Jeuken et al. 2009). In the series of 29 BILs, we found an abundance of 15 introgressions that conferred plant stage-dependent quantitative resistance against all 6 tested *B. lactucae* isolates (Zhang et al. 2009a). The genetic size of these *L. saligna* introgressions ranged from 20 to 80 cM (Jeuken et al. 2008). At the, most relevant, adult plant stage in the field (ADT_F) eight BILs showed reduced infection levels, but their 30–50 % infection reduction is not sufficient for practical application in commercial breeding and cultivation (Jeuken and Lindhout 2004; Zhang et al. 2009a). In search for a desired race non-specific very strong or absolute resistance in the field, we studied the potential of stacking the quantitative resistances of BILs. For the purpose of the present paper, each introgression segment conferring quantitative resistance is considered as one QTL, because for the majority of the resistant BILs no information on the number and position of resistance gene(s) within the introgressions is available. For three of the introgression segments used in this study, namely, 2.2, 4.2 and 8.2, information from substitution mapping revealed the presence of multiple linked sub-QTL (den Boer et al. 2013).

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

Materials and methods

Stacking of introgressions from BILs

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

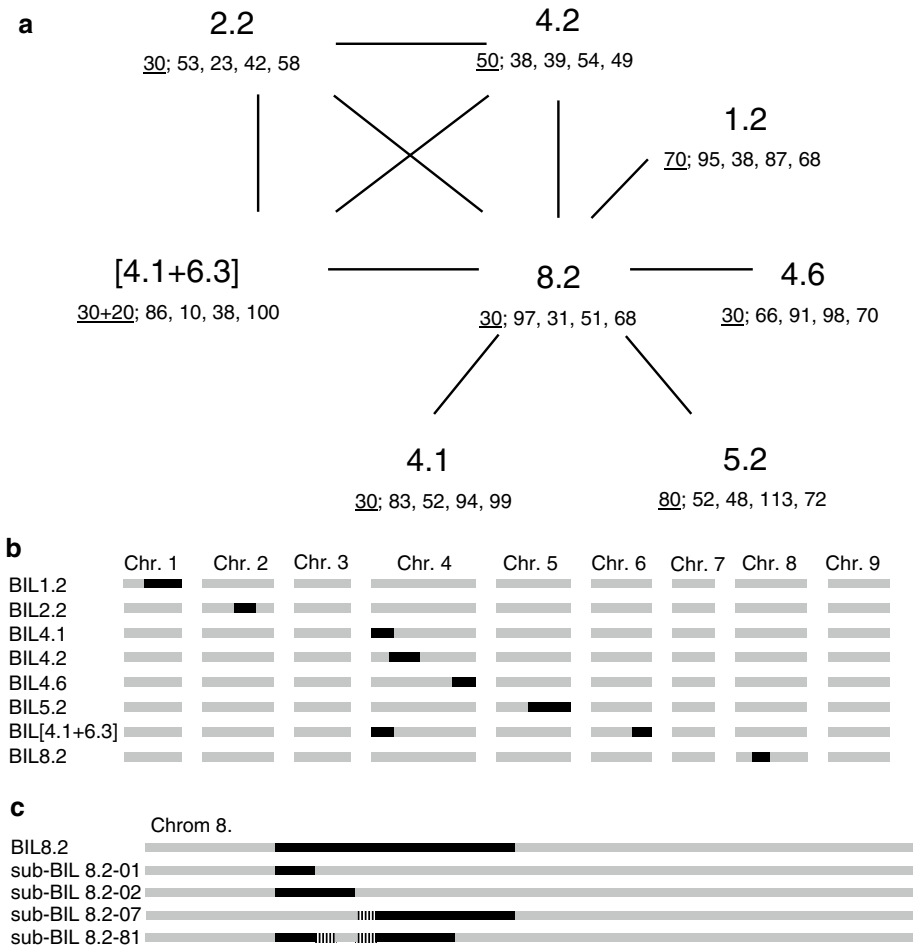


Fig. 1 Overview of intercrossed backcross inbred lines (BILs) and their genotype. **a** *Connecting lines* indicate successful stacking of introgression segments from two BILs. Per BIL five consecutive numbers indicate: the length of the *L. saligna* introgression segments in cM (*underlined* and rounded to tens); relative infection severity level (relative to *L. sativa* cv. Olof, which is set at 100 % infection) in a seedling disease test (SDT), young plant disease test (YDT), adult disease test in greenhouse (ADT_G) and adult disease test in field (ADT_F) (from 11 locations in the Netherlands and France ADT_{F-C}) respectively as reported in Zhang et al. (2009a). **b** A schematic genotype presentation is shown per BIL; *bar colors* in Fig. 1b, c represent the genotype: *black* homozygous *L. saligna*; *gray* homozygous *L. sativa*; *vertical striped* unknown genotype. **c** A schematic genotype presentation is shown for four used sub-BILs of BIL8.2. Exact marker genotypes are shown in den Boer et al. 2013

introgression segments of BIL[4.1+6.3] and of some of its derived sub-BILs (which are lines with smaller donor segments than the parental BIL), indicates that plants carrying a *L. saligna* segment on Chromosome 6 always require a *L. saligna* segment on Chromosome 4. In other words, for BIL[4.1+6.3] the 6.3 *L. saligna* introgression segment does not segregate independently from the 4.1 introgression segment, which indicates at a hybrid incompatibility between the donor segment at Chromosome 6 and the recurrent *L. sativa* alleles at Chromosome 4.

