

Virus-Induced Gene Silencing-Based Functional Characterization of Genes Associated with Powdery Mildew Resistance in Barley¹

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We successfully implemented virus-induced gene silencing (VIGS) in barley (*Hordeum vulgare*) for the functional characterization of genes required for *Mla13*-mediated resistance toward the biotrophic barley pathogen *Blumeria graminis* f. sp. *hordei*. Initially, barley cultivars were screened for their ability to host the barley stripe mosaic virus (BSMV)-VIGS vector by allowing its replication and systemic movement without causing excessive symptoms. *Phytoene desaturase* silencing leading to photobleaching was used as a phenotypic marker alongside reverse transcription-PCR data to characterize the silencing response at the molecular level. Barley cultivar Clansman, harboring the *Mla13* resistance gene, was chosen as the most suitable host for BSMV-VIGS-based functional characterization of *Rar1*, *Sgt1*, and *Hsp90* in the *Mla*-mediated resistance toward powdery mildew. BSMV-induced gene silencing of these candidate genes, which are associated in many but not all race-specific pathways, proved to be robust and could be detected at both mRNA and protein levels for up to 21 d postinoculation. Systemic silencing was observed not only in the newly developed leaves from the main stem but also in axillary shoots. By examining fungal development from an incompatible mildew strain carrying the cognate *Avr13* gene on plants BSMV silenced for *Rar1*, *Sgt1*, and *Hsp90*, a resistance-breaking phenotype was observed, while plants infected with BSMV control constructs remained resistant. We demonstrate that *Hsp90* is a required component for *Mla13*-mediated race-specific resistance and that BSMV-induced VIGS is a powerful tool to characterize genes involved in pathogen resistance in barley.

One of the best-studied cereal pathosystems for investigating the genetic and molecular bases of monocotyledonous plant-pathogen interactions is the association between the obligate biotrophic powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*) and its natural host barley (*Hordeum vulgare*). Powdery mildew is one of the most important and devastating diseases of barley worldwide. Genetic resistance in barley to *Bgh* can be either race specific or nonrace specific. Race-specific resistance is conditioned by the interactions of resistance (*R*) gene products in the host, such as those encoded by the complex *Mla* locus on

chromosome 1H, and the products of cognate *Avr* genes in races of the fungus (Jørgensen, 1992).

Mutational analysis and map-based cloning in barley have identified genes that are necessary to establish *Mla*-mediated resistance to *Bgh*. This approach identified RAR1, a small zinc-binding protein with two highly similar domains, CHORD-I and -II (Cys- and His-rich domain), which is conserved in almost all eukaryotes except for yeasts and metazoans. In the latter species, equivalent proteins differ from plant homologs by carrying an additional C-terminal domain, the CS motif, with homology to the *Saccharomyces cerevisiae* protein SGT1 (for suppressor of G-two allele of *Skp1*; Shirasu et al., 1999). It has been shown that RAR1 interacts with SGT1 protein both in yeast two-hybrid assays and in planta. In plants, vertebrates, and yeast, SGT1 associates with SCF (Skp1-Cullin-F-box)-type E3 ubiquitin-ligase complexes potentially linking disease resistance signaling cascades to ubiquitination-dependent processes (Azevedo et al., 2002; Liu et al., 2004). The CS domain, a central region of human SGT1, exhibits a tertiary structure that is related to the cochaperone p23 and binds to cytosolic heat shock protein 90 (HSP90; Lee et al., 2004). It has been

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demonstrated that HSP90 interacts with RAR1 and SGT1 and is essential for RPS2-mediated resistance in *Arabidopsis* (*Arabidopsis thaliana*; Takahashi et al., 2003). In addition, HSP90 also interacts with other *R* gene products such as the resistance proteins N in *Nicotiana benthamiana* and RPM1 in *Arabidopsis* (Hubert et al., 2003; Liu et al., 2004). Many, but not all, of the powdery mildew *Mla* *R* genes require the *Rar1* gene for their function (Freialdenhoven et al., 1994; Peterhansel et al., 1997; Schulze-Lefert and Vogel, 2000), and *Rar1* independence is determined by a single-amino acid substitution within the Leu-rich repeat of the barley MLA6 and MLA13 proteins (Halterman and Wise, 2004).

Sgt1 and *Rar1* are required in multiple *R* gene-mediated and nonhost resistance responses to a variety of pathogens (Peart et al., 2002; Schornack et al., 2004). So far, functional proof that *Hsp90* is a required component in disease resistance signaling pathways has been demonstrated only for dicotyledonous plants, using virus-induced gene silencing (VIGS) and *Arabidopsis* mutants (Lu et al., 2003, 2004; Takahashi et al., 2003). This highlights the need to develop novel approaches allowing the functional characterization of molecular mechanisms associated with host and nonhost resistance mechanisms in cereals.

