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ORIGINAL PAPER

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

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

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Abstract

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

Abstract Previous studies on the genetic dissection of the complete resistance of wild lettuce, *Lactuca saligna*, to downy mildew revealed 15 introgression regions that conferred plant stage dependent quantitative resistances (QTLs). Three backcross inbred lines (BILs), carrying an individual 30–50 cM long introgression segment from *L. saligna* in a cultivated lettuce, *L. sativa*, background, reduced infection by 60–70 % at young plant stage and by 30–50 % at adult plant stage in field situations. We studied these three quantitative resistances in order to narrow down their mapping interval and determine their number of loci, either single or multiple. We performed recombinant screenings and developed near isogenic lines (NILs) with smaller overlapping *L. saligna* introgressions (substitution mapping). In segregating introgression line populations, recombination was suppressed up to 17-fold compared to the original *L. saligna* × *L. sativa* F_2 population. Recombination suppression depended on the chromosome region and was stronger suppressed at the smallest introgression lengths. Disease evaluation of the NILs revealed that the resistance of all three BILs was not explained by a single locus but by multiple sub-QTLs. The 17 *L. saligna*-derived sub-QTLs had a smaller and plant stage dependent resistance effect, some segments reducing; others even promoting downy mildew infection. Implications for lettuce breeding are outlined.

Abbreviations

BIL	Backcross inbred line (*L. saligna* introgression, 20–80 cM long, in a lettuce, *L. sativa*, genetic background)
NIL	Near isogenic line (*L. saligna* introgression, <20 cM long, in a lettuce, *L. sativa*, genetic background)
QTL	Quantitative trait locus
SDT	Seedling disease test
YDT	Young plant disease test
ADT_G	Adult plant disease test in the greenhouse
ADT_F	Adult plant disease test in the field

Introduction

Most knowledge on resistance in plants to (hemi)-biotrophic specialized plant pathogens exists on race-specific qualitative resistance that is conferred by *R* genes with the NBS-LRR (nuclear binding site and leucine-rich repeat) motifs of which many are nowadays cloned (Gururani et al. 2012). This resistance by *R* genes relies on the direct or indirect recognition of pathogen-delivered effectors. Much less is known about the genes underlying polygenic and/or quantitative and/or non-race specific resistance, of which

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E. den Boer and N. W. Zhang contributed equally to this paper.

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E. den Boer · N. W. Zhang · K. Pelgrom · R. G. F. Visser · R. E. Niks · M. J. W. Jeuken (✉)
Laboratory of Plant Breeding, Wageningen University, P.O.Box 386, 6700 AJ Wageningen, The Netherlands
e-mail: marieke.jeuken@wur.nl

very few genes are cloned like e.g. *mlo, Yr36*, *Pi21, Lr34* and *Rgh4* (Büschges et al. 1997; Fu et al. 2009; Fukuoka et al. 2009; Krattinger et al. 2009; Liu et al. 2012). The resistance conferred by genes like *Lr34*, *Lr46* and the *mlo* gene, which are not classical *R*-genes, seems to be durable as the resistance remains effective for a long time (Fu et al. 2009; Fukuoka et al. 2009; Jørgensen 1992; Kolmer 1996; Lillemo et al. 2008; Risk et al. 2012; William et al. 2003).

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

Earlier research included histological and genetic studies. Histology on the infection process indicated that the resistance response of *L. saligna* was based mainly on pre-hyphal resistance (Zhang et al. 2009b). Research on a small F_2 population of 126 plants revealed three resistance QTLs, each explaining 12–26 % of phenotypic variance, and a resistance caused by a digenic interallelic interaction that leads to hybrid necrosis (Jeuken and Lindhout 2002; Jeuken et al. 2009). Fertility limitations of the F_2 hampered further inbreeding, and prompted us to develop a set of 29 backcross inbred lines (BILs) (Jeuken and Lindhout 2004). Those homozygous introgression lines, with *L. saligna* introgression segments from 20 to 80 cM in a *L. sativa* background, represented together 96 % of the *L. saligna* genome. The BILs were tested in four types of disease test, namely on seedlings (SDT), young plants (YDT), adult plants in the greenhouse (ADT_G) and adult plants in the field (ADT_F) (Zhang et al. 2009a). Fifteen BILs with quantitative resistance were detected. The three F_2-QTLs were not confirmed in this set of BILs (Jeuken et al. 2008). Of the fifteen resistant BILs, only two BILs, 2.2 and 4.2, showed resistance at all plant stages. BIL8.2 showed resistance in young and adult plant tests, but not in seedling tests (Zhang et al. 2009a). BIL 2.2, 4.2, and 8.2 showed an infection reduction of 60–70 % at young plant stage and 30–50 % at adult plant stage in field situations (most relevant for lettuce cultivation). Those three BILs were selected for further fine mapping (Zhang et al. 2009a). A preliminary study on stacking resistances showed that some combined introgression segments of BIL2.2, 4.2 and 8.2 led at young plant stage to an increased level of resistance compared to their respective individual segments (Zhang et al. 2009b). Stacking large *L. saligna* introgressions implies that also more genes for undesired traits are introgressed. Therefore, lines with smaller introgressions with only the gene conferring the resistance trait from *L. saligna* are preferred for further stacking strategies in resistance breeding.