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

Development and stacking of smaller introgression segments

For the 8.2 introgression (30 cM segment), sub-BILs, which are lines derived from a parental BIL with a smaller donor segments than the parental BIL, were already available (den Boer et al. 2013). For stacking of smaller introgressions, we selected four sub-BILs with predominately overlapping introgressions, 8.2-01 (7 cM segment), 8.2-02 (12 cM segment), 8.2-07 (~18 cM segment) and 8.2-81 (2 segments of ~10 and ~8 cM) for intercrossing with genotypes carrying smaller introgressions from [4.1+6.3] (den Boer et al. 2013, Fig. 1c). The overlap of introgressions between sub-BIL 8.2-02 and 8.2-07 and 8.2-81 in the interval of 30.2–35.6 cM is uncertain due to lack of markers. We designated a line that contains two or more stacked donor introgressions derived from either BIL or sub-BIL a ‘combi-line’. As no sub-BILs for the [4.1+6.3]

introgression were available yet, a recombinant screening was performed. We focused mainly on the 6.3 introgression because at adult plant stage in a greenhouse test (ADT_G) it conferred quantitative resistance by reducing the relative infection severity (RIS) to 38 % compared to the susceptible reference and the 4.1 introgression did not (RIS 94 %) (Jeuken et al. 2008; Zhang et al. 2009a).

An inbred progeny of 2,100 plants from preBIL[4.1+6.3] (heterozygous for both introgressions) was screened for recombinants using markers NL1151 and NL0897 at 4.1 and LE1126 and LE1211 at 6.3 (Table S1, Fig. 2). Fourteen hundred plants were screened for 6.3 alone and 700 plants were screened for 4.1 and 6.3. During the process of recombinant screening and development of homozygous lines (sub-BILs), we observed that certain recombinants in 6.3 were not dependent on the presence of 4.1 introgression segments. Sub-BILs of these specific recombinants allowed us to fine map the hybrid incompatibility loci on 6.3 and 4.1 as well as the locus for resistance on 6.3. For the stacking of smaller introgressions from 4.1, 6.3, and 8.2, a few sub-BILs per introgression segment were selected. We followed the same stacking procedure as described above for the combination of BIL introgressions.

Genotyping

DNA was isolated by NaOH method (Wang et al. 1993) or modified CTAB method (Jeuken et al. 2001). Co-dominant DNA-markers were used to screen for recombinants, to determine the position of the recombination, to distinguish between inbred and outcrossed progeny and for selection of stacked homozygous *L. saligna* introgressions in the combi-lines. Primer sequences for markers covering the 1.2, 4.1, 4.6, 5.2 and 6.3 BIL introgressions are listed in Table S1 and markers for 2.2, 4.2 and 8.2 introgressions are described in den Boer et al. 2013. SSR markers were kindly provided by Syngenta BV and EST markers were developed on lettuce EST sequences of the *Compositae* Genome Project Database (CGPDB), (http://compgenomics.ucdavis.edu) and McHale et al. (2009). Polymorphisms between PCR products of *L. saligna* and *L. sativa* alleles were visualized by high-resolution melting curve differences on a LightScanner System (Idaho Technology) or by size differences on agarose gels (directly or after enzymatic digestion) as described previously (Jeuken et al. 2008).

Assessment of resistance to *B. lactucae*

To determine the effects of stacked (sub-)introgressions on resistance levels in the field, all combi-lines were tested at adult plant stage in the field (ADT_F) in the presence of control lines. Control lines were: parental BILs and sub-BILs, susceptible control *L. sativa* cv. Olof and reference resistant control *L. sativa* cv. Iceberg [with an average RIS of 40 % compared to the severity on susceptible controls in field tests, reported by Grube and Ochoa (2005) and den Boer et al. (2013)]. ADT_F were performed in 3 years (2009, 2010 and 2011) with three to five experiments (locations) per year and four or six replications per experiment (Table S2). Artificial or natural infection occurred. Symptoms of *B. lactucae* infection were recognized by at least two independent and experienced observers. Infected leaf material was collected to isolate the pathogen strains. These isolates were applied to the differential set and tested for their virulence