To date, functional studies of genes associated with host and nonhost resistance in barley utilized reverse-genetics approaches based on gain-of-function (transient gene expression; Schweizer et al., 1999) or loss-of-function using double-stranded RNA interference (Schweizer et al., 2000), which is a form of post-transcriptional gene silencing. These approaches are restricted to biolistic delivery of gene or double-stranded RNA constructs into single cells (for review, see Panstruga, 2004). The single cell-restricted inhibition of endogenous gene expression confirmed *Mlo* as a suppressor of broad-spectrum *Bgh* resistance (Schweizer et al., 2000), and the requirement of *Rar1* and *Sgt1* as part of signaling cascades leading to race-specific *Bgh* resistance (Azevedo et al., 2002). The resistance-breaking phenotypes obtained using this assay comprise successful *Bgh* penetration and haustorium formation, elongating aerial hyphae, and, eventually, secondary hyphae formation restricted to the close vicinity of the silenced cell (Schweizer et al., 2000; Panstruga, 2004). However, such a silencing approach relies on efficient delivery, minimal damage to the bombarded epidermal cells, and a relatively fast turnover of preexisting endogenous protein (Panstruga, 2004).

Reverse-genetics approaches based on VIGS mediate a homology-dependent degradation of the target RNA within multiple cells and cell layers of systemic upper uninoculated leaves and organs from the challenged plants. Previous studies have indicated that the VIGS systemic silencing response is concomitant with viral replication and is closely associated with systemic leaves supporting virus invasion and replication. This was illustrated by using a potato virus X (PVX) VIGS vector with a deleted 25-kD open reading frame (PVX.Δ25K) resulting in phloem restriction of the virus

(Himber et al., 2003). When PVX.Δ25K was used as a VIGS vector to silence endogenous mRNA, such as *phytoene desaturase* (*Pds*) or *Rubisco*, whose down-regulation leads to a visible silencing phenotype, the silencing response was confined to veins and 10 to 15 adjacent cells, indicating that the silencing response exhibits short-range movement (Himber et al., 2003).

Barley stripe mosaic virus (BSMV) has recently been developed as a VIGS vector for monocots (Holzberg et al., 2002; Lacomme et al., 2003) and generates a robust silencing response in barley. Previous studies have highlighted the invasion pattern of a green fluorescent protein (GFP)-tagged BSMV-based vector (Haupt et al., 2001). It was observed that the virus escaped first from major veins, and, following cell-to-cell spread through the mesophyll tissues, the epidermal cells were then infected (Haupt et al., 2001). The invasion of the epidermis is an important characteristic of BSMV and its further use as a reverse-genetic tool for the characterization of genes associated with *Bgh* resistance, as fungal structures are observed only on epidermal cells.

We report here the use of a BSMV-VIGS vector for the characterization of genes known to be associated with powdery mildew resistance in barley and show a requirement for HSP90 in *Rar1*- and *Sgt1*-dependent, *Mla13*-mediated resistance.

RESULTS

Identification of Barley *Mla13* Cultivars Most Suitable for BSMV-VIGS Functional Studies

The efficiency of BSMV-induced gene silencing is dependent on the ability of the host plant to tolerate virus accumulation. As the inheritance of resistance to BSMV has been reported (Vasquez et al., 1974; Carroll et al., 1979), it is necessary to identify appropriate barley cultivars that provide a suitable genetic background to study molecular mechanisms associated with powdery mildew resistance (cultivars harboring *Mla* *R* genes) and that tolerate the substantial levels of BSMV accumulation required to elicit a significant VIGS response. Barley cultivars Spire, Tyne, Clansman, Pallas near-isogenic line P11, and Digger (Cereal Pathogen Resistance Allele database <http://www.drastic.org.uk>) harboring the *Mla13* *R* gene were screened to identify genotypes capable of supporting BSMV replication and allowing functional characterization of genes associated with host resistance to the *Bgh* isolate 139 (National Institute of Agricultural Botany [NIAB], Cambridge, UK), carrying the corresponding avirulence gene *AvrMla13*.

We used a previously described BSMV.hpPDS_{Hv60} construct (Fig. 1; Lacomme et al., 2003) designed to silence *Pds*, a visual marker of gene silencing, to assess *Mla13* barley cultivars for their ability to develop a BSMV VIGS response. All the above-mentioned cultivars exhibited photobleaching symptoms when challenged with BSMV.hpPDS_{Hv60}. However, symptoms triggered by BSMV infection ranged from mild chlo-

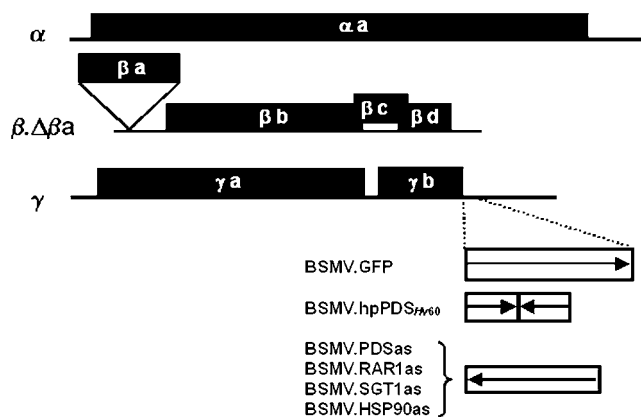


Figure 1. Schematic representation (not drawn to scale) of the BSMV genome organization and BSMV constructs engineered to express GFP and *Pds*, *Rar1*, *Sgt1*, and *Hsp90* cDNA fragments as inverted repeats or in antisense orientation. Genomic organization of BSMV RNAs α , β , and γ were as described previously (Holzberg et al., 2002). RNA β is a βa (coat protein) deletion mutation.

