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Rin4 Causes Hybrid Necrosis and Race-Specific Resistance in an Interspecific Lettuce Hybrid [W]

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Some inter- and intraspecific crosses may result in reduced viability or sterility in the offspring, often due to genetic incompatibilities resulting from interactions between two or more loci. Hybrid necrosis is a postzygotic genetic incompatibility that is phenotypically manifested as necrotic lesions on the plant. We observed hybrid necrosis in interspecific lettuce (*Lactuca sativa* and *Lactuca saligna*) hybrids that correlated with resistance to downy mildew. Segregation analysis revealed a specific allelic combination at two interacting loci to be responsible. The allelic interaction had two consequences: (1) a quantitative temperature-dependent autoimmunity reaction leading to necrotic lesions, lethality, and quantitative resistance to an otherwise virulent race of *Bremia lactucae*; and (2) a qualitative temperature-independent race-specific resistance to an avirulent race of *B. lactucae*. We demonstrated by transient expression and silencing experiments that one of the two interacting genes was *Rin4*. In *Arabidopsis thaliana*, RIN4 is known to interact with multiple *R* gene products, and their interactions result in hypersensitive resistance to *Pseudomonas syringae*. Site-directed mutation studies on the necrosis-eliciting allele of *Rin4* in lettuce showed that three residues were critical for hybrid necrosis.

INTRODUCTION

During evolution, ancestral species can diverge into several derived species that become genetically isolated from each other due to pre- and postzygotic barriers, reducing the capacity for hybridization (Mallet, 2006; Rieseberg and Willis, 2007). In plants, one of the best described postzygotic barriers is hybrid necrosis (Bomblies and Weigel, 2007). This type of genetic incompatibility is manifested as necrotic lesions in seedlings or adult plants and is often associated with phenotypes such as wilting, chlorosis, stunted growth, and lethality. Hybrid necrosis has been reported in both interspecific and intraspecific plant crosses. As these phenotypes are not observed in the parental genotypes, hybrid necrosis must be a result of interactions between two or more genes (negative epistasis or Bateson-Dobzhansky-Muller incompatibilities) brought together from different species or genotypes (Bomblies and Weigel, 2007). However, the mechanisms underlying hybrid necrosis are still poorly understood. Studies on interspecific hybrids of tomatoes (*Solanum lycopersicum*) and intraspecific hybrids in *Arabidopsis thaliana* indicate that some forms of hybrid necrosis result from particular alleles encoding resistance (R) proteins inducing autoimmunity-like responses when combined with particular alleles of genes elsewhere in the genome (Krüger et al., 2002; Wulff et al., 2004; Bomblies et al., 2007; Alcázar et al., 2009).

We study disease resistance in interspecific hybrids between cultivated lettuce (*Lactuca sativa*) and a distantly related, wild species (*Lactuca saligna*). The latter is considered a nonhost species to *Bremia lactucae*, a causal agent of lettuce downy mildew (Bonnier et al., 1992). The sexually compatible gene pool of lettuce consists of *Lactuca serriola*, which is closely related and probably the progenitor of *L. sativa*, and more distantly related species, such as *L. saligna* and *Lactuca virosa* (De Vries, 1997; Koopman et al., 2001). These *Lactuca* species are autogamous and therefore predominantly homozygous. We generated two interspecific F2 populations (cross 1 and 2) between different accessions of *L. saligna* and cultivars of *L. sativa* (Jeuken et al., 2001). From cross 1, we developed a set of 29 backcross inbred lines (BILs), each with a single introgressed chromosomal segment from *L. saligna* in the *L. sativa* genetic backgrounds; this set of BILs represented 96% of the *L. saligna* genome (Jeuken et al., 2001, 2008; Jeuken and Lindhout, 2004; Zhang et al., 2009b).

In previous studies that were focused on disease resistance, we observed the possible functional correlation of several traits: lethality, temperature-dependent necrotic lesions on leaves, retarded growth, quantitative resistance, and complete resistance associated with hypersensitivity to downy mildew on the hybrid progeny and introgression lines; all of these traits mapped to one locus at the top of chromosome 9 at 9 centimorgans (cM) (hereafter referred to as the C9 locus; see Supplemental Figure 1 online) (Jeuken et al., 2001, 2008; Jeuken and Lindhout, 2002, 2004). Lethality was observed in seedlings that were homozygous for the *L. saligna* C9 introgression in the *L. sativa* genetic background (BIL9.1) (Jeuken and Lindhout, 2004). These seedlings were extremely necrotic and died within a week, suggesting

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a lethal homozygous allelic combination at two (or more) independent loci (Figure 1). Plants that were heterozygous for the C9 introgression (designated preBIL9.1; the "pre" suffix indicates that the introgression was heterozygous) were viable and fertile but developed necrotic lesions on their leaves and stems at a later stage and showed retarded growth (Jeuken and Lindhout, 2004). These phenotypes were more extreme in winter. Variable quantitative resistance to race BI:14 of *B. lactucae* was observed in preBIL9.1 plants (Jeuken et al., 2008). Complete hypersensitive resistance to downy mildew (formerly designated *R39*) was observed in the interspecific F2 populations and in preBIL9.1 plants; this resistance was effective against race BI:16 and not against race BI:14 (Jeuken and Lindhout, 2002; Jeuken et al., 2008).

The availability of hybrid plant materials (F2 progeny and a set of BILs) allowed for the use of a forward genetic approach to dissect and validate the loci determining hybrid necrosis. In this article, we provide evidence for two interacting loci determining hybrid necrosis and the identification of *Rin4* as one of the genes responsible. In addition, we demonstrate a relationship between hybrid necrosis and resistance to *B. lactucae*.

RESULTS

Two Epistatic Loci Are Involved in Hybrid Necrosis

To identify the epistatic loci determining hybrid necrosis, we first focused on the lethality phenotype. Genotyping had shown that the nonviable BIL9.1 plants were homozygous for the C9 introgression from *L. saligna* (Figure 1). Plants homozygous *L. saligna* for the C9 locus will be referred to as having the genotype *9sal*, heterozygotes will be referred to as *9het*, and homozygotes *L. sativa* for the C9 locus will be referred to as *9sat*. In the original interspecific F2 populations (*L. saligna* × *L. sativa* crosses 1 and 2), we compared genotypic composition of all plants that were homozygous *L. saligna* for the C9 locus but were viable. We identified one (cross 1) and seven (cross 2) viable *9sal* plants (see Supplemental Table 1A online, right column). None of these eight plants were homozygous for *L. sativa* alleles in a region from ~30 to 35 cM on chromosome 6 (hereafter referred to as the C6 locus, and similar to C9, genotypes on chromosome 6 are referred to as *6sal*, *6sat*, and *6het*; see Supplemental Table 1A online; equivalent to chromosome 8 of the consensus map for *Lactuca* spp; Truco et al., 2007). Thus, a homozygous combination of *L. sativa* alleles on the C6 locus and *L. saligna* alleles on the C9 locus (*6sat9sal*) was implicated as causing necrosis leading to lethality. Consistent with this, the C9 locus showed distorted segregation in cross 1 (deficiency of *L. saligna* alleles) and the C6 locus in cross 2 (deficiency of *L. sativa* alleles; see Supplemental Table 1A online).

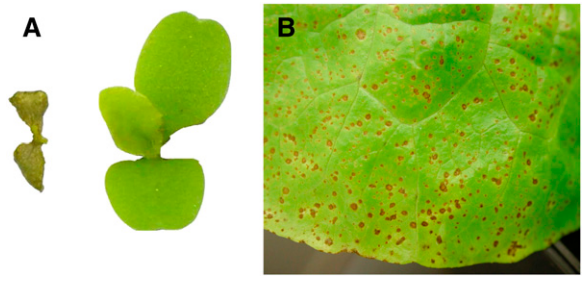

Figure 1. Hybrid Necrosis Phenotypes in Lettuce.

(A) Left: Completely necrotic seedling that is unable to survive; this backcross introgression line is homozygous for the *L. saligna* allele at the C9 locus and the *L. sativa* allele at the C6 locus (*6sat9sal*). Right: Normal wild-type seedling phenotype; genotype is homozygous *L. sativa* at both the C6 and C9 loci (*6sat9sat*). Both seedlings were grown at 15°C for 12 d.

(B) Detail of leaf with a high density of necrotic lesions in a 6-week-old plant of preBIL9.1, which was heterozygous at the C9 locus (*6sat9het*).

To confirm this interaction, we crossed two other introgressed lines derived from cross 1, preBIL9.1b (*6sat9het*) and BIL6.1 (*6sal9sat*), which differed for the putative interactive C6 and C9 loci and were homozygous *L. sativa* for the rest of the genome. Plant materials are diagrammed in Figure 2. BIL6.1 was homozygous for an *L. saligna* introgression from 0 to 40 cM on C6 (*6sal9sat*). PreBIL9.1b plants were *6sat9het* and had identical phenotypes for hybrid necrosis and resistance to *B. lactucae* as preBIL9.1 plants but they had a smaller heterozygous C9 segment (0 to 11 cM instead of 0 to 48 cM). We identified an F1 plant that was heterozygous at the C6 and C9 loci. Its F2 progeny segregated for the two loci and produced the nine expected genotype classes, which were identified by DNA marker analysis (plant material column, Figure 2). Seeds were collected from eight different F2 genotypes. Plants of the ninth genotype class, *6sat9sal* genotype (BIL9.1b), died after 1 week (Figure 2). Plants from six genotypic classes as well as the *6sat9sal* plants (while they lived) were characterized for their phenotypes compared with the parental lines *L. sativa* cv Olof and *L. saligna* CGN05271. Of the six genotypes, four contained at least one interacting pair of C6 + C9 *L. sativa* and *L. saligna* alleles that was expected to lead to at least some degree of necrosis (Figure 2). The symptoms of hybrid necrosis (necrotic lesions and reduced growth) were extreme for the homozygous *6sat9sal* genotype, severe for *6sat9het*, low for *6het9sal*, and lacking for *6het9het*, *6sal9sat*, and *6sal9sal* genotypes (Figure 2). The most extreme and lethal hybrid necrosis phenotype was observed for the homozygous allelic combination with two *L. sativa* alleles at C6 and two *L. saligna* alleles at C9 (BIL9.1, *6sat9sal*). The levels of necrosis in the two genotypes that had opposite heterozygous-homozygous allelic combinations, *6sat9het* and *6het9sal*, were not lethal but showed a large difference in the level of necrosis (~20-fold, severe versus low, respectively; Figure 2). The genotype that was heterozygous at both loci, *6het9het*, did not show symptoms of hybrid necrosis. These four levels of hybrid necrosis indicated a gene dosage effect of the alleles at the two interacting loci.

A similar interaction was observed between C6 and C9 loci in the progeny of a BC_4S_1 line from cross 2; this line has the *6het9sal* genotype and has been introgressed into the *L. sativa* background. This suggests the same epistatic interaction for hybrid necrosis as in progeny from cross 1, which were derived from a different accession of *L. saligna*.

Hybrid Necrosis Is Temperature Sensitive

To check for the temperature sensitivity that had been observed earlier for resistance (Jeuken and Lindhout, 2004), we tested

Plant material			Hybrid necrosis			Resistance levels					
						T=17°C [h]		T=19°C [i], 6 hr ↑ 24°C			
specific name	genetic label[k]	genotype[m] C6 C9	macrosc. necrosis level	microsc. necrosis level[a]	dry weight[c]	rAUDPC[df] Bl:14	rAUDPC[d] Bl:16	rAUDPC[eg] Bl:14	rAUDPC[eg] Bl:16	IS[j] Bl:14	IS[j] Bl:16
BIL9.1b	*6sat9sal*		+++ lethal[b]	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
preBIL9.1b	*6sat9het*		++ severe	12.28 a	0.4 a	0.03 a	0.00 a	0.24 a	0.00 a	21	0
	6het9sal		+ low	0.72 b	9.2 b	0.32 ab	0.08 a	1.02 b	0.04 a	82	2
	6het9het		−	0.50 bc	13.3 c	0.81 abc	0.25 a	1.10 bc	0.15 a	82	6
BIL6.1	*6sal9sat*		−	0.39 bc	13.9 c	2.68 e	2.35 c	1.24 bc	1.81 c	89	99
	6sal9het		−	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
6.1+9.1	*6sal9sal*		−	0.39 bc	12.6 c	1.35 cd	1.13 b	1.30 c	1.88 c	96	98
Lactuca sativa			−	0.01 c	13.2 c	1.00 bc	1.00 b	1.00 b	1.00 b	80	58
Lactuca saligna			−	0.29 bc	15.6 c	0.00 a	0.00 a	0.00 a	0.00 a	0	0
BIL4.4			−	N.D.	N.D.	2.11 de	1.49 b	1.21 bc	1.88 c	92	98

Figure 2. Hybrid Necrosis and Resistance Levels.

Plant material is described in genotype labels and diagrams (columns 1 to 3). Hybrid necrosis symptoms are described as quantity of necrotic lesions at macroscopical and microscopical level and as retarded growth (columns 4 to 6). Resistance levels are described as relative area under disease progress curve (rAUDPC) and infection severity (IS) against *B. lactucae* races Bl:14 and Bl:16 at two temperatures (columns 7 to 12). Letters in common within a column indicate that the values are not significantly different ($\alpha = 0.05$, Tukey honestly significant difference [HSD] procedure). N.D., not determined. a, Percentage of necrotic leaf area. Two leaf segments × three plants per genotype were examined. b, Seedling gets completely necrotic and shrivels after several days. c, Dry weight in grams from the shoots of 11-week-old plants grown in the greenhouse ($n = 7$). d, Relative AUDPC from YDT in climate chamber calculated from observations for infection severity at 8, 10, and 12 d after inoculation (DAI). *L. sativa* cv Olof was set at 1.00. e, Relative AUDPC from YDT in greenhouse calculated from observations for infection severity at 8, 9, 10, and 11 DAI. *L. sativa* cv Olof was set at 1.00. f, Similar results were observed in disease tests with race Bl:14 on cotyledons of these genotypes. g, Similar results were observed in four disease tests with races Bl:14 and Bl:16 on detached leaf discs from 9- and 12-week-old plants of these genotypes. h, In the climate chamber with day/night cycles of 19/12°C (block intervals) and an average temperature of 17°C. i, In the greenhouse with an average temperature of 19°C, minimal night temperature of 15°C, and the day temperature was 6 h above 24°C and 12 h above 22°C. j, Average infection severity level of downy mildew at 11 DAI on young plants (YDT) scored as percentage of leaf area showing sporangiophores. k, sat = homozygous *L. sativa*, sal = homozygous *L. saligna*, and het = heterozygous. m, graphical genotype of C6 and C9; white means *L. sativa* allele, and black means *L. saligna* allele. The C6 and C9 introgressions are 40 and 11 cM long, respectively.

plants at 15 and 30°C. At 15°C, plants showed the degrees of hybrid necrosis as described above (Figure 2); however, at 30°C, all plants, even the lethal *6sat9sal* genotype, grew normally, similar to *L. sativa* cv Olof (Figure 3). After 7 weeks, we transferred plants of four of the genotypes (*6sat9sal*, *6sat9het*, *6sat9sat*, and *6sal9sal*) that had been grown at 30 to 15°C. The first symptoms of hybrid necrosis were brown necrotic lesions in the youngest leaf, particularly close to the major veins; these symptoms were seen 48 h after the transfer for *6sat9sal* and at 80 h for *6sat9het* (see Supplemental Figure 2 online). Ultimately, the *6sat9sal*

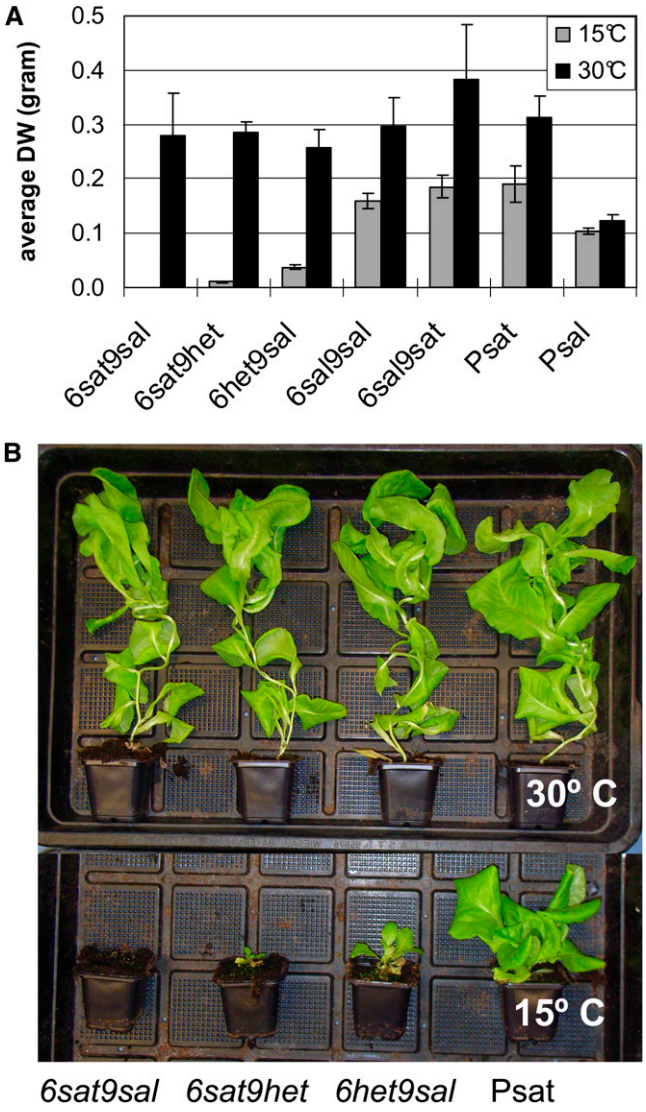

Figure 3. Temperature Sensitivity of Hybrid Necrosis.

(A) Plant growth as measured by average shoot dry weight (n = 5, 45 d old). Psat, *L. sativa* cv Olof (*6sat9sat*); Psal, *L. saligna* CGN05271 (*6sal9sal*); 95% confidence intervals are shown.

(B) Phenotypes of 7-week-old plants of indicated C6C9 genotypes, grown at 30 and 15°C.

plants became completely necrotic and died after 8 d. Plants with the *6sat9het* genotype died after 20 d (see Supplemental Figure 3 online).

Hybrid Necrosis and Resistance to *B. lactucae* Involve the Same Loci

The colocalization of race-specific resistance (formerly designated *R39* and derived from *L. saligna*; Jeuken and Lindhout 2002) with the C9 hybrid necrosis locus and evidence from other studies of involvement of *R* genes in hybrid necrosis (Krüger et al., 2002; Bomblies et al., 2007; Alcázar et al., 2009) suggested a possible relationship between hybrid necrosis and this resistance. To investigate whether there is a relationship between resistance and hybrid necrosis, we inoculated the same range of C6 and C9 genotypes used to characterize hybrid necrosis with two races of *B. lactucae*, Bl:14 and Bl:16. These races differ in their virulence phenotypes and have been tested also on the two original interspecific F2 populations (see Introduction). Seedlings, young plants, and adult plants were tested. The results for young plants are shown in Figure 2. The results were very similar for all three plant stages.

In the disease tests with race Bl:14 at 17°C, the genotype *6sat9het* showed low levels of infection severity, *6het9sal* showed medium levels of infection severity, and *6het9het* showed almost the same high level as the susceptible parent, *L. sativa* cv Olof (Figure 2). At a 2°C higher temperature, the severity of infection rose significantly for all genotypes, which resulted in only *6sat9het* still exhibiting resistance, while *6het9sal* and *6het9het* were as susceptible as *L. sativa* cv Olof (Figure 2). Therefore, the resistance to Bl:14 was quantitative, showed different levels for different doses of alleles at the C6 and C9 loci, and depended upon temperature. Interestingly, the resistance levels to Bl:14 paralleled the different levels of hybrid necrosis (necrosis and reduced dry weight) observed in uninoculated plants (Figure 2). This is consistent with the digenic interaction triggering a resistance response resulting in necrotic leaf lesions and a decrease of the spread of pathogen.

In the disease tests with race Bl:16, only very low or very high levels of infection were observed. Surprisingly, the *6sal9sal* genotype was susceptible to both races of *B. lactucae*, indicating that the *L. saligna* C9 allele does not lead to resistance per se. Complete resistance to Bl:16 was observed in the *6sat9het*, *6het9sal*, and *6het9het* genotypes that did not parallel the levels of resistance to Bl:14 and hybrid necrosis and did not change with temperature. At least one *L. sativa* allele on C6 with at least one *L. saligna* allele on C9 was sufficient to give complete resistance to Bl:16. This race-specific resistance seemed not to be a consequence of the triggered autoimmune hypersensitive response (HR) but another outcome of the same allelic interaction. The *6het9het* genotype was remarkable in that it was completely resistant to race Bl:16 but completely susceptible to race Bl:14 and did not show necrotic lesions.

In the F2 population derived from *L. saligna* × *L. sativa* cross 1, we detected only one peak logarithm of the odds (LOD) value for resistance to Bl:16 at the C9 locus and none at the C6 locus (Jeuken and Lindhout, 2002) (see Supplemental Figure 1 online). In hindsight, failure to detect a significant LOD peak on C6 in this F2 might have resulted from the distorted segregation of the C9 locus with a deficiency of *L. saligna* alleles (see Supplemental Table 1B online).

Rin4 Is the Candidate Gene on C9

The EST-derived marker most closely associated with the resistance to race Bl:16 in the interspecific F2 populations was LE0478 (see Supplemental Figure 1 online). Primers for this marker were based on a contig of lettuce ESTs (QG_CA_Contig7104; CGP1 database; The Compositae Genome Project;

http://compgenomics.ucdavis.edu). This contig had the highest sequence similarity to *RPM1 INTERACTING PROTEIN4* of *Arabidopsis* (*RIN4*; At3g25070) and was therefore designated *Rin4*. Extensive studies of RIN4 have provided support for the guard mechanism of action of R proteins that detect the activity of pathogen effectors indirectly by their effects on key proteins, such as RIN4 (Mackey et al., 2002, 2003). RIN4 is a 211–amino acid, acylated plasma membrane–associated protein that acts as a negative regulator of basal defense (Mackey et al., 2002; Kim et al., 2005b). It is a target of at least three effectors from *Pseudomonas syringae* (AvrRpm1, AvrB, and AvrRpt2) and is guarded by two independent R proteins (RPM1 and RPS2) (Mackey et al., 2002, 2003; Axtell and Staskawicz, 2003; Kim et al., 2005a).

We determined the cDNA sequences of the *Rin4* alleles from our parental lines of cross 1, *L. sativa* cv Olof and *L. saligna* CGN05271, and from the wild species *L. virosa* CGN05978. From each accession, we detected two versions of *Rin4* transcripts with open reading frames of 735 and 732 bp (designated Transcript1 and Transcript2) due to a CAG indel at base pair position 705; the inferred amino acid sequences are consequently 244 and 243 amino acids long, respectively. Following sequencing of the genomic DNA of *L. sativa* cv Olof and *L. saligna* CGN05271, we detected only one version of *Rin4*, which included the CAG sequence. The CAG polymorphism between the transcripts occurred at an intron splice site; therefore, an alternative splicing event seems to be responsible for these two versions of this *Rin4* transcript (see Supplemental Figure 4 online). We detected five synonymous single nucleotide polymorphisms, five nonsynonymous single nucleotide polymorphisms, and a 3-bp replacement (in addition to the indel) between the *Rin4* cDNA sequences of *L. sativa* cv Olof and *L. saligna* CGN05271 resulting in inferred Rin4 amino acid sequences that differ for six amino acids (Figure 4A). At these six polymorphic sites, *L. virosa* CGN05978 encoded four amino acids that were identical to *L. sativa* and two that were identical to *L. saligna* (Figure 4A).

Only the *Rin4salT2* Transcript Caused Necrosis

To examine whether one or both Rin4 proteins from *L. saligna* CGN05271 elicited the hybrid necrosis, different *Rin4* transcripts were expressed in planta using *Agrobacterium tumefaciens*–mediated transient assays. The shorter Transcript2, but not Transcript1, of the *L. saligna* allele of *Rin4* (*Rin4salT2*) caused a severe necrotic reaction in *L. sativa* plants harboring the *L. sativa* allele at the C6 locus (Figure 4B; see Supplemental Table 2A online). Neither of the *Rin4* transcripts from *L. sativa* and *L. virosa* elicited necrosis.

We used site-directed mutagenesis of *Rin4T2* to determine which of the six polymorphic amino acids between *L. saligna* and *L. sativa* were critical for hybrid necrosis. Replacement of six *L. saligna* residues by their corresponding *L. sativa* residues indicated that the last three polymorphic residues (positions 167, 217, and 234) are required for hybrid necrosis elicited by *Rin4sal* (Figure 4; see Supplemental Table 3 online). However, replacement of the last three polymorphic *L. sativa* residues in Rin4sat by their corresponding *L. saligna* residues indicated that the three residues alone or in combination were not sufficient to convert Rin4sat into a necrosis-inducing protein.

Silencing of *Rin4* Impaired Resistance to Race BI:16

To validate the involvement of *Rin4* in the resistance in preBIL9.1b (*6sat9het*) to *B. lactucae* BI:16, we reduced *Rin4* transcript levels by virus-induced gene silencing (VIGS) and challenged these plants with race BI:16. The silencing of *Rin4* did not result in abnormal plant phenotypes in any of the tested genotypes. Silencing of *Rin4* rendered preBIL9.1b (*6sat9het*) susceptible (Figure 4C). This result confirms that *Rin4* is involved in this resistance. We conclude, therefore, that the *L. saligna* allele of *Rin4* located at C9 is responsible for both the hybrid necrosis and the resistance to *B. lactucae* race BI:16.

Rin4 Amino Acid Haplotypes in *Lactuca* spp

To learn more about the diversity of Rin4 amino acid haplotypes among species of *Lactuca*, we sequenced cDNAs of *Rin4* from a diverse panel of 38 additional accessions comprising 12 *Lactuca* species (six to eight accessions for each of *L. sativa*, *L. serriola*, *L. saligna*, and *L. virosa* and one to two accessions for the other *Lactuca* species; see Supplemental Table 4 online). Sequence analysis of 41 accessions (38 + the previous three; Figure 4) showed that all species expressed both the T1 and T2 transcripts of *Rin4*; *L. tatarica* expressed two additional *Rin4* transcripts (see Supplemental Figure 5 online). We focused on the T2 transcript because *Rin4salT2* elicited hybrid necrosis. The accessions from *L. sativa*, *L. serriola*, *L. aculeata*, *L. dregeana*, and *L. altaica* were monomorphic for *Rin4* at the inferred amino acid level (see Supplemental Figure 5 online), which was consistent with their close taxonomic affiliations. *L. saligna* showed intraspecific amino acid polymorphism; three Rin4T2 amino acid haplotypes were detected among eight accessions of *L. saligna*, with six or five polymorphic residues relative to *L. sativa*. The three haplotype groups reflected their origin (France-Italy, Russia-Georgia, and Israel). *L. saligna* CGN05271 (parent of cross 1) and *L. saligna* CGN11341 (parent of cross 2) both had the France-Italy haplotype that had six polymorphic residues relative to *L. sativa.* No intraspecific amino acid polymorphism was detected among the six accessions of *L. virosa*. *L. virosa* was the only species that had any (two) amino acids in common with *L. saligna* at the amino acid sites that were polymorphic between *L. sativa* and *L. saligna* (T89 and Q115, Figure 4; see Supplemental Figure 5 online).

No Necrosis Was Induced by *Rin4salT2* in Species Other Than *L. sativa*

Agrobacterium-mediated transient expression of *Rin4salT2* from accession CGN05271 in 36 accessions comprising nine species in the genus *Lactuca* and two species from genera related to *Lactuca* demonstrated that a severe necrotic reaction was observed in all 10 *L. sativa* cultivars but was not observed in any of the other eight *Lactuca* species nor in two species from genera related to *Lactuca* (see Supplemental Table 2B online). Therefore, the hybrid necrosis is the result of a specific

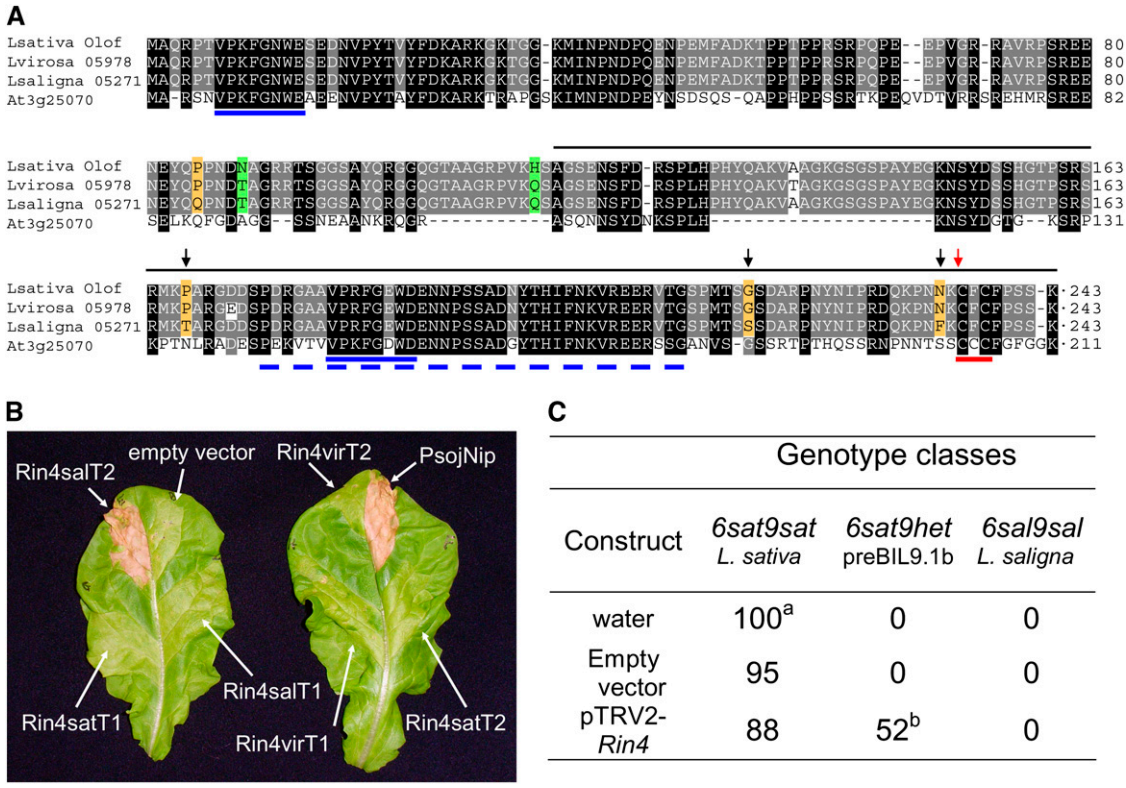

Figure 4. *Rin4* Is the Candidate Gene on C9.

(A) Rin4 from *L. sativa* cv Olof, *L. saligna* CGN05271, *L. virosa* CGN05978, and RIN4 from *Arabidopsis* (At3g25070). The deduced amino acid sequences of lettuce *Rin4* transcript2 were aligned using the ClustalW software. Transcript1 has one extra amino acid, a Gln, Q, between Lys-235 and Cys-236 (red arrow). Conserved residues between *Arabidopsis* and *Lactuca* species are shaded in black, and conserved residues within *Lactuca* species are shaded in gray. Orange-shaded amino acids highlight amino acid differences between *L. saligna* and *L. sativa*/*L. virosa.* Green-shaded amino acids highlight amino acid differences between *L. saligna*/*L. virosa* and *L. sativa*. Arrows point at critical residues for hybrid necrosis in Rin4salT2 based on site-directed mutagenesis studies. The solid blue lines represent AvrRpt2 cleavage sites in RIN4 of *Arabidopsis*, RCS1 and RCS2; the dashed blue line represents a RIN4 P142-G179 fragment that interacts with AvrB; the red line represents a RIN4 putative palmitoylation site at C203-C205 (Kim et al., 2005a). The black line marks the DNA sequence that was engineered into pTRV2 vector for VIGS experiments.
(B) Transient expression with *Rin4* alleles/transcripts infiltrated in *L. sativa* cv Olof 8 DAI. Five plants × two leaves per genotype class. Alleles: sat = *L. sativa*, sal = *L. saligna*, and vir = *L. virosa.* T1 and T2 are *Rin4* transcript versions 1 and 2.
(C) Relative infection severity to *B. lactucae* Bl:16 of *Rin4*-silenced lettuce genotypes by VIGS. [a] The absolute infection severity of *6sat9sat*, *L. sativa* cv Olof, was 82% at 10 DAI in the young plant disease test. [b] The infection severity was estimated for entire leaves, although we could not observe whether the entire leaf was silenced for *Rin4*. Therefore, the presented relative infection severity value may underestimate the effect of the silencing.

interaction between the products of the *Rin4sal* and the *L. sativa* C6 alleles.

DISCUSSION

Hybrid necrosis and resistance to *B. lactucae* in an interspecific lettuce hybrid is due to the specific combination of an *L. sativa* allele on C6 and an *L. saligna* allele on C9 (hereafter referred to as the interacting allele pair). The C9 gene was shown to be *Rin4*, which interacts with an unknown gene on C6. In *Arabidopsis*, the phosphorylation or disappearance of RIN4 elicits a necrotic HR dependent on the R proteins RPM1 or RPS2 (Axtell and Staskawicz., 2003; Mackey et al., 2003). Therefore, the association of resistance with this digenic interaction suggests that the unknown C6 gene may also be an *R* gene. Even in the absence of infection, the interaction of C6sat with Rin4sal triggers a macroscopic necrotic phenotype. A similar resistance response has been observed in tomato; a specific interaction between alleles originating from different species at the loci encoding Rcr3 and the R protein Cf-2 results in weak necrosis (Krüger et al., 2002).

The *Rin4sal* and *C6sat* hybrid necrosis response is quantitative, temperature dependent, and causes increased resistance to the otherwise virulent Bl:14 race of *B. lactucae* in a gene dosage-dependent manner. Different combinations of interacting alleles elicited different levels of hybrid necrosis and resistance. The temperature dependence and association with increased pathogen resistance has been observed in several

other instances of hybrid necrosis (Bomblies et al., 2007; Bomblies and Weigel, 2007; Alcázar et al., 2009). In addition, several resistances to pathogens are temperature sensitive (Wang et al., 2009).

The *C6sat-Rin4sal* pair elicits hybrid necrosis and confers complete HR-mediated resistance to infection with the incompatible BI:16 race of *B. lactucae*. The double heterozygote *6het9het* was sufficient to provoke complete resistance to BI:16 (Figure 2); however, it was not sufficient to elicit hybrid necrosis. Resistance to BI:16 was not dependent upon temperature and was only mildly dependent upon the level of hybrid necrosis and the doses of the interacting alleles. This resistance required the heterologous *C6sat-Rin4sal* pair of alleles; the homologous combinations of alleles *C6sal-Rin4sal* and *C6sat-Rin4sat* and the other heterologous combination, *C6sal-Rin4sat*, did not confer resistance. This is different from previously reported situations. In the case of interaction between alleles of *Rcr3* and *Cf-2* from *Solanum pimpinellifolium* and *S. lycopersicum* (formerly *Lycopersicon esculentum*), *Cf-2*^{pim}-*Rcr3*^{esc} caused an HR-mediated resistance to *Avr2*-containing races of *Cladosporum fulvum* and a weak autonecrosis; however, the allelic combination from *S. pimpinellifolium*, *Cf-2*^{pim}-*Rcr3*^{pim}, also conferred race-specific resistance to *Avr2*-containing races (Jones et al., 1993; Krüger et al., 2002; Rooney et al., 2005).

Molecular Considerations

Only six residues in Rin4 were polymorphic between *L. saligna* and *L. sativa* that might render the innocuous Rin4sat isoform into an autoimmunity-inducing, BI:16-incompatible isoform in the C6sat-Rin4sal combination. Mutant studies with reciprocal replacement of these six residues in one Rin4 parental protein by their corresponding residues in the other Rin4 parental isoform showed that three residues were critical but not sufficient individually or in combination for hybrid necrosis (see Supplemental Table 3 online). It remains to be determined which of the additional polymorphic residues are required for hybrid necrosis and which of the polymorphic residues are critical to convert the BI:16-susceptible C6sat-Rin4sat combination into a resistant C6sat-Rin4sal combination. Interestingly, none of these polymorphic residues are at positions previously characterized as being important for the function of RIN4 in *Arabidopsis* (Kim et al., 2005a). However, the last critical polymorphic residue in Rin4sal at position 234 is two residues away from the putative palmitoylation site at C203 to C205 in *Arabidopsis* RIN4 and therefore may influence membrane localization. Also, the polymorphisms at positions 167 and 217 in lettuce Rin4 flank by seven amino acids the region in *Arabidopsis* RIN4 that was identified as interacting with AvrB using yeast two-hybrid analysis (Figure 4A; Kim et al., 2005a).

Transcript1 of *Rin4* is one amino acid longer than transcript 2 and is present in all the *Lactuca* species tested. It therefore seems to be the product of an evolutionarily conserved alternative splicing event. The functional significance of the two protein products remains unknown. One of several possibilities is that one isoform might fulfill the role in resistance of a decoy target in effector perception as hypothesized in the decoy model (Van der Hoorn and Kamoun, 2008).

Genetic Aspects of Hybrid Necrosis Levels

Four specific *C6sat-Rin4sal* allelic combinations caused different levels of hybrid necrosis. These four levels seemed to depend on the dose of the necrosis-inducing heterologous *C6sat-Rin4sal* pair of alleles. Decreasing numbers of combinations of *C6sat* and *Rin4sal* alleles, four (*6sat9sa*l), three (*6sat9het* and *6het9sal*), and two (*6het9het*), showed decreasing levels of hybrid necrosis from lethal to severe to low to nil, respectively (Figure 2). The lack of hybrid necrosis in the *6het9het* genotype that contains one pair of interacting alleles requires further explanation. Assuming a guard-guardee relationship between a putative C6 R protein and the Rin4 protein, four possible guard-guardee associations could occur in the *6het9het* genotype: the non-necrosis–inducing C6sal-Rin4sal, C6sal-Rin4sat, and C6sat-Rin4sat and the necrosis-inducing C6sat-Rin4sal. If pairs of the guard-guardee isoforms from the same species (C6sat-Rin4sat and C6sal-Rin4sal) preferentially interact compared with heterologous pairs of isoforms, the *6het9het* would tend not to develop hybrid necrosis (see Supplemental Figure 6 online). In the other three genotypes with a pair of heterologous necrosis-inducing *C6sat-Rin4sal* alleles (*6het9sal*, *6sat9het*, and *6sat9sal*), one or both of the non-necrosis–inducing isoforms, C6sal and Rin4sat, are absent. As a consequence, C6sat or Rin4sal have no alternative but to form a guard-guardee association with a heterologous protein, leading to hybrid necrosis. The lower the doses of non-necrosis–inducing alleles the more heterologous guardee-guard associations are formed, leading to higher levels of hybrid necrosis (see Supplemental Figure 6 online).

In conclusion, we showed specific isoforms of Rin4 induced hybrid necrosis and resistance in combination with a heterologous gene product from a marginally sexually compatible species. Together with previous studies of other species, our findings raise several interesting questions. Is the *C6sat* allele a functional *R* gene encoding a nucleotide binding–leucine-rich repeat protein? Has it evolved to protect against *B. lactucae* or other pathogen species? Why is it widespread in *L. sativa* but lacking in other species?

METHODS

Lettuce Material

Cross 1 comprised *Lactuca saligna* CGN05271 × *Lactuca sativa* cv Olof. Cross 2 comprised *L. saligna* CGN11341 × *L. sativa* cv Norden. From both crosses one F1 plant was selfed and backcrossed to its recurrent *L. sativa* parent. F2 populations consisted of 126 and 54 plants from crosses 1 and 2, respectively. The F2 genetic linkage map and the infection severity levels in adult F2 plants to downy mildew were described previously (Jeuken et al., 2001; Jeuken and Lindhout, 2002). Both BC_1 populations were further backcrossed with the respective cultivated *L. sativa* parent until the BC_4 generation. For cross 2, we selfed the BC_4 and genotyped the BC_4S_1. For cross 1, we developed a set of BILs from the $BC_4S_{1\text{-}2}$ and $BC_5S_{1\text{-}2}$ using marker-assisted selection. This resulted in a set of 29 BILs with a total of 96% of the *L. saligna* genome introgressed into *L. sativa* (Jeuken and Lindhout, 2004; Jeuken et al., 2008). BILs were genotyped with >700 DNA markers (amplification fragment length polymorphism [AFLP], ESTs, and simple sequence repeats (SSRs)). Most of these BILs contained one homozygous introgression fragment of *L. saligna* with an average genetic length of 33 cM (~20 to 40% of a

chromosome) in an *L. sativa* cv Olof background. For some lines it was not possible to obtain the introgression in a homozygous state and the best alternative, a line with the introgression in heterozygous state, was used. We designated such lines preBILs. An overview of the *L. sativa* × *L. saligna* hybrid material is shown in Supplemental Table 5 online.

In this study, we focused on preBIL9.1b and BIL6.1. PreBIL9.1b showed hybrid necrosis and contained one heterozygous introgression, from 0 to 11 cM on chromosome 9. It was derived from preBIL9.1 (introgression from 0 to 48 cM). BIL6.1 harbored one homozygous introgression from 0 to 40 cM on chromosome 6.

Pathogen Material

The virulence phenotypes of *Bremia lactucae* races Bl:14 and Bl:16 have been previously described (Jeuken and Lindhout, 2002) and in the evaluation report by the International Bremia Evaluation Board (http://www.plantum.nl/ibeb.html). Pathogen maintenance, inoculum preparation, and the method of inoculation were performed as described previously (Jeuken and Lindhout, 2002).

Linkage Analyses, Genotyping, and Quantitative Trait Loci Mapping

Analyses of additional markers were performed on the F2 population of cross 1 to saturate and improve the genetic linkage map of the *L. saligna* × *L. sativa* cross (Jeuken et al., 2001), especially near the interacting loci. Markers consisted of AFLP markers from two primer combinations, E48M59 (primer+CAC and Primer+CTA) and E33M59 (Primer+AAG and Primer+CTA) and SSR markers and candidate gene markers that were developed from lettuce EST sequences as part of the Compositae Genome Project (see Supplemental Table 6 online; http://compgenomics.ucdavis.edu/; McHale et al., 2009). Polymorphisms between *L. saligna* and *L. sativa* in EST and SSR markers were visualized by the size differences of their PCR products on agarose gels (directly or after enzymatic digestion) as previously described (Jeuken et al., 2008) or by high-resolution melting curve differences visualized on a LightScanner System (Idaho Technology).

Linkage analyses were performed using JoinMap 4.0 software (Van Ooijen, 2006) on the F2 population of cross 1 with the following mapping conditions. For grouping, regression mapping was used with weak linkages recombination and LOD thresholds of 0.45 and 0.05. Markers were assigned to nine linkage groups at a LOD threshold of 8. Calculations of the linkage maps were done using all pairwise recombination estimates smaller than 0.40, LOD scores higher than 1, a jump threshold of 5, and Haldane's mapping function. As the integration of former linkage maps for F2 populations from cross 1 and 2 showed high colinearity with respect to marker order and distance (Jeuken et al., 2001), we consider the linkage map for cross 2 to be identical. To fine-map the resistance to *B. lactucae* Bl:16 to the new F2 linkage map, we performed quantitative trait loci mapping procedures like simple interval mapping and approximate multiple quantitative trait loci mapping using MapQTL 5.0 (Van Ooijen, 2004).

The C6 and C9 plant material was genotyped with a minimum of eight DNA markers (combinations of EST, SSR, and AFLP markers) per introgression segment at an early plant stage to select the desired genotypes.

Phenotyping Hybrid Necrosis

Plants were phenotyped by observing cotyledons or leaves for macroscopically visible necrotic lesions. Plants were categorized based on abundance and size of necrotic lesions compared with other plants under the same conditions.

We quantified the level of necrotic leaf area through microscopy evaluation. Three plants per genotype, randomly placed, were grown in a greenhouse at 15° for 5 weeks. The 4th true leaf was then sampled from each by cutting two leaf segments near the mid-leaf, 1 × 2 cm in size. Leaf segments were discolored for 3 d in acetic acid/ethanol solution (v:v = 1:3), cleared and stored in saturated chloral hydrate solution (5g/2 mL), and finally mounted in 70% glycerol. Slides were observed under the light microscope, and the necrotic areas were recognized by cytoplasm granulation and darkening with a yellow or brown color. Each necrotic area was visualized through a digital camera and measured using AxioVison LE 4.6 (Carl Zeiss) in μm^2. The percentage of necrotic area per leaf segment was calculated. For multiple comparisons of the percentage of necrotic area per genotype, we used two-way analysis of variance (ANOVA) and the Tukey HSD test ($\alpha = 0.05$).

To quantify the retardation of growth, the shoot dry weights of 11-week-old plants that had been grown in a randomized block design in a greenhouse were measured in grams. Seven plants per genotype were examined. Aboveground parts were harvested and dried for 16 h at 105°C.

For multiple comparisons of the shoot dry weights between genotypes, we used one-way ANOVA and the Tukey HSD test ($\alpha = 0.05$).

Disease Tests

The levels of infection severity in response to *B. lactucae* races Bl:14 and Bl:16 were determined using seedling disease tests (SDTs) (14 seedlings per genotype), young plant disease tests (YDTs) (eight plants per genotype), and adult plant disease tests in the greenhouse (ADT_G) (seven plants × eight leaf discs per genotype). For each genotype, these tests were performed on plants at three different developmental stages as described previously (Jeuken and Lindhout, 2002; Zhang et al., 2009a).

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

For YDTs, attached leaves were tested from young, 3- to 4-week-old plants. One test with race Bl:14 and one test with Bl:16 were performed in a climate chamber. In parallel, one test with Bl:14 and one test with Bl:16 were performed in a greenhouse compartment. The same inoculum was given for the tests with Bl:14 and for the tests with Bl:16. The temperature was controlled during growth and after inoculation at both locations but was inevitably more variable in the greenhouse due to the natural day/night cycle (17 h day/7 h night in June during the tests) and natural light conditions. In the greenhouse, the temperature varied gradually between a low point of 15°C during the night to a peak of 29°C at noon; the average was 18.6°C. In the climate chamber, the temperature shifted directly from 19.6°C during the artificial day of 16 h to 12.3°C in the artificial night, with an average temperature of 17.2°C. Representative leaves of young plants were scored (as described above) at 8, 9, 10, and 11 DAI.

Rin4 Sequences

The 3′ end of a lettuce *Rin4* was identified from an EST sequence (QG_CA_Contig7104; CGP1 database; The Compositae Genome Project; http://compgenomics.ucdavis.edu) based on sequence similarity to *RIN4* from *Arabidopsis thaliana.* We used the Qiagen RNeasy plant mini kit with a DNase column treatment per the manufacturer's directions for RNA isolation and iScript enzyme (Bio-Rad). The SMART RACE cDNA Amplification kit (Clontech) was used according to the manufacturer's instructions to identify the 5′ cDNA sequence from *L. sativa* cv Salinas. Oligonucleotide primers in the 5′ and 3′ untranslated regions (Rin4_UTR) as well as in the internal DNA sequence (Rin4_INT1, Rin4 INT2, and

Rin4_INT3) were designed to the *RIN4* cDNA sequence (see Supplemental Table 7 online). Genomic DNA from three lettuce genotypes, *L. sativa* cv Salinas, *L. serriola* accession UC96US23, and *L. saligna* CGN5322, were amplified with these oligonucleotides using Amplitaq Gold Polymerase with a 57°C annealing temperature (Applied Biosystems). Products were sequenced. Additional cDNA sequences of ESTs from *L. virosa* and *L. saligna* that showed sequence similarity to these *Rin4* cDNA sequences were selected by BLAST analysis from the Compositae Genome Project Database (http://compgenomics.ucdavis.edu/). Based on all of these sequences, two oligonucleotide primers at the start and the end of the cDNA sequence of the *RIN4* gene (Rin4_TOT) were designed (see Supplemental Table 7 online). In subsequent PCR experiments, the cDNA and genomic sequences spanning the open reading frames of *Rin4* were obtained for *L. sativa* cv Olof, *L. saligna* CGN05271, and *L. virosa* CGN05978. In the same way, *Rin4* homologous cDNA sequences from 38 additional accessions comprising 12 *Lactuca* species were obtained (see Supplemental Table 4 online). Four to six clones were sequenced per accession using Sanger sequencing. The inferred amino acid sequences of *RIN4* transcripts were aligned using the ClustalW software from DNASTAR Lasergene8.

Phylogenetic Analysis

A midpoint rooted neighbor-joining phylogenetic tree was generated using MEGA version 4 and bootstrap value 1000 (Tamura et al., 2007).

Agrobacterium tumefaciens–Mediated Transient Assays

Transient assays were executed to overexpress different wild-type and mutant *Rin4* transcripts. Total RNA isolation and cDNA syntheses were performed as described above. Full-length cDNAs encoding the *Rin4* alleles (*L. sativa* = *sat*, *L. saligna* = *sal*, and *L. virosa* = *vir*) and transcript versions (T1 and T2) were amplified from lettuce cDNA prepared from leaves of *L. sativa* cv Olof, *L. saligna* CGN05271, and *L. virosa* CGN05978. The *rin4* mutants were created using the Quick-Change site-directed mutagenesis kit (Stratagene), according to the manufacturer's instructions. PCR products, prepared using the proofreading enzyme Phusion DNA polymerase (Finnzymes), were cloned into pENTR/D-TOPO entry vector (Invitrogen) and then recombined into the GATEWAY T-DNA binary vector pK7WG2 (Karimi et al., 2002) using LR clonase (Invitrogen). The resulting binary vectors, pK7WG2 with target genes under the control of a 35S promoter, were electroporated into *A. tumefaciens* strain C58C1 (pGV2260).The *A. tumefaciens* strain C58C1 (pGV2260) and the strain containing the *PsojNIP* gene cloned in the binary vector pB7WG2 were provided by G. Van den Ackerveken (Utrecht University). PsojNIP is a necrosis-inducing protein from *Phytophthora sojae* (Qutob et al., 2002). Luria-Bertani medium (10 g/L bacteriological peptone, 10g/L NaCl, and 5 g/L yeast extract) was used for liquid and solid (15 g/L agar) bacterial cultures. Spectinomycin (50 mg/L) was used to maintain pB7WG2 in *A. tumefaciens*.

Rin4 alleles were tested in two independent experiments performed with 10 replications each on *L. sativa* cv Olof, *L. sativa* cv Norden, *L. saligna* CGN05271, *L. saligna* CGN11341, BIL6.1, and BIL6.1 +9.1. A third experiment was performed on 36 accessions comprising nine *Lactuca* species and two other species (*Cichorium intybus* and *Mycelis muralis*) and with four replications of each transient test. The *rin4* mutants were tested in at least two independent experiments on at least five plants each from *L. sativa* cv Olof and BIL6.1. Plants were grown in a greenhouse at 21° in the daytime and 19° at night until they attained the sixth to seventh leaf stage. The 5th and 6th true leaves were then infiltrated; the culture preparations and leaf infiltrations were performed as described by Wroblewski et al. (2005).

VIGS

In initial experiments to develop a VIGS system for lettuce, we examined the ability of the tobacco rattle virus (TRV)-based VIGS vector from D. Kumar (Yale University) to suppress the expression of the endogenous phytoene desaturase gene of lettuce (*PDS*) in *L. sativa* and *L. saligna*, following the protocol used for tomato (*Solanum lycopersicum*; Liu et al., 2002a, 2002b). Lettuce is a host for tobacco rattle virus (Mojtahedi et al., 2003). Two independent sets of infiltrations were performed, with 20 *L. sativa* and 20 *L. saligna* plants infiltrated with a mixture of *Agrobacterium* culture containing the pTRV2-*PDS* and pTRV1. The same numbers of plants were also infiltrated with the pTRV1 and empty pTRV2 (empty vector control) and mock-infiltrated with water. Twenty-three days after agroinfiltration, the expected photobleaching phenotype, caused by inhibition of carotenoid synthesis, was observed on the 4th to 14th leaves in *L. sativa* and on the 3rd to 10th leaves in *L. saligna* on at least 75% of the plants (see Supplemental Figure 7 online). The effect of *PDS* suppression was visible uniformly throughout each entire leaf (see Supplemental Figure 7 online), especially for leaves 5 to 8 of *L. sativa* and 3 to 5 of *L. saligna*. We validated the silencing by measuring the transcript levels for *PDS* using quantitative RT-PCR. Primers that anneal to the *PDS* gene outside the region targeted for silencing were used (primer pair Ls-PDS-RT3; see Supplemental Table 7 online). The experiment was conducted using an iCycler MyiQ detection system (Bio-Rad), using the iQ SYBR Green Super mix (Bio-Rad). Assays were done in duplicate. Relative quantification of the *PDS* transcript level was normalized to results obtained using the lettuce ubiquitin transcript as a control by applying the $2^{-\Delta Ct}$ formula (Livak and Schmittgen, 2001). Three plants representing each group and RNA target were analyzed. The Tukey HSD test with α = 0.05 was applied for pairwise multiple comparisons between the groups.

After confirming the effectiveness of the VIGS approach (see Supplemental Figures 7 and 8 online), a new TRV construct, pTRV2-*Rin4*, was made with a 285-bp fragment of lettuce *Rin4* (Figure 4). In several independent experiments, plants were agroinfiltrated with pTRV2-*Rin4*, and 30 d after agroinfiltration, the plants were challenged with *B. lactucae* race BI:16 as in standard disease tests of young plants (see description of disease tests above). Infection severities were measured and analyzed as described above. Similar trends were observed in two experiments. Detailed results of one experiment are presented (see Results).

VIGS Plasmid Constructions

pTRV1 and pTRV2 VIGS vectors have been described previously (Liu et al., 2002a). pTRV2-PDS was created as follows. A 315-bp fragment of a cDNA fragment corresponding to bases 1334 to 1648 of the lettuce *PDS* gene (contig CLS_S3_Contig8919; CGP1 database; The Compositae Genome Project, http://compgenomics.ucdavis.edu) was PCR amplified from *L. sativa* cv Olof cDNA using Taq DNA polymerase and the primer pair Ls-PDS1 (see Supplemental Table 7 online). The resulting PCR product was cloned into the pGEM-T Easy vector as described by the manufacturer (Promega) and later ligated into *Eco*RI-cut pTRV2.

pTRV2-Rin4 was created as follows. A 285-bp fragment corresponding to bases 451 to 735 of lettuce Rin4satT1 cDNA (Figure 4) was PCR amplified from lettuce cDNA using Taq DNA polymerase and the primer pair Rin4_TOT (see Supplemental Table 7 online). The resulting PCR product was digested with *Eco*RI, and the 285-bp fragment was ligated into *Eco*RI-cut pTRV2 using standard methods.

Agroinfiltrations

For *Agrobacterium*-mediated virus infection, cultures of *A. tumefaciens* GV3101 containing pTRV1, empty pTRV2 vector control, and each of the constructs derived from pTRV2 were grown, harvested, and subsequently infiltrated as described (Van der Hoorn et al., 2000; Bai et al.,

2008). The infiltration was performed on the abaxial side of both cotyledons of each lettuce seedling at 9 d after sowing using a needleless syringe. The infiltrated plants were grown under normal greenhouse conditions (20°C daytime and 18°C nighttime) and were checked for virus symptoms at regular intervals. The only symptom of TRV infection observed was restricted plant growth compared with mock-infiltrated plants.

Temperature Sensitivity Tests

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

Accession Numbers

Sequence data from *Rin4T2* from 41 *Lactuca* accessions from this article can be found in the GenBank/EMBL data libraries under accession numbers GQ497773 to GQ497814.

Author Contributions

M.J.W.J. designed and performed research, analyzed data, and wrote the article. K.P., L.K.M., and E.D.B. performed research. N.W.Z. designed and performed research and analyzed data. P.L. and R.G.F.V. contributed to biological interpretation, and R.E.N. and R.W.M. contributed to biological interpretation and writing.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Map Positions of Hybrid Necrosis Loci.

Supplemental Figure 2. Hybrid Necrosis Symptoms in Youngest Leaf after Temperature Shift.

Supplemental Figure 3. Hybrid Necrosis Symptoms in Whole Plants after Temperature Shift.

Supplemental Figure 4. Proposed Alternative Splicing of *Rin4* Transcripts.

Supplemental Figure 5. Rin4 Amino Acid Haplotypes in *Lactuca* spp.

Supplemental Figure 6. Proposed Genetic and Molecular Model for Hybrid Necrosis Levels in Lettuce.

Supplemental Figure 7. Silencing of PDS in Lettuce by VIGS.

Supplemental Figure 8. Real-Time PCR Expression Data of *PDS* in Lettuce Leaves.

Supplemental Table 1. F2 Segregation Ratios and Infection Severities.

Supplemental Table 2. Transient Expression of *Rin4* Alleles and Transcripts.

Supplemental Table 3. Transient Expression of *rin4* Mutants.

Supplemental Table 4. *Lactuca* Diversity Panel.

Supplemental Table 5. *L. sativa*-*L. saligna* Hybrid Material.

Supplemental Table 6. DNA Markers at C6 and C9 Loci.

Supplemental Table 7. Primer Pairs for *Rin4* Sequencing, Cloning, and RT-PCR.

Supplemental Data Set 1. Text File Corresponding to the Alignment in Supplemental Figure 5.

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