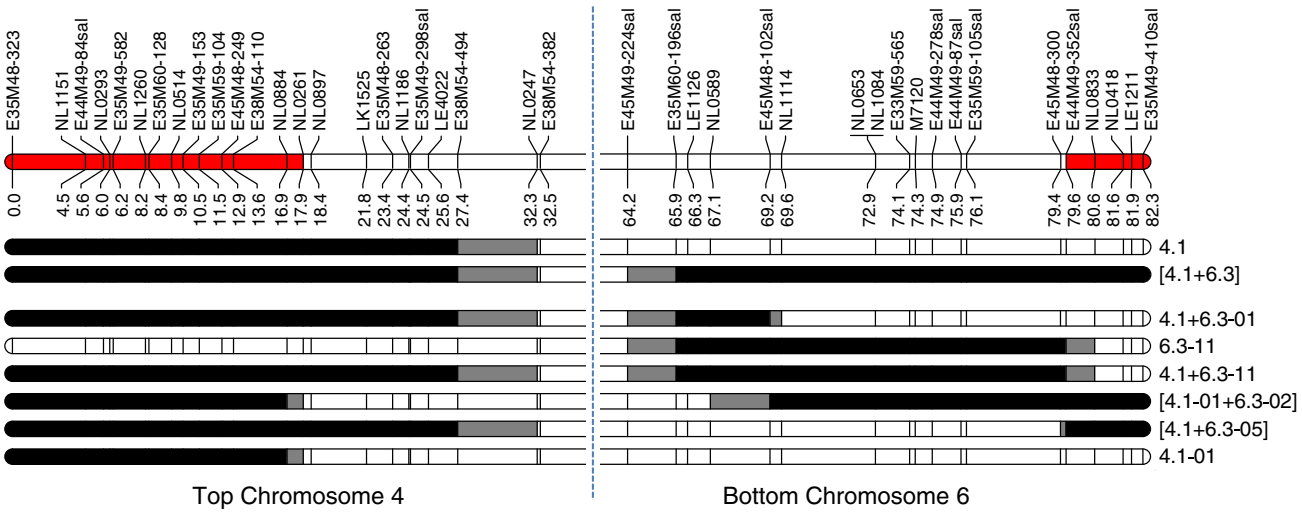


Fig. 2 Genotypes of sub-BILs derived from BIL[4.1+6.3]. *Black bar* represents genotype homozygous *L. saligna*, *white* means homozygous *L. sativa* and *gray* represents marker intervals where a recombination event resides. *Square brackets* around introgression segments, like for BIL[4.1+6.3], indicates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

spectrum. The following *B. lactucae* races were identified: Bl:22, 24, 25, 26 and three mixtures. The virulence spectrum of these three mixtures was complex and not informative enough to lead to the identification of the constituent races or of possibly novel races. The number of randomized replications, plants per replicate (8–25 plants), the location of the field test, the *B. lactucae* infection (natural or artificial and detected races), and the plant age at time of observation for each experiment are shown in Table S3. The infection severity level (ISL) per replicate was evaluated as an average infection score for whole plants in a scale from zero (no infection symptoms) to nine (maximum infection symptoms) on adult plants as described in Zhang et al. (2009a).

