### Title

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### Journal

Plant Biotechnology Journal, 13(7)

### ISSN

1467-7644



### Authors

Govindarajulu, Manjula
Epstein, Lynn
Wroblewski, Tadeusz
et al.



### Publication Date

2015-09-01

### DOI

10.1111/pbi.12307

Peer reviewed

Plant Biotechnology Journal

*Plant Biotechnology Journal* (2014), pp. 1–9 doi: 10.1111/pbi.12307

# Host-induced gene silencing inhibits the biotrophic pathogen causing downy mildew of lettuce

Manjula Govindarajulu[1], Lynn Epstein[2], Tadeusz Wroblewski[1] and Richard W. Michelmore[1,3,*]

[1]*Genome Center, University of California, Davis, CA, USA*

[2]*Department of Plant Pathology, University of California, Davis, CA, USA*

[3]*Departments of Plant Science, Molecular & Cellular Biology, Medical Microbiology & Immunology, University of California, Davis, CA, USA*

*Received 28 July 2014;*
*revised 18 October 2014;*
*accepted 4 November 2014.*
**Correspondence* (Tel 530 752 1729;
fax 530 752 0436;
email rwmichelmore@ucdavis.edu)

**Keywords:** disease resistance, RNA interference, gene silencing, HIGS, *Bremia lactucae*, *Lactuca sativa*.

## Summary

Host-induced gene silencing (HIGS) is an RNA interference-based approach in which small interfering RNAs (siRNAs) are produced in the host plant and subsequently move into the pathogen to silence pathogen genes. As a proof-of-concept, we generated stable transgenic lettuce plants expressing siRNAs targeting potentially vital genes of *Bremia lactucae*, a biotrophic oomycete that causes downy mildew, the most important disease of lettuce worldwide. Transgenic plants, expressing inverted repeats of fragments of either the *Highly Abundant Message #34* (*HAM34)* or *Cellulose Synthase* (*CES1*) genes of *B. lactucae*, specifically suppressed expression of these genes, resulting in greatly reduced growth and inhibition of sporulation of *B. lactucae*. This demonstrates that HIGS can provide effective control of *B. lactucae* in lettuce; such control does not rely on ephemeral resistance conferred by major resistance genes and therefore offers new opportunities for durable control of diverse diseases in numerous crops.

## Introduction

RNA interference (RNAi) is a potent tool for silencing genes in a broad range of organisms. A form of RNAi involves expression of a double-stranded RNA (dsRNA) or small interfering RNA (siRNAs) that trigger degradation of target mRNA sequences by post-transcriptional gene silencing (PTGS). RNAi-based, host-induced gene silencing (HIGS) is a potentially powerful new strategy for disease control in various organisms. In plants, HIGS has potential for control of insects (Huvenne and Smagghe, 2010) and nematodes (Fairbairn *et al.*, 2007) and has been shown to control the western corn rootworm, *Diabrotica virgifera,* in corn (Baum *et al.*, 2007) and *Heterodera schachtii*, the sugar beet cyst nematode in *Arabidopsis* (Huang *et al.*, 2006; Sindhu *et al.*, 2009; Yadav *et al.*, 2006). HIGS of parasitic weeds, such as *Tryphysaria* spp. which form invasive haustoria in host roots, has been reported; movement of siRNAs into *Tryphysaria* spp. was demonstrated by suppression of β-glucuronidase (GUS) expression in *Tryphysaria* after it had invaded host roots that expressed dsRNA from the *GUS* gene (Tomilov *et al.*, 2008). However, this is a very different type of haustorial interface from that between fungal or oomycete pathogens and plants (Aly, 2013).

There are clearly a large number of biochemical exchanges, in both directions, between plants and pathogens (Spanu, 2012). Several studies involving transient expression have demonstrated that host plants that express siRNAs targeting the transcripts of vital pathogen genes can inhibit the expression of these genes in pathogens. Transient induction of silencing using virus-induced gene silencing (VIGS) to induce HIGS in cereals (Nowara *et al.*, 2010; Panwar *et al.*, 2013; Pliego *et al.*, 2013; Yin *et al.*, 2011; Zhang *et al.*, 2012) reduced growth of obligate fungal (powdery mildew and rust) pathogens. Recently, HIGS has been reported in stable transgenic plants. HIGS of *Fusarium oxysporum* genes resulted in control of *Fusarium* wilt in transgenic banana (Ghag *et al.*, 2014). Tobacco plants expressing siRNAs that targeted *GUS* silenced the *GUS* gene in transgenic *F. verticillioides,* a fungal necrotroph (Tinoco *et al.*, 2010) and HIGS of genes of the hemibiotroph *F. graminearum* resulted in control in barley and *Arabidopsis* (Koch *et al.*, 2013). Most recently, HIGS targeting the Avr3a-like gene (PcAvr3a1) in *Phytophthora capsici* allowed infection of otherwise resistant tobacco (*Nicotiana tabacum*), implicating a component of nonhost resistance to *P. capsici* in tobacco (Vega-Arreguín *et al.*, 2014). In contrast, HIGS against genes of the hemibiotrophic oomycete *Phytophthora parasitica* failed to induce silencing in transgenic *Arabidopsis* (Zhang *et al.*, 2011). It was unknown whether siRNAs produced in stable transgenic plants would move from the plant into biotrophic pathogens such as *Bremia lactucae* in sufficient quantities to induce gene silencing and thereby render the plants resistant.

The obligate biotrophic oomycete pathogen, *B. lactucae* infects through an epidermal cell and then a coenocytic mycelium grows intercellularly through the mesophyll, obtaining nutrients via haustoria that invaginate the plant plasmalemma (Michelmore *et al.*, 2009). Successful infection results in profuse sporulation on the leaf surface giving the downy appearance characteristic of the disease. Current approaches to combat *B. lactucae* rely on genetic resistance and fungicides. Any of 25 major *Downy mildew* (*Dm*) genes identified in lettuce (*Lactuca sativa* L.) can provide complete resistance against specific isolates of *B. lactucae* in a gene-for-gene manner (Crute and Johnson, 1976; Farrara

Please cite this article as: Govindarajulu, M., Epstein, L., Wroblewski, T. and Michelmore, R.W. (2014) Host-induced gene silencing inhibits the biotrophic pathogen causing downy mildew of lettuce. *Plant Biotechnol. J.*, doi: 10.1111/pbi.12307

*et al.*, 1987; Hulbert and Michelmore, 1985; Michelmore *et al.*, 2009); however, typical of major resistance genes, *Dm* genes have remained effective for only limited periods of time due to rapid changes in pathogen virulence to overcome resistance. Chemical protection can be used but several protectants are either being withdrawn from agricultural use due to concerns over their safety and environmental impact or have been rendered ineffective due to development of insensitivity in *B. lactucae* (Brown *et al.*, 2004; Schettini *et al.*, 1991). Consequently, alternative, more durable control strategies are needed.

Lettuce is readily transformed using *Agrobacterium tumefaciens* allowing the rapid generation of large numbers of stable transgenics. RNAi can be used to efficiently silence resistance genes in stably transformed lettuce plants (Wroblewski *et al.*, 2007). Genomic resources are also available for *B. lactucae* (Michelmore *et al.*, 2009). We therefore conducted proof-of-concept experiments that demonstrated HIGS against an obligate biotrophic pathogen in stable transgenic plants.

## Results and discussion

To investigate the potential of HIGS against *B. lactucae* in lettuce, seven genes that represented a range of biological activities likely to be vital to *B. lactucae* was selected as targets for silencing. *Highly Abundant Message #34* (*HAM34*) is a single copy, conserved, constitutively highly expressed gene encoding a protein of unknown function (Judelson and Michelmore, 1990). *HAM34* is present in other oomycetes but not plants; no homologous gene is detectable by BLAST in the lettuce genome. *Cellulose synthase (CES1)* encodes a protein involved in cell wall biosynthesis (Blum *et al.*, 2010). No homologue of the *CES1* target sequence was detected in the lettuce genome. *Actin* (*ACT*) and β-*tubulin* (*TUB*) encode cytoskeleton proteins; the latter is the target site of the fungicide benomyl (Davidse, 1986). *Elongation factor* (*ELF1*) encodes a protein integral for protein synthesis (van't Klooster *et al.*, 2000). *NLP2* and *NLP3* encode Nep 1-like proteins that are possibly involved in pathogenesis (Gijzen and Nürnberger, 2006). To induce RNAi, ~230- to 400-bp trigger sequences that were located near the 3′ end of the coding sequences were selected for each *B. lactucae* gene. The seven selected fragments were checked by BLAST to confirm the absence of stretches of 14 nt or more of identity in the lettuce genome to minimize the possibilities of off-target effects (Elbashir *et al.*, 2002; Jackson *et al.*, 2003) on lettuce gene expression. The *B. lactucae* gene fragments were amplified and cloned as inverted repeats of the RNAi trigger sequences fused to a 400-bp fragment of the *UidA* gene (Figure S1, Wroblewski *et al.*, 2014). The UidA fragment served as a reporter for silencing using *A. tumefaciens*-mediated transient β-glucuronidase (GUS) assays, which allowed us to select transgenics exhibiting high levels of silencing (Wroblewski *et al.*, 2007, 2014).

Eleven to 15 stable transgenic lettuce plants, cv. Cobham Green, were generated with each of the seven hairpin (hp) RNAi-mediated HIGS constructs (Table 1). Cobham Green expresses no known genes for resistance to downy mildew (*Dm* genes) and is therefore fully susceptible to nearly all isolates of *B. lactucae*. Using the transient GUS assays (Figure 1), one to five $T_1$ transformants expressing siRNAs were identified (Table 1) amongst the plants transformed with six of the seven constructs and these plants were studied further. Of the 12 $T_1$ plants transformed with the construct targeting *ELF1,* none exhibited GUS silencing and no further experiments were conducted with those plants (Figure 1). The $T_2$ seedlings and leaves of mature $T_2$ plants were then challenged with two isolates of *B. lactucae* to assay for resistance. Profuse sporulation was observed on wild-type cv. Cobham Green controls and all of the plants transformed with RNAi constructs targeting *TUB*, *ACT*, *NLP2* and *NLP3*. Therefore, there was no evidence for HIGS elicited by these four constructs.

In contrast, seedlings and mature leaves in four and two $T_2$ families carrying RNAi constructs designed to silence *HAM34* and *CES1,* respectively, segregated for resistance, indicative of HIGS (Table 1, Figure 2). In two of the resistant $T_2$ families representing transgenics designed to silence *HAM34* and in both resistant *CES1* families, resistance segregated 1:2:1 for profuse sporulation, sparse sporulation and no sporulation, respectively, consistent with the segregation of a single effective integration event in progeny of hemizygous $T_1$ transgenics (Table 2). The other two families silenced for HAM34 had only one of 25 $T_2$ plants with no sporulation and a few plants with sparse sporulation (Table 2); the number of plants with reduced sporulation was consistent with a 1:3 segregation ratio indicative of the reduced sporulation phenotypes only occurring in transgenics homozygous for the transgene. Results for individual plants were always consistent: the same plants that were resistant at the seedling stage as indicated by a lack of sporulation were also resistant in the

**Table 1** Numbers of transgenic plants tested for silencing and disease phenotypes

| Target gene* | Number of $T_1$ transgenic plants generated | Number of transgenic $T_1$ plants with silencing of GUS† | Number of $T_2$ families with cosegregation of silencing of GUS and no disease | Number of $T_2$ families with 1:2:1 segregation for silencing of GUS and no disease |
|---|---|---|---|---|
| *ELF1* | 12 | 0 | 0 | 0 |
| *HAM34* | 13 | 5 | 4 | 2 |
| *CES1* | 14 | 5 | 2 | 2 |
| *ACT* | 13 | 3 | 0 | 0 |
| *TUB* | 11 | 2 | 0 | 0 |
| *NLP2* | 15 | 1 | 0 | 0 |
| *NLP3* | 15 | 4 | 0 | 0 |

**Elongation factor (ELF1), Highly Abundant Message #34 (HAM34), Cellulose synthase (CES1), Actin (ACT), β-tubulin (TUB), (Nep1)-like protein 2 (NLP2)* and *(Nep1)-like protein 3 (NLP3)*.

†Blue stain = no silencing of GUS. Unstained = silencing of GUS.

 

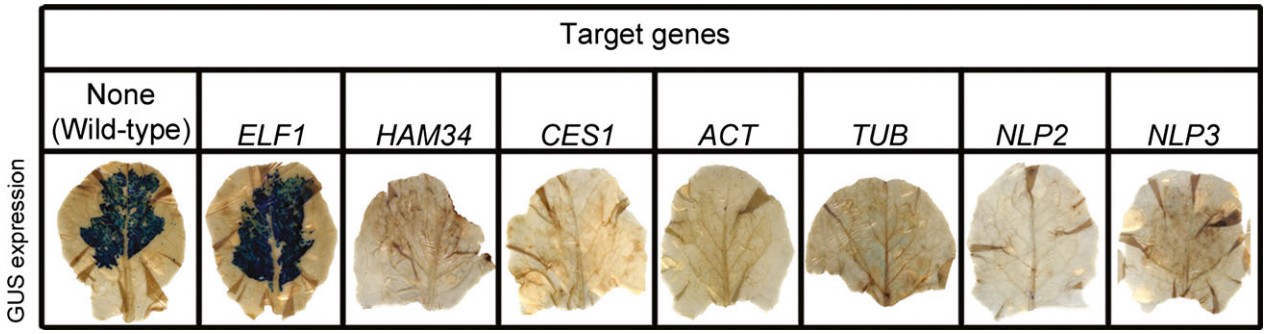


**Figure 1** Stable $T_1$ transgenic lines screened for silencing using *Agrobacterium*-mediated transient expression of the *GUS* (*UidA*) gene. Transient expression of GUS provided a fast and reliable method to identify stable transgenic lines of lettuce that were actively silencing genes. Absence of GUS expression identified the plants that were expressing the silencing construct. *Elongation factor (ELF1), Highly Abundant Message #34 (HAM34), Cellulose synthase (CES1), Actin (ACT), β-tubulin (TUB), (Nep1)-like protein 2 (NLP2)* and *(Nep1)-like protein 3 (NLP3)*.

detached leaf assays (Figure 2). Silencing of GUS correlated with resistance: $T_2$ plants exhibiting GUS activity and therefore not silenced were susceptible to *B. lactucae*, while those completely lacking GUS activity were fully resistant and those with sparse sporulation exhibited incomplete silencing as evidenced by dark blue dots in the leaf (Figure 2). Progeny tests of $T_3$ families derived from each of the fully resistant $T_2$ plants confirmed the results for silencing of *HAM34* and *CES1* (Figure 3). Five to seven fully resistant plants for each transgenic $T_2$ family were selfed to produce $T_3$ seed. Susceptible seedlings died following inoculation and therefore could not be selfed; consequently, sib plants from transgenic $T_2$ families that were 100% susceptible were selfed without inoculation. At 9 days postinoculation (dpi), $T_3$ plants silenced for *GUS* in the *HAM34* and *CES1* transgenics (Figure 3c, d) had no or only sparse sporulation (Figure 3h,i) but never profuse sporulation in contrast to 100% of seedlings with profuse sporulation on the wild-type control (Figure 3f) and on GUS-expressing, and therefore non-silenced, $T_3$ seedlings derived from the susceptible/nonsilenced families (Figure 3g). Again, those $T_3$ plants with no sporulation exhibited no staining with GUS, and those with sparse sporulation had dark blue dots indicating partial silencing.

To assess the HIGS phenotype, wild-type control, nonsilenced GUS-expressing $T_3$ sibs, RNAi-HAM34, RNAi-CES1 and lettuce cv. Diana were examined microscopically over time. *B. lactucae* induced a typical incompatible, hypersensitive response in cv. Diana due to the activity of *Dm3* (Shen *et al.*, 2002) with growth and development limited to the penetrated epidermal cell; penetrated cells exhibited autofluorescence typical of a hypersensitive response (HR) by 4 dpi (Figure S2). Amongst the other four genotypes, at 1.5 dpi, there were no observable differences in the development of primary and secondary vesicles in epidermal cells or of initial intercellular hyphae and haustoria, that is the two HIGS-associated genotypes appeared to exhibit typical susceptible interactions at 1.5 dpi (Figure 4a–d). Although the pathogen continued to grow in the two HIGS-exhibiting transgenic plants, growth was retarded in both at 4, 6 and 9 dpi, (Figure 4g–h; Figure S3c-d); however, haustoria appeared normal (Figure 4k–l) and autofluorescence characteristic of *Dm*-mediated resistance was not observed. In the control and non-silenced GUS-expressing $T_3$ sibs, sporophores first appeared at 4 dpi and there was profuse sporulation by 9 dpi. No sporophores were observed at any time point on the HIGS-exhibiting transgenic plants, and therefore, the lifecycle of *B. lactucae* was not completed.

Quantitative PCR of *B. lactucae* genomic DNA was used to estimate *B. lactucae* biomass at 6 and 9 dpi (Figure 5). Estimates of DNA based on *B. lactucae actin* and *β-tubulin* were highly correlated ($r = 0.98$, $P < 0.0001$). Biomass in the wild-type control and the nonsilenced, GUS-expressing $T_3$ controls were not significantly different ($\alpha = 0.05$). Based on the detransformed estimates of mass of *B. lactucae,* DNA averaged from the actin and β-tubulin standards, and compared to the wild type at 6 and 9 dpi, respectively, RNAi-HAM34 had $10 \pm 4$ and $4 \pm 1$% of the control biomass, RNAi-CES1 had $3 \pm 1$ and $4 \pm 2$% of the biomass, cv. Diana had $0.04 \pm 0.02$ and $0.02 \pm 0.01$% of the biomass, and the transformed nonsilenced $T_3$ controls had $130 \pm 31$ and $95 \pm 10$%. In the RNAi transgenic plants, the biomass of *B. lactucae* was greater than in the resistant cv. Diana but less than in the susceptible wild-type control (Figure 5), congruent with the microscopic observations (Figures 4 and S3). The reduced growth of the pathogen in the transgenic lines is consistent with HIGS.

To demonstrate specific silencing of pathogen genes by HIGS, expression levels of the two *B. lactucae* genes targeted, *HAM34* and *CES1*, were assayed in the $T_3$ seedlings that exhibited silencing at 6 and 9 dpi using quantitative real-time reverse transcriptase PCR (qRT-PCR). Transcript levels of *HAM34* and *CES1* in the infected transgenic plants were normalized relative to the *B. lactucae* actin gene and compared to expression of *HAM34* and *CES1* in nonsilenced wild-type controls. Gene silencing was detected for *HAM34* and *CES1* at both 6 and 9 dpi only in those transgenics expressing the cognate RNAi sequence (Figure 6). The expression of each of the genes at both time points was unaffected in the nonsilenced, GUS-expressing $T_3$ sibs and the $T_3$ transgenic nontarget RNAi lines. In contrast, *HAM34* showed highly significantly ($P < 0.0001$) decreased expression relative to actin specifically in the RNAi-HAM34 lines at both 6 and 9 dpi (Figure 6). Similarly, *CES1* specifically showed highly significantly ($P < 0.0001$) decreased expression in RNAi-CES1 lines at 6 and 9 dpi. This specific decrease in the abundance of *B. lactucae* gene transcripts indicates that translocation of siRNA molecules from the host cell to the pathogen occurs in sufficient quantities to induce gene silencing in the pathogen and that the resistant phenotype is not a nonspecific consequence of high levels of expression of a ihpRNA construct in the plant.

 

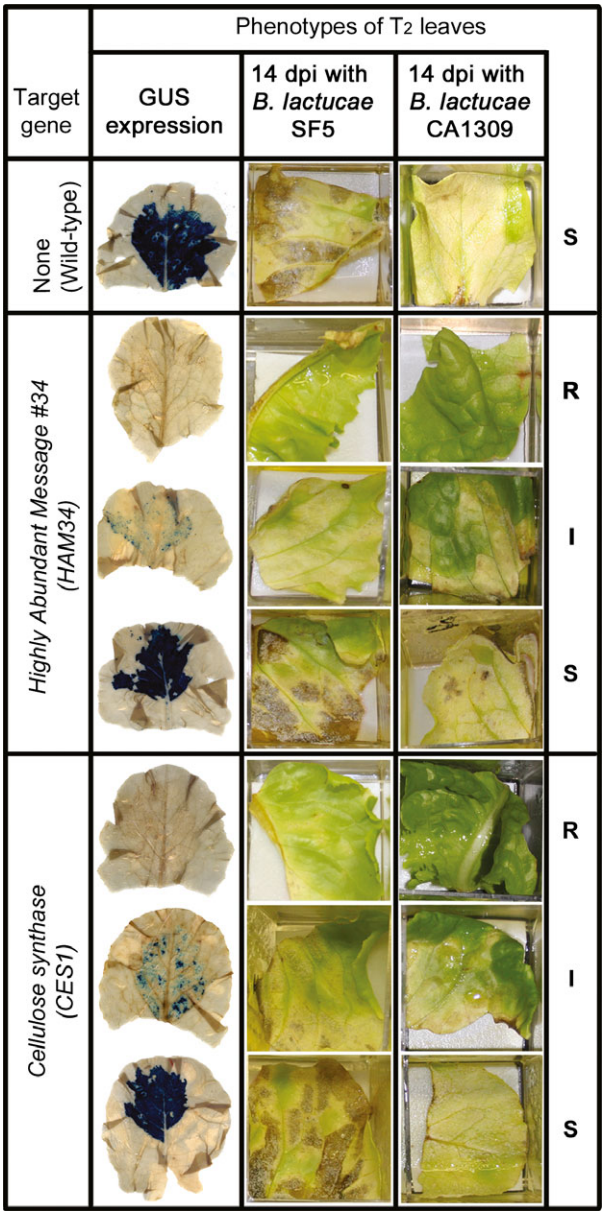


**Figure 2** Analysis of GUS activity and sporulation on leaf pieces from $T_2$ plants that segregated for disease resistance and silencing of GUS in seedlings. The $T_2$ plants segregated 1:2:1 with one-quarter of the plants with no sporulation (R, resistant) and no GUS activity, half of the plants with sparse sporulation (I, partially resistant) and weak GUS activity, and one-quarter of the plants with profuse sporulation (S, susceptible) and strong GUS activity. The $T_2$ seedlings with GUS activity, and therefore no silencing, were susceptible to both isolates of *Bremia lactucae*.

This study provides a clear demonstration of HIGS controlling an obligate biotrophic oomycete and one of the first demonstrations of HIGS against a pathogen in stably transformed plants. Our data are consistent with dsRNA or siRNAs moving from the host to the pathogen, probably across the haustorial interface (Nowara *et al.*, 2010; Panwar *et al.*, 2013; Yin *et al.*, 2011), in sufficient quantities to inhibit the growth of *B. lactucae*. However, the form and mechanism of the movement of the silencing signal from plant to pathogen is currently unknown. It is possible that small RNA signals move in both directions as part of natural interactions between plants and pathogens. Small RNA virulence effectors from *Botrytis cinerea* were recently shown to compromise plant RNAi machinery by binding to *Arabidopsis* Argonaute 1 (AGO1) proteins, which in turn silence host immunity genes, and thus facilitate infection (Weiberg *et al.*, 2013). One possibility is that dsRNA is generated in the host nucleus, cleaved into siRNAs by DICER in the plant cytoplasm and delivered across the haustorial interface into *B. lactucae*. Effectors secreted by oomycete pathogens may enter host cells via receptor-mediated endocytosis (Kale and Tyler, 2011). It is possible that the siRNAs move in the reverse direction using a similar mechanism. Our data also present the opportunity for using HIGS as an experimental tool to test the function and importance of genes in biotrophic pathogens that are recalcitrant to transformation.

This study serves as a proof-of-concept for controlling other biotrophic diseases. Orthologous *CES1* and *HAM34* sequences are present in other oomycetes and therefore are good candidates for triggering HIGS and disease control in other pathosystems. New methods of control are particularly important for multiple rust diseases of wheat, including the new virulent Ug99 race of *Puccinia graminis* (Singh *et al.*, 2011). It also will be interesting to determine whether HIGS will be effective against other types of diseases such as wilts caused by *Verticillium dahliae*, *Fusarium oxysporum* and *Botrytis cinerea* in lettuce. Pathogen control conferred by HIGS is likely to be more durable than resistance mediated by major genes for resistance. Targeting ~400 bp of genes that are vital to the pathogen means that it would require a major change in the pathogen to negate the effect of HIGS. The evolutionary hurdle required for re-establishing virulence could be further enhanced by concatenating trigger sequences that target multiple genes vital to the pathogen. HIGS may also be subject to reduced regulatory hurdles and be more readily accepted by the public because no new proteins are made as they are in other transgenic strategies.

## Experimental procedures

### Design and generation of RNAi constructs

Seven *B. lactucae* genes were targeted for RNA interference (RNAi)-based host-induced gene silencing (HIGS). To induce RNAi, ~230- to 400-bp fragments were selected for each gene from regions present in other oomycetes. These fragments were checked using BLAST (CLC Genomic Workbench 4.0 software; http://www.clcbio.com/) to confirm the absence of stretches of 14 or more nucleotides of identical sequence in the lettuce genome. Inverted-repeat silencing constructs that included a 400-bp fragment of the reporter gene encoding beta-glucuronidase (Wroblewski *et al.*, 2007, 2014) (*UidA,* Figure S1) were generated to silence each of the *B. lactucae* target genes (Table 1, Figure 1). The seven *B. lactucae* gene fragments: *ELF1* (bases 309–676), *HAM34* (bases 988–1223), *CES1* (bases 2315–2694), *ACT* (bases 534–770), *TUB* (bases 439–768), *NLP2* (bases 31–426) and *NLP3* (bases 55–446) located near the 3′ end of the coding region were PCR-amplified from genomic DNA of *B. lactucae* isolate SF5 (see Table S1 for primers used) and cloned into the pENTR™ vector using the D-TOPO® Cloning Kit (http://www.lifetechnologies.com). All constructs were verified by DNA sequencing, and the fragments transferred into the modified binary pGSA1165 vector (www.chromdb.org). The cloning resulted in inverted-repeat constructs in which the fragments of the *B. lactucae* trigger sequence were adjacent to the intron-3 from *Flaveria trinervia*

 

**Table 2** Segregation for resistance to *Bremia lactucae* in $T_2$ families of lettuce expressing RNAi targeting *Cellulose synthase (CES1)* or *Highly Abundant Message #34 (HAM34)* in *B. lactucae*

| Target gene (and $T_2$ family designation) | Disease phenotype | | | Segregation ratio tested with $\chi^2$ and *P*-value |
|---|---|---|---|---|
| | Fully resistant Number of progeny | Partially resistant | Susceptible | |
| *RNAi CES1* (C1) | 5 | 12 | 8 | $P_{1:2:1} = 0.68$ |
| *RNAi CES1* (C2) | 6 | 13 | 6 | $P_{1:2:1} = 0.98$ |
| *RNAi HAM34* (H1) | 7 | 13 | 5 | $P_{1:2:1} = 0.84$ |
| *RNAi HAM34* (H2) | 6 | 12 | 7 | $P_{1:2:1} = 0.94$ |
| *RNAi HAM34* (H3) | 1 | 5 | 19 | $P_{1:3} = 0.76$ |
| *RNAi HAM34* (H4) | 1 | 7 | 17 | $P_{1:3} = 0.47$ |

*pyruvate orthophosphate dikinase* (*pdk*) gene (Rosche and Westhoff, 1995; Wesley *et al.*, 2001).

## Generation and analysis of stable transgenic lettuce plants

Silencing constructs were transformed into the LBA4404 strain of *A. tumefaciens* (Hoekema *et al.*, 1984). *Agrobacterium tumefaciens*-mediated transformation of *L. sativa* cv. Cobham Green (Michelmore *et al.*, 1987) was performed at the UC Davis plant transformation facility. Eleven to 15 primary stable transformants ($T_1$ plants) were generated with each construct. Each $T_1$ plant was assayed using *A. tumefaciens*-mediated transient assays for β–glucuronidase (GUS) activity (Schob *et al.*, 1997; Wroblewski *et al.*, 2005 and Wroblewski *et al.*, 2007). *Agrobacterium tumefaciens* C58C1 carrying pTFS40, which encodes a functional *UidA* gene, was suspended in water ($OD_{600}$ 0.5–0.6) and leaves were infiltrated when plants were at the 2–3 leaf stage (Wroblewski *et al.*, 2005). Leaves were collected 4 days postinfiltration and assayed for GUS activity (Jefferson *et al.*, 1987). The lack of GUS activity was indicative of silencing. From 0 to 38% of the $T_1$ plants, depending on the hp RNAi gene construct, they had been transformed with, exhibited silencing (Table 1, Figure 1). All silenced and nonsilenced $T_1$ plants were self-pollinated to generate $T_2$ seeds.

## Analysis of transgenic plants: disease assays

$T_2$ progeny from each silenced and nonsilenced $T_1$ plant were challenged with *B. lactucae*. A suspension of conidia of isolate SF5 was sprayed onto 7-day-old $T_2$ seedlings (25 seedlings/$T_2$ family) as well as wild-type lettuce cv. Cobham Green as a control. Seedlings were scored 2 weeks later for resistance using a three-state scale: resistant, zero to trace amounts of sporulation on up to 25% of seedlings; partially resistant, 25–75% of seedlings with light to moderate sporulation; susceptible, profuse sporulation on 75–100% of seedlings. Progeny for which there was sporulation on <75% of the seedlings were grown further and assayed for GUS at the 2–3 leaf stage as described above (Figure 2). The $T_2$ seedlings with GUS activity, and therefore no

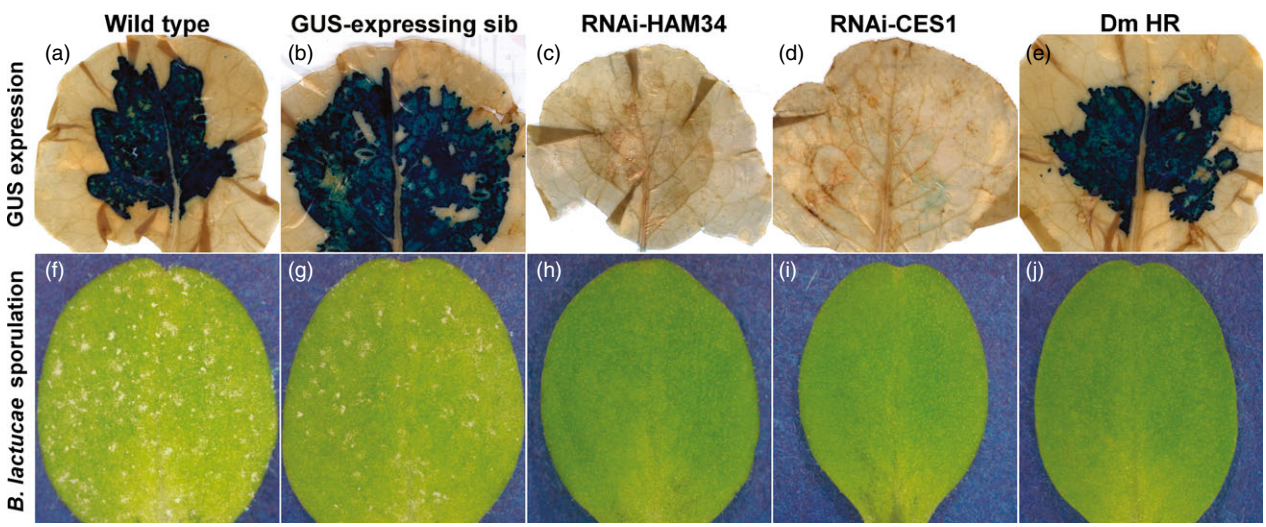


**Figure 3** Five lettuce genotypes tested for host-induced gene silencing (HIGS). (a and f) Wild-type *Lactuca sativa* cv. Cobham Green control; (b and g) Nonsilenced GUS-expressing $T_3$ control (GUS-expressing sib); (c and h) $T_3$ with the *Bremia lactucae HAM34* gene silenced (RNAi-HAM34); (d and i) $T_3$ with the *B. lactucae cellulose synthase* gene silenced (RNAi-CES1); (e and j) nontransformed *L. sativa* cv. Diana carrying the *Dm3* major resistance gene (*Dm* HR). (a-e) Transient *Agrobacterium tumefaciens* GUS assays on leaves showing silencing of *UidA* in two lines (c and d) and not in the infiltrated area of the three controls (a, b and e). (f-j) 9 days after lettuce cotyledons were infected with *B. lactucae* isolate SF5, sporulation was present on (f) and (g), but not on any of the three other genotypes.

 

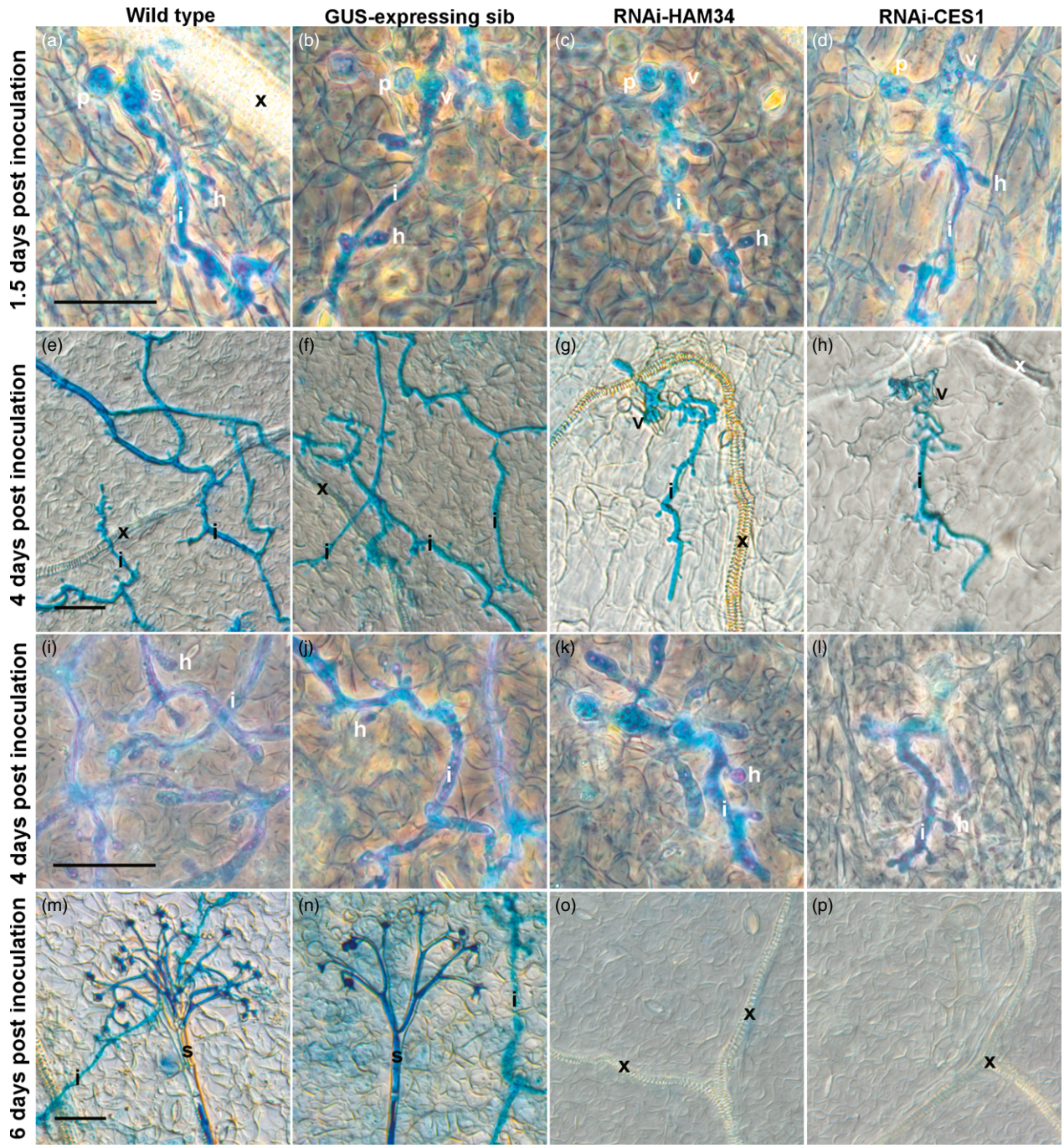


**Figure 4** Z-images, taken using light microscopy, of trypan blue-stained *Bremia lactucae* in four lettuce genotypes at 1.5, 4 and 6 days postinoculation (dpi) with *B. lactucae*. (a–d) Phase contrast images at 1.5 dpi. No differences were observed in the development of the primary vesicles (p) and secondary vesicles (v) in the epidermal cells or in the intracellular hyphae (i) or the haustoria (h) in the mesophyll. (e–h) Differential interference contrast images at 4 dpi. There was more *B. lactucae* growth in the nonsilenced genotypes (left-most two columns) than in the silenced genotypes. (i–l) Phase contrast images at four dpi, at higher magnification than (e–h); silenced lines exhibited reduced growth. (m–p) Differential interference contrast images at 6 dpi. Pronounced sporulation in the non-silenced genotypes and large uncolonized areas in the two silenced genotypes. s, sporophore; x, xylem. Bars = 50 μm.

silencing, were susceptible to both isolates of *B. lactucae*. Fully susceptible plants died at the seedling stage and could not be tested further. Additional tests for resistance were also performed using isolates SF5 and CA1309 inoculated onto leaf discs (Figure 2). Five to seven $T_2$ plants of two families segregating for *RNAi-HAM34* and *RNAi-CES1* (Figure 2) were self-pollinated to produce $T_3$ seeds (four families total). The $T_3$ families for *RNAi-HAM34* and *RNAi-CES1* were phenotyped for disease resistance as described above. A suspension of isolate SF5 conidia was sprayed onto 7-day-old $T_3$ seedlings (20 seedlings/$T_3$ family) of the following genotypes: wild-type *L. sativa* cv. Cobham Green as a control, GUS-expressing $T_3$ seedlings as a nonsilenced control, transgenic RNAi-HAM34 and RNAi-CES1 $T_3$ seedlings and *L. sativa* cv. Diana (incompatible with *B. lactucae* isolate SF5 due

 

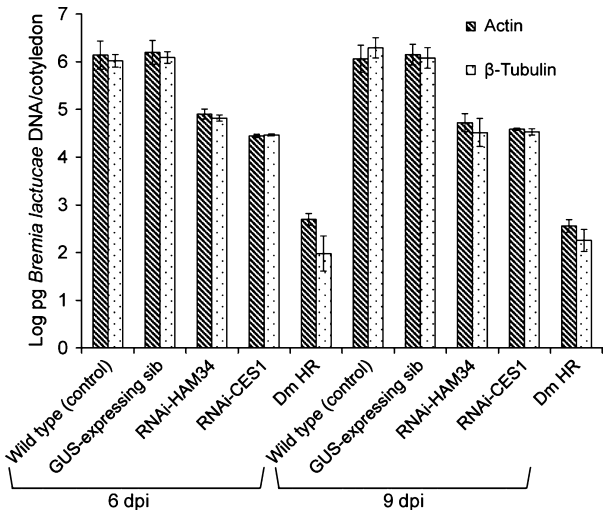


**Figure 5** The quantity of *Bremia lactucae* DNA in five lettuce genotypes at 6 and 9 dpi. DNA was measured by qPCR using *B. lactucae*-specific primers for β-*tubulin* and *actin* and an external standard of *B. lactucae* genomic DNA. Error bars are two SEM.

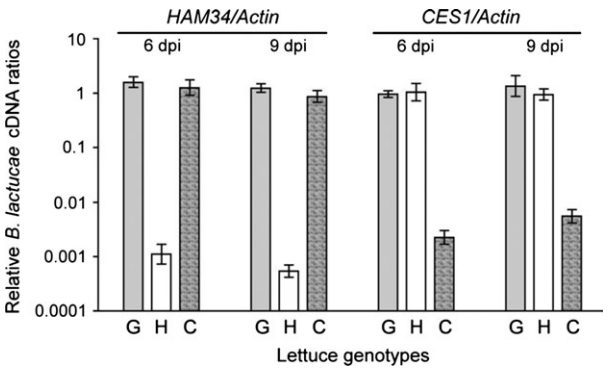


**Figure 6** Relative ratios of *Bremia lactucae HAM34* and *CES1* transcripts in transgenic lines relative to wild-type lettuce estimated by real-time reverse transcriptase PCR 6 and 9 days post inoculation (dpi). The ratios of the target gene/*actin* were computed for the wild type and each genotype and then the ratio for each genotype was divided by the ratio for the wild-type. Ratios were then log-transformed to achieve homoscedasticity for ANOVA. The values on the *y*-axis were detransformed to show the relative suppression relative to levels in the wild type. Error bars represent two SEM. Light grey G, GUS-expressing sibs. White H, RNAi-HAM34. Mottled darker grey C, RNAi-CES1.

to the *Dm3* gene). These seedlings were grown further and assayed for GUS activity at the 2–3 leaf stage as described above.

### Plant materials and *Bremia lactucae* inoculation for microscopy and extractions of DNA and RNA

Seven-day-old seedlings of five lettuce genotypes, wild-type *L. sativa* cv. Cobham Green as control, GUS-expressing $T_3$ seedlings as controls, transgenic RNAi-HAM34 and RNAi-CES1 $T_3$ seedlings, and *L. sativa* cv. Diana, were inoculated with *B. lactucae* isolate SF5 or mock-inoculated with water. For each replicate, for each assay, twelve cotyledons for nucleic acid analysis and six to eight cotyledons for microscopy were randomly selected. There were two independent trials each with two independent biological replicates for each time point per trial. Statistical inferences were consistent between the two trials; data are shown for $n = 4$.

### *Bremia lactucae* staining and microscopy

Six to eight lettuce cotyledons were collected for microscopy 1.5, 4, 6 and 9 dpi with *B. lactucae*. For visualization of *B. lactucae*, infected cotyledons were stained using trypan blue; the staining protocol (van Wees, 2008) was modified by incubating the cotyledons at 65 °C for 5 min followed by 15 min at room temperature, and destaining three times at 2-h intervals with chloral hydrate. Images of *B. lactucae* in or on the cotyledons were obtained with a Leica DM5000B microscope with LAS V4.3 Live Z builder software. Z-images were captured using either differential interference contrast optics with the 20× objective or phase contrast with the 40× objective. Autofluorescence was observed with epifluorescence with a filter cube with 385–425 nm excitation and 515–555 emission.

### qPCR-based quantification of *Bremia lactucae* growth *in planta*

DNA for a standard curve was extracted from *B. lactucae* spores using a modified CTAB method (Bernatzky and Tanksley, 1986). Standard curves were prepared with five serial log dilutions from 10 pg to 100 ng DNA. Infected cotyledons were collected at 6 and 9 days postinoculation (dpi) as described above, and genomic DNA was extracted using the same modified CTAB method. DNA was quantified with a Qubit 2.0 Fluorometer (www.lifetechnologies.com) and quality assessed using NanoDrop 2000 (www.nanodrop.com) and observations after 2% agarose gel electrophoresis. The quantity of *B. lactucae* DNA per cotyledon was estimated by measuring the amount of *actin* and β-*tubulin* using an Applied *Biosystems ViiA 7™* Real-Time PCR System (www.lifetechnologies.com). The qPCR reactions (12 μL final volume/sample) consisted of 2 μL target genomic DNA (5 ng/μL) added to a 10 μL master mix containing 6 μL of SYBR® Green PCR Master Mix, 0.2 μL of each 10 μM primer (Table S2) and 3.6 μL of nuclease-free water. The qPCR conditions were 2 min at 50 °C, followed by 10 min at 95 °C with forty cycles of 15 s at 95 °C and 1 min at 60 °C. The qPCR assays contained no-template controls (cycle threshold values >36) as negative controls for each *B. lactucae* gene. All genomic DNA qPCRs were performed with three technical replicates for each gene/time point/biological replicate. The cycle threshold values from the *B. lactucae* genomic DNA standard were used to estimate the log picograms of *B. lactucae* DNA per cotyledon at 6 and 9 dpi. An ANOVA followed by a Tukey–Kramer's HSD (JMP 10; SAS Institute Cary, NC, USA) was used to compare quantities of log mass DNA in the different genotypes. Estimates of the biomass in the transgenic plants in comparison to the controls were based on the detransformed geometric mean values.

### RNA extraction and cDNA synthesis

Inoculated and mock-inoculated cotyledons were harvested 6 and 9 dpi as described above and used for total RNA extractions. Total RNA was extracted from frozen powdered tissues using Qiagen Plant RNeasy kit (www.Qiagen.com) according to the manufacturer's instructions. All total RNA samples were digested with DNase I (www.thermoscientificbio.com) as recommended by the manufacturer before cDNA synthesis. DNA-

 

free, total RNA samples were quantified using a Qubit 2.0 Fluorometer (www.lifetechnologies.com) and quality assessed using NanoDrop 2000 (www.nanodrop.com) and observation after 2% agarose gel electrophoresis. First-strand cDNA was synthesized using RevertAid First-Strand cDNA Synthesis Kit (www.thermoscientificbio.com) following the manufacturer's instructions.

### Quantitative reverse transcriptase PCR

Expression levels of *B. lactucae actin* and the two target *B. lactucae* genes for silencing (*HAM34* and *CES1*) were quantified by comparative quantitative reverse transcriptase PCR (qRT-PCR). Primer sequences used for qRT-PCR are listed in Table S2. The qRT-PCR was performed in 12-μL reactions using the SYBR® Green PCR Master Mix (www.lifetechnologies.com). Transcript levels were measured using Applied *Biosystems ViiA 7™* Real-Time PCR System (www.lifetechnologies.com). The qPCR reactions were conducted with three technical replicates for each gene/time point/biological replicate. The cycling conditions were as follows: 2 min at 50 °C, followed by 10 min at 95 °C with 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The efficiency of each primer (≥84%) was determined by dilution and included in the calculation for relative expression (Pfaffl, 2001). The ratios of the target gene/*actin* were computed for the wild type and each genotype and then the ratio for each genotype was divided by the ratio for the wild type following the Pfaffl's method (Pfaffl, 2001). Ratios were then log-transformed to achieve homoscedasticity for ANOVA (or MANOVA). For each of the genotypes, there was no significant ($\alpha = 0.05$) correlation between log *HAM34*/*Actin* cDNA and *CES1*/*Actin* cDNA concentration: GUS-expressing sibs, $r = -0.15$, $P = 0.72$; RNAi-HAM34, $r = 0.55$, $P = 0.72$; and RNAi-CES1, $r = 0.22$, $P = 0.60$. Consequently, we analysed *HAM34* and *CES1* expression independently. For both *HAM34* and *CES1*, a two-way ANOVA indicated no significant ($\alpha = 0.05$) dpi*genotype and dpi effects and a highly significant ($P < 0.0001$) genotype effect. Tukey's HSD was used to compare gene expression between genotypes.

## Acknowledgements

We thank Oswaldo Ochoa for cultures of *B. lactucae* and assistance in screening and Pauline Sanders for help in the greenhouse. This work was supported by the Bill and Melinda Gates Foundation.

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## Supporting information

Additional Supporting information may be found in the online version of this article:

**Figure S1** Schematic of the interfering hairpin RNA (ihpRNA) construct in modified binary vector pGOLLUM (Wroblewski, 2007; Wroblewski, 2014).

**Figure S2** Microscopic Z-images of the hypersensitive resistance response in lettuce cv. Diana mediated by *Dm3*, 4 days post-inoculation with *B. lactucae.*

**Figure S3** Z-images of differential interference contrast microscopy of trypan blue-stained *Bremia lactucae* in four lettuce genotypes 9 days postinoculation.

**Table S1** Primers used for PCR amplification of *Bremia lactucae*-specific sequences for cloning.

**Table S2** Primers used in *Bremia lactucae* gene expression studies using qRT-PCR and *B. lactucae* DNA quantification using qPCR.

 