rotic stripes to necrotic areas in upper leaves, and these were also observed in plants challenged by BSMV.GFP and BSMV.hpPDS_{Hv60} constructs (data not shown). Spire and Clansman displayed the strongest photobleaching with mild BSMV infection symptoms in systemic leaves (Fig. 2A, left and right sections), as opposed to Digger, where weak photobleaching and strong BSMV infection symptoms were observed (data not shown).

To gain a better insight into the level of *Pds* silencing, we estimated the efficacy of the silencing response triggered by BSMV.hpPDS_{Hv60} in these cultivars at the molecular level. Semiquantitative reverse transcription (RT)-PCR was performed to assess the relative *Pds* mRNA levels using an endogenous gene (*ubiquitin*) as an internal standard as described previously (Lacomme et al., 2003).

RT-PCR tests were carried out on samples from leaves with the most pronounced silencing phenotype. Samples were taken from three plants infected with each construct. *Pds* mRNA levels were assessed by amplification of a portion of *Pds* upstream of the sequences introduced into the viral vectors ("Materials and Methods"; Lacomme et al., 2003). Lower levels of *Pds* mRNA were detected from silenced tissues of BSMV.hpPDS_{Hv60}-infected plants than from control BSMV.GFP-infected tissues in all cases (Fig. 2B, top section). For all samples, *ubiquitin* mRNA levels were comparable (Fig. 2B, bottom section). The strongest reduction of *Pds* RT-PCR product accumulation between control and silenced samples was detected in leaves from Spire, Tyne, and Clansman (Fig. 2B, cultivars 1, 2, and 3, top and bottom sections), in line with the observation that these cultivars developed the strongest phenotypic *Pds* VIGS response. As BSMV infection symptoms were less obvious in Clansman (Fig. 2A, plants challenged by BSMV.GFP), this cultivar was chosen to investigate *Bgh* *Mla13*-mediated resistance.

Systemic Silencing of *Sgt1*, *Rar1*, and *Hsp90*

To demonstrate the efficacy and robustness of the BSMV VIGS system for functional characterization of genes associated with *Bgh* resistance, we targeted genes known to be involved in *Mla*-mediated resistance pathways (*Rar1* and *Sgt1*) and *Hsp90*, which is associated with many *Rar1*- and *Sgt1*-dependent resistance pathways in dicotyledonous plants, but for which there is no such evidence in monocots (Azevedo et al., 2002; Hubert et al., 2003; Takahashi et al., 2003).

We generated VIGS constructs with antisense cDNA fragments from *Sgt1*, *Rar1*, and *Hsp90* (BSMV.SGT1as, BSMV.RAR1as, and BSMV.HSP90as; Fig. 1). First leaves from the barley cultivar Clansman were then inoculated (Fig. 3A) with each of these and control (BSMV.GFP) constructs. Systemic leaves numbers 2 and 3 (Fig. 3A) were harvested at different time points after BSMV infection ranging from 7 d post inoculation (dpi) to 21 dpi. The silencing response of the targeted genes was monitored at both the mRNA and protein levels.

By 7 dpi, reduced levels of *Sgt1*, *Rar1*, and *Hsp90* mRNA were detected in systemic leaves from plants