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

Materials and methods

Plant material

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

Genetic map

An extended genetic map from a F_2 population ($n = 126$) between *L. saligna* CGN05271 × *L. sativa* cv. Olof is available and contains about 1,000 markers. The markers are rather evenly spread over the linkage groups without clear clustering (Jeuken et al. 2001). The set of 29 BILs was previously genotyped with 780 markers, and *L. saligna*-derived alleles were only detected on the expected BIL segments, therefore it is unlikely that the BILs contain besides the original selected introgression segment additional *L. saligna* introgression segments.

Map saturation

To saturate the three BIL introgression regions with markers, additional markers were developed and initially their

positions were mapped on the F_2 population ($n = 126$), (Jeuken et al. 2001). New amplified fragment length polymorphism (AFLP) assays with two primer combinations E48M59 (selective nucleotides CAC and CTA) and E33M59 (selective nucleotides AAG and CTA) were performed. EST markers were developed on lettuce EST sequences from the *Compositae* Genome Project Database (CGPDB, compgenomics.ucdavis.edu) (McHale et al. 2009) and on EST sequences provided by R. Michelmore (Davis, California, USA). Additionally, SSR markers were developed and mapped by Syngenta Seeds B.V, The Netherlands.

To saturate our three target introgression regions with EST based markers, we aligned our F_2 map with the latest version of the RIL (Salinas × *L. serriola*) map (CGPDB) and selected and tested the EST sequences in intervals between common markers within the introgression segment regions for polymorphisms.

Recombinant screening and line development

Recombinant screenings were performed to obtain plant genotypes with smaller, overlapping *L. saligna* introgressions than in the parental BILs. To select recombinant plants that have a crossover site within the introgression, we used the selfed segregating populations from the original heterozygous recombinant backcross-plant (also called preBIL) that was used to develop the homozygous BIL. Two co-dominant PCR-markers nearest to the ends of the introgression were used to genotype the plants and screen for recombinants. Per introgression segment an initial recombinant screening on 200–400 plants was performed, and for the 8.2 introgression additional recombinant screenings on 5,148 plants were performed on earlier detected recombinant plants.

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

DNA extraction and genotyping

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

The polymorphisms of the PCR products from the EST and SSR markers were initially visualised by size differences on agarose gels (directly or after enzymatic digestion) as previously described (Jeuken et al. 2008) and later visualized by high-resolution melting curve differences on a LightScanner System (Idaho Technology, USA). AFLP analyses were run as described previously (Jeuken et al. 2001; Vos et al. 1995).

Disease evaluation

Plant materials used in the disease evaluation are shown in Table 1. Three to nine independent young plant disease tests (YDT) were performed on each line as described (Jeuken et al. 2008; Zhang et al. 2009a). In each experiment six plants per line were used and at 8–10 dpi the infection severity level (ISL) as percentage of sporulating area was evaluated on two leaves. We applied *B. lactucae* race Bl:14 on all YDT, except on one experiment in 2009 and two experiments in 2010 where we applied Bl:21.

Fourteen adult plant disease tests in the field (ADT_F) were performed by breeding companies at seven locations in the autumn of 2008, 2009, 2010 and 2011 (Table 2). Artificial or natural infection or both occurred. Symptoms of *B. lactucae* infection were recognized by at least two independent and experienced observers. Infected leaf material was collected and the isolates were tested for their resistance spectrum on a differential set. The following *B. lactucae* races were identified: Bl:18, 22, 24, 25, 26 and four mixtures. The resistance spectrum of these four mixtures was complex and not informative enough to lead to the identification of a mix of individual races or of possibly novel isolates. The number of randomized replications, plants per replicate (8–25 plants), the location of the field test, the *B. lactucae* infection (natural or artificial and detected races), and the plant age at time of observation for each experiment are shown in Table 2. For the 2.2 and 4.2 introgression one set of NILs was tested in 2008. For the 8.2 introgression four different sets of NILs were tested in 2008, 2009, 2010, and 2011. The following 8.2 NILs were tested in all four sets (years): NIL8.2–01, 02, 06 and 07 (more details in Table 2). Within each year identical sets were tested in all locations. The ISL per replicate was evaluated as an average infection score for whole plants in a scale from zero (no infection symptoms) to nine (maximum infection symptoms) on adult plants.

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

Lines used per introgression region	YDT		ADT_F	
2.2 introgression	Year exp.[a]	Replicates[b]	Year exp.[a]	Replicates[c]
L. sativa Olof, BIL2.2, NIL2.2–01 to NIL2.2-11	08	3 × 6 = 18	08	18
BIL2.1 and BIL2.3	08	3 × 6 = 18	N.D.	N.D.
4.2 introgression				
L. sativa Olof, BIL4.2, NIL4.2-01 to NIL4.2-11	08	3 × 6 = 18	08	18
BIL4.1 and BIL4.3	08	3 × 6 = 18	N.D.	N.D.
8.2 introgression				
L. sativa Olof, BIL8.2, NIL8.2-01, 02, 06	08; 09; 10	9 × 6 = 54	08; 09; 10; 11	76
BIL8.1	08; 09; 10	9 × 6 = 54	09; 10; 11	58
NIL8.2-03	08; 09	6 × 6 = 36	09	22
NIL8.2-04, 09, 10, 11	08	3 × 6 = 18	08; 09	40
NIL8.2-05	08; 09	6 × 6 = 36	08; 09	40
NIL8.2-07	08; 09	6 × 6 = 36	08; 09; 10; 11	76
NIL8.2-08	08; 09; 10	9 × 6 = 54	08; 09	40
NIL8.2-12	08	3 × 6 = 18	09	22
NIL8.2-13, 14, 15 to 21, 24, 26	09	3 × 6 = 18	09	22
NIL8.2-14, 22, 23, 25, 27	09; 10	6 × 6 = 36	09; 10	40
NIL8.2-28, 29, 33, 37, 38, 39, 40, 41, 44, 49, 52, 56, 58, 60, 62, 64, 65, 66, 70, 71, 75, 79	10	3 × 6 = 18	10	18
NIL8.2-81	N.D.	N.D.	10; 11	36
NIL8.2-80, 82, 83	N.D.	N.D.	10	18
NIL8.2-59, 63, 73, 84, 85	N.D.	N.D.	11	18
NIL8.2-201 to NIL8.2–217	N.D.	N.D.	11	18

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

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

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

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

Data analysis of disease tests

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

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

Another analysis was executed in which at each marker locus the average ISL were compared between the group of lines with the homozygous *L. sativa* genotype and the group with the homozygous *L. saligna* genotype using the same mixed model analysis as described above. *P* values from this analysis were graphed as −log (*P*) or as log (*P*) when the homozygous *L. saligna* or homozygous *L. sativa* genotype respectively showed the lowest average ISL (Fig. 2, S1 and S2). Therefore the highest or lowest peaks, that exceed the threshold levels at $\alpha = 0.01 = -\text{LOG } 2$ and LOG-2, indicate the most likely QTL positions and indicate the allele associated with the resistance (Monforte and Tanksley 2000). All statistics was calculated by statistical package IBM SPSS statistics version 19 or GenStat 14.

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

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

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

[a] 'mix.' or 'mixture' means that individual races could not be determined because of a complex mixture of races or due to presence of not described and/or new isolates

[b] In 2008 the correlations are shown individually for the different sets of NILs from the 2.2, 4.2, and 8.2 introgression segments respectively

Results

Recombinant screening and development of NILs

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

The recombination frequency in 8.2-preNILs was 2–3.5 times more suppressed than in the preBIL8.2, suggesting that the smaller the introgression, the larger the suppression of recombination (Table 3). A nine times higher recombination frequency was observed for the double recombinant preNIL8.2–73 with a ~10 cM *L. sativa* segment between two *L. saligna* segments of 3.0 and 8.5 cM, compared to its counterpart preNIL8.2–6 with an 21.5 cM introgression with equal outermost introgression extremities and no intermediate *L. sativa* segment (Table 3). The recombination frequency within preNIL8.2–73 was even two times higher than in the *L. saligna* × *L. sativa* F_2 population (Table 3). This increased recombination frequency suggests that crossovers occur at much higher frequency in a homozygous segment (from *L. sativa*) than in a chromosome stretch that is heterozygous and non-homologous (one homologue from *L. sativa* and one from *L. saligna*).

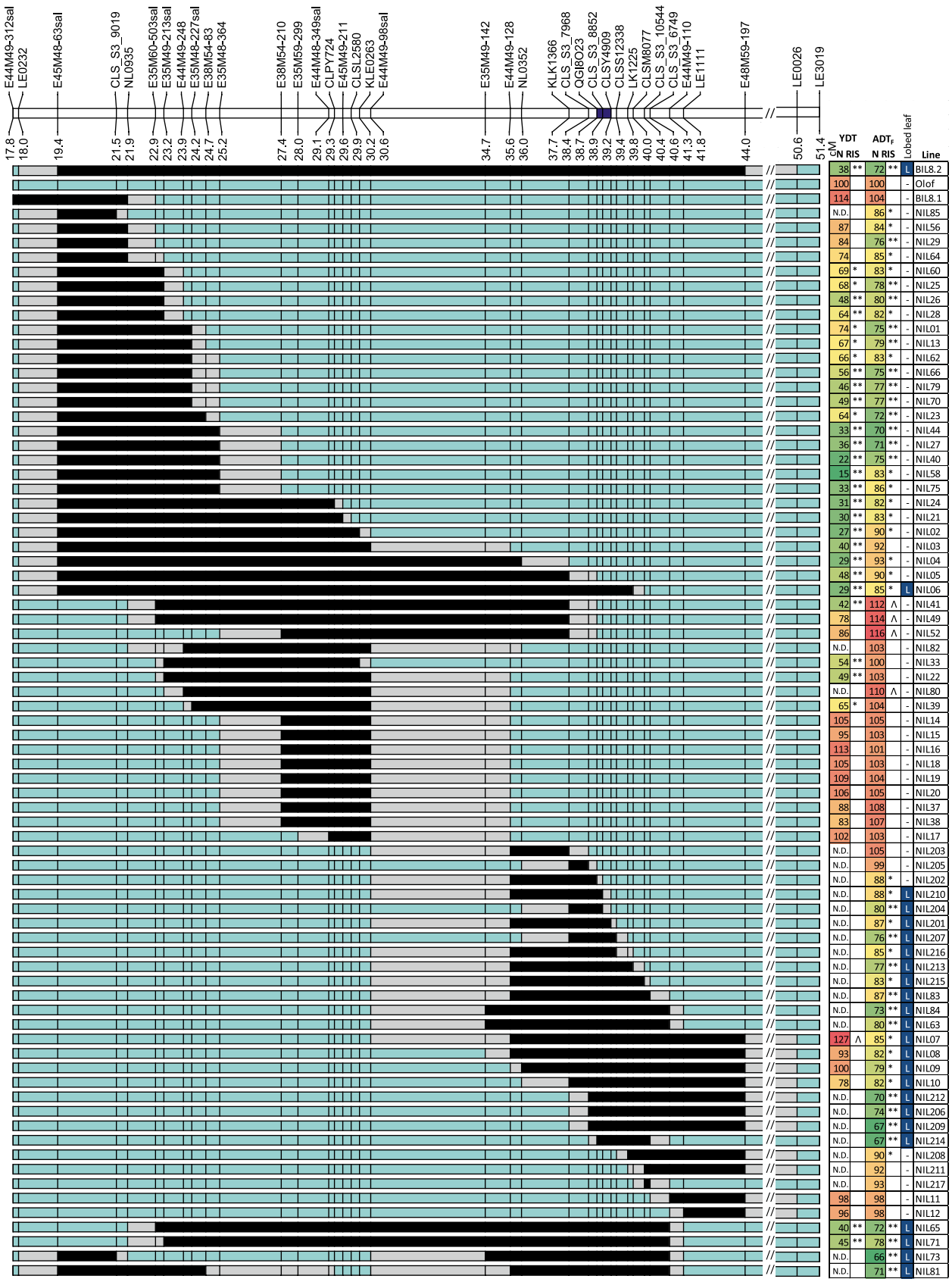
Fig. 1 Genotypes and disease evaluations at young plant (YDT) and adult plant stage (ADT_F) of lettuce NILs with smaller *L. saligna* introgressions than in BIL8.2. Genetic map of Chromosome 8, 17–51 cM and genotype graphs of tested lines. *Light blue bars* represent homozygous *L. sativa*, *solid black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

