Functional Contribution of Chorismate Synthase, Anthranilate Synthase, and Chorismate Mutase to Penetration Resistance in Barley–Powdery Mildew Interactions

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Plant processes resulting from primary or secondary metabolism have been hypothesized to contribute to defense against microbial attack. Barley chorismate synthase (*HvCS*), anthranilate synthase α subunit 2 (*HvASa2*), and chorismate mutase 1 (*HvCM1*) occupy pivotal branch points downstream of the shikimate pathway leading to the synthesis of aromatic amino acids. Here, we provide functional evidence that these genes contribute to penetration resistance to *Blumeria graminis* f. sp. *hordei*, the causal agent of powdery mildew disease. Single-cell transient-induced gene silencing of *HvCS* and *HvCM1* in mildew resistance locus *a* (*Mla*) compromised cells resulted in increased susceptibility. Correspondingly, overexpression of *HvCS*, *HvASa2*, and *HvCM1* in lines carrying mildew resistance locus *o* (*Mlo*), a negative regulator of penetration resistance, significantly decreased susceptibility. Barley stripe mosaic virus–induced gene silencing of *HvCS*, *HvASa2*, and *HvCM1* significantly increased *B. graminis* f. sp. *hordei* penetration into epidermal cells, followed by formation of haustoria and secondary hyphae. However, sporulation of *B. graminis* f. sp. *hordei* was not detected on the silenced host plants up to 3 weeks after inoculation. Taken together, these results establish a previously unrecognized role for the influence of *HvCS*, *HvASa2*, and *HvCM1* on penetration resistance and on the rate of *B. graminis* f. sp. *hordei* development in *Mla*-mediated, barley–powdery mildew interactions.

The plant immune system plays a crucial role in preventing microbial invasion and growth. Pathogen-associated molecular patterns (PAMPs) are presented by diverse pathogens which trigger the activation of nonspecific basal defense mechanisms.

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These responses include the transcription of thousands of stress-related genes as well as the induction of antimicrobial metabolites and peptides during early stages of pathogen invasion. By contrast, effector-triggered immunity generally follows gene-for-gene interactions, where a specific resistance (R) protein initiates a signal cascade when it recognizes, either directly or indirectly, its corresponding avirulence (AVR) effector delivered by an invading pathogen (Flor 1971; Jones and Dangl 2006). Whereas specific R protein–AVR effector recognition often leads to rapid hypersensitive cell death at the invasion site (Boyd et al. 1995; Caldo et al. 2004; Jørgensen 1994; Thordal-Christensen et al. 1997), nonspecific PAMP-triggered immunity may confer quantitative defense against a wider range of pathogens (Jones and Takemoto 2004). In barley–powdery mildew interactions, basal defense responses, such as early autofluorescence and cytoplasmic aggregation in the host cell subjacent to the conidium, are detected as early as 2 h after a fungal spore lands on the plant surface (Aist and Bushnell 1991). By contrast, R proteins appear to function 14 to 20 h after inoculation (hai) after appressorium peg penetration into host epidermal cells (Aist and Bushnell 1991; Boyd et al. 1995; Ellingboe 1972).

Secondary metabolites are nonessential components for the primary metabolic process but are required for plant development and environmental responses. Components of secondary metabolic function, such as alkaloids, isoflavonoids, lignin, and indole-derivatives, play important roles in pathogen resistance (Fig. 1). For example, in the pea–*Nectria haematococca* interaction, pea produces the isoflavonoid antimicrobial product pisatin. Pathogenicity is dependent on fungal pisatin demethylating ability (*PDA*) genes to detoxify pisatin (Reimann and VanEtten 1994). Moreover, deficiency of certain antimicrobial end products often results in enhanced susceptibility in compatible interactions. The *Arabidopsis* phytoalexin deficiency (*pad*) mutants that interfere with camalexin biosynthesis result in enhanced susceptibility to the necrotrophic fungi *Alternaria brassicicola* (Nafisi et al. 2007; Thomma et al. 1999) and *Botrytis cinerea* (Ferrari et al. 2007).

The diversity in the makeup of metabolites in different species makes it difficult to derive a general mechanism of plant defense. In order to characterize conserved functions in the defense response, we focused on the pivotal enzyme chorismate synthase (CS) (EC 4.2.3.5), as well as its downstream branch-point enzymes, anthranilate synthase (AS) (EC 4.1.3.27) and chorismate mutase (CM) (EC 5.4.99.5). CS is a key enzyme in

the synthesis of aromatic amino acids (Fig. 1). CS converts 5-enolpyruvylshikimate-3-phosphate (EPSP) into chorismate, which is the common precursor for the phenylalanine/tyrosine (Phe/Tyr) or tryptophan (Trp) biosynthetic pathways. CM catalyzes conversion of chorismate to prephenate, the first regulatory step in Phe/Tyr biosynthesis. In higher plants, there are usually two or three CM isoenzymes located in different subcellular compartments, as well as possessing unique enzyme kinetics (d'Amato et al. 1984; Eberhard et al. 1996; Mobley et al. 1999). CM1 and CM3 are feedback-inhibited by free Phe and Tyr but activated by Trp, whereas CM2 is insensitive to any of the three amino acids (Eberhard et al. 1996; Gilchrist et al. 1972; Kuroki and Conn 1988; Mobley et al. 1999). AS, composed of α and β subunits, is a branch-point enzyme converting chorismate to anthranilate, leading to the synthesis of Trp and other indole alkaloid derivatives. The α subunit has a catalytic domain of anthranilate synthesis and feedback regulatory domain for Trp inhibition. The AS β subunit serves as a holoenzyme, supplying a nitrogen donor via glutamine and ammonia. In plants, the AS α subunit has two isoforms, $\alpha 1$ and $\alpha 2$ (Bohlmann et al. 1996; Niyogi and Fink 1992; Tozawa et al. 2001). In *Arabidopsis* and *Ruta graveolens*, AS $\alpha 2$ is sensitive to Trp feedback inhibition while AS $\alpha 1$ is elicitor inducible and insensitive to free Trp (Bohlmann et al. 1996; Niyogi and Fink 1992).

In this study, we present functional analysis of genes encoding three pivotal metabolic enzymes in barley–powdery mildew interactions in the presence or absence of the *mildew resistance locus a* (*Mla*) resistance gene. We provide evidence for a previously unrecognized role of *HvCS*, *HvASa2*, and *HvCM1* in penetration resistance to *Blumeria graminis* f. sp. *hordei*. We also show that these genes influence the rate of *B. graminis* f. sp. *hordei* development in *Mla*-mediated barley–powdery mildew interactions.