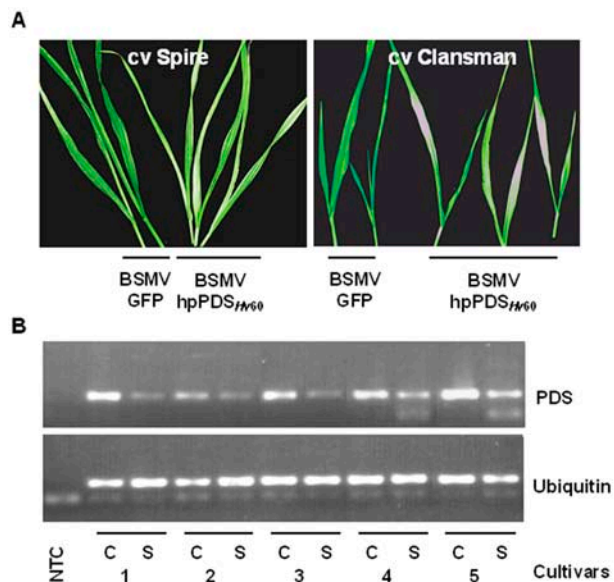


Figure 2. Initial screening of barley cultivars for BSMV VIGS response. A, Phenotypic assessment of virus symptoms and silencing abilities of BSMV.hpPDS_{Hv60} in barley cultivars Spire (left) and Clansman (right) is shown. Symptoms were recorded at 14 dpi with BSMV.GFP control and BSMV.hpPDS_{Hv60} VIGS constructs. B, RT-PCR-based assessment of the silencing response obtained in barley cultivars. Semiquantitative RT-PCR was carried out on cDNA originated from BSMV.GFP-infected (C) and BSMV.hpPDS_{Hv60}-infected (S) barley cultivars Spire (1), Tyne (2), Clansman (3), Pallas (4), and Digger (5). PCR products from the silenced gene *Pds* (top) were run alongside *ubiquitin* (bottom) to ensure equal amounts of template cDNA were used. Products were visualized after 30 cycles and 40 cycles of PCR amplification for, respectively, *ubiquitin* and *Pds* by gel electrophoresis alongside no template control (NTC). The most silenced leaves and the corresponding leaves from, respectively, BSMV.hpPDS_{Hv60}- and BSMV.GFP-infected plants from three different plants were pooled.

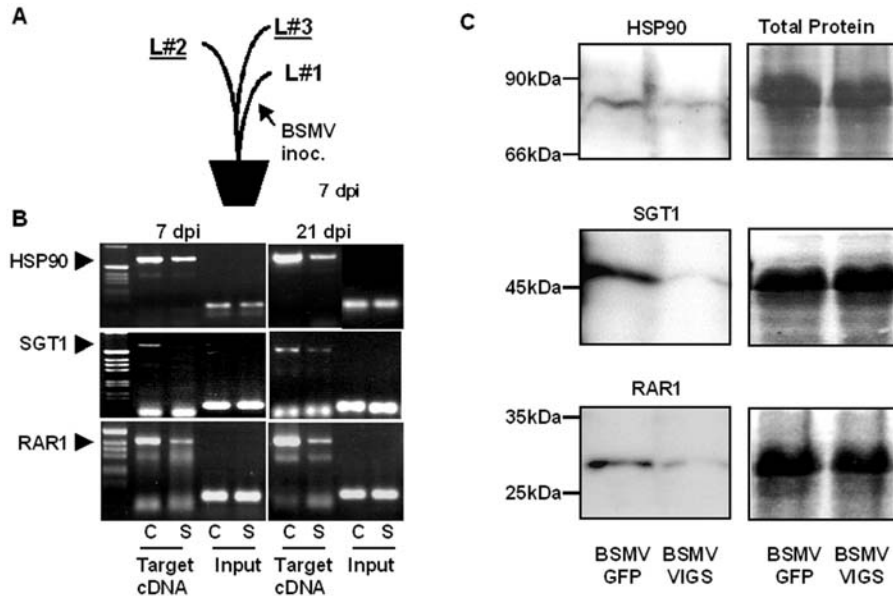


Figure 3. Molecular assessment of BSMV-based VIGS efficacy on main stem leaves of barley cultivar Clansman. A, Schematic representation of young barley plants 7 d post BSMV infection. The arrow points to the first emerging barley leaf on the main stem that was used for virus infection. Systemic leaves 2 (L#2) and 3 (L#3) were harvested and prepared for RT-PCR and western-blot analysis (not drawn to scale). B, RT-PCR results for *Hsp90*, *Sgt1*, and *Rar1* following amplification with gene-specific primers 7 dpi (left) and 21 dpi (right) with BSMV constructs for silencing target genes (S) and in control plants infected with BSMV.GFP (C). Equal template input is shown by PCR amplification with *ubiquitin*-specific primers. Systemic leaves (either L#2, or L#2 and L#3) from at least two different plants were pooled. C, Western-blot analysis for HSP90, SGT1, and RAR1 on leaf number 2 at 7 dpi with BSMV.GFP as a control and BSMV VIGS vectors using the target gene-specific sequence (left). The position of molecular mass standard Broad Range (for HSP90 and SGT1 western blotting; Bio-Rad) or DualVue Markers (for RAR1 western blotting; Amersham) is indicated. Protein loading is shown by Ponceau staining (right). For both RT-PCR and western-blotting analyses, pooled leaves from three different plants were sampled.

challenged with corresponding BSMV VIGS construct, compared to leaves from BSMV.GFP-infected control plants (Fig. 3B, left section). No significant difference in level of *ubiquitin* or *Pds* (data not shown), included as internal control for the input of RNA, was observed (Fig. 3B, left section). Reduced levels of *Sgt1*, *Rar1*, and *Hsp90* mRNA were detected up to 21 dpi in systemic silenced leaves, indicating that the silencing response was stable for longer periods of time (Fig. 3B, right section).

