

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

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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.

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

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

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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 T₁ 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 T₁ plants transformed with the construct targeting *ELF1*, none exhibited GUS silencing and no further experiments were conducted with those plants (Figure 1). The T₂ seedlings and leaves of mature T₂ 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 T₂ 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 T₂ 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 T₁ transgenics (Table 2). The other two families silenced for *HAM34* had only one of 25 T₂ 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
<i>ELF1</i>	12	0	0	0
<i>HAM34</i>	13	5	4	2
<i>CES1</i>	14	5	2	2
<i>ACT</i>	13	3	0	0
<i>TUB</i>	11	2	0	0
<i>NLP2</i>	15	1	0	0
<i>NLP3</i>	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.

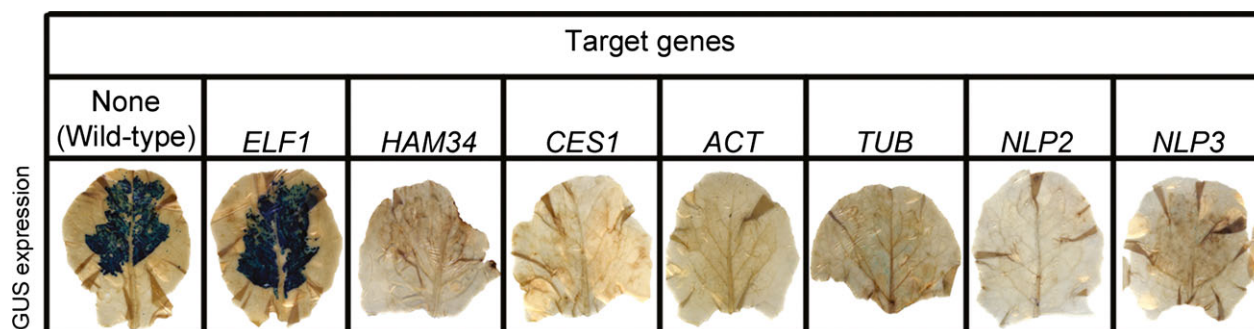


Figure 1 Stable T₁ transgenic lines screened for silencing using *Agrobacterium*-mediated transient expression of the *GUS* (*Uda*) gene. Transient expression of *GUS* provided a fast and reliable method to identify stable transgenic lines of lettuce that were actively silencing genes. Absence of *GUS* 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: T₂ 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 T₃ families derived from each of the fully resistant T₂ plants confirmed the results for silencing of *HAM34* and *CES1* (Figure 3). Five to seven fully resistant plants for each transgenic T₂ family were selfed to produce T₃ seed. Susceptible seedlings died following inoculation and therefore could not be selfed; consequently, sib plants from transgenic T₂ families that were 100% susceptible were selfed without inoculation. At 9 days postinoculation (dpi), T₃ 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, T₃ seedlings derived from the susceptible/nonsilenced families (Figure 3g). Again, those T₃ 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 T₃ 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 T₃ 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 T₃ 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 ± 4 and $4 \pm 1\%$ of the control biomass, RNAi-*CES1* had 3 ± 1 and $4 \pm 2\%$ of the biomass, cv. Diana had 0.04 ± 0.02 and $0.02 \pm 0.01\%$ of the biomass, and the transformed nonsilenced T₃ controls had 130 ± 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 T₃ 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 T₃ sibs and the T₃ 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 hpRNA construct in the plant.

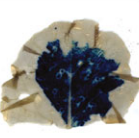



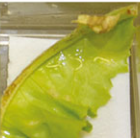

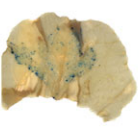

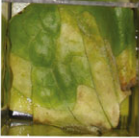

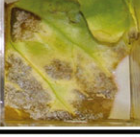




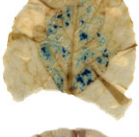





Target gene	Phenotypes of T ₂ leaves			
	GUS expression	14 dpi with <i>B. lactucae</i> SF5	14 dpi with <i>B. lactucae</i> CA1309	
None (Wild-type)				S
Highly Abundant Message #34 (HAM34)				R
				I
				S
Cellulose synthase (CES1)				R
				I
				S

Figure 2 Analysis of GUS activity and sporulation on leaf pieces from T₂ plants that segregated for disease resistance and silencing of GUS in seedlings. The T₂ 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 T₂ 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 T₂ 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 χ^2 and <i>P</i> -value
	Fully resistant Number of progeny	Partially resistant	Susceptible	
<i>RNAi CES1</i> (C1)	5	12	8	$P_{1:2:1} = 0.68$
<i>RNAi CES1</i> (C2)	6	13	6	$P_{1:2:1} = 0.98$
<i>RNAi HAM34</i> (H1)	7	13	5	$P_{1:2:1} = 0.84$
<i>RNAi HAM34</i> (H2)	6	12	7	$P_{1:2:1} = 0.94$
<i>RNAi HAM34</i> (H3)	1	5	19	$P_{1:3} = 0.76$
<i>RNAi HAM34</i> (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 (T₁ plants) were generated with each construct. Each T₁ 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 (OD₆₀₀ 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 T₁ plants, depending on the hp RNAi gene construct, they had been transformed with, exhibited silencing (Table 1, Figure 1). All silenced and nonsilenced T₁ plants were self-pollinated to generate T₂ seeds.

Analysis of transgenic plants: disease assays

T₂ progeny from each silenced and nonsilenced T₁ plant were challenged with *B. lactucae*. A suspension of conidia of isolate SF5 was sprayed onto 7-day-old T₂ seedlings (25 seedlings/T₂ 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 T₂ seedlings with GUS activity, and therefore no

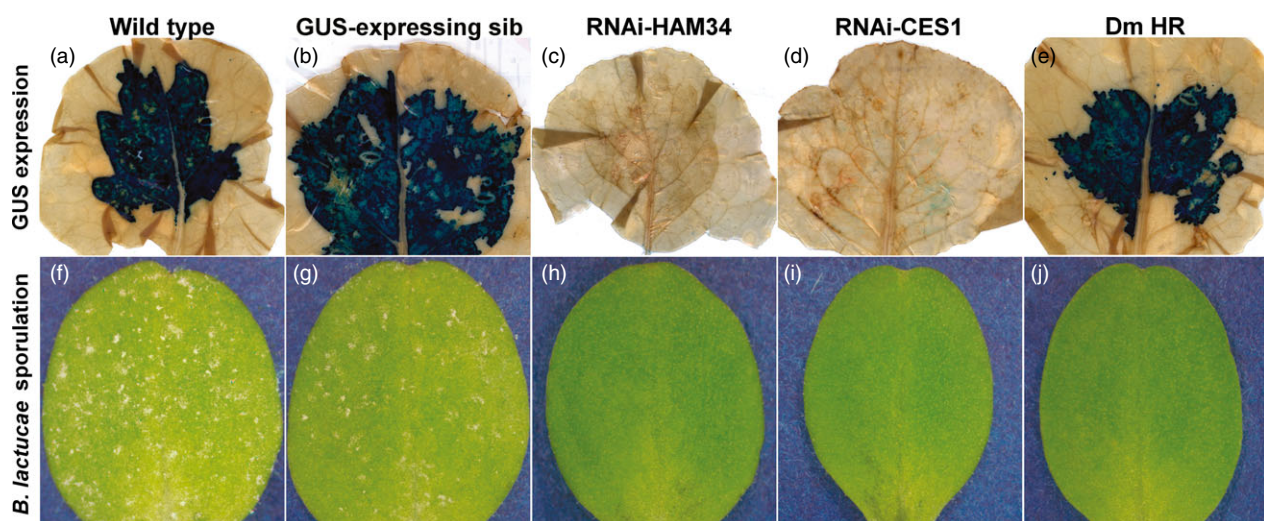


Figure 3 Five lettuce genotypes tested for host-induced gene silencing (HIGS). (a and f) Wild-type *Lactuca sativa* cv. Cobham Green control; (b and g) Nonsilenced GUS-expressing T₃ control (GUS-expressing sib); (c and h) T₃ with the *Bremia lactucae* *HAM34* gene silenced (RNAi-HAM34); (d and i) T₃ 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.

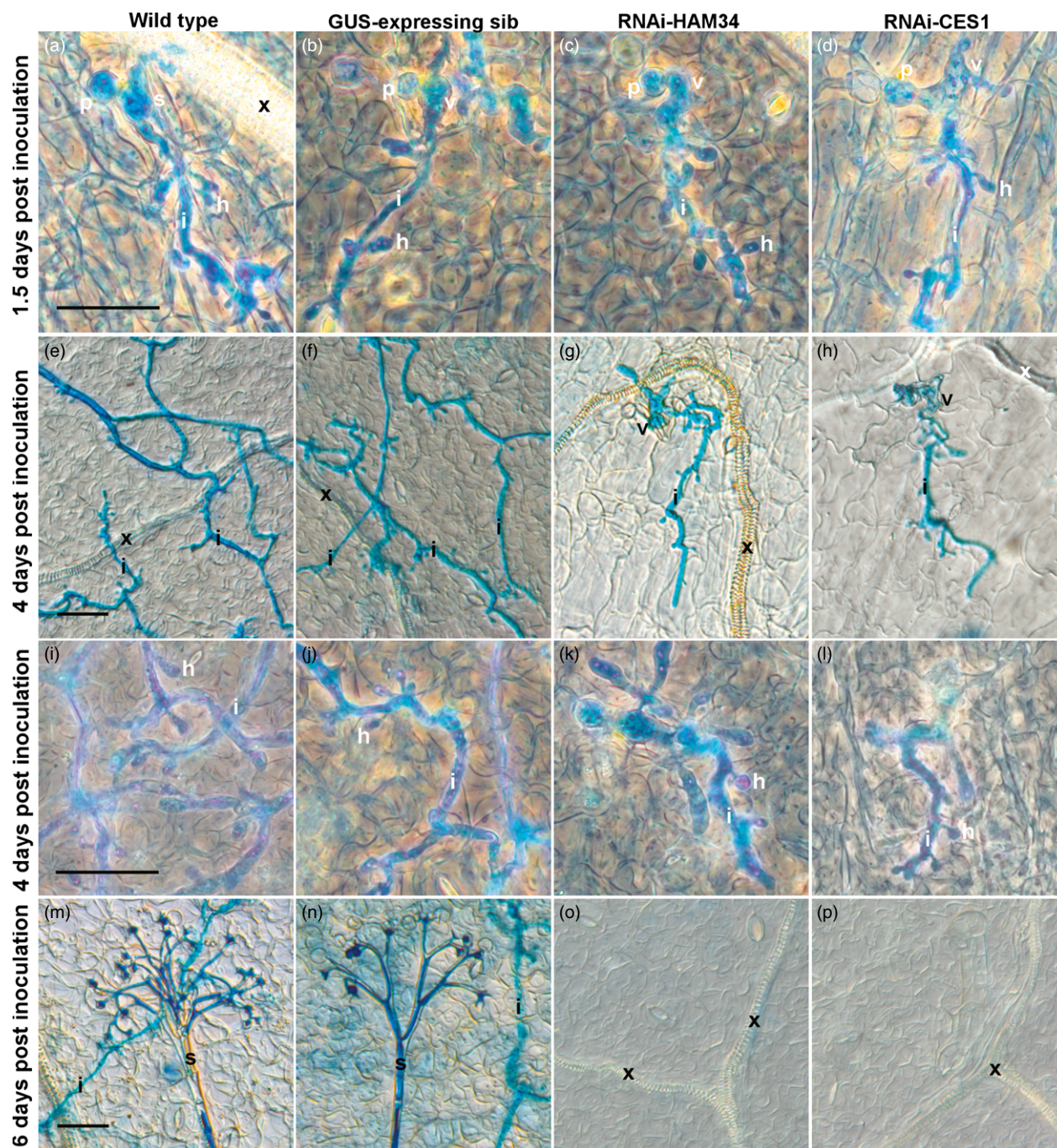


Figure 4 Z-images, taken using light microscopy, of trypan blue-stained *Bremia lactucae* in four lettuce genotypes at 1.5, 4 and 6 days postinoculation (dpi) with *B. lactucae*. (a–d) Phase contrast images at 1.5 dpi. No differences were observed in the development of the primary vesicles (p) and secondary 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 μ m.

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

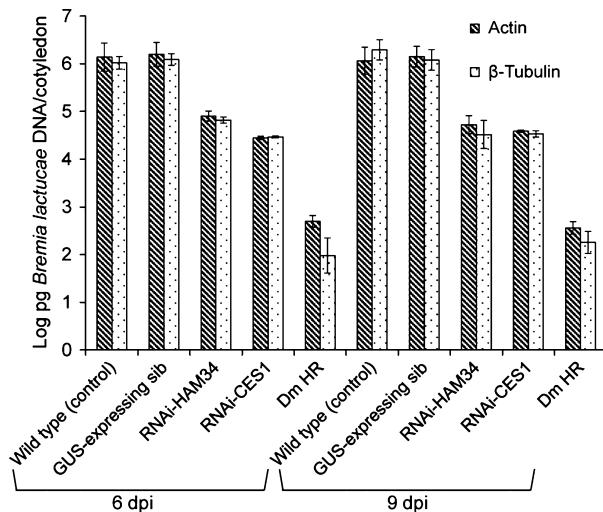


Figure 5 The quantity of *Bremia lactucae* DNA in five lettuce genotypes at 6 and 9 dpi. DNA was measured by qPCR using *B. lactucae*-specific primers for β -tubulin and actin and an external standard of *B. lactucae* genomic DNA. Error bars are two SEM.

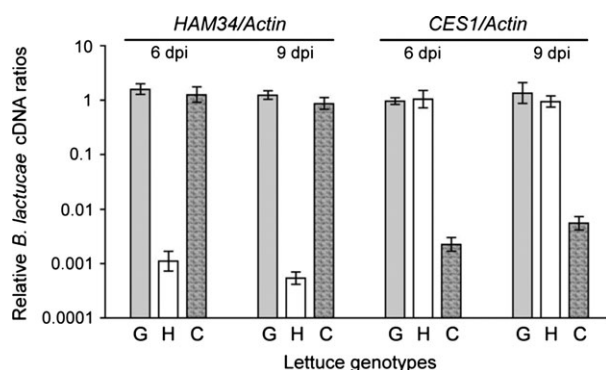


Figure 6 Relative ratios of *Bremia lactucae* HAM34 and CES1 transcripts in transgenic lines relative to wild-type lettuce estimated by real-time reverse transcriptase PCR 6 and 9 days post inoculation (dpi). The ratios of the target gene/actin were computed for the wild type and each genotype and then 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 T₃ seedlings as controls, transgenic RNAi-HAM34 and RNAi-CES1 T₃ 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 Tankley, 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 μ L final volume/sample) consisted of 2 μ L target genomic DNA (5 ng/ μ L) added to a 10 μ L master mix containing 6 μ L of SYBR® Green PCR Master Mix, 0.2 μ L of each 10 μ M primer (Table S2) and 3.6 μ L 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- μ L 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 ($\geq 84\%$) 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.

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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.