Specific In Planta Recognition of Two GKLR Proteins of the Downy Mildew *Bremia lactucae* Revealed in a Large Effector Screen in Lettuce

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Submitted 17 May 2013. Accepted 12 July 2013.

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

The lettuce downy mildew pathogen, *Bremia lactucae*, causes large losses in susceptible host plants and has been classified as a pathogen with a high risk of quick adaptation to lettuce resistance traits and chemical control (Brown et al.

2004; McDonald and Linde 2002). Its large effective population size, high gene flow, and mixed (both sexual and asexual) reproductive system contribute greatly to this risk. *B. lactucae* belongs to the order Peronosporales, an order of the oomycetes that includes downy mildew and *Phytophthora* spp. The downy mildews are obligate biotrophs that are found on many plant species, including *Arabidopsis* (*Hyaloperonospora arabidopsidis*), cucurbits (*Pseudoperonospora cubensis*), grapevine (*Plasmopara viticola*), and sunflower (*Plasmopara halstedii*). Obligate biotrophs depend on the living host for their growth and reproduction (Kemen and Jones 2012; Spanu 2012). Therefore, for successful infection, it is of prime importance that biotrophs cope with inducible defenses of the host, which can be described as consisting of two overlapping layers of plant immunity (Boller and He 2009; Jones and Dangl 2006; Thomma et al. 2011). The first is triggered by the recognition of pathogen-derived molecules termed pathogen-associated molecular patterns (PAMPs) by transmembrane pattern recognition receptors, and is referred to as PAMP-triggered immunity (PTI). Though pathogens can avoid inducing PTI (e.g., by masking PAMPs), a more common mechanism is the suppression of PTI within the host cell (Jones and Dangl 2006; O'Connell and Panstruga 2006). Pathogens can achieve this by translocating proteins (effectors) into host intracellular compartments where they can manipulate cellular processes (e.g., the suppression of plant defense responses), leading to effector-triggered susceptibility. Gram-negative bacterial pathogens deploy a type III secretion system to bring effectors into the host cytoplasm by means of a pilus-like structure (Büttner and He 2009). Oomycetes get in close contact with host cells by penetrating the plant cell wall and invaginating the plant cell membrane to form haustoria, feeding structures that are thought to contribute to pathogenicity (Avrova et al. 2008; Kemen and Jones 2012; Whisson et al. 2007). From the haustoria, effectors are secreted from the pathogen before they cross the host membrane (Whisson et al. 2007). In the case of the order Peronosporales, two main classes of effectors that enter host cells have been defined: Crinklers and RXLR proteins (Stassen and Van den Ackerveken 2011). The canonical RXLR effector contains an N-terminal signal peptide and a translocation domain that contains an RXLR amino acid motif and, optionally, a dEER motif. The C-terminal part of the protein, following the translocation domain, is referred to as the effector domain. Variations in the presence and exact sequence of motifs in the translocation domain have been observed, e.g., QXLR motifs in *P. cubensis*

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*The e-Xtra logo stands for “electronic extra” and indicates that four supplementary figures, four supplementary tables, and additional supplementary information is published online. Also, Figure 7 appears in color online.

effectors (Tian et al. 2011) or the presence of an EER motif but not of an RXLR motif in *H. arabidopsis* ATR5 (Bailey et al. 2011).

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

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

Cultivated lettuce (*Lactuca sativa*) can be crossed, with some difficulty, with wild lettuce species that include *Lactuca* spp. that are considered *B. lactucae* nonhosts (e.g., *L. saligna*). These wild lettuce species provide a pool of genetic material from which new *Dm* genes and resistance quantitative trait loci (QTLs) have been identified (Jeuken and Lindhout 2002; Van Treuren et al. 2011; Zhang et al. 2009a). Dominant *R* genes have been extensively used to breed *B. lactucae*-resistant lettuce cultivars. However, turnover of *Dm* genes has been rapid, because *B. lactucae* is quick to adapt to newly introduced *R* genes. To understand the molecular basis of ETI in the lettuce–*B. lactucae* interaction and to identify new *R* genes for resistance breeding, we deployed a selection of 34 *B. lactucae* candidate RXLR and RXLR-like effectors, which were previously identified by transcriptome sequencing (Stassen et al. 2012), to screen a large collection of lettuce breeding lines for new recognition specificities. We discovered two proteins, containing the newly found RXLR-like variant GKLR that are recognized in planta, one of which is recognized by *L. sativa* cultivars containing the *Dm2* gene.

RESULTS

Screening *Lactuca* accessions for recognition of 34 *Bremia* effectors.

Previously, we predicted 77 potential RXLR and RXLR-like effectors from our *B. lactucae* transcriptome data (Stassen et al. 2012). From these, we cloned a set of 16 full-length effector candidates and, from previous *B. lactucae* transcript assemblies, we cloned and verified 12 potential effectors. Finally, we obtained the full coding sequence of six potential effectors that were not full-length in the assembled 454-transcript data by using short reads of spore-derived genomic DNA or by 3' rapid amplification of cDNA ends. The coding sequences of all 34 *B. lactucae* effector candidates were cloned in a GATEWAY ENTRY plasmid and confirmed by Sanger sequencing (the nucleotide and protein sequences can be found in Supplementary Information S1).