Because protein stability may mask the silencing effect observed at the mRNA level we therefore assessed the accumulation of SGT1, RAR1, and HSP90 proteins in control and silenced leaves. Total protein extracts sampled from control and silenced Clansman leaves were analyzed by protein gel blots using antisera to SGT1, RAR1, or HSP90. By 7 dpi significantly lower levels of SGT1, RAR1, and HSP90 (approximately 45 kD, 28 kD, and 80 kD, respectively) were detected in leaves of plants challenged with the corresponding BSMV VIGS construct in comparison to controls (Fig. 3C). These results indicate that the VIGS response triggered by BSMV leads to a down-regulation of *Sgt1*, *Rar1*, and *Hsp90* genes detectable at both the RNA and protein levels.

We previously observed that the BSMV VIGS response was also observed in emerging leaves originating from axillary shoots by 21 dpi with BSMV.PDSas

(Holzberg et al., 2002; data not shown). We investigated the efficacy of the VIGS response in these tissues by monitoring HSP90, SGT1, and RAR1 protein levels. By 21 d post BSMV inoculation, axillary leaves were fully developed (up to three axillary leaves; Fig. 4A). Protein extracts from silenced and control plants were prepared using these axillary leaves. Reduced levels of HSP90 (Fig. 4B) SGT1 (Fig. 4C), and RAR1 (Fig. 4D) proteins were detected in axillary leaves challenged with BSMV VIGS constructs, in comparison to control axillary leaves. This indicates that axillary leaves still display a significant silencing response and could provide an alternative source of silenced tissues to characterize molecular mechanisms associated with powdery mildew resistance.

Resistance-Breaking Phenotypes of Powdery Mildew on BSMV-Silenced Barley Leaves

As the different BSMV VIGS constructs were used to assess the functional involvement of selected genes in *Mla13*-mediated resistance to *Bgh*, the development of fungal structures was observed and used as a criterion to phenotypically assess resistance levels.

Leaves 2, 3, and 4 from silenced plants exhibiting mild virus symptoms were detached at 7 dpi and 14 dpi from the main stem, and leaves 1, 2, and 3 from axillary

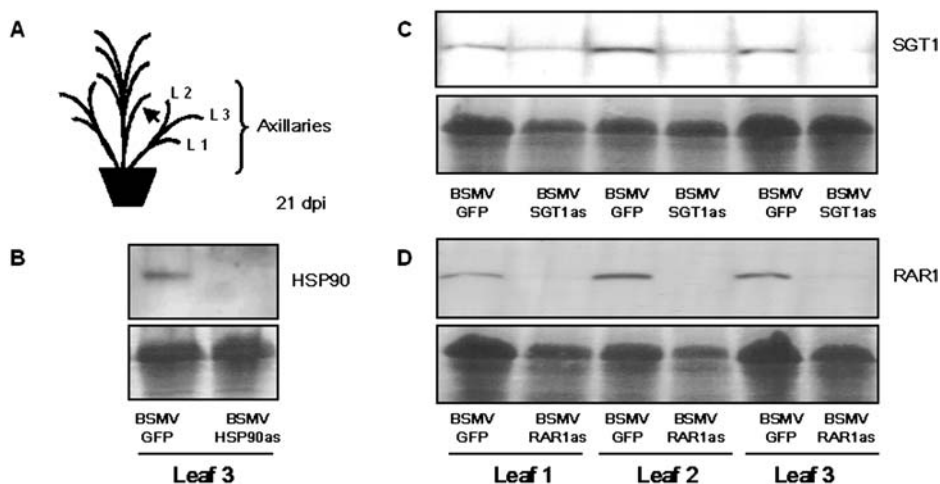


Figure 4. Molecular assessment of BSMV-VIGS efficacy on axillary leaves of barley cultivar Clansman. A, Schematic representation of young barley plants 21 d post BSMV infection (not drawn to scale). The arrow points to the first leaf on the main stem (corresponding to L1 from Fig. 3A) that was used for virus inoculation. B to D, Western-blot analysis for HSP90 (B), SGT1 (C), and RAR1 (D) protein levels from axillary leaves at 21 dpi after BSMV.GFP, BSMV.HSP90as, BSMV.SGT1as, or BSMV.RAR1as inoculation. Systemic leaves 1 (L1), 2 (L2), and 3 (L3; SGT1 and RAR1) or axillary leaf number 3 (HSP90) pooled from three different plants was harvested and prepared for western-blot analysis. Protein loading is shown by Ponceau staining (lower sections).