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

Disease evaluations at young and adult plant stage

The three BILs,the recurrent parent *L. sativa* cv. Olof and additional control lines showed in all YDT and ADT_F a similar relative infection level as in previous experiments (Fig. 3, Zhang et al. 2009a). Between YDT experiments the new NILs showed similar relative infection levels (RIS). For ADT_F the new NILs showed similar RIS between locations within a year and between the years. Similar preliminar QTL positions were observed between individual experiments of YDT and between individual experiments of ADT_F, with occasional variances in the strength of the effect (details of final QTL positions of pooled data are described in the next paragraphs).

Some infection level differences were observed between ADT_F experiments, but those were mainly due to the evaluation moment. Low infestation levels resulted in large differences, and high infection levels in smaller differences between susceptible and quantitatively resistant lines (Table 2). In all experiments significant differences between the NILs, BIL and *L. sativa* cv. Olof were observed. Between the different experiments within YDT and within ADT_F the ISL of the lines were significantly correlated ($r = 0.5 - 0.9$, Table 2), even though different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LE0232
E45M48-63sal
CLS_S3_9019
NL0935
E35M60-503sal
E35M49-213sal
E44M49-248
E35M48-227sal
E38M54-83
E35M48-364
E38M54-210
E35M59-299
E44M48-349sal
CLPY724
E45M49-211
CLSL2580
KLE0263
E44M49-98sal
E35M49-142
E44M49-128
NL0352
KLK1366
CLS_S3_7968
QGI8O23
CLS_S3_8852
CLSY4909
CLSS12338
LK1225
CLSM8077
CLS_S3_10544
CLS_S3_6749
E44M49-110
LE1111
E48M59-197
LE0026
LE3019
cM
YDT
N RIS
ADT_F
N RIS
Lobed leaf
Line
17.8 18.0 19.4 21.5 21.9 22.9 23.2 23.9 24.2 24.7 25.2 27.4 28.0 29.1 29.3 29.6 29.9 30.2 30.6 34.7 35.6 36.0 37.7 38.4 38.7 38.9 39.2 39.4 39.8 40.0 40.4 40.6 41.3 41.8 44.0 50.6 51.4
38 ** 72 ** L BIL8.2
100 100 - Olof
114 104 - BIL8.1
N.D. 86 * - NIL85
87 84 * - NIL56
84 76 ** - NIL29
74 85 * - NIL64
69 * 83 * - NIL60
68 * 78 ** - NIL25
48 ** 80 ** - NIL26
64 ** 82 * - NIL28
74 * 75 ** - NIL01
67 * 79 ** - NIL13
66 * 83 * - NIL62
56 ** 75 ** - NIL66
46 ** 77 ** - NIL79
49 ** 77 ** - NIL70
64 * 72 ** - NIL23
33 ** 70 ** - NIL44
36 ** 71 ** - NIL27
22 ** 75 ** - NIL40
15 ** 83 * - NIL58
33 ** 86 * - NIL75
31 ** 82 * - NIL24
30 ** 83 * - NIL21
27 ** 90 * - NIL02
40 ** 92 - NIL03
29 ** 93 * - NIL04
48 ** 90 * - NIL05
29 ** 85 * L NIL06
42 ** 112 Λ - NIL41
78 114 Λ - NIL49
86 116 Λ - NIL52
N.D. 103 - NIL82
54 ** 100 - NIL33
49 ** 103 - NIL22
N.D. 110 Λ - NIL80
65 * 104 - NIL39
105 105 - NIL14
95 103 - NIL15
113 101 - NIL16
105 103 - NIL18
109 104 - NIL19
106 105 - NIL20
88 108 - NIL37
83 107 - NIL38
102 103 - NIL17
N.D. 105 - NIL203
N.D. 99 - NIL205
N.D. 88 * - NIL202
N.D. 88 * L NIL210
N.D. 80 ** L NIL204
N.D. 87 * L NIL201
N.D. 76 ** L NIL207
N.D. 85 * L NIL216
N.D. 77 ** L NIL213
N.D. 83 * L NIL215
N.D. 87 ** L NIL83
N.D. 73 ** L NIL84
N.D. 80 ** L NIL63
127 Λ 85 * L NIL07
93 82 * L NIL08
100 79 * L NIL09
78 82 * L NIL10
N.D. 70 ** L NIL212
N.D. 74 ** L NIL206
N.D. 67 ** L NIL209
N.D. 67 ** L NIL214
N.D. 90 * - NIL208
N.D. 92 - NIL211
N.D. 93 - NIL217
98 98 - NIL11
96 98 - NIL12
40 ** 72 ** L NIL65
45 ** 78 ** L NIL71
N.D. 66 ** L NIL73
N.D. 71 ** L NIL81

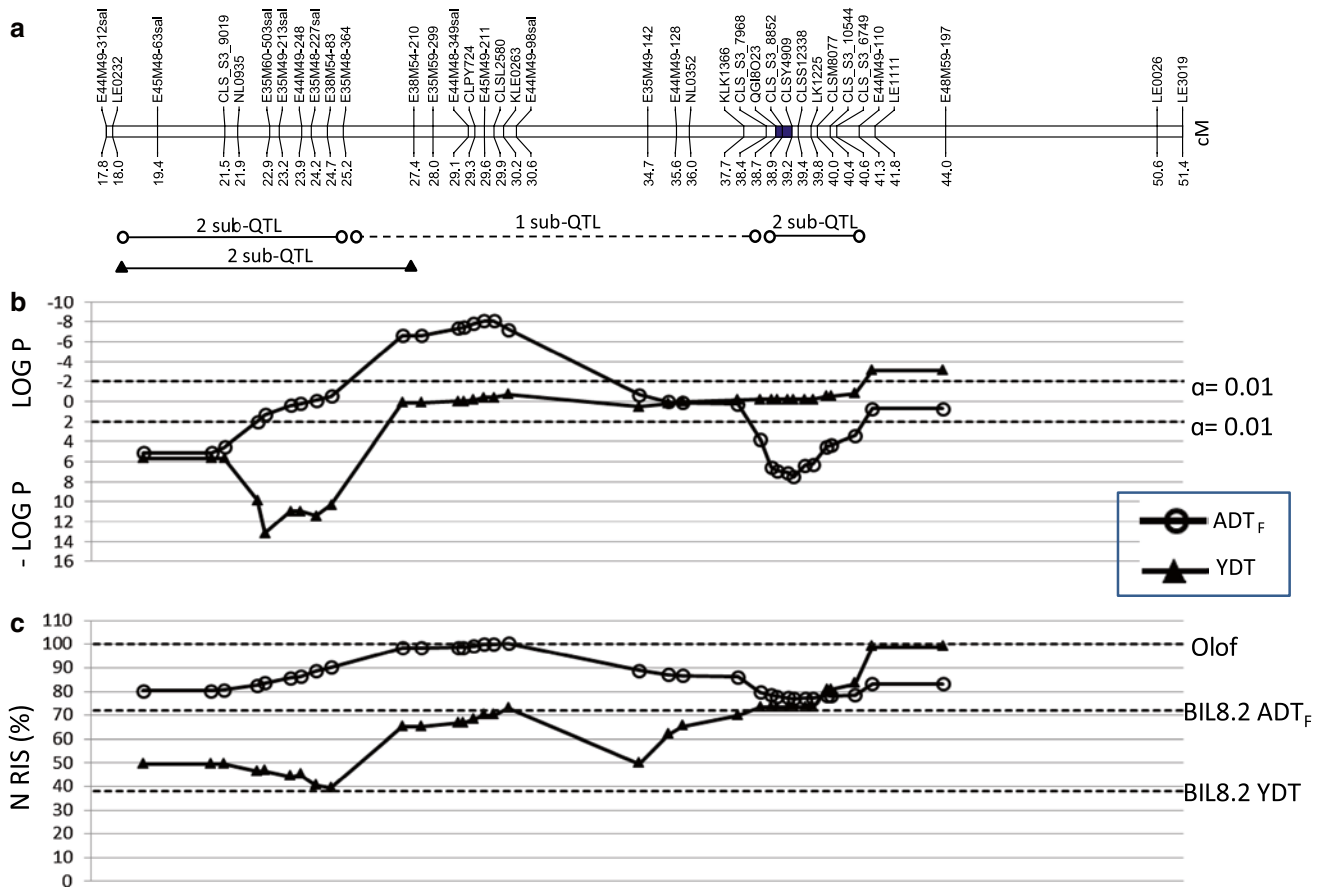

Fig. 2 Fine mapping QTLs within the *L. saligna* introgression of BIL8.2 at the young and adult plant stage. Four graphs are aligned according marker positions. From top to bottom: **a** genetic map of Chromosome 8, 17–51 cM; Locations of sub-QTL regions for both the ADT_F (*lines with open circle*)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

B. lactucae races were used. Based on these and earlier field experiments with six different isolates (Zhang et al. 2009a), we assume that the resistances are race nonspecific. When comparing natural or artificial infections for ADT_F, similar correlations were observed between natural × natural (average $r = 0.8$, $n = 4$), natural × artificial (average $r = 0.7$, $n = 9$) and artificial × artificial ($r = 0.7$, $n = 1$), which implies no inoculation method effects (Table 2). The significantly correlated experiments allowed us to pool the data from the different experiments with the same lines within YDT and within ADT_F. The correlation of the ISL of the common NILs between the YDT and ADT_F was rather low, 2.2 $r = 0.52$, 8.2 $r = 0.39$ and 4.2 $r = 0.67$. This result suggests that the genes for resistance are growth stage specific in their effect. The data from the two different plant stages (YDT and ADT_F) was not pooled but analysed separately.

Mapping resistance loci within the 8.2 introgression

Preliminary results from YDT (Zhang et al. 2008) and new ADT_F results in 2008 with the first set of 12 NILs indicated two sub-regions involved in resistance. One sub-region was effective at both young and adult plant stage and ranges from ~19 to 30 cM (lower RIS in YDT and ADT_F for NIL01, 02, 03, 04, 05, and 06 in Fig. 1). The other sub-region was effective at adult plant stage only and ranges from ~36 to 41 cM (lower RIS in ADT_F for NIL07, 08, 09 and 10 in Fig. 1). Lines with a smaller heterozygous introgression than BIL8.2, overlapping these two sub-regions

Table 3 Summary of recombination screenings and their recombination frequencies Rec. = Recombinant; Rec. freq. = recombinant frequency; Rec. suppr. = times recombination suppression compared to the F2 population

Parental plant	Chr.	Fig. L. saligna intr. and marker pos.[a]	*L. saligna* intr. length # cM	Dist. betw. rec. scr. markers # cM	Plants #	Rec.[b] #	Rec. freq. per cM BIL/NIL	Rec. freq. per cM F_2	Rec suppre. BIL/NIL vs. F_2[c]
preBIL2.2	C2	8 9	28.5	20.8	442	11	0.0006	0.0091	15.2 × * †
preBIL4.2	C4	10 // 11	49.2	29.3	258	8	0.0005	0.0092	17.3 × * †
preBIL8.2	C8	3 7	28.6	11.6	361	29	0.0035	0.0074	2.1 × *
preNIL8.2-03[d]	C8	2 3	14.4	8.3	1267	45	0.0021	0.0077	3.6 × *
preNIL8.2-05	C8	2 4	19.5	14.1	348	20	0.0020	0.0077	3.8 × *
preNIL8.2-07,10,83	C8	5 6	10.8[e]	2.9	3047	17	0.0010	0.0070	7.2 × * †
preNIL8.2-06[d]	C8	2 4	21.5	14.1	359	17	0.0017	0.0077	4.6 × * † ^
preNIL8.2-73[f]	C8	1 4	3.0+8.5	14.5	127	65	0.0176	0.0081	0.5 × * † ^

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

[b] All recombinants were single cross-overs except for three double recombinants in the offspring of preNIL8.2-73

[c] Fisher's exact test on recombination suppression differences between populations, α = 0.05: Recombinant frequency per cM is significantly different between preBIL/preNIL offspring and F_2 population*; between offspring preBIL/preNIL and preBIL8.2†; between offspring preNIL8.2-73^ and preNIL8.2-06

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

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

[f] The preNIL8.2-73 progeny segregation revealed that preNIL8.2-73 has two cross-over events in coupling phase (*cis*), originating from one recombinant gamete with two cross-over events instead of two recombinant gametes with each one different cross-over event (*trans*)

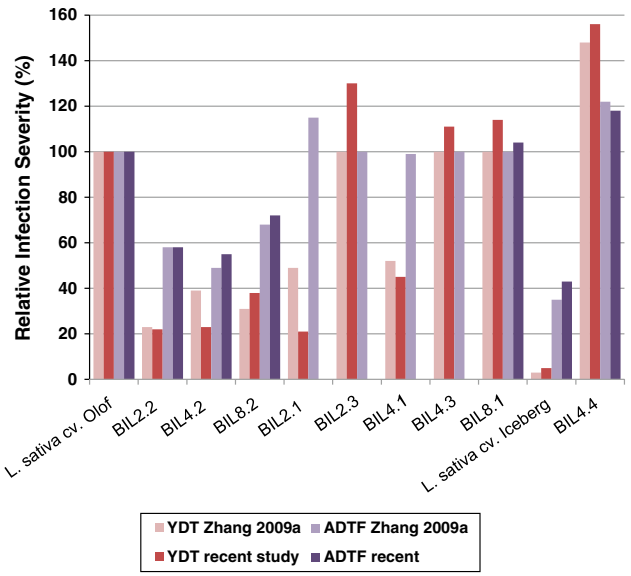

Fig. 3 Comparison of average RIS values of common control lines in the recent study (*dark color*) and a former study by Zhang et al. (2009a) (*light color*). Disease assessments at young plant stage (YDT, *red color*) and adult plant stage in the field (ADT_F, *purple color*) are shown. For field test data of Zhang et al. (2009a), the ADT_{F-C} dataset (11 locations) of Table 1 was used. No recent field test data were available for BIL2.1, 2.3, 4.1 and 4.3

were used to develop new NILs for further fine mapping. The same procedure was followed in subsequent recombinant screenings. NILs were numbered in order of the time they were developed (Fig. 1). The detected QTL positions in different sets of NILs were always verified in later evaluated NIL subsets.