Fig. 1. Biosynthetic pathways leading to the production of secondary metabolites and antimicrobial compounds. Antimicrobial products derived from secondary metabolism are highly induced during the plant immune response (Dixon 2001; Glazebrook and Ausubel 1994), including phenylpropanoid and lignin derivatives during the defense response of barley to *Blumeria graminis* f. sp. *hordei* (Aist et al. 1988; Burhenne et al. 2003; Caldo et al. 2006; von Ropenack et al. 1998). The three (boxed) candidate genes, chorismate synthase (cs), anthranilate synthase (as), and chorismate mutase (cm), are differentially expressed in the comparison of compatible versus incompatible interactions of barley with *B. graminis* f. sp. *hordei*.

RESULTS

Previously, we utilized the 22K Affymetrix Barley1 GeneChip to analyze time-course expression profiles among incompatible and compatible interactions between barley and powdery mildew. In each of three independent biological replications, a three-by-two matrix design of three near-isogenic lines, harboring introgressed *Mla1*, *Mla6*, and *Mla13* coiled-coil nucleotide-binding site leucine-rich repeat resistance alleles, were challenged with either *B. graminis* f. sp. *hordei* isolate 5874 (*AVR_{a1}* and *AVR_{a6}*) or K1 (*AVR_{a1}* and *AVR_{a13}*) and harvested at 0, 8, 16, 20, 24, and 32 hai for a total of 108 (host $\times$ pathogen $\times$ time) experimental units (Caldo et al. 2004).

In all, 22 differentially expressed genes in compatible and incompatible barley–*B. graminis* f. sp. *hordei* interactions were identified by a mixed linear model at a cutoff *P* value < 0.0001 and a false discovery rate (FDR) $< 7\%$. Transcript accumulation of the identified genes was equivalent in both interactions from 0 to 16 hai, when *B. graminis* f. sp. *hordei* conidiospores germinate and appressoria are formed. Later, during establishment of the periaustorial interface between penetrating *B. graminis* f. sp. *hordei* and host epidermal cells, divergent expression of these transcripts occurs, where compatible interactions lead to lower accumulation of transcripts compared with paired incompatible interactions. Many of the 22 genes are positioned at various steps in the shikimate and its downstream amino acid biosynthesis pathway (Caldo et al. 2004, 2006). Therefore, we focused on the pivotal branch-point enzymes chorismate synthase (*HvCS*) (represented by probeset Barley1_05108), chorismate mutase 1 (*HvCM1*) (represented by probeset Barley1_07705), and anthranilate synthase α subunit 2 (*HvASa2*) (represented by probeset Barley1_48443) for further investigation.

Phylogeny of *B. graminis* f. sp. *hordei*-inducible *HvASa2* and *HvCM1*.

In higher plants, genes encoding the α subunit of both anthranilate synthase and chorismate mutase have two or more copies, which differentiate into two major groups: genes associated with steady-state functions of the cell and genes that are induced by stress (Bohlmann et al. 1996; Matsukawa et al. 2002; Mobley et al. 1999; Tozawa et al. 2001). In barley, there are also two anthranilate synthase α subunits, *HvASa1* (Barley1_11179) and *HvASa2* (Barley1_48443), and two chorismate mutase genes, *HvCM1* (Barley1_07705) and *HvCM2* (Barley1_17185), among which *HvASa2* and *HvCM1* have been identified as significantly differentially expressed in compatible versus incompatible interactions (Caldo et al. 2004). Based on phylogenetic analysis, *HvASa2* is closely related to *OsASa2*, which is also elicitor inducible (Fig. 2A) (Tozawa et al. 2001). *HvCM1* is in a monophyletic clade with *Arabidopsis* elicitor-inducible *AtCM1* (Fig. 2B). Both *HvASa1* and *HvCM2* fall in clades with elicitor noninducible genes (Fig. 2A and B). The above phylogenetic results confirm previous Barley1 GeneChip expression analysis, indicating that elicitor-inducible and not housekeeping isoforms are likely candidates involved in defense against *B. graminis* f. sp. *hordei* (Caldo et al. 2004).

Functional analysis of *HvCS*, *HvCM1*, and *HvASa2* via transient-induced gene silencing.

Because *HvCS*, *HvASa2*, and *HvCM1* were differentially expressed in compatible versus incompatible interactions, we were interested to test whether the resistant phenotype would be compromised upon silencing of these genes. We utilized an RNAi-based, transient-induced gene silencing (TIGS) assay to functionally test these three genes in barley–powdery mildew interactions (Douchkov et al. 2005). TIGS is a high-throughput single-cell assay by which one can evaluate hundreds of trans-

formed cells in a single experiment. Hairpin constructs of candidate gene fragments are co-bombarded with a Ubi:β-glucuronidase (GUS) expression vector into barley epidermal cells. Because host cell–*B. graminis* f. sp. *hordei* interactions are mainly a cell-autonomous event, lack of *B. graminis* f. sp. *hordei* haustoria indicates proper resistance activity whereas presence of haustoria indicates that the single cell silenced for the target gene compromises host resistance (Schweizer et al. 2000; Shirasu et al. 1999a). The penetration efficiency is calculated as the ratio of transformed susceptible cells to the total transformed cells attacked by *B. graminis* f. sp. *hordei*, thus quantifying the influence of silencing candidate genes. *HvCS*, *HvASa2*, and *HvCM1* gene fragments were cloned into the hairpin vector, pIPKTA30 (Douchkov et al. 2005), and were designated as hpCS, hpASa2, and hpCM1, respectively. The hairpin constructs were co-bombarded with a Ubi:GUS expression vector into barley epidermal cells and transformed cell–*B. graminis* f. sp. *hordei* interaction phenotypes were ex-

amined after GUS staining. In all, 12,651 individual GUS-marked cells were scored from three independent replications of the experiment. Silencing of individual *HvCS*, *HvCM1*, or *HvASa2* in barley C.I. 16151 (*Mla6*) cells did not compromise resistance compared with the empty vector control in the barley–*B. graminis* f. sp. *hordei* incompatible interaction (Table 1). However, because the *R* gene, *Mla6*, is a strong resistance component, we inferred that the metabolic gene-mediated defense is overridden by the function of *Mla6* in the incompatible interaction. We hypothesized that, by silencing these genes in cells where *Mla6* had been compromised, cells might become super susceptible, as shown by increased penetration efficiency. We used a *B. graminis* f. sp. *hordei*–susceptible fast-neutron mutant, *m1a6-m9472* (Meng et al. 2009), to investigate whether GUS-marked barley cells become supersusceptible when transformed with hpCS, hpASa2, or hpCM1. However, variation in penetration efficiency among replicates was larger than 25% in the *B. graminis* f. sp. *hordei*–susceptible, *m1a6-m9472* genotype, which made it difficult to make any significant inference. We encountered the same intrinsic variation in penetration efficiency when using the *B. graminis* f. sp. *hordei*–susceptible *rar1-1* and *rar1-2* mutants (Freialdenhoven et al. 1994; Shirasu et al. 1999b) that interfere with MLA protein stability (Bieri et al. 2004). Therefore, we decided to investigate these genes by silencing *Mla6* in the resistant line, C.I. 16151, to generate single GUS-marked, compromised cells to alleviate the influence of surrounding susceptible cells in our assay.

Epidermal cells silenced for *HvCS*, *HvCM1*, or *HvASa2* in combination with *Mla6* were approximately 10 to 20% more susceptible compared with silencing *Mla6* only, as shown by the difference in penetration efficiency (Table 1). A significant difference in penetration efficiency was observed by silencing *HvCM1* together with *Mla6* or *HvCS* with *Mla6* compared with silencing *Mla6* alone, with *P* values of 0.044 and 0.0011, respectively (Table 1). However, experiments to silence *HvASa2* combined with *Mla6* resulted in a nonsignificant *P* value of 0.0614, suggesting that the Tyr/Phe biosynthetic pathway may contribute incrementally more to defense than the Trp pathway in the barley–*B. graminis* f. sp. *hordei* interaction. The combined effects of *HvCS*, *HvCM1*, and *HvASa2* genes are shown in Table 2. A combination of hpCS with hpCM1, hpCS with hpASa2, or hpCM1 with hpASa2 showed no significant difference in susceptibility compared with the empty vector control. Including hpMla6 in the combinations increased the penetration index approximately 10% compared with silencing *Mla6* only. All the combinations with hpMla6 showed highly significant differences in susceptibility compared with silencing *Mla6* alone, with cutoff *P* values of <0.001 (Table 2). It should be noted that silencing becomes less efficient as more constructs are co-transferred into plant cells (Douchkov et al. 2005). This may explain

Fig. 2. Phylogenetic analysis of plant anthranilate synthase α subunit and chorismate mutase. **A**, Peptide sequences of anthranilate synthase α subunit and **B**, chorismate mutase were used for phylogenetic tree construction by the neighbor-joining method with 1,000 bootstrap replicates. Trees were rooted using **A**, yeast anthranilate synthase TRP component I and **B**, chorismate mutase ARO7. Gene locus identifiers and Barley1 exemplar names are indicated in parentheses. Subcellular localization in the cytosol or chloroplast are designated as cyto or chloro, respectively. Bootstrap values are shown at nodes. Branch length represents number of amino acid substitutions per site.

Table 1. *Blumeria graminis* f. sp. *hordei* penetration efficiency (PE) in transient-induced gene silencing assay I

Construct	Number of cells ^a		PE ^d	<i>P</i> value ^b	
	Attacked ^c	With haustoria		Empty vector vs. others	hpMla6 vs. others
pIPKTA30	375	15	2.61	n.a.	<0.0001
hpAS	465	12	2.28	0.4486	<0.0001
hpCM	465	33	6.5	0.8661	<0.0001
hpCS	588	12	1.76	0.9481	<0.0001
hpMla6	621	249	39.54	<0.0001	n.a.
hpMla6 + hpAS	1,197	597	49.52	<0.0001	0.0614
hpMla6 + hpCM	1,455	804	56.05	<0.0001	0.0044
hpMla6 + hpCS	864	531	59.36	<0.0001	0.0011

^a Raw numbers indicate the combined total of three independent experiments.

^b *P* values were obtained using a mixed linear model to test for a significant difference of the PE by comparing the test constructs versus the empty vector negative control or the hpMla6 positive control. A *P* value < 0.05 indicates that the PEs are significantly different; n.a. = not available.

^c Attacked with *B. graminis* f. sp. *hordei*.

^d PE is shown as the mean of the PE from three independent replications.

why there is no increase in penetration efficiency when silencing multiple genes in combination with *Mla6* compared with silencing a single gene and *Mla6*. The above results illustrate that silencing both chorismate synthase and chorismate mutase make possible *B. graminis* f. sp. *hordei* penetration, implying that branches of the aromatic amino acid biosynthesis pathway leading to lignin and phenylpropanoid phytoalexins contribute to plant defense.

Virus-induced gene silencing of *HvCS*, *HvAsa2*, and *HvCM1* increase formation of *B. graminis* f. sp. *hordei* secondary hyphae but not conidiation in *Mla6*-mediated resistant plants.

The use of *Barley stripe mosaic virus*-induced gene silencing (BSMV-VIGS) has been shown to be an effective tool for reverse genetic functional analysis in whole seedling leaves (Hein et al. 2005; Scofield et al. 2005). In contrast to the single-cell TIGS assay, BSMV-VIGS can be used to silence large contiguous regions and, therefore, is useful for investigation of non-cell-autonomous effects. In this study, the BSMV-VIGS system was modified by cloning each of the three BSMV sub-genome segments into an expression vector with the 35S promoter (Meng et al. 2009). The modified BSMV constructs are optimized for biolistic transformation and eliminate the need for in vitro transcription reactions. First, empty virus vector BSMVm:00 and BSMVm:PDS, which carries a fragment of the phytoene desaturase (PDS) gene in its γ genome segment, were examined for silencing efficiency. Seven-day-old barley seedlings were infected with BSMVm:00 or BSMVm:PDS via bombardment. The chlorotic mosaic symptoms of BSMV infection were first detectable on the second leaf by 6 days after bombardment (dab). The chlorotic infection symptom was also displayed on the tip of the third leaf approximately 10 to 12 dab. Mosaic symptoms were rarely observed on the fourth leaf. Consistent with virus infection symptoms, BSMVm:PDS, which results in a photobleaching phenotype after silencing, was visible 7 to 9 dab on the second leaf and the tip of the third leaf 12 to 14 dab. Therefore, at 9 dab, barley second leaves were used for investigation in VIGS experiments.

To study the function of *HvCS*, *HvAsa2*, and *HvCM1* in barley-*B. graminis* f. sp. *hordei* interactions, the *HvCS*, *HvAsa2*, and *HvCM1* gene fragments were cloned into the BSMV γ sub-genome and designated as BSMVm:CS, BSMVm:AS, and BSMVm:CM, respectively (discussed below). *B. graminis* f. sp. *hordei* resistance gene *Mla6* was also cloned into the virus as BSMVm:Mla6, which served as a positive control. At 9 dab, second leaves of BSMV-VIGS-treated barley cv. C.I. 16151 (*Mla6*) were inoculated with *B. graminis* f. sp. *hordei* isolate 5874 (*AVR_{ab}*). Seven days after inoculation (dai) with *B. graminis* f. sp. *hordei*, plants were scored for infection types.

Negative control plants infected with BSMVm:00 displayed the typical C.I. 16151 (*Mla6*)-mediated resistant infection type, whereas the positive control infected with BSMVm:Mla6 exhibited typical susceptible symptoms with *B. graminis* f. sp. *hordei* sporulation on the leaf surface (Fig. 3). When plants were inspected macroscopically, no colonization of *B. graminis* f. sp. *hordei* was detected up to 3 weeks after inoculation of plants infected with BSMVm:CS, BSMVm:AS, and BSMVm:CM (Fig. 3). However, at the microscopic level, BSMVm:CS-, BSMVm:AS-, and BSMVm:CM-treated plants displayed elongating secondary hyphae (ESH) at 72 hai (Fig. 4), indicating functional haustoria within the epidermal cell (Ellingboe 1972). A susceptibility index was calculated as the ratio of cells with haustoria to the total cells attacked by *B. graminis* f. sp. *hordei* in silenced regions (discussed below). Barley leaves treated with BSMVm:CS, BSMVm:AS, or BSMVm:CM displayed a signifi-

Fig. 3. Phenotypes of *Barley stripe mosaic virus*-induced gene silencing (BSMV-VIGS)-treated plants. C.I. 16151 plants infected with BSMV-VIGS empty vector (BSMVm:00) and test constructs **A**, before and **B**, 7 days after *B. graminis* f. sp. *hordei* 5874 (*AVR_{ab}*) inoculation. BSMV-VIGS empty vector and constructs containing target gene fragments *HvCS*, *HvCM1*, *HvAsa2*, and *Mla6* are abbreviated as 00, CS, CM, AS, and *Mla6*, respectively. Photographs were taken **A**, 9 days and **B**, 16 days post bombardment of BSMV-VIGS constructs.

Table 2. *Blumeria graminis* f. sp. *hordei* penetration efficiency (PE) in transient-induced gene silencing assay II

Construct	Number of cells ^a		PE ^d	P value ^b	
	Attacked ^c	With haustoria		Empty vector vs. others	hpMla6 vs. others
pIPKTA30	426	30	6.69	n.a.	<0.0001
hpAS + hpCM	450	15	3.01	0.1338	<0.0001
hpAS + hpCS	396	15	3.42	0.1788	<0.0001
hpCM + hpCS	540	21	3.90	0.3202	<0.0001
hpMla6	864	453	52.43	<0.0001	n.a.
hpMla6 + hpAS + hpCM	930	570	61.39	<0.0001	0.0012
hpMla6 + hpAS + hpCS	1,062	672	63.14	<0.0001	0.0004
hpMla6 + hpCM + hpCS	1,953	1,200	61.41	<0.0001	0.0017

^a Raw numbers indicate the combined total of three independent experiments.

^b P values were obtained using a mixed linear model to test for a significant difference of the PE by the test constructs versus the empty vector negative control or the hpMla6 positive control. A P value < 0.05 indicates that the PEs are significantly different; n.a. = not available.

^c Attacked with *B. graminis* f. sp. *hordei*.

^d PE is shown as the mean of the PE from three independent replications.

cant difference in penetration efficiency from the BSMVm:00 empty vector control (Table 3). The susceptibility of BSMVm:CS treated plants was 24%, on average, compared with 48% for BSMVm:Mla6-treated (positive control) plants. BSMVm:AS- and BSMVm:CM-infected plants displayed a lower susceptibility index of 13 and 15%, respectively (Table 3). No colonization of *B. graminis* f. sp. *hordei* was detected after macroscopic observation (Fig. 3); therefore, we inferred that *B. graminis* f. sp. *hordei* can form haustoria and secondary hyphae but fail to propagate in the silenced plants. Finally, inspection of 7-dai BSMVm:CS-, BSMVm:AS-, or BSMVm:CM-treated plants by microscopy revealed that only elongated secondary hyphae were detected without sporulation (Fig. 4B), whereas abundant *B. graminis* f. sp. *hordei* sporulation was observed 7 dai on BSMVm:Mla6 *R*-gene-silenced plants or the Manchuria

non-virus-infected susceptible control (Fig. 4B). Mla6-mediated resistance normally halts further development of *B. graminis* f. sp. *hordei* after penetration, whereas the above results indicate that silencing genes encoding metabolic enzymes facilitate *B. graminis* f. sp. *hordei* penetration and secondary hyphae development, indicating that expression of these genes influences the rate of *B. graminis* f. sp. *hordei* development in *Mla*-mediated barley–powdery mildew interactions.

Quantitative reverse-transcription polymerase chain reaction of target gene mRNA from VIGS-treated plants.

To confirm whether a specific candidate gene's mRNA had been targeted, quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed. Second leaves of BSMV-treated plants were used for RT-PCR assays 7 dai with

A 72 hai

B 7 dai

Fig. 4. *Blumeria graminis* f. sp. *hordei* infection structures in *Barley stripe mosaic virus*-induced gene silencing (BSMV-VIGS)-silenced and control barley leaves. C.I. 16151 (*Mla6*) plants infected with BSMV-VIGS constructs were inoculated with *B. graminis* f. sp. *hordei* isolate 5874 (AVRa6). Barley leaves were fixed at **A**, 72 h after inoculation (hai) and **B**, 7 days after inoculation (dai). Manchuria barley was included as a susceptible inoculation control. BSMV-VIGS constructs and Manchuria check are designated at the lower-left corner of each picture. The bars represent 100 μ m in length.

B. graminis f. sp. *hordei*. Actin mRNA was used as an internal quantitative control for all samples. mRNA levels of *HvCS*, *HvAsa2*, *HvCM1*, and *Mla6* from plants treated with matching BSMV constructs decreased efficiently compared with mRNAs from BSMVm:00 controls (Fig. 5). For most silenced genes, there are no detectable amplicons at 25 and 30 cycles. However, amplicons could be detected at 35 PCR cycles, indicating that trace mRNA of the target genes was still present and silencing was not 100% efficient. This is consistent with the observed heterogeneous silencing pattern observed in BSMVm: PDS plants (Scofield et al. 2005). In order to test whether cross-silencing had occurred in BSMVm:CM- and BSMVm:AS-treated plants, specific primers were designed to amplify the

Fig. 5. Barley stripe mosaic virus–induced gene silencing (BSMV-VIGS)–induced mRNA degradation of target transcripts. Degradation of target transcripts was assayed via quantitative reverse-transcription polymerase chain reaction of C.I. 16151 plants treated with BSMV-VIGS empty vector (BSMVm:00) compared with corresponding test constructs. Plants were inoculated with *Blumeria graminis* f. sp. *hordei* 9 days after attempted silencing. Tissue was harvested for RNA preparation 72 h after *B. graminis* f. sp. *hordei* inoculation. Gene-specific primers were used to amplify *HvCS*, *HvAsa2*, *HvAsa1*, *HvCM1*, *HvCM2*, and *Mla6* transcripts. “NC” of each sample RNA represents the negative control without reverse transcription. Lanes designated 20, 25, 30, and 35 represent amplification cycles performed for each sample. *Actin* transcripts were used as a quantitative control.

nontargeted family members, *HvCM2* and *HvAsa1*. qRT-PCR performed with primers specific for *HvCM2* showed no difference in mRNA levels from the controls, indicating a low probability of cross-silencing with *B. graminis* f. sp. *hordei*–inducible *HvCM1*. However, decreased amplification of *HvAsa1* RNAs from the BSMVm:AS-silenced plants indicated that some cross-silencing had occurred (Fig. 5).

***HvCS*, *HvCM1*, and *HvAsa2* contribute to *mlo*-mediated broad-spectrum resistance.**

The barley *mlo* gene, which encodes a seven-transmembrane protein (Devoto et al. 1999), is a negative defense regulator in barley–*B. graminis* f. sp. *hordei* interactions. Transient overexpression of *Mlo* in wild-type *Mlo* plants results in supersusceptibility (Kim et al. 2002). In contrast, mutant *mlo* derivatives mediate non-race-specific resistance early during pathogen penetration (Büschges et al. 1997; Consonni et al. 2006; Freialdenhoven et al. 1996; Wolter et al. 1993).

Our previous TIGS and VIGS results indicate that silencing genes in secondary metabolism influences fungal penetration. We reasoned that, if defense components derived from Tyr/Phe or Trp synthetic pathways are involved in penetration defense, overexpression of the three genes would quantitatively decrease penetration efficiency. A single-cell transient overexpression assay was performed to investigate whether *HvCS*, *HvAsa2*, and *HvCM1* could lessen *Mlo*-mediated susceptibility to *B. graminis* f. sp. *hordei*. Expression constructs containing the open reading frames of *HvCS*, *HvAsa2*, or *HvCM1*, each preceded by the ubiquitin promoter, were tested for function in *mlo*-5 BC₇ Ingrid seedlings by co-bombardment with the V26UMUG vector, which encodes both the GUS and *Mlo* genes (Shirasu et al. 1999a). In this assay, GUS-marked epidermal cells were rendered susceptible to *B. graminis* f. sp. *hordei* due to the presence of wild-type *Mlo*, contained within the V26UMUG vector, whereas neighboring nontransformed cells retained broad-spectrum *mlo*-5 resistance (Halterman and Wise 2004).

Results of three independent overexpression experiments indicated that the three genes had significantly decreased penetration efficiency compared with the V26UMUG (GUS-*Mlo*) control (Table 4). The average *B. graminis* f. sp. *hordei* penetration efficiency in leaves bombarded with the GUS-*Mlo* reporter plasmid, V26UMUG, was 80.44%. Bombardment with *HvCS* decreased the penetration efficiency by 26.5% on average compared with the control, with a *P* value of 0.0004. Transformation with *HvCM1* and *HvAsa2* reduced *B. graminis* f. sp. *hordei* penetration efficiency by 14.7 and 12.0% on average, respectively. Including both *HvCM1* and *HvAsa2* in the bombardment had effects in reducing *B. graminis* f. sp. *hordei* penetration efficiency equivalent to overexpressing *HvCM1* alone, suggesting that i) there is no additive effect of combining *HvCM1* and *HvAsa2* expression and ii) *HvCS* appears to have a greater effect than *HvCM1* or *HvAsa2* on *B. graminis* f. sp. *hordei* penetration efficiency.

Table 3. *Blumeria graminis* f. sp. *hordei* penetration efficiency (PE) in C.I. 16151 (*Mla6*) treated with Barley stripe mosaic virus (BSMV) constructs

Treatment	Number of cells ^a			<i>P</i> value ^b	
	Attacked ^c	With haustoria	PE ^d	BSMVm:00 vs. others	BSMVm:Mla6 vs. others
BSMVm:00	1,971	54	3.15	n.a.	<0.0001
BSMVm:AS	1,791	219	13.04	0.0083	<0.0001
BSMVm:CM	1,281	198	15.45	0.0024	<0.0001
BSMVm:CS	2,475	609	24.03	<0.0001	<0.0001
BSMVm:Mla6	1,707	861	48.03	<0.0001	n.a.

^a Raw numbers indicate the combined total of three independent experiments.

^b *P* values were obtained using a mixed linear model to test for a significant difference of the PE by comparing the test BSMV constructs versus the BSMVm:00 empty vector or the BSMVm:Mla6 positive control. A *P* value < 0.05 indicates that the PEs are significantly different; n.a. = not available.

^c Attacked with *B. graminis* f. sp. *hordei*.

^d PE is shown as the mean of the PE from three independent replications.

BSMV-VIGS interferes

with *mlo*-mediated resistance to powdery mildew.

To investigate whether silencing the three genes compromises *mlo*-mediated broad-spectrum resistance, the BSMVm:00, BSMVm:CS, BSMVm:AS, and BSMVm:CM constructs were tested on the penetration-resistant line *mlo*-5 BC₇ Ingrid. Interestingly, sporulation of *B. graminis* f. sp. *hordei* was observed on plants at 7 dai treated with both BSMV empty vector and BSMV constructs containing the three candidate genes, indicating that BSMV itself interferes with *mlo*-mediated resistance to powdery mildew (Supplementary Fig. 1). The *mlo*-mediated resistance requires vesicle transport and a membrane fusion event (Bhat et al. 2005; Collins et al. 2003). Some plant virus-encoded cell-to-cell movement proteins are bound to the endoplasmic reticulum (ER) and are involved in vesicle transport (Ploubidou and Way 2001; Verchot-Lubicz et al. 2007). For example, *Potato virus X* encodes TGBp2 which, upon infection, induces ER-derived granular-type vesicles that are critical for the virus movement (Verchot-Lubicz et al. 2007). BSMV TGB1, TGB2, and TGB3 proteins are required for cell-to-cell movement (Lawrence and Jackson 2001b). Subcellular localization revealed that TGB1 is associated with ER and TGB2 is associated with membrane and cell wall fractions, suggesting that BSMV cell-to-cell movement requires viral protein cytoplasmic membrane association (Lawrence and Jackson 2001a,b). We hypothesize that BSMV infection changes the host membrane system and, thus, interferes with *mlo*-mediated resistance, resulting in a susceptible phenotype in BSMV-treated plants.

DISCUSSION

Secondary metabolic enzymes contribute to penetration resistance.

Previously, we showed that several genes encoding secondary metabolic enzymes are nonspecifically induced in both compatible and incompatible interactions before *B. graminis* f. sp. *hordei* haustorium formation and then differentially expressed after penetration (Caldo et al. 2004, 2006). Our functional analysis in this study demonstrated that genes at pivotal branch points are involved in penetration resistance, which is one of the major components in basal defense. TIGS results demonstrated that, in *Mla*-compromised cells, impairment of *HvCS* and *HvCM1* functions to increase *B. graminis* f. sp. *hordei* penetration efficiency, indicating that these enzymes and their downstream components contribute to host defense. Overexpression of *HvCS*, *HvAsa2*, and *HvCM1* in the presence of *MLO*, a negative regulator of basal defense, significantly decreased the penetration efficiency compared with overexpression of *Mlo* alone. The *mlo* mutants confer resistance during *B. graminis* f. sp. *hordei* penetration, characterized as early and large papillae formation. It is believed that *B. graminis* f. sp. *hordei* recruits *MLO* as a virulence target to suppress host basal defense (Büschges et al. 1997; Devoto et al. 1999). Bhat and associates (2005) showed that *MLO* protein accumulates beneath fungal appressoria upon *B. graminis* f. sp. *hordei* penetration and the association between *MLO* and its interacting calmodulin is coincident with successful invasion of *B. graminis* f. sp. *hordei*. The decreased penetration efficiency in *Mlo*-transformed cells via overexpression of *HvCS*, *HvAsa2*, and *HvCM1* indicates that these metabolic enzymes contribute to host penetration resistance.

In this study, silencing and overexpression of *HvCS* showed prominent effects on penetration resistance. We should note that abolishment of CS function may dramatically disturb plant metabolism, resulting in some pleiotropic effects. At this time, we cannot differentiate between effects on penetration resistance in *HvCS*-silenced plants from possible pleiotropic

effects on defense due to disturbance in aromatic amino acid synthesis and the resulting metabolic sink. However, *HvAsa2* and *HvCM1* are *B. graminis* f. sp. *hordei* inducible, while genes encoding their isoenzymes *HvAsa1* and *HvCM2* mediate steady-state secondary metabolic functions. In this scenario, even if the functions of *HvAsa2* or *HvCM1* or both are disabled, steady-state secondary metabolic functions could still be operating through *HvAsa1* and *HvCM2*. qRT-PCR results showed a low probability of cross-silencing between housekeeping gene *HvCM2* and *B. graminis* f. sp. *hordei*-inducible *HvCM1* (Fig. 5). However, decreased amplification of *HvAsa1* RNAs from the BSMVm:AS-silenced plants indicated that cross-silencing had occurred. It is unclear whether cross-silencing of *HvAsa1* would have an effect on the penetration phenotype, because time-course GeneChip expression profiles displayed a clear downregulation of *HvAsa1* (Barley1_11179), in contrast to the significant induction of *HvAsa2* (Barley1_48443). It is possible that silencing *HvAS* α subunit expression affects the metabolic flow in Trp biosynthetic pathway and interferes with basic cell functions, resulting in a decreased defense response.

Host resistance to fungal pathogens can act at either the invasion or post-invasion stage. Gene-for-gene interactions usually function during post invasion. Though, in plant–biotrophic pathogen interactions, it is still not clear how AVR effectors are recognized by R proteins, it is widely believed that either direct or indirect recognition between AVR and R proteins occurs inside the host cell (Catanzariti et al. 2007; Ridout et al. 2006; van den Burg et al. 2006). The subsequent signaling cascade often results in a hypersensitive response (HR), which halts further development of the pathogen. *mlo*-Mediated broad-spectrum resistance is categorized under invasion defense, in which haustoria or HR are not observed (Peterhansel et al. 1997). Recent studies on penetration resistance reveal that it is highly associated with transportation of vesicles and other toxic components at the pathogen-attacked sites (Bhat et al. 2005; Collins et al. 2003; Lipka et al. 2005; Stein et al. 2006).

Arabidopsis does not support the growth and asexual reproduction of *B. graminis* f. sp. *hordei*. Conidia will germinate and form appressoria but the majority will not penetrate the epidermal cell wall and papillae. A series of mutations belonging to three complementation groups (*pen1*, *pen2*, and *pen3*) allow increased penetration by *B. graminis* f. sp. *hordei*. *PEN1* encodes a syntaxin (SYP121) (Collins et al. 2003), *PEN2* encodes a glycosyl hydrolase (Lipka et al. 2005), and *PEN3* encodes the putative ATP-binding cassette (ABC) transporter PDR8 (Stein et al. 2006). Current hypotheses envisage that

Table 4. *Blumeria graminis* f. sp. *hordei* penetration efficiency (PE) with overexpression of *HvCS*/*HvCM1*/*HvAsa2* and β -glucuronidase (GUS)–mildew resistance locus *o* (*Mlo*) in *mlo*-5 plants

Construct	Number of cells ^a		PE ^c	P value ^d
	Attacked ^b	With haustoria		
GUS- <i>Mlo</i> ^e	772	596	80.44	n.a.
GUS- <i>Mlo</i> + AS	513	360	68.47	0.0415
GUS- <i>Mlo</i> + CM	764	488	65.67	0.0106
GUS- <i>Mlo</i> + CS	898	490	53.92	0.0004
GUS- <i>Mlo</i> + CM + AS	284	170	66.26	0.0128

^a Raw numbers indicate the combined total of three independent experiments.

^b Attacked with *B. graminis* f. sp. *hordei*.

^c PE is shown as the mean of the PE from three independent replications.

^d P values (GUS-*Mlo* vs. others) were obtained using a mixed linear model to test for a significant difference of the PE by comparing the test constructs versus the GUS-*Mlo* control. A P value < 0.05 indicates that the PEs are significantly different; n.a. = not available.

^e Control GUS-*Mlo* construct was co-bombarded with the overexpression empty vector to obtain a mole equivalence with other treatments.

antimicrobial components produced by the PEN2 glycosyl hydrolase are transported to pathogen-attacked sites by the PEN3 ABC transporter (Stein et al. 2006). It is possible that some antimicrobial products derived from the secondary metabolic pathway are one of the targets for PEN3, and decreasing of certain antimicrobial compounds weakens penetration resistance. These antimicrobial products could be phenylpropanoid or indole derivatives; data presented in this report demonstrates that impairment of the Tyr/Phe biosynthetic pathway increased *B. graminis* f. sp. *hordei* haustorial formation, an indicator of penetration efficiency. One candidate from the phenylpropanoid pathway is p-coumaroyl-hydroxyagmatine (p-CHA), which attains levels 25-fold higher in *mlo-3* and *mlo-5* than in wild-type *Mlo* plants (von Ropenack et al. 1998). Von Ropenack and colleagues (1998) showed that p-CHA has antimicrobial activity in vitro and in vivo, which reduced *B. graminis* f. sp. *hordei* appressoria and haustoria formation. In this scenario, it is possible that p-CHA at the invasion sites is toxic to *B. graminis* f. sp. *hordei* and contributes to penetration resistance.

Silencing genes in the aromatic amino acid synthetic pathway will affect more than one single metabolic product and its corresponding penetration resistance pathway. Lignin, which serves as a mechanical barrier to pathogens, may affect penetration efficiency. Cell-wall-bound phenylpropanoid derivatives also contribute to penetration resistance. In *mlo* plants, cell wall apposition papilla is usually larger and forms earlier than in wild-type *Mlo* plants (Collins et al. 2003). Cell-wall-bound phenolics around papilla in *mlo* plants display resistance to saponification 2 h earlier than in corresponding *Mlo* wild-type plants (von Ropenack et al. 1998), indicating that early papilla formation is important for invasion resistance.

Virus-induced silencing of metabolic genes interferes with the rate of *B. graminis* f. sp. *hordei* development in *Mla*-mediated barley–powdery mildew interactions.

Penetration resistance is a complex and dynamic process, involving multiple positive and negative factors (Bhat et al. 2005; Collins et al. 2003; Lipka et al. 2005; Stein et al. 2006). BSMV-VIGS of *HvCS*, *HvAsa2*, and *HvCMI* in C.I. 16151 (*Mla6*) plants displayed fully developed *B. graminis* f. sp. *hordei* haustoria and ESH, but without sporulation. However, in typical *Mla6*-mediated resistance, developing haustoria are terminated and ESH are not observed (Halterman et al. 2001; Wise and Ellingboe 1983). These results provide for a temporal division between the formation of haustoria or secondary hyphae and *Mla*-mediated resistance. Either early establishment of *B. graminis* f. sp. *hordei* infection inside of the host cells or delayed *Mla* resistance will allow *B. graminis* f. sp. *hordei* enough time to form haustoria and secondary hyphae before the manifestation of HR. In the first scenario, the weakened penetration defense in plants silenced for *HvCS*, *HvCMI*, or *HvAsa2* may facilitate rapid *B. graminis* f. sp. *hordei* penetration and enough lag time to establish its haustoria and secondary hyphae before *Mla*-triggered HR. Alternatively, silencing the three genes interferes with *Mla*-mediated resistance, resulting in a delayed defense response. Shen and associates (2007) demonstrated that barley MLA10 regulates basal defense through WRKY transcription factors. It was shown that the recognition between MLA10 and its corresponding pathogen effector, *AVR_{Alto}*, induces the association between MLA10 and WRKY1/2, blocking WRKY function, resulting in derepression of basal defenses. In this scenario, *Mla*-mediated resistance still requires a set of basal defense gene functions. Further research is necessary to determine the diversity of antimicrobial products that function in the barley–powdery mildew defense response.

MATERIALS AND METHODS

Plant materials and fungal isolates.

Seven-day-old seedlings of C.I. 16151 (*Mla6*) (Moseman 1972) and *mlo-5* BC₇ Ingrid were used for the single-cell transient assays and VIGS assays. Virus-infected barley was maintained in a growth chamber (Percival Scientific, Perry, IA, U.S.A.) at 24°C with 16 h of light (550 µmol/m²/s) and 8 h of darkness at 20°C. *B. graminis* f. sp. *hordei* isolate 5874 (*AVR_{Al}* and *AVR_{as}*) was propagated on *Hordeum vulgare* cv. Manchuria at 18°C (16 h of light and 8 h of darkness).

***HvCS*, *HvAsa2*, and *HvCMI* full-length cDNA cloning.**

The *HvCMI* full-length cDNA clone was obtained from CUGI (Clemson University Genomics Institute) EST Hv_CEB0009G14. Full-length cDNAs of *HvCS* and *HvAsa2* were obtained from 5' rapid amplification of cDNA ends (RACE) using the GeneRace kit (Invitrogen, Carlsbad, CA, U.S.A.). *HvCS* and *HvAsa2* nucleotide sequences were extended into the 5' untranslated region by three rounds of PCR with the nested primer (provided by the manufacturer) and gene-specific primers, listed in Supplementary Table 1. Sequence information from 5' RACE was used to design gene-specific 5' primers, which were used to amplify full-length cDNAs of *HvCS* and *HvAsa2* by SuperScript One-Step RT-PCR with the Platinum Taq System. RT-PCR products were ligated into the pCR2.1-TOPO vector as TOPO-CS and TOPO-AS.

Plasmid construction.

Primers used for TIGS and VIGS are listed in Supplementary Table 2. Barley gene fragments were cloned into the hairpin vector pPKTA30 for TIGS assays (Douchkov et al. 2005). The BSMV-VIGS vector (Scofield et al. 2005) was modified for biolistic transfer (Meng et al. 2009). For VIGS assays, *HvCS*, *HvAsa2*, and *HvCMI* gene fragments were cloned into the BSMVm:γ vector as BSMVm:CS, BSMVm:AS, and BSMVm:CM, respectively. The UbiNos_2-3 vector, which was modified from a UbiNos vector (Shirasu et al. 1999a) by inserting the multiple cloning site (5'-AGCTTCTGCAGCCC GGGGATCCGAGCTCG-3') between *Hind*III and *Eco*RV, was used to express *HvCS*, *HvAsa2*, and *HvCMI*. Open reading frames of *HvCS*, *HvCMI*, and *HvAsa2* were cloned into UbiNos_2-3.

Single-cell transient assays.

Bombardment of barley leaves was carried out via BioRad (Hercules, CA, U.S.A.) PDS-1000/He Biolistic transfer system. Gold particles (BioRad) were coated as described (Douchkov et al. 2005). For TIGS assays, 7-day-old barley seedlings were bombarded at 650 pounds per square inch using a hepta adaptor. Seedlings were then grown in the greenhouse for 6 days before inoculation. Leaves were cut and placed randomly on 1% Phytoagar (Ducheva, Haarlem, The Netherlands) in a 22.5-by-22.5-cm Nunc plate for inoculation. For overexpression, detached barley leaves were prepared for biolistic transfer as described previously (Halterman and Wise 2004; Halterman et al. 2001). The inoculated leaves were incubated at 18°C (16 h of light and 8 h of darkness) for 5 days. Leaves then were immersed in a GUS solution (0.1 M Na₂HPO₄/NaH₂PO₄, pH 7.0; 10 mM Na-EDTA; 5 mM potassium hexacyanoferrat [II] and potassium hexacyanoferrat [III]; 5-bromo-4-chloro-3-indoxyl-b-d-glucuronic acid at 1 mg/ml; cyclohexylammonium salt; 0.1% (vol/vol) Triton X-100; 20% [vol/vol] methanol) at 37°C overnight and stained with Coomassie blue (0.3% [wt/vol] Coomassie blue and 7.5 and 30% [vol/vol] methanol) as described (Halterman and Wise 2004; Halterman et al. 2001). Scoring for susceptibility was performed under light microscopy

by counting the number of GUS cells with haustoria to the total GUS cells with attached *B. graminis* f. sp. *hordei* conidiospores.

VIGS.

Seven-day-old seedlings of barley cv. C.I. 16151 were infected with BSMV via co-bombardment of BSMV α , BSMV β , and modified BSMV γ containing the designated target gene fragment. Biolistic transfer procedure was identical to that in TIGS assays described previously. The seedlings were then placed in the VIGS growth chamber (Percival Scientific) for silencing 9 days prior to *B. graminis* f. sp. *hordei* inoculation. Plant-*B. graminis* f. sp. *hordei* interaction phenotypes were scored 7 to 9 dai. For microscopic observation, virus-infected leaves were cut and placed randomly on 1% Phytoagar (Ducheve) in a 22-by-22-cm Nunc plate for inoculation. The inoculated leaves were incubated at 18°C (16 h of light and 8 h of darkness) for 72 h. Leaves were fixed in a ethanol/acetic acid (3:1, vol/vol) solution overnight and subsequently washed with deionized water twice and stained with Coomassie blue (0.3% [wt/vol] Coomassie blue and 7.5 and 30% [vol/vol] methanol) as described (Halterman and Wise 2004; Halterman et al. 2001). Barley-*B. graminis* f. sp. *hordei* interaction phenotypes were scored by light microscopy.

qRT-PCR of BSMV-VIGS-treated plants.

Second leaves from each BSMV-VIGS-treated plant that displayed a typical mosaic virus infection symptom were sampled for RT-PCR. Barley total RNA was isolated using a hot (60°C) phenol/guanidine thiocyanate method as described previously (Caldo et al. 2004) and treated with DNase I. Two μ g of RNA was transcribed into cDNA with an oligodT primer by SuperScript III reverse transcriptase (Invitrogen). First-strand cDNAs were used as templates for amplifying target gene fragments at cycling conditions of 92°C for 20 s, 58°C for 20 s, and 68°C for 15 s with 20, 25, 30, 35, and 40 cycles. *Actin* was used as internal constitutive expression control for cDNA quantitative normalization.

Data collection and statistical analysis.

In single-cell transient assays, scoring for susceptibility was performed under light microscopy by counting the number of GUS cells with haustoria compared with the total GUS cells attacked by *B. graminis* f. sp. *hordei*, as evidenced by an attached conidiospore. For each experiment, eight leaves per treatment were counted. At least three independent replicates were performed. The ratio of susceptible cells to the total attacked GUS cells was calculated per treatment per experiment for statistical analysis. A completely randomized design was applied to each experiment. *P* values were calculated with a mixed linear model ($y_{ij} = \tau_i + \eta_j + \varepsilon_{ij}$, where y_{ij} is the penetration efficiency for the (*i*)th TIGS treatment in the (*j*)th replicate, τ_i is the fixed effect of (*i*)th treatment with TIGS hairpin constructs, η_j is the random effect of (*j*)th replicate, and ε_{ij} is the error residual) via SAS (Statistical Analysis Software; SAS Institute Inc., Cary, NC, U.S.A.). In VIGS assays, BSMV-VIGS-treated barley plants displayed a mosaic silencing phenotype in restricted regions, which is consistent with BSMV movement (Lawrence and Jackson 2001a,b). Therefore, a counting unit was defined as a 5-mm-long section between two veins. Per leaf, regions with the most susceptible cells were selected as counting units for which conidiospores with fully developed appressoria were counted. Susceptibility was calculated as the ratio of conidiospores with haustoria or secondary hyphae to the total conidiospores per treatment per experiment. Three independent experiments were performed where at least 600 conidiospores were counted per treatment. A completely randomized design was applied to each experiment. *P* values were calculated with a

mixed linear model via SAS as above, except substituting BSMV-VIGS in the place of TIGS in the formula.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Clemson University Genomics Institute: www.genome.clemson.edu
 PLEXdb (Plant Expression database): www.plexdb.org/