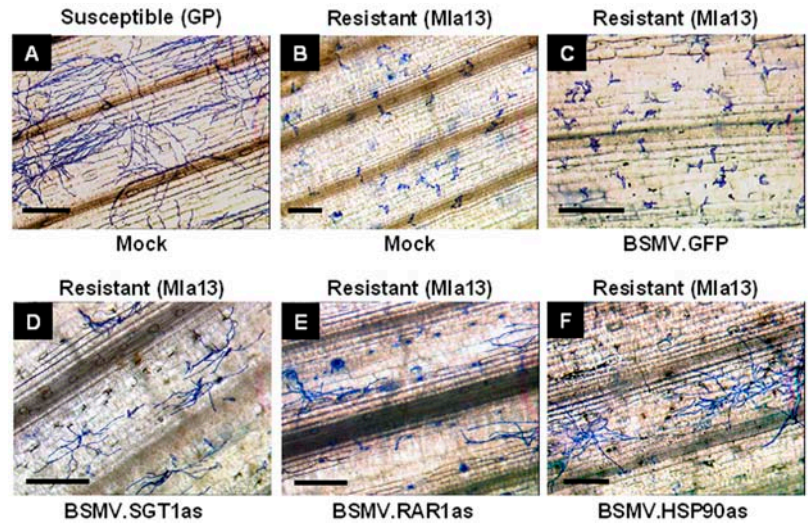
shoots at 21 dpi (Figs. 3A and 4A) and inoculated with *Bgh*. After 4 to 5 d post *Bgh* inoculation, leaves were fixed and fungal structures stained (described in “Materials and Methods”). Our observations focused on the primary infection process of powdery mildew, which consists of six morphologically identifiable stages: spore germination, formation of appressorial initials, maturation of appressoria, formation of penetration pegs, formation of haustoria, and formation of secondary hyphae. The formation of mature haustoria, which is generally observed by 34 to 36 h post *Bgh* inoculation and the development of secondary hyphae, which form by 3 to 4 dpi, are prerequisites for establishment of a compatible interaction between host and parasite (Ellingboe, 1972). The percentage of spores developing mycelial growth (hyphae) out of 100 observed spores per leaf was used as an indicator of resistance levels. We compared the proportions of spores developing hyphae on the genetically susceptible barley cultivar Golden Promise (corresponding to 100% of relative penetration) to the genetically resistant cultivar Clansman (not BSMV infected) and to Clansman plants infected with various BSMV constructs. The experiment was conducted three times for main stem leaf tests, assessing hyphae growth on four leaves of each type (main stem leaf numbers 2, 3, and 4) from four plants per construct. The experiment was repeated twice for axillary leaves, sampling three leaves of each type (axillary leaf numbers 1, 2, and 3) and from three plants per construct.

Typical results are shown in Figure 5, and the levels of susceptibility compared with the susceptible cultivar Golden Promise are presented in Figure 6. On main stems, hyphae growth was observed on all leaves from the susceptible barley cultivar Golden Promise

lacking the cognate *Mla13 R* gene (Fig. 5A). On average, 20 ($\pm 1.5\%$) out of every 100 spores developed hyphae in this compatible interaction and were defined as 100% of relative penetration efficiency (Fig. 6). Further data are relative to the 100% of relative penetration in Golden Promise. In contrast, the majority of *Bgh* spores developed only primary germ tubes and appressoria prior to fungal growth arrest in the resistant cultivar Clansman harboring the cognate *Mla13 R* gene (Fig. 5B). Indeed, only 10% ($\pm 3\%$) of spores inoculated on to leaves harvested from main stem leaves could overcome the *Mla13* resistance and establish hyphae (Fig. 6, left section). Similarly, leaves harvested from the barley cultivar Clansman inoculated with BSMV.GFP and BSMV.PDSas prior to mildew challenge revealed a *Bgh* resistance comparable to that observed in non-BSMV-challenged plants, and only 13% ($\pm 5\%$) and 10% ($\pm 2.5\%$) of spores developed hyphal structures, respectively. Figure 5C shows a typical fungal growth arrest in BSMV.GFP-infected plants following *Bgh* inoculation. In contrast, Clansman plants challenged with BSMV.SGT1as, BSMV.RAR1as, and BSMV.HSP90as (Fig. 5, D–F) displayed a significant increase in mycelial growth in all systemically BSMV-infected tissues. The percentages of relative penetration were 68% ($\pm 9\%$), 36% ($\pm 6.5\%$), and 40% ($\pm 5.5\%$) for plants challenged by BSMV.SGT1as, BSMV.RAR1as, and BSMV.HSP90as, respectively (Fig. 6, left section).

As the silencing response is observed in axillary leaves by 21 dpi (Fig. 4), we also investigated the effect of *Sgt1*, *Rar1*, and *Hsp90* silencing in this tissue. As observed for leaves of the main stem by 7 dpi and 14 dpi, control plants infected by BSMV.GFP or BSMV.PDSas displayed no significant difference in

Figure 5. Observation of fungal structures in silenced and control leaves from main stem leaves 5 d post *Bgh* inoculation. Fungal structures from *Bgh* isolate 139 carrying *AvrMla13* were visualized by aniline blue staining on leaves from (A) non-BSMV-infected susceptible barley cultivar Golden Promise showing highly developed hyphae, and (B) non-BSMV-infected resistant barley cultivar Clansman (*Mla13*) showing fungal growth arrest after primary germ tube and appressorial germ tube development. Potential effects of BSMV infection on *Bgh* resistance were assessed by inoculating the barley cultivar Clansman with the BSMV.GFP construct (C). Resistance breakdown was observed on barley leaves of plants infected with BSMV.SGT1as (D), BSMV.RAR1as (E), and BSMV.HSP90as (F). The bars equal 200 μ m.



mycelial growth compared to non-BSMV-challenged resistant Clansman plants. Specifically, 3% ($\pm 3\%$), 3% ($\pm 1.5\%$), and 6% ($\pm 6\%$) of relative penetration were observed on unchallenged, BSMV.GFP-infected, and BSMV.PDSas-infected Clansman, respectively (Fig. 6, right section). In comparison to the main stem, significantly lower percentages of relative penetration were observed on the axillary leaves.