We tested 49 and 74 NILs in YDT and ADT_F respectively. We distinguished four infection classes: 'resistant' with an ISL as the resistant parental BIL, 'susceptible' with an ISL like *L. sativa* cv. Olof, 'intermediate' with an ISL between the resistant BIL and the susceptible *L. sativa* cv. Olof and 'super susceptible' with an ISL more than *L. sativa* cv. Olof. In young and adult plant stage the infection levels of the majority of the 8.2 NILs were distributed over three classes: resistant, susceptible and intermediate (Fig. 1). A minority of one NIL in YDT and four NILs in ADT_F fell in the class 'super susceptible' (Fig. 1). The many NILs with an intermediate ISL at young and adult plant stage suggested that the resistance was explained by multiple sub-QTLs instead of one or two loci. The classification of the NILs in one of the four classes was often not similar between YDT and ADT_F, which suggests plant stage dependent resistance. For example NIL41 is resistant in YDT and super susceptible

in ADT_F, while NIL07 is susceptible in YDT and resistant in ADT_F (Fig. 1).

The plots of the *P* value and the average RIS per marker position for the homozygous *L. saligna* genotype indicated which segments of the introgression of *L. saligna* conferred a decrease in RIS, namely where the Log *P* had negative values. For the 8.2 introgression, the resistance in the young plant stage was located at around 23 cM and in the adult plant stage at around 20 and 39 cM (Fig. 2). In YDT, the NILs with a longer *L. saligna* introgression coming from the top side extending to 27.4 cM (like NIL 02, RIS = 27 %), had a lower RIS than the NILs with shorter introgressions like NIL01(RIS = 74 %) (Fig. 1). Multiple comparison (Duncan test, $\alpha = 0.05$) between NILs and control lines indicated presence of at least two sub-QTLs between 18.0 cM and 27.4 cM effective at young plant stage (Figs. 1, 2).

Also for ADT_F certain longer introgressions conferred a lower RIS than short introgressions as illustrated at the top side by NIL01 (RIS = 75 %) and NIL85 (RIS = 86 %) and at the bottom side by NIL209 (RIS = 67 %) and NIL208 (RIS = 90 %). At the adult plant stage the resistance was explained by at least two sub-QTLs between 18.0 and 25.2 cM and two sub-QTLs between 38.4 and 41.3 cM (Figs. 1, 2). The magnitude of the infection reduction was around 10 % for the four individual sub-QTLs and around 30 % for both two linked sub-QTLs compared to *L. sativa* Olof. Two sub-QTL at the top are present in NIL01 (RIS 75 %) and two sub-QTLs at the bottom are present in NIL209 and NIL214 (both RIS 67 %). The resistance was probably not associated with leaf morphology trait 'lobed leaf' because NIL202 and NIL208 showed resistance but did not have the *L. saligna* allele for 'lobed leaf' (Fig. 1).

Neutralising effect genes in ADT_F

Four NILs, NIL41, 49, 52 and 80, showed at the adult plant stage a higher ISL than susceptible parent *L. sativa* cv. Olof (super susceptible, Fig. 1). Furthermore we observed several susceptible NILs with high RIS and a long *L. saligna* introgression that completely overlapped smaller introgressions from NILs that had a lower RIS, for example; NIL02 with 90 % and NIL01 with 75 % RIS (Fig. 1). The tendency of NILs to be relatively susceptible if they had the 25–38 cM region derived from *L. saligna* suggests that in that region *L. saligna* carries a gene conferring susceptibility or neutralising resistance (Figs. 1, 2). This conclusion is also drawn from the *P* value plot (peak with positive Log *P* values, Fig. 2). The resistance gene conferring a 18–22 cM region of the 8.2 introgression (RIS of 86 % in NIL85) also occurs in BIL8.1. Still, BIL8.1 was completely susceptible (RIS = 104 %). This suggests that the *L. saligna* chromosome 8 also contains one or more genes to the left of marker E45M48–63 that neutralise the resistance present in the said segment.

Mapping QTLs within the 2.2 and 4.2 introgression

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

Within the 4.2 introgression plant morphological traits long-narrow leaf and non-heading co-localise with each other and with a resistance locus that may or may not explain both the resistance and plant morphology. Plant morphological traits dark green and twisted leaf co-localise with each other but not with resistance because NIL4.2-11, has no dark green and twisted leafs but is moderately resistant.

Discussion

Disintegration of the resistance

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

sub-QTLs (Brouwer and Clair D 2004); fine mapping with substitution mapping of *Rphq2*, barley QTL for resistance to leaf rust (*Puccinia hordei*), in a window of 0.11 cM also did not indicate sub-QTLs (Marcel et al. 2007).

Plant stage dependent QTLs

Of the 17 suggested sub-QTLs in all three BIL introgression segments together, probably only two sub-QTLs might explain resistance at both plant stages (a sub-QTL in 8.2, Fig. 2; a sub-QTL in 4.2, Fig. S2).The resistance levels of the complete 2.2, 4.2 and 8.2 introgression segments at both plant stages might be explained by interactions among the detected plant stage dependent sub-QTLs or by epistatic interactions among unknown loci. The detection of plant stage dependent sub-QTLs within the three BIL introgression segments corresponds with the result of the set of 29 BILs within the whole lettuce genome, in which the majority of the 15 resistant BILs showed plant stage dependent resistance (Zhang et al. 2009a). Developmental plant stage dependent quantitative resistance has been found in multiple studies, in multiple plant species (Castro et al. 2002; Eenink and Jong 1982; Mallard et al. 2005; Monteiro et al. 2005; Prioul et al. 2004; Qi et al. 1998; Wang et al. 2010).

Neutralizing effect genes

In all three introgression segments (2.2, 4.2 and 8.2) a subregion was detected that had a negative or neutralizing effect on the resistance level. This effect can be caused by infection promoting genes from *L. saligna* or by absence of possible resistance genes from *L. sativa*. One of the resistance sub-QTLs within the 8.2 introgression was also located within the overlapping *L. saligna* introgression of BIL8.1. BIL8.1, which is as susceptible as *L. sativa* cv. Olof, should contain therefore besides the shared resistance sub-QTL with BIL8.2 also at least one negative or neutralizing QTL. This fact suggests that the 13 BILs which in the study of Zhang et al. (2009b), were at all plant stages (at least) as susceptible as *L. sativa* cv. Olof may contain QTLs for resistance that are neutralized by genes with an opposite effect within the same BIL introgression.

Recombination suppression

Recombination suppression was observed in the recombinant screenings on lines with heterozygous introgressions (preBILs and preNILs). The level of recombination suppression varied from 2 to 17-fold (compared to the F_2 population) and depended on the region and on the size of the heterozygous introgression. Smaller introgressions showed more suppression. Recombination suppression in plants that were heterozygous for a donor introgression was also described in interspecific introgression lines of tomato (Brouwer and Clair 2004; Paterson et al. 1990) and barley (Johnston et al. 2013), but not in intra-specific NILs of maize (Graham et al. 1997) and rice (Wissuwa and Ae 2001). These findings suggest that recombination frequencies tend to get lower, when the introgressed parent species is rather distantly related from the recurrent parent. Brouwer and Clair D (2004), Johnston et al. (2013) and Canady et al. (2006) also reported a stronger suppression of recombination within smaller sizes than within larger sizes of introgression segments.

The nine times increased recombination frequency of double recombinant preNIL73 compared to that of its counterpart preNIL06 suggested that: (1) there is less recombination in a segment that is heterozygous for DNA from different species than in a homozygous segment. (2) As an interstitial segment is homozygous and is accompanied at both sides by heterozygous regions, recombination events accumulate in the interstitial homozygous segment. Similar findings were observed in an *Lycopersicon esculentum* × *Lycopersicon pennellii* F_2 population (Canady et al. 2006).

Gene cloning perspectives

Fragmentation of the resistance into mostly smaller effects and into plant developmental stage specific sub-QTLs makes cloning of the genes probably very difficult and of limited use. Most sub-QTLs conferred only a reduction in field infection severity of around 10 %, which requires many replications within disease tests to conclude differences in resistance phenotype between lines with and without the quantitative resistance allele.

In some studies substitution mapping of a QTL might lead to the ultimate cloning of the responsible gene like for *Pi21, Yr36, Lr34* and *Rgh4* (Fu et al. 2009; Fukuoka et al. 2009; Krattinger et al. 2009; Liu et al. 2012). But in our case the genetics was more complex and further attempts for gene cloning of sub-QTLs with effects of about 10 % seems not useful at this moment.

Breeding perspectives

Although the resistance within the BILs fragmented into multiple sub-QTLs, some NILs showed a similar effect as the parental BIL and without undesired plant morphological traits, like NIL44, of which the 10 cM introgression length is only a third of the 8.2 introgression. NIL214, of which the introgression length is only 3 cM, also shows a similar effect as the parental BIL at adult plant stage but it also contains the lobed leaf *L. saligna* allele. If the lobed leaf trait can be implemented as a positive morphological trait of a lettuce variety, NIL214 can be a very interesting

quantitative resistance donor in breeding. The resistances of NIL44 and NIL214 have been proven to be functional against four of the newest *B. lactucae* races under different environmental conditions in the field. The similar resistance levels of NILs, like NIL44 and 214, to the parental BIL line, was explained by the presence of a part of the sub-QTLs for resistance and the absence of negative or neutralizing *L. saligna*-introgression regions. For resistance breeding the effects of the single sub-QTLs alone are too small to be of practical interest. By stacking multiple sub-QTLs of smaller introgressions within or among 2.2, 4.2 and 8.2 introgressions, it might be possible to obtain lines with a higher or complete resistance and without undesired morphological traits. The effect of stacking has to be studied in detail to elucidate if and which specific combination of (sub-) QTLs can explain the nonhost resistance of *L. saligna* and to assess its value for practical use.

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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.

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