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

Gene action across loci analysis

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

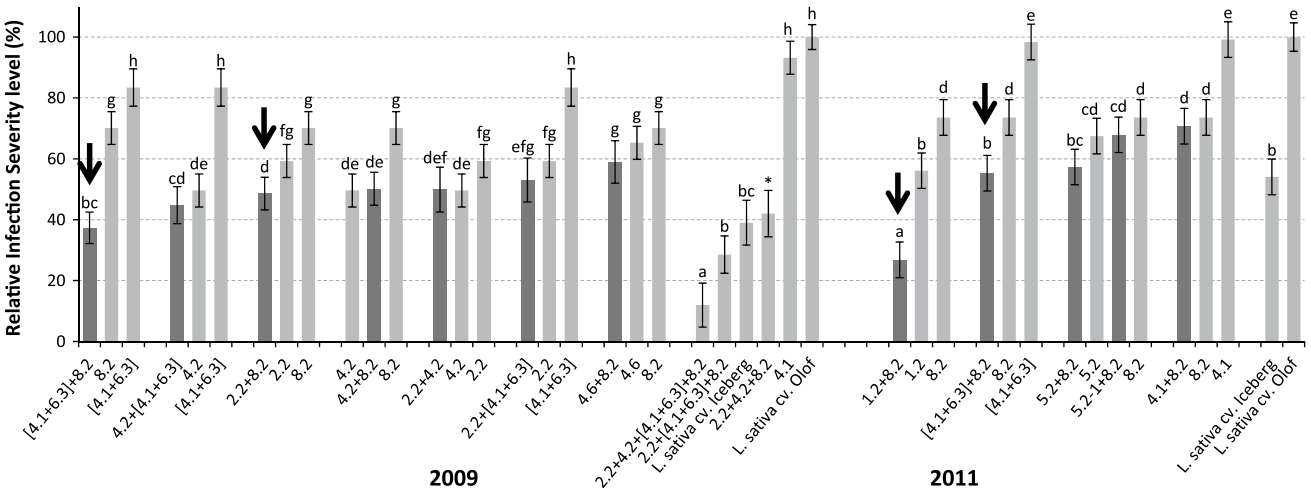


Fig. 3 Comparison of relative infection severity level (RIS) of combi-lines (stacked introgressions) with parental BILs (individual introgressions in field tests (ADT_F) in 2009 and 2011. For ease of comparison combi-line values (*dark grey bar*) are grouped with their parental line values (*light grey bar*). Control lines are aligned on the right hand per year. *Arrows* point to combi-line values that are significantly different from both parental BILs. Susceptible parent *L. sativa* cv. Olof has RIS 100 %. *Error bars* represent 95 % confidence intervals and *letters* in common indicate no significant difference (Duncan's multiple range test, $\alpha = 0.05$). *Combi-line2.2+4.2+8.2 was tested at three locations in 2008, RIS of this line was corrected to RIS in 2009 by its relative position between combi-line[4.1+6.3]+8.2 and *L. sativa* cv. Olof

Table 1 Infection level comparison of combi-line[4.1+6.3]+8.2 and its derived combi-lines with less and/or smaller introgression segments (color online)

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

Data of Fig. 4 are arranged to visualize the effect of stacking introgressions. See legend Fig. 4. Gradual color scale is used to visualize differences in RIS values, within each disease test type (from '*green*' low infection to '*yellow*' intermediate infection to '*red*' high infection). The susceptible control *L. sativa* cv. Olof has a RIS of 100 %. Within each column, RIS values followed by letters in common are not significantly different (Duncan's multiple range test, $\alpha = 0.05$). YDT means young plant disease test, ADT_F means adult plant disease test in the field

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

[b] Combi-line represents the line with the stacked introgression derived from a cross between line 1 and line 2, and its name is mentioned in the first column. For example: in combi-line4.1+8.2, line 1 is BIL4.1 and line 2 is BIL8.2

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

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

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

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

across loci was concluded to be epistatic. About the direction of gene action across loci: if the infection is further decreased (i.e. more resistant), the epistasis is positive; if the infection is increased (more susceptible), the epistasis is negative. About the magnitude of the effects: the additive effect is the sum of its individual components; the magnitude of positive epistatic effects is described as 'less-than-additive' or 'more-than-additive', respectively, as used in Eshed and Zamir (1996).

Results

Infection levels of stacked segments with quantitative resistance

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

The 14 combi-lines and their parental BILs were tested and evaluated in 2 sets (of 10 and 5 combi-lines) in field tests in 2009 (4 locations) and in field tests in 2011 (3 locations) (Table S2). In both years, five lines were in common: *L. sativa* cv. Olof, *L. sativa* cv. Iceberg, BIL4.1, BIL8.2 and combi-line[4.1+6.3]+8.2.

Three of the 12 field experiments (locations) had a lower overall infection level, but within the 3 locations similar differences between lines were observed as in the other 9 locations. The average ISL of susceptible control cv. Olof was in 9 locations value 7 or higher and in 3 locations around value 5 (see Table S3). Within each year, the field test results were highly correlated between

Table 2 Overview of additive and epistatic gene action effects of stacked introgression segments of BILs (a) and sub-BILs (b)

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

In general the effects of stacking are only analysed for gene action when the combi-line showed a deviating reduction in infection level from both individual parental lines (shown in Fig. 3; Table 1), except for combi-line2.2+4.2 as an example

YDT young plant disease test, ADT_F adult plant disease test in the field

[a] In brackets experimental year is indicated, in twentieth century, (09 = year 2009)

[b] Lines 1 and 2 represent the lines with a single introgression. Their numbering is based on the order in the name of the combi-line. For example: in combi-line1.2+8.2, line 1 is BIL1.2 and line 2 is BIL8.2. Significant reduction in relative infection severity levels (RRIS) (relative in percentage from susceptible parent *L. sativa* cv. Olof) are presented from line 1 and line 2. Non-significant reduction is indicated by 'ns' (Duncan's multiple range test, $\alpha = 0.05$)

[c] *Exp. Add.* expected RRIS when gene action across loci is additive (sum RRIS line 1 and line 2)

[d] *Obs.* observed RRIS from combi-line, RRIS is compared to expected and *P* values from LSD test are indicated: * <0.05, ** <0.01, *** <0.001

[e] Effect on RIS of combi-line compared to the individual lines

[f] Reduction in infection was not different from both individual parental lines (line 1 and 2)

[g] Both parental lines did not show an significant reduction in infection from *L. sativa* cv. Olof, therefore effect of additivity is not tested

[h] *ND* not defined because expected additive effect exceeds absolute resistance (RRIS higher than 100 %) and therefore the magnitude of the effect cannot be estimated

[i] An experiment × effect interaction was detected between three locations. For the individual locations the effects were: 1 × additive, 1 × less-than-additive, 1 × more-than-additive

[j] An experiment × effect interaction was detected between four locations. For the individual locations the effects were: 3 × additive and 1 × more-than-additive

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

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

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

Three combi-lines, 1.2+8.2, 2.2+8.2 and [4.1+6.3]+8.2, showed a significantly lower RIS than the most resistant parental BIL (Fig. 3). Combi-line1.2+8.2 had the lowest RIS (27 %), which was lower than that of the reference resistant control *L. sativa* cv. Iceberg. Combi-line[4.1+6.3]+8.2, with an average RIS of 48 % over 3 years, had a similar infection level as *L. sativa* cv. Iceberg. The RIS of combi-line2.2+8.2 was only 10 RIS

units (percentage points) lower than that of the RIS of BIL2.2. Stacking three introgressions did not result in significantly lower RIS than for the respective lines with two stacked introgressions, except for the comparison of 2.2+[4.1+6.3]+8.2 with 2.2+8.2. Stacking four introgressions, 2.2+4.2+[4.1+6.3]+8.2, resulted in a very low RIS of 12 %, which was significantly lower than that for the respective lines with three and two stacked introgressions (Fig. 3).

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

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

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

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

By comparison of their infection levels at young plant stage (Fig. 4c), we mapped this low infection level at Chromosome 6, between 79.4 and 80.6 cM. This conclusion is based on the observation that both combi-lines4.1+6.3-11 and sub-BIL[4.1+6.3-05] had a similar low RIS (18 and 13 %, respectively) and overlapping introgression region of 1.2 cM. The resistance on this short interval on 6.3 apparently interacts with a *L. saligna*-derived gene on 4.1, since a low RIS like in [4.1+6.3-05] and 4.1+6.3-11 was not found in the individually tested 6.3-11 (RIS 66 %) where the whole chromosome 4 was *L. sativa*-derived (Fig. 4c).

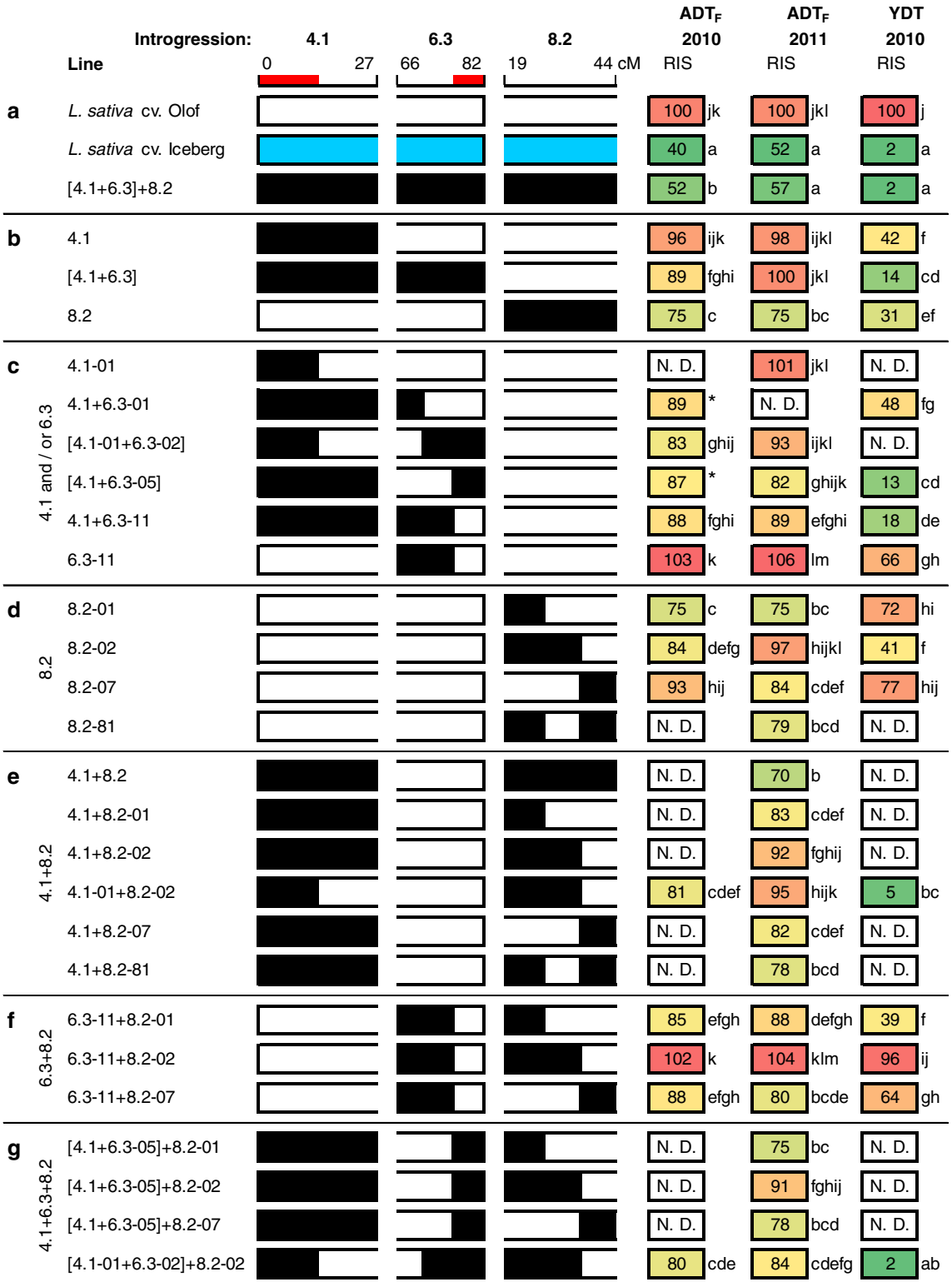
Fig. 4 Genetic dissection of the low infection level of combi-line[4.1+6.3]+8.2. The genotypes and infection severity levels of individual and stacked lines are shown. A schematic genotype presentation is shown for introgression segments 4.1, 6.3 and 8.2 at Chromosomes 4, 6 and 8, respectively; *bar color* represents the genotype of the introgression segments: *black* homozygous *L. saligna*; *white* homozygous *L. sativa* cv. Olof. The strong resistance control line *L. sativa* cv. Iceberg is shown in *blue*. Hybrid incompatibility loci are shown in *red map intervals*. Relative infection severity levels (RIS) are presented from five adult disease tests in field (ADT_F) in 2010, three ADT_F in 2011 and four young plant disease tests (YDT). *Gradual color scale* is used to visualize differences in RIS values, within each year (from '*green*' low infection to '*yellow*' intermediate infection to '*red*' high infection). *Letters* in common within each type of disease test, indicate no significant difference between the lines tested in 1 year (Duncan's multiple range test, $\alpha = 0.05$). *ND* not determined. *Asterisk* means lines were tested in two out of five ADT_F experiments in 2010 (in spring) (color figure online)

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

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

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

There were many interesting interactions between the stacked (sub-)introgressions. For example, one of the most resistant combi-lines in the YDT was combi-line4.1-01+8.2-02 (RIS 5 %), which was obviously more resistant than the line with 8.2-02 alone (RIS 41 %) and 4.1 alone (RIS 42 %). Strikingly, the resistance conferred by 8.2-02 alone (RIS 41 %) was completely cancelled, when combined with 6.3-11 in combi-line6.3-11+8.2-02 (RIS 96 %) (Fig. 4d, f). This high susceptibility in combi-line6.3-11+8.2-02 was at least partly due to a central segment of 8.2, since combi-lines6.3-11+8.2-01



and 6.3-11+8.2-07, which only differed from combi-line6.3-11+8.2-02 by shorter introgressions of 8.2, were again medium resistant (RIS 39 and 64 %, respectively) (Fig. 4f).

In 2010 and 2011, the set of lines was evaluated in field tests. The control lines and BILs had an infection level as expected (Zhang et al. 2009a). Within each year, the field test results were significantly correlated between all locations

(all correlations, $P = <0.001$; 2010: average $r = 0.82$; 2011: average $r = 0.66$) and therefore combined. Between years, the field test results were also significantly correlated for the 18 lines in common (correlation, $P = <0.001$, $r = 0.91$). Combi-line[4.1+6.3]+8.2 showed on average a RIS of 48 % over 3 years (2009: 37 %, 2010: 52 %, and 2011: 57 %; Figs. 3, 4a), which is similar to the RIS on our reference resistant line *L. sativa* cv. Iceberg (average RIS 43 %).

At the adult plant stage none of the sub-introgression combinations showed a significantly lower RIS than one of its parental lines and none of the lines had a similar low RIS as combi-line[4.1+6.3]+8.2 (Fig. 4; Table 1). Therefore, we were neither able to narrow down the high resistance of combi-line[4.1+6.3]+8.2 to smaller marker intervals nor to identify the underlying epistatic loci.

Discussion

The diversity in joint effects of quantitative resistances

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

The stacking of 4.1, 6.3 and 8.2 sub-introgressions resulted in some cases in superior levels of resistance at young plant stage. At adult plant stage in the field these combinations of sub-introgressions did not lead to as high levels of resistance as in combi-line[4.1+6.3]+8.2. Surprisingly, in one combination we found a negative epistatic interaction, i.e. two introgressions that individually conferred resistance but in combination (combi-line6.3-11+8.2-02 in YDT) resulted in high susceptibility. A variety of gene interactions across loci as found in the present study were also reported among quantitative traits in other plant species: QTLs for yield and yield trait components in rice showed both additive and epistatic effects (Zhuang et al. 2002), in rice epistatic interactions between heading date genes *Hd1* and *Hd2*, and *Hd2* and *Hd3* were reported in stacked QTL-NILs (Lin et al. 2000) and mainly additive effects but also epistatic with 'less-than-additive' effects (28 %) were detected in yield associated traits in tomato in 180 studied interactions (Eshed and Zamir 1996). In the scarce studies on stacking quantitative disease resistances also a variety of interactions between stacked QTLs were observed. In a stacking study of three resistance QTLs in wheat against *Fusarium* head blight, the best performing line showed a 'less-than-additive' epistatic interaction between two QTLs (Miedaner et al. 2006). Additive gene actions across loci were observed in two studies in barley: by stacking three QTLs to barley stripe rust at the adult plant stage (Castro et al. 2003) and by stacking two QTLs to barley leaf rust (Marcel et al. 2007). No (significant) additional decrease in infestation was observed when two root knot nematode (*Meloidogyne hapla*) resistance QTLs were combined in potato (Tan et al. 2009). Whether the lack of additive effects in QTLs for resistance as shown in our present study is rule or exception is not yet clear. It is conceivable that studies with no increased resistance effects by stacking have a lower probability to be published because of the negative result.

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

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

For the resistance at young plant stage, the interactive loci of [4.1+6.3]+8.2 seem to be located at the segments of combi-line4.1-01+8.2-02 that has three times less *L. saligna* genome than combi-line[4.1+6.3]+8.2 (3 vs. 9 % based on genetic map lengths) and a similar level of resistance. At young plant stage the 4.1 introgression interacts with other loci, like with the 1.2 cM interval on 6.3 and with 8.2-02, and thereby significantly reduces infection levels.

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

In the substitution mapping study of individual introgression segments 8.2, 2.2 and 4.2, similar plant stage dependence and complex inheritance, based on interactions between sub-QTLs or possibly CNVs, was observed (den Boer et al. 2013). Plant stage-dependent effectiveness of partial resistance genes has also been reported in other plant pathosystems like in barley-rust and barley-powdery mildew (Aghnoum et al. 2009; Wang et al. 2010).

Implications for breeding

The stacking of the resistance QTLs under study did not lead automatically to substantially increased levels of resistance. The effect of seven out of ten developed double introgression combinations did not deviate from that of individual introgressions (and did not lead to higher levels of resistance). For breeding, the joint effect of additive or epistatic 'more-than-additive' QTL effects, which is observed for combi-line1.2+8.2 and combi-line[4.1+6.3]+8.2, respectively, are the most valuable/interesting. The genetic dissection of combi-line[4.1+6.3]+8.2 led to lines with lower *L. saligna* genome percentages and similar low infection levels in young plant stage, but not in field test at adult plant stage. The complex inheritance for field resistance of combi-line[4.1+6.3]+8.2 makes this line unpromising for practical application in breeding.

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

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

Non-host resistance from *L. saligna*

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

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

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

The observed diverse interactions between and within (sub-)introgressions on resistance levels of lettuce to *B. lactucae* might suggest the following: (1) the genetic basis of the non-host resistance from *L. saligna* CGN05271 is very complex, and/or (2) epistatic and/or additive interactions between yet untested combinations of genes explain non-host resistance, and/or (3) non-host resistance of *L. saligna* is caused by a gene(s) of which the action was not discovered in the set of BILs (for instance because of 5 % missing *L. saligna* genome in the set of BIL, and/or due to close linkage to regions involved in hybrid incompatibilities). The QTLs detected in the BILs and in the present study may affect physiological qualities of the plants, leading to rather marginal variation in levels of suitability as nutrient source for *B. lactucae*.

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

Acknowledgments The authors acknowledge support from the foundation TTI Green Genetics (TTI-GG), and contributions in field tests by five breeding companies. The authors want to thank Chris Maliepaard for suggestions on the statistical analysis.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The experiments comply with the current laws of the Netherland, in which they were performed.

References

Aghnoum R, Marcel TC, Johrde A, Pecchioni N, Schweizer P, Niks RE (2009) Basal host resistance of barley to powdery mildew: connecting quantitative trait loci and candidate genes. Mol Plant Microbe Interact 23:91–102

Bonnier FJM, Reinink K, Groenwold R (1991) New sources of major gene resistance in *Lactuca* to *Bremia lactucae*. Euphytica 61:203–211

Breen MS, Kemena C, Vlasov PK, Notredame C, Kondrashov FA (2012) Epistasis as the primary factor in molecular evolution. Nature 490:535–538

Carlborg O, Haley CS (2004) Epistasis: too often neglected in complex trait studies? Nat Rev Genet 5:618–625

Castro AJ, Chen X, Corey A, Filichkina T, Hayes PM, Mundt C, Richardson K, Sandoval-Islas S, Vivar H (2003) Pyramiding and validation of quantitative trait locus (QTL) alleles determining resistance to barley stripe rust: effects on adult plant resistance. Crop Sci 43:2234–2239

Clair DA (2010) Quantitative disease resistance and quantitative resistance loci in breeding. Annu Rev Phytopathol 48:247–268

Cook DE, Lee TG, Guo X, Melito S, Wang K, Bayless AM, Wang J, Hughes TJ, Willis DK, Clemente TE, Diers BW, Jiang J, Hudson ME, Bent AF (2012) Copy number variation of multiple genes at *Rhg1* mediates nematode resistance in soybean. Science 338(6111):1206–1209

Coupland G (1995) Genetic and environmental control of flowering time in Arabidopsis. Trends Genet 11:393–397

den Boer E, Zhang NW, Pelgrom K, Visser RGF, Niks RE, Jeuken MJW (2013) Fine mapping quantitative resistances to downy mildew in lettuce revealed multiple sub-QTLs with plant stage dependent effects reducing or even promoting the infection. Theor Appl Genet 126:2995–3007

Ding H-F, Li G-X, Liu X, Jiang M-S, Li R-F, Wang W-Y, Zhang Y, Zhang X-D, Yao F-Y (2010) Detection of epistatic interactions of three QTLs for heading date in rice using single segment substitution lines. Russ J Plant Physiol 57:137–143

Eshed Y, Zamir D (1996) Less-than-additive epistatic interactions of quantitative trait loci in tomato. Genetics 143:1807–1817

Grube R, Ochoa O (2005) Comparative genetic analysis of field resistance to downy mildew in the lettuce cultivars 'Grand Rapids' and 'Iceberg'. Euphytica 142:205–215

Jeuken MJ, Lindhout PL (2002) *Lactuca saligna*, a non-host for lettuce downy mildew (*Bremia lactucae*), harbors a new race-specific *Dm* gene and three QTLs for resistance. Theor Appl Genet 105:384–391

Jeuken MJW, Lindhout P (2004) The development of lettuce backcross inbred lines (BILs) for exploitation of the *Lactuca saligna* (wild lettuce) germplasm. Theor Appl Genet 109:394–401

Jeuken M, van Wijk R, Peleman J, Lindhout P (2001) An integrated interspecific AFLP map of lettuce (*Lactuca*) based on two *L. sativa* × *L. saligna* F_2 populations. Theor Appl Genet 103:638–647

Jeuken M, Pelgrom K, Stam P, Lindhout P (2008) Efficient QTL detection for nonhost resistance in wild lettuce: backcross inbred lines versus F_2 population. Theor Appl Genet 116:845–857

Jeuken MJW, Zhang NW, McHale LK, Pelgrom K, den Boer E, Lindhout P, Michelmore RW, Visser RGF, Niks RE (2009) *Rin4* causes hybrid necrosis and race-specific resistance in an interspecific lettuce hybrid. Plant Cell 21:3368–3378

Lebeda A, Boukema IW (1991) Further investigation of the specificity of interactions between wild *Lactuca* spp. and *Bremia lactucae* isolates from *Lactuca serriola*. J Phytopathol 133:57–64

Lin HX, Yamamoto T, Sasaki T, Yano M (2000) Characterization and detection of epistatic interactions of 3 QTLs, Hd1, Hd2, and Hd3, controlling heading date in rice using nearly isogenic lines. Theor Appl Genet 101(7):1021–1028

Marcel TC, Aghnoum R, Durand J, Varshney RK, Niks RE (2007) Dissection of the barley 2L1.0 region carrying the 'Laevigatum' quantitative resistance gene to leaf rust using near-isogenic lines (NIL) and subNIL. Mol Plant Microbe Interact 20:1604–1615

McHale L, Truco M, Kozik A, Wroblewski T, Ochoa O, Lahre K, Knapp S, Michelmore R (2009) The genomic architecture of disease resistance in lettuce. Theor Appl Genet 118:565–580

Miedaner T, Wilde F, Steiner B, Buerstmayr H, Korzun V, Ebmeyer E (2006) Stacking quantitative trait loci (QTL) for *Fusarium* head blight resistance from non-adapted sources in an European elite spring wheat background and assessing their effects on deoxynivalenol (DON) content and disease severity. Theor Appl Genet 112:562–569

Tan M, Alles R, Hutten R, Visser R, van Eck H (2009) Pyramiding of *Meloidogyne hapla* resistance genes in potato does not result in an increase of resistance. Potato Res 52:331–340

Wang H, Qi M, Cutler AJ (1993) A simple method of preparing plant samples for PCR. Nucleic Acids Res 21:4153–4154

Wang L, Wang Y, Wang Z, Marcel TC, Niks RE, Qi X (2010) The phenotypic expression of QTLs for partial resistance to barley leaf rust during plant development. Theor Appl Genet 121:857–864

Yano M, Sasaki T (1997) Genetic and molecular dissection of quantitative traits in rice. Plant Mol Biol 35:145–153

Zhang NW (2008) PhD-thesis. Genetic dissection of nonhost resistance of wild lettuce, *L. saligna*, to downy mildew. Wageningen University and Research Center, Wageningen

Zhang NW, Lindhout P, Niks RE, Jeuken MJW (2009a) Genetic dissection of *Lactuca saligna* nonhost resistance to downy mildew at various lettuce developmental stages. Plant Pathol 58:923–932

Zhang NW, Pelgrom KTB, Niks RE, Visser RGF, Jeuken MJW (2009b) Three combined quantitative trait loci from nonhost *Lactuca saligna* are sufficient to provide complete resistance of lettuce against *Bremia lactucae*. Mol Plant Microbe Interact 22:1160–1168

Zhuang JY, Fan YY, Rao ZM, Wu JL, Xia YW, Zheng KL (2002) Analysis on additive effects and additive-by-additive epistatic effects of QTLs for yield traits in a recombinant inbred line population of rice. Theor Appl Genet 105:1137–1145