In silenced axillary leaves, the percentages of relative penetration were 22% ($\pm 2\%$), 23% ($\pm 8\%$), and 46% ($\pm 24\%$) for BSMV.SGT1as-, BSMV.RAR1as-, and BSMV.HSP90as-challenged plants, respectively. Our results confirm that, in these tested foliar tissues,

BSMV-VIGS is an efficient approach to characterize the functions of genes such as *Sgt1* and *Rar1* that are required components of the *Mla13*-mediated resistance response to *Bgh*. Furthermore, the resistance-breaking phenotype observed in BSMV.HSP90as-silenced tissues also implicates *Hsp90* as a required component of *Bgh* *Mla*-mediated resistance in barley.

DISCUSSION

In this study, we demonstrate that BSMV-VIGS is a robust approach to identify genes associated with

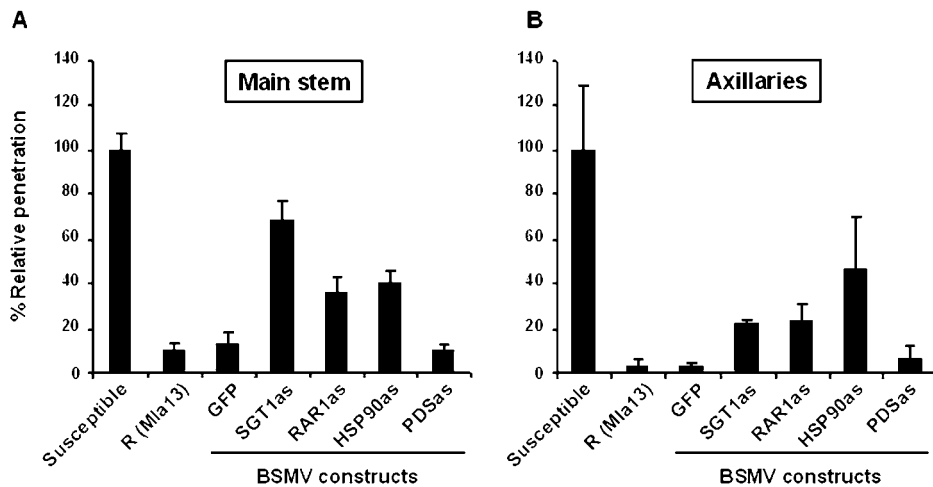


Figure 6. Percentage of relative penetration following BSMV-mediated silencing of *Sgt1*, *Rar1*, and *Hsp90* compared to control plants. Note that 100% of relative penetration is defined as that observed in the susceptible barley cultivar Golden Promise. Similarly, the number of spores developing hyphae for mock-inoculated cultivar Clansman (Resistant [*Mla13*]), for Clansman infected with BSMV control constructs (BSMV.GFP and BSMV.PDSas), and for Clansman infected with BSMV VIGS constructs (BSMV.SGT1as, BSMV.RAR1as, and BSMV.HSP90as) were reported as relative values with respect to Golden Promise controls. Results presented are from three independent VIGS experiments using four different plants per construct and experiment (\pm se), corresponding to (A) main stem leaves (combined data from leaves 2 and 3 harvested at 7 d or leaves 2, 3, and 4 at 14 d post BSMV challenge) and (B) axillary leaves (combined data from leaves 1, 2, and 3 harvested at 21 d post BSMV challenge).

fungus resistance in barley and report that *Hsp90* is required for *Bgh* *Mla*-mediated resistance. We confirm the requirement for *Sgt1*, *Rar1*, and *Hsp90* genes in the *Mla13*-mediated resistance response to powdery mildew in barley, as previously observed in other *R* gene-mediated responses in dicot species including *Arabidopsis* and *Nicotiana*.

The involvement of *Rar1* and *Sgt1* in *R*-gene pathways has been demonstrated in different plants through either mutant analysis or transient loss-of-function assays. The previously characterized barley *rar1* mutants are no longer resistant to *Bgh* in an *Mla12* background (Freialdenhoven et al., 1994). For both *Rar1* and *Sgt1*, in planta functional studies in dicots using VIGS (Liu et al., 2004) and in barley using single-cell RNAi (Azevedo et al., 2002) have confirmed their requirement in host resistance response. The BSMV-VIGS approach used in this study to silence *Sgt1* and *Rar1* results in a resistance-breaking phenotype leading to the development of secondary hyphae in silenced leaves by 7 and 21 dpi with BSMV VIGS constructs. The observed resistance-breaking phenotype was comparable for both *Rar1* and *Hsp90*, irrespective of the development stage. Indeed, the percentage of relative penetration was comparable in silenced leaves from the main stem (by 7 dpi) and axillaries (by 21 dpi). However, despite a clear role of *Sgt1* in *Mla13*-mediated resistance, a significant difference in the percentage of relative penetration was observed for *Sgt1* silencing in the main stems ($68\% \pm 6.5\%$) versus the axillary leaves ($22\% \pm 2\%$). Further experiments are required to conclude if a differential tissue requirement for SGT1 could account for these contrasting levels of resistance-breaking phenotype in different types of leaves.