A summary of features of the candidate effector proteins is given in Table 1 for 30 *B. lactucae* RXLR (BLR) proteins that contain the canonical RXLR motif and 4 candidates with an alternative RXLR-like motif that were predicted based on their similarity to effectors of other oomycetes and by comparison with a hidden Markov model based on the amino acid sequence surrounding the RXLR motifs of the BLRs. In three of these RXLR-like motifs, the first residue is not an arginine but a glycine (named BLG01, BLG02, and BLG03) and, in the fourth RXLR-like protein, this residue is a glutamine (named BLQ01). EER-like domains (rich in E, Q, D, or N residues, preferably ending in R or K) were found in the 25 of the 34 effector candidates, including in the BLG proteins that have a conserved DER sequence. The length of the predicted effector proteins varied from 65 to 463 amino acids, with an average of just under 180 amino acids.

Recognition of effectors in planta can be efficiently tested by transient expression of the corresponding *B. lactucae* coding sequences using *Agrobacterium tumefaciens* for delivery to plant cells. Because all known RXLR(-like) effectors that are recognized by plant R proteins are host-translocated, we engineered the coding sequences such that the signal peptide sequences were removed (Δ SP) and replaced by new start codons in front of the predicted signal peptide cleavage sites. Infiltration of lettuce leaves with *Agrobacterium* spp. carrying a T-DNA vector with the Δ SP coding sequences under control of the 35S *Cauliflower mosaic virus* promoter results in production of the Δ SP-effector protein in the plant cell cytoplasm, the same location at which the protein is thought to arrive when secreted from *B. lactucae* and translocated into the host cell. Intracellular recognition of effectors by *R*-gene-encoded proteins is expected to induce a clearly visible cell death response, also referred to as the HR. Because no *B. lactucae* effectors are known, thus far, that trigger HR in *Lactuca* spp., we made use of the cell-death-inducing protein PsojNIP of the oomycete *P. sojae* that was previously used as a positive control for transient expression in *L. sativa* (Jeuken et al. 2009). Necrotic lesions developed, starting from 2 days post-*Agrobacterium* spp. infiltration, in response to transient expression of PsojNIP but not in response to expression of either yellow fluorescent protein (YFP) or β -glucuronidase (GUS). Results were similar in all other *Lactuca* spp. tested (e.g., in *L. saligna* and *L. aculeata*) (Supplementary Fig. S1).

In total, 129 *Lactuca* accessions or lines were selected, representing a broad range of *B. lactucae* resistances, including dominant *Dm* genes and partial or quantitative resistance traits, as well as additional genetically unknown forms of resistance to *B. lactucae* (Supplementary Table S1). The collection of 129 *Lactuca* lines was screened for effector recognition by *Agrobacterium* infiltration of the 34 different effector constructs.

Agrobacterium-mediated transient expression was robust in nearly all tested lines, as can be seen from the cell death response that is induced by PsojNIP expression. None of the tested lines showed a strong response to *Agrobacterium* with the YFP construct. Average scores were given per lettuce–effector combination based on the presence of no or few visible symptoms (0), strong yellowing of the leaf (1), or cell death (2). The responses of the *Lactuca* lines to each of the 34 effectors, defined as an average score, are summarized in Supplementary Table S2. Two of the 34 effectors, BLG01 and BLG03, induced clear cell death responses (average symptoms scores ≥ 1.3) in several *Lactuca* lines. Four other effectors were found to induce cell death in a single line in one experiment; however, the specific recognition could not be confirmed for these combinations.

Recognition of the RXLR-like proteins BLG01 and BLG03.

A variant of the mature (without signal peptide) protein, BLG01^E—based on an allele from *B. lactucae* isolates NL519 and F703—was found to induce a stronger response than the protein encoded by our reference strain Bl:24 (BLG01^A). Of the 129 tested lines, 41 gave a strong cell death response to BLG01^E. Weak or inconsistent responses to BLG01^A and BLG01^E were observed for 16 and 8 *Lactuca* lines, respectively (reviewed in Supplementary Figure S2). The higher number of BLG01^E-responsive lines is likely due to a stronger response that is more easily detected, because most lines that showed a weak response to BLG01^A showed a strong response to BLG01^E. Additionally, the response of 16 lines is only visible

with BLG01^E but not with BLG01^A. Most of the *Lactuca* lines in our collection that showed a response to BLG01 were of the *L. saligna* species. BLG01 recognition in a few responsive *L. serriola* and *L. virosa* lines could not be confirmed in additional transient expression experiments (Supplementary Table S3), suggesting that BLG01 recognition is specific for *L. saligna*.

A second effector, BLG03, was specifically recognized in two *L. sativa* lines, Amplus and UCDM2 (Fig. 1). In all other lines, BLG03 did not induce responses that could be distinguished from the GUS negative control (e.g., in ‘Olof’). The

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

Table 1. Overview of cloned *Bremia lactucae* effector candidates^z

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

^z The first RXLR or RXLR-like motif and position within 100 amino acids from the start codon are indicated. The first EER or EER-like motif and position of which the first amino acid is within 20 amino acids of the first amino acid of the RXLR motif are shown. Source: M = main assembly, 3 = 3' rapid amplification of cDNA ends, E = transcript extended with SOLiD data, and P = preliminary assemblies.

set of cultivated lettuce lines (*L. sativa*) included in the screen contains differential lines that provide a wide range of genetically known *R* genes and can be used to determine *R*-gene specificities. Both Amplus and UCDM2 contain the *Dm2* resistance specificity that is absent in all other *L. sativa* lines tested. Therefore, *Dm2* could mediate the recognition of BLG03.

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

BLG01, BLG02, and BLG03 do not have significant homology to any sequences in the National Center for Biotechnology Information nonredundant protein database (e value < 1e-3), nor are there any significant matches to Pfam domains (Finn et al. 2010). The best BLAST matches in a combined database of oomycete proteins (*H. arabidopsidis*, *P. infestans*, *P. ramorum*, *P. sojae*, *Pythium ultimum*, and *Saprolegnia parasitica*) were to a putative *Phytophthora infestans* RXLR effector (PITG_15128, e value = 0.037) for BLG01, a *P. sojae* RXLR effector (Ps_133875, e value = 0.81) for BLG03, and a *P. ramorum* RXLR effector (Pr_97351, e value = 0.032) for BLG02 (Supplementary Information S2).

BLG01 and BLG03 expression.

In order to be recognized in planta, these G^K/_RLR effectors need to be expressed during the infection process. Expression in planta was already observed, because the effector transcripts were previously identified by transcriptome sequencing of infected lettuce leaves (Stassen et al. 2012). To determine the changes in expression during the different stages of infection, we analyzed a time series by quantitative polymerase chain reaction (qPCR) (Fig. 3). *L. sativa* 'Olof' seedlings were spray-inoculated with spores of *B. lactucae* isolate BL:24, after which samples were taken every 24 h, starting immediately after spraying. Within the first 24 h, the majority of spores germinated and *B. lactucae* had penetrated the epidermis. Substantial *B. lactucae* hyphal growth and formation of haustoria in mesophyll cells occurred over the next 4 days. At 6 days postinoculation (dpi), conidiophores formed and sampling was stopped. The expression of *B. lactucae* actin relative to *L. sativa* actin shows the substantial relative growth of *B. lactucae* throughout the entire time course (Fig. 3). Expression of BLG03 increases immediately after inoculation and appears to be stable from 1 dpi onward. BLG01 gene expression decreases slightly during the course of infection, with expression levels comparable with those of BLG03 at the later stages of infection. BLG02 shows an increase of expression at the first day after inoculation similar to that of BLG03. After 1 dpi, expression levels of BLG02 decline, approaching the level seen immediately after inoculation. Similar expression in time was detected in an independent biological replicate. These results confirm that the three BLG

	<--Signal peptide --><-----Translocation domain----->	
BLG01	MVRVYVAALTGFLALSASASATLQLTSVNESLADAYDSTAPARGKLRAYAATNVESDERA	60
BLG02	MVRIYVAALTVVLAFAFSASCSATSPLTAKATPVTANGSGGRAQGRRAHTTTNVEIDERS	60
BLG03	MVRVYVAALAAIFALSA--SATLHLTLANASSVTTEEGDGRPQGKLRVNAATNVESDER-	57
	:**: .:*** ** : : . : . . :*:***. :***** **	
BLG01	FSNLLHLKTLNVLFSPLSKERMEAAISKSDSSVVKHLSHGAVNEKKLGIGRDGVKAFE	120
BLG02	ISDVIYAIARQVADHRSNLVQAPKRTYTTTLATLDSLQVLEQNWSELRLLY--VKQTK	118
BLG03	FLDGLKALVRGFYDLTPFAPRFQKDTYSNLLKLNLSFEQFSGKDTLQLRRLYDAARRHN	117
	: : : : . . : : . : : : : : : *	
BLG01	PAKVSQVKMILEDIFRKPYNKILLKVLKANGGERNLVRNLAQAEMLGKVFVFLKSTLAS	180
BLG02	RKRPKDFVSMYELLVKK-----YGLLKLTDMMKK	147
BLG03	KVNPTNPVSVYTGLVEK-----YGEFEIVNMVYS	146
	. . : :.* *	
BLG01	KWERDGVSLMDVWSYICKDVKAPTEEEMKIFSHWCYAFVLSAKGKFSAEETVIEKTLLK	240
BLG02	PDYISLSET-DNFKYLYDEASVYWR-KNSVLK---QYASELYDSKGNKTFDVTDFESLQ	202
BLG03	LKHAQSSSRDVLKRLGKEEKWYWKDKKDAGK---MYAEALDLGNEFTVKNMVSKLSKLE	203
	. . * . : . : . . . ** *	
BLG01	VHNDNKNKKGKLAINMLAQIHLRSRKFVDANFFELYDPSSSLKLLLELEKSPRACDL	300
BLG02	PFFERIGRRGD-----YLEITAAEHEKWK---NKLKRP-----	233
BLG03	QFLNRIDQKASEEQLKALVVADIERVKTKK---ESVSPSDVEM-----	243
	. . . : . : : *	
BLG01	VELYRLLKQAVTDDKFMKGIWDRSTKKPEQPNKRN-	336
BLG02	-----	
BLG03	-----	

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

genes are expressed from 0 to 5 dpi, suggesting that the effector proteins are produced at all stages of infection.

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

The recognition of the BLG01 and BLG03 effectors is thought to occur in the host cell cytoplasm. To test whether the GKLR and DER motifs are needed for effector recognition, we investigated whether the effector domains of BLG01 and BLG03 are sufficient for recognition when expressed in lettuce. Using the same *Agrobacterium*-mediated transient transformation system as used to screen the collection of lettuce lines, we expressed BLG01⁶⁰⁻³³⁶ in the responsive *L. saligna* line CGN05271 and BLG03⁵⁸⁻²⁴³ in the responsive UCDM2 line. Cell death responses in the zones infiltrated with BLG01⁶⁰⁻³³⁶ did not differ from those infiltrated with BLG01 with translocation domain (BLG01²²⁻³³⁶) (Fig. 4), indicating that the translocation domain (BLG01²²⁻⁶⁰) is not required for in planta recognition in CGN05271. Similarly, the recognition of BLG03 is also independent of the translocation domain (BLG03²⁰⁻⁵⁸), as shown by the cell death response induced by BLG03⁵⁸⁻²⁴³ (Fig. 5).

To test whether the GKLR- and DER-containing domains mediate translocation of the effectors, we tested the translocation domain of BLG01 and BLG03 in the AVR3a-translocation assay in *P. capsici* (Schornack et al. 2010). The assay is based on recognition of transgenic *P. capsici* strains by *Nicotiana benthamiana* plants expressing R3a. The *P. capsici* strains express a fusion of the signal peptide and translocation domain of interest to the effector domain of AVR3a, which is recognized intracellularly by R3a, only if the translocation domain is able to mediate translocation. *P. capsici* transformed with an empty vector was able to spread from the initial infection site in 100% of wild-type *N. benthamiana* plants and 70% of R3a-expressing plants (Fig. 6). The CRN16:Avr3a fusion (Schornack et al. 2010) was used as positive control and transformants containing this construct caused spreading lesions from 95% of infection sites on wild-type plants but only in 12.5% of infection sites on R3a-expressing plants. *P. capsici* transformants expressing BLG01:AVR3a and BLG03:AVR3a fusions spread from 58 and 46% of infection sites, respectively, on wild-type plants. By contrast, no spreading lesions were observed on R3a-expressing *N. benthamiana* plants, strongly suggesting that the BLG01 and BLG03 GKLR- and DER-containing domains mediate translocation. Transformants expressing BLG01:AVR3a and BLG03:AVR3a constructs in which the GKLR motif was substituted by AAAA caused more spreading lesions on R3a

plants than the nonmutated BLG:AVR3a transformants, suggesting that disruption of the GKLR motif prohibits efficient translocation (Supplementary Fig. S3).

Allelic diversity of GXL^R effectors.

Allelic diversity of the three G^K_RLR effectors in a selection of *B. lactucae* isolates was investigated to test whether the proteins showed signs of selection. A set of eight *B. lactucae* isolates was chosen as a group with high diversity based on their *R* gene specificities. Because *B. lactucae* is diploid, a single isolate can contain different alleles. To determine possible alleles, PCR products were cloned and, from each isolate, at least eight clones per gene were sequenced. Seven different alleles were found for BLG01 and BLG03 and six alleles for BLG02. The distribution of alleles is represented in Table 2 and the protein translations of the different alleles can be found in Supplementary Information S3. Two striking observations can be made regarding BLG01. First, as can be seen from the allele distribution, isolate Bl:5 appears to possess four alleles of this effector, which could be due to gene duplication or polyploidy. Second, the allele sequences reveal that no functional BLG01 proteins are encoded in isolates NL519 and F703 due to a nonsense mutation in the fifth codon (TAC→TAA, Y→stop). The same stop is found in one of the alleles of Bl:5, whereas two other Bl:5 alleles have premature stops at other positions. In one of these alleles, the stop (CAG→TAG, Q→stop) is at amino acid position 24, the third residue after the predicted signal peptide cleavage site (SA|TL); in the other case, a two-nucleotide deletion in the effector domain (amino acid position 286) induces a frame shift that reads into a stop after two amino acids. In contrast to NL519 and F703, a full-length copy of the effector is present in Bl:5. For most isolates, two different alleles of BLG02 and BLG03 were found. BLG02^A and BLG02^B encode the same protein sequence and only have synonymous nucleotide variants. All amino acid differences encoded in BLG02 alleles were found in the effector domain. This is in contrast to BLG03, in which amino acid polymorphisms are also found in the signal peptide and translocation domain. Although one allele has an insertion of two amino acids in the signal peptide, all BLG03 alleles are predicted to encode a signal peptide. BLG03^F, only present in Bl:17, has a premature stop codon in the effector domain. Furthermore, BLG03^G, found in Bl:5, is more sequence divergent from the Bl:24 reference sequence than all other BLG03 alleles. None of our effectors are predicted to be under positive selection to maintain amino acid diversity based on the ratio of synonymous (dS) to nonsynonymous (dN) substitutions (codon-based test of positive selection averaging over all sequence pairs:

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

BLG01, Z score = 0.05790, $p = 0.47696$; BLG02, Z score = 0.15217, $P = 0.43965$; and BLG03, Z score = -1.31461, $P = 1.0$). However, the many different alleles and, in particular, the nonsense alleles suggest that BLG01 and BLG03 have been under selective pressure.

The different BLG01 alleles were next used to make constructs of the effector without signal peptide for in planta expression. Constructs with the effector domains of all BGL01 alleles except F were tested in *L. saligna* CGN5271 to determine their potential to trigger cell death. BLG01^D and BLG01^G did not appear to induce cell death in CGN5271. Strikingly, the effector domain of BLG01^E triggered the strongest responses when expressed in *L. saligna* CGN05271 and, as a result, provided more clear-cut scoring (Supplementary Fig. S4). However, because there is a premature stop in the signal peptide of

BLG01^E, this protein will not be produced by the *B. lactucae* isolates F703 and NL519.

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

To genetically map the locus responsible for the response to BLG01, backcross inbred lines (BIL), which cover 96% of the *L. saligna* CGN05271 genome in an *L. sativa* ‘Olof’ background (Jeuken and Lindhout 2004), were tested by transient *Agrobacterium*-mediated expression of the BLG01 alleles A and E. None of the BIL showed a cell death response to BLG01 (Table 3). Because 4% of the *L. saligna* genome was absent in the set of 28 BIL, the locus could be located in one of the four chromosomal regions (bottom chromosome 3, top chromosome 5, top chromosome 7, or bottom chromosome 9) that are not represented in the BIL (Jeuken and Lindhout 2004; Jeuken et al. 2008). From the original *L. saligna* CGN05271 × *L. sativa* ‘Olof’ F2 mapping population (Jeuken et al. 2001), viable F3, F4, and BC1S1 families were obtained from selfed parental

Fig. 4. Recognition of BLG01 without signal peptide (BLG01²²⁻³³⁶) and without signal peptide and translocation domain (BLG01⁶⁰⁻³³⁶) in *Lactuca saligna* CGN05271. β-Glucuronidase (GUS) and PsojNIP serve as controls for responses to *Agrobacterium* and successful T-DNA transfer, respectively. Pictures were taken 8 days postinoculation.

Fig. 5. Recognition of BLG03 with (+TD; BLG03²⁰⁻²⁴³) and without (-TD; BLG03⁵⁸⁻²⁴³) translocation domain in *Lactuca sativa* ‘UCDM’ but not in *L. sativa* ‘Olof’. β-Glucuronidase (GUS) and PsojNIP serve as controls for responses to *Agrobacterium* and successful T-DNA transfer, respectively. Pictures were taken 6 days postinoculation.

Table 2. Distribution of alleles of BLG01, BLG02, and BLG03 over eight *Bremia* isolates^w

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

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

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

lines that were heterozygous or homozygous *L. saligna* at one of the four regions that were not represented in the BIL. Several families were found to segregate for cell death in response to both allele BLG01^A and allele BLG01^E (Table 3). Most F3 plants that were responsive to the stronger BLG01^E allele were also responsive to the BLG01^A allele. Only six plants responsive to BLG01^E were not visually scored as responsive to BLG01^A, probably because of the weaker response to BLG01^A. This segregation was also observed in F3 plants from a cross between the responsive *L. saligna* parent CGN11341 and the nonresponsive *L. sativa* parent ‘Norden’ (Table 3). Comparing the parental genotypes from the families that showed cell death revealed that the response to BLG01 was linked to a region at the bottom of chromosome 9 that was always homozygous or heterozygous *L. saligna*, indicating that the trait is dominant. This was confirmed in four F1 plants obtained from a cross between CGN05271 and ‘Olof’ that were all responsive to the effector (Table 3). The position of the locus on chromosome 9 was further confirmed and more precisely positioned using F3 families, of which the F2 parent had a recombination event near the candidate region. Thus, the locus could be mapped to a region of 4.4 centimorgans (cM) between markers CLSX3110 and CLS_S3_4656, as shown by the genotype of four informative F3 plants (Fig. 7).

The BLG01-triggered response resembles ETI and, therefore, was expected to be causally linked to *B. lactucae* resistance. The responsive *L. saligna* line CGN05271 is a nonhost for *B. lactucae*, because of multiple quantitative resistance loci (Zhang et al. 2009a and b); therefore, we tested segregating families for linkage of the response to BLG01 and the *B. lactucae* resistance phenotype. Both BC1S2 families obtained from BC1S1 plants 90_2 and 90_6 that were responsive to BLG01 were as susceptible to isolate Bl:24 as the *L. sativa* ‘Olof’ parent (Table 4). We conclude that the locus for responsiveness to BLG01 does not confer resistance to *B. lactucae* isolate Bl:24 that expresses the recognized effector protein.

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

BLG03 is recognized by lettuce lines Amplus and UCDM2 (Fig. 1), which carry known resistance loci to *B. lactucae*. Amplus contains two *R* genes, *Dm2* and *Dm4*, whereas UCDM2

contains a single locus, *Dm2*. Therefore, these two lines have the *Dm2* locus in common, which is absent from the other tested lines. This raised the possibility that the *Dm2*-encoded R protein mediates BLG03 recognition. We investigated whether resistance to *B. lactucae* isolate Bl:5, to which lettuce *Dm2* confers resistance, co-segregated with the ability to recognize BLG03 in an F2 population of a UCDM2 × Cobham Green cross, in which the *Dm2* resistance locus segregates. The response to BLG03 was determined by *Agrobacterium* infiltration in leaves, and resistance to *B. lactucae* Bl:5 was determined in a leaf disc assay. Of 143 tested F2 plants, all but 28 developed a clear cell death in response to transient expression

Fig. 7. Locus of the BLG01 cell-death response in *Lactuca saligna* CGN05271 at the bottom of chromosome 9. Genotype graphs for four F3 plants with the closest recombination are shown. Blue is homozygous *L. sativa* ‘Olof’, yellow is heterozygous, gray represents intervals with a recombination event, and white means unknown genotype. Green indicates the smallest region in which the cell-death response to BLG01 is fine mapped.

Table 3. Response to BLG01 transient expression in *Lactuca saligna* and *L. sativa* parental lines and progeny^z

Lines, populations	Assay 1 and 2			Assay 3		
	Plants tested	Responsive plants		Plants tested	Responsive plants	
		BLG01 ^E	BLG01 ^A		BLG01 ^E	Remark
<i>L. sativa</i> ‘Olof’	6	0	0	3	0	Nonresponsive parent
<i>L. saligna</i> CGN05271	6	6	6	3	3	Responsive parent
Set of 28 BIL	84	0	0	...	...	...
BC1S1	5	2 (1)	1 (1)	...	...	...
F3	48	15 (11)	13 (9)	74	19 (8)	...
F4	3	3 (1)	2 (1)	...	...	...
F1	...	...	...	4	4	...
<i>L. sativa</i> ‘Norden’	4	0	0	2	0	Nonresponsive parent
<i>L. saligna</i> CGN11341	7	7	7	3	3	Responsive parent
F3	14	7 (4)	5 (4)	3	3 (2)	...

^z Numbers in parentheses indicate the number of families. BIL = backcross inbred lines.

Table 4. Disease assessment by detached leaf assay at adult plant stage, ADT_G, with *Bremia lactucae* Bl:24 on genotyped BC1S2 populations from two BC1S1 plants that showed a cell-death response to BLG03^E

Plant	Genotype bottom C9	Number of plants tested	Number of leaf segments	Race Bl:24 ISL
BC1S2 90_2	Homozygous <i>Lactuca sativa</i>	3	18	84
	Heterozygous	6	36	75
BC1S2 90_6	Homozygous <i>L. sativa</i>	3	18	75
	Heterozygous	6	36	92
<i>L. sativa</i> ‘Olof’	Homozygous <i>L. sativa</i>	3	27	84

of BLG03 (four sites per plant), indicating that the ability to recognize the effector is a dominant trait. All plants that recognized BLG03 were resistant to Bl:5. Conversely, all plants that did not recognize BLG03 were susceptible to Bl:5. This indicates that *Dm2* or a closely linked gene from the *Dm2* background is required for recognition of BLG03.

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

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

DISCUSSION

In planta effector recognition.

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

Wild *Lactuca* spp. can be used to introgress *R* genes or QTLs into lettuce cultivars. For example, the *Dm3* gene originates from a *L. serriola* accession but is very rare in natural populations, with only a single accession of 1,033 tested from 49 natural populations having an intact *Dm3* gene (Kuang et al. 2006). *Dm3* is a fast-evolving *R* gene from the RGC2 locus. This locus encodes two types of *R* gene candidates, a fast-evolving type and a type that evolves at a much slower rate and is more conserved among different accessions (Kuang et al. 2004). Recognition of the *B. lactucae* effector BLG01 appears to be relatively common in *L. saligna* species and to only occur sporadically in other species. The wide recognition of BLG01 may indicate recognition by a slowly evolving or more ancient *R* gene. The exact nature of the recognition of BLG01 in *L. saligna* and whether this recognition is dependent on the same gene in all lines remains to be determined.

The recognition of BLG01 in *L. saligna* CGN05271 was mapped at the bottom of chromosome 9, where no *R* gene clusters to *B. lactucae* in *L. serriola* and *L. sativa* are known thus far (Kesseli et al. 1994; Truco et al. 2007). None of the 23

lettuce expressed sequence tag (EST) sequences from the Lettuce SFP Chip Project that are mapped within the 4.4-cM region between our flanking markers (CLSX3110 and CLS_S3_4656) show homology to NB-LRR-like resistance proteins. We are aware of only three *R* genes from *L. saligna* that have been introgressed into *L. sativa*, none of which are located on chromosome 9 (Moreau 1994; Segura et al. 2011; Van Etteken and Van der Arend 1999). Interestingly, the cell death response to the *P. syringae* effector AvrPto did map in the same region on chromosome 9 in a *L. sativa* × *L. serriola* recombinant inbred line (RIL) population (McHale et al. 2009).

BLG03 is recognized in only two lettuce breeding lines of the initial screen. These lines share the *Dm2* resistance locus, which maps in or near the RGC2 locus (Meyers et al. 1998). The *Dm2* locus provides resistance to Bl:5, and recognition of BLG03 correlated with resistance to Bl:5 in 143 F2 plants of a UCDM2 × Cobham Green cross, in which *Dm2* segregates. This indicates that the response to BLG03 maps to the RGC2 locus in UCDM2. Sequencing of BLG03 in eight different isolates revealed seven different alleles. Of the sequenced *B. lactucae* isolates, Bl:5 and F703 are unable to successfully infect *Dm2*-containing hosts. We were not able to amplify BLG03 from *B. lactucae* isolate F703, although the quality of DNA was not a problem because we could amplify BLG01 and BLG02. Both Bl:5 BLG03 alleles (B and G) were not found in other sequenced *B. lactucae* isolates. One of the Bl:5 alleles (BLG03^G) did not induce a visible response in *Dm2*-containing lines but the other (BLG03^B) was recognized in the same lines as the Bl:24 reference allele (BLG03^A). The fact that resistance to isolate Bl:5 and recognition BLG03^B are fully linked in a segregating F2 population strongly suggests that the effector could be the avirulence protein Avr2 that is recognized by the *Dm2* gene.

Effector recognition versus resistance.

In most reported cases, effector recognition is linked to resistance. Recognition of BLG01, however, was not linked to resistance to Bl:24 in laboratory assays, and BLG03 was cloned from a *B. lactucae* isolate that can successfully infect the lettuce lines in which BLG03 is recognized. Examples of a lack of correlation between recognition of an effector and resistance to pathogen isolates that express the effector have previously been reported. Screening of 54 *Phytophthora infestans* effectors in *Solanum* spp. by PVX expression uncovered two interactions that were not correlated to resistance. In an F2 population of a cross between a resistant and susceptible *Solanum* sp., no correlation between the ability to recognize certain *P. infestans* effectors and resistance to *P. infestans* was found, despite perfect correlation between recognition of other *P. infestans* effectors and resistance (Vleeshouwers et al. 2008). Likewise, not all *Arabidopsis thaliana* accessions that recognize ATR39-1 or ATR1Emco5 are resistant to isolates that encode these alleles (Goritschnig et al. 2012; Krasileva et al. 2011; Rehmany et al. 2005).

An explanation for the lack of resistance in plants that can recognize individual *B. lactucae* effectors is that *B. lactucae* uses additional effectors to suppress ETI. A study that investigated crosses of virulent and avirulent *B. lactucae* isolates revealed a possible locus that inhibits avirulence triggered by Avr5/8 but no evidence for other inhibitors of avirulence in *B. lactucae* (Ilott et al. 1989). The study highlights that inhibitor loci exist but that they are polymorphic. Since this study was performed, over 20 years of selective pressure on *B. lactucae* has given rise to new isolates that break various resistances. However, none of our 34 tested effectors were able to suppress BLG01- or BLG03-induced cell death. We cannot rule out the existence of suppressors of cell death because our selection of

effectors is nonexhaustive and suppressors may even be non-RXLR effectors, which we did not investigate.

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

MATERIALS AND METHODS

Cloning and sequencing.

Effector predictions were sequence verified from PCR product using flanking primers and sequenced by Macrogen Inc. (Seoul, Korea). All primers used in this study are listed in Supplementary Table 4. Verified effector candidates were TOPO cloned into the pENTR vector using the pENTR/D-TOPO Cloning kit (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer’s instructions starting from the sequence after the predicted signal peptide cleavage site of the effectors, unless otherwise indicated, and were preceded by a newly introduced start codon. Constructs were then recombined into the pK2GW7 vector (Plant-Systems Biology VIB, Ghent, Belgium). Clones were electrotransformed into *Agrobacterium tumefaciens* C58C1 (pGV2260). The PsojNIP and YFP constructs are described by Cabral and associates (2012). For sequencing of effector alleles, DNA was PCR amplified using primers flanking the coding sequence, blunt-end ligated into pJET1.2 (Fermentas, Vilnius, Lithuania), and transformed into *Escherichia coli* DH5 α by heat-shock. Plasmid isolation and sequencing was carried out by Macrogen Inc.

Lettuce lines for the effector recognition screen.

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

Agrobacterium-mediated transient transformation assay.

A. tumefaciens strains containing the 35S-effector T-DNA were grown overnight in selective media at 28°C and 220 rpm. Cells were spun down at 2,500 $\times$ g for 10 min and resuspended in induction medium (1 $\times$ M9 salts, 1% glucose, and 50 μ M

acetosyringone) with appropriate antibiotics. After growth for 4 h at 28°C and 220 rpm, cells were spun down at 25,00 $\times$ g for 10 min and resuspended in infiltration medium (0.5 $\times$ Murashige and Skoog salts, 10 mM morpholinoethanesulfonic acid, 0.5% fructose, 0.5% sucrose, and 150 μ M acetosyringone). Strains were then pressure infiltrated into leaves using a needleless syringe. Responses in lettuce lines were scored 8 dpi, unless otherwise indicated. To test whether any of the 34 effectors are recognized in planta, we pressure infiltrated suspensions of the *Agrobacterium* strains carrying the effector constructs into leaves of the selected lettuce lines. Visual responses to transient expression of effector candidates were scored 5 to 8 days after *Agrobacterium* infiltration. We included strains carrying a YFP- or GUS-containing vector as a negative control, and a necrosis-inducing protein (NIP) gene-containing vector as a positive control. Expression of YFP is not expected to elicit visible cell death; therefore, any response seen after infiltration with *Agrobacterium* carrying the YFP construct is considered background. The NIP gene encoding PsojNIP that is derived from *P. sojae* induces a cell death response in lettuce that is visible 1 to 2 days after infiltration of the *Agrobacterium* strain and develops into a dark necrotic lesion after 48 h.

Time course qPCR.

For time-course experiments, 1-week-old *L. sativa* ‘Olof’ seedlings were spray inoculated with 150 spores μ l⁻¹ until runoff was imminent. Seedlings were grown under high humidity (closed tray with transparent lid) at 17°C with 9 h of light (100 μ E/m²/s) and kept under these conditions for the duration of the experiment. Samples were taken and snap-frozen immediately after spraying and every 24 h until 5 dpi. Total RNA was extracted using the Spectrum plant total RNA kit (Sigma Life Science, St. Louis) and treated with DNase (Fermentas). cDNA was synthesized using RevertAid H minus Reverse Transcriptase (Fermentas) and Oligo(dT)15. Cycle thresholds (C_T) were determined in triplicate per transcript using the ABI PRISM 7900HT or the Life Technologies ViiA7 system using SYBR Green as reporter dye. Expression levels were determined as the number of qPCR cycles required for the abundance of each amplicon to reach C_T level, and were normalized to *L. sativa* actin or *B. lactucae* actin (resulting in Δ C_T values).

Effector translocation assay.

The method for testing the delivery of the effector domain of Avr3a (originally from *P. infestans*) from transgenic *P. capsici* into cells of *N. benthamiana* was performed essentially as described by Schornack and associates (2010). The coding sequences of BLG01 and BLG03, encoding the N-terminal region of the proteins containing the signal peptide and GKLR-DER domain, were amplified from cDNA and cloned into the pTOR::CRN16-AVR3a vector (Schornack et al. 2010) digested with *Cla*I/*Asc*I to replace the CRN16 sequence with the BLG sequences. Transformation of *P. capsici* strain LT3112 was performed as described by Huitema and associates (2011). *P. capsici* transformants were inoculated on detached *N. benthamiana* leaves in the form of agar plugs from the border of radially grown colonies on V8 agar plates, and scored 4 to 7 dpi. The reduction in the percentage of spreading lesions on R3a plants relative to that on wild-type plants was calculated as 1 – (% spreading lesions R3a/% spreading lesions wild type) $\times$ 100, and was compared using analysis of variance (ANOVA) and *t* tests.

Codon-based test for positive selection.

Insertions and deletions were removed from sequences and sequences with internal stop codons were removed. dS and dN substitution rates were calculated using Nei and Gojobori’s

method (Nei and Gojobori 1986), using MEGA4 (Tamura et al. 2007). Standard error was determined by 500 bootstrap replications. The null hypothesis of no selection ($dN = dS$) versus the positive selection hypothesis ($dN > dS$) was determined using the Z test: $Z = (dN - dS) / \sqrt{[Var(dS) + Var(dN)]}$.

Materials for mapping the BLG01 response.

Two crosses of *L. saligna* and *L. sativa* were previously made: cross 1 of *L. saligna* CGN05271 $\times$ *L. sativa* 'Olof' and cross 2 of *L. saligna* CGN11341 $\times$ *L. sativa* Norden (Jeuken et al. 2001). Materials for assays 1, 2, and 3 were as follows. Assay 1: three replicates of 28 BIL derived from cross 1 that, together, cover 96% of the *L. saligna* CGN5271 genome (Jeuken and Lindhout 2004) and the parental lines of cross 1 and cross 2. Assay 2: F3, F4, and BC1S1 plants derived from cross 1 and cross 2. Assay 3: parental lines of cross 1 and cross 2, F1 offspring of cross 1, and F3 offspring of F2 plants of both cross 1 and cross 2, with a recombination near the C9 locus.

Marker development and genotyping.

For fine mapping, new markers were developed and selected to saturate the region. Based on alignment of our F2 map (improved version of Jeuken and associates [2001], with the *L. sativa* $\times$ *L. serriola* RIL map by the Lettuce SFP Chip Project and the Compositae Genome Project Database), we selected candidate EST sequences and markers. Primers were developed, tested, and, in the case of polymorphism, run on the segregating populations for mapping. Polymorphisms between *L. sativa* and *L. saligna* PCR products of the EST markers were visualized by high-resolution melting curve differences on a LightScanner System (Idaho Technology, Salt Lake City, UT, U.S.A.). All plants with a cell death response in transient assay 2 and all the plants from assay 3 were genotyped using the new markers.

Disease test on adult plants.

A detached-leaf assay was conducted on adult plants (37 days after sowing) according to the protocol of ADT_G, as previously described (Jeuken et al. 2008). Two genotyped BC1S2 populations from a BC1S1 plant that showed a cell death response toward BLG01 were tested with *B. lactucae* Bl:24. From each plant, at least six leaf squares were collected (2.5 by 2.5 cm). Leaf squares were inoculated with inoculum from *B. lactucae* isolate Bl:24 containing 4×10^5 spores per milliliter. At 9 dpi, the percentage of the area of each leaf square that showed *B. lactucae* sporulation was determined. One-way ANOVA was used to analyze the data, with line (offspring from one parent with genotype at C9 locus) as fixed factor and each different plant as block factor. For comparison between the heterozygous and homozygous *L. sativa* genotypes from each BC1S1 parent with each other, a Duncan's multiple range test ($\alpha = 0.05$) was performed with GenStat (14th edition) software.

ACKNOWLEDGMENTS

This project was supported by the foundation TTI Green Genetics (TTI GG, The Netherlands) and was a collaboration among Utrecht University, Wageningen University, and five vegetable breeding companies: Enza zaden (Enkhuizen, The Netherlands), Nunhems (Nunhem, The Netherlands), Rijk-Zwaan (De Lier, The Netherlands), Syngenta (Enkhuizen, The Netherlands), and Vilmorin (La Mènitric, France). We thank H. van Pelt for photography, E. Huitema (University of Dundee, Scotland) for providing materials and protocols for *P. capsici* transformation, and V. Vleeshouwers (University of Wageningen, The Netherlands) for providing *N. benthamiana* R3 seed.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- The International Bremia Evaluation Board on the website of the International Seed Federation: www.worldseed.org/istf/ibeb.html
- University of California Davis Compositae Genome Project database: compgenomics.ucdavis.edu
- University of California Davis Lettuce Genome Resource database: lgr.genomecenter.ucdavis.edu
- University of California Davis Lettuce SFP Chip Project website: chipllett.ucdavis.edu
- Wageningen University's Center for Genetic Resources, the Netherlands website: www.wageningenur.nl/en/Expertise-Services/Legal-research-tasks/Centre-for-Genetic-Resources-the-Netherlands-1.htm