The BSMV VIGS approach has unequivocally established the requirement of *Hsp90* in *Mla*-mediated resistance of barley to *Bgh*. The requirement of *Hsp90* in host and nonhost resistance response was previously reported using different approaches in model plant systems. A high-throughput VIGS-based reverse-genetic screen in *N. benthamiana* has implicated *Hsp90* as a required component of N-, Rx-, and Pto-mediated resistance (Lu et al., 2003). *Hsp90* was also shown to be involved in a nonhost response in *N. benthamiana* (Kanzaki et al., 2003). The physical interaction of HSP90 with RAR1, SGT1, and the resistance protein N has been demonstrated (Liu et al., 2004). However, despite the fact that HSP90 interacts with RAR1, SGT1, and the resistance protein RPM1 to modulate its function, RAR1 and SGT1b can associate independently with HSP90 (Hubert et al., 2003) in *Arabidopsis*. Recently, it has been reported that the stability of barley MLA resistance proteins (both MLA1 and MLA6) is dependent on RAR1 (Bieri et al., 2004). A similar RAR1 dependence was observed for the stability of Rx protein in *N. benthamiana* providing extreme resistance to PVX virus (Bieri et al., 2004). Therefore, interactions among these proteins can regulate R-protein function.

VIGS-based approaches allow the characterization of genes such as *Hsp90*, for which down-regulation leads in some cases to severe morphological defects, as reported for *N. benthamiana* using tobacco rattle virus (TRV) VIGS vectors (Liu et al., 2004) or PVX (Lu et al., 2003), and therefore are likely to be lethal using RNAi transgenic lines. No such severe phenotypes have been observed in *Hsp90*-silenced barley. One could hypothesize that either *Hsp90* has different roles in *N. benthamiana* and barley or that different levels of *Hsp90* down-regulation have been achieved, resulting in different symptom severity. It is noteworthy that, in the case of *Sgt1* silencing, *N. benthamiana* *Sgt1*-silenced plants using a TRV VIGS vector were shorter and more branched than control plants (Peart et al., 2002). No such obvious branching phenotype was observed in barley despite significant reduction in SGT1 accumulation in the main stem and axillary leaves. This could simply be due to the architectural differences between monocots and dicots or because *Sgt1* is required for pathogen resistance but not for plant development in barley. Alternatively, this could be explained by the ability of TRV to invade efficiently meristematic tissues (Ratcliff et al., 2001; Valentine et al., 2004) and therefore cause silencing at earlier stages of shoot development than BSMV. However, BSMV strain ND18, from which BSMV VIGS vectors were derived, is seed transmissible, and other BSMV seed-transmitted strains (such as MI-1) invade reproductive meristems very early in their development (Johansen et al., 1994). In our case, the BSMV VIGS vector used is deleted from its coat protein (β a open reading frame). Despite the fact that no data are available on the seed transmissibility and meristem invasion of such mutants, it has been reported that seed transmission determinants are mapped on RNA γ and RNA β (Edwards, 1995). Considering the regulatory role of the γ b gene on RNA β expression (Petty et al., 1990), it is conceivable that such a coat protein mutant may display a reduced meristem invasion and prevent severe developmental phenotypes associated with *Sgt1* silencing.

Another important factor that may influence functional characterization and the silencing response is the gene family complexity of *Rar1*, *Sgt1*, and *Hsp90* in barley. In *Arabidopsis*, *Sgt1* and *Rar1* have been identified as, respectively, two copies (Austin et al., 2002; Azevedo et al., 2002) or a single-copy gene (Muskett et al., 2002), whereas *Hsp90* has seven copies (four in the cytoplasmic subfamily; Krishna and Gloor, 2001). It is believed that *Sgt1* and *Rar1* are both single-copy genes in barley (Takahashi et al., 2003). *Hsp90* is likely to belong to a multiple-gene family in barley as for *Arabidopsis*. Indeed, a BLASTN search (Altschul et al., 1997) for barley expressed sequence tags (ESTs) at the National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov/BLAST/>) with homology to the 383-bp cytosolic *HvHsp90* sequence used for the silencing experiments revealed 380 ESTs, from which 248 shared at least 25 nt

consecutive homology and therefore could be targeted by the BSMV.HSP90as VIGS construct. Using Cap3 software (Huang and Madan, 1999), these ESTs could be grouped into three different contigs, each homologous to cytosolic HSP90. Therefore, at least three barley *Hsp90* cytosolic genes and another yet unidentified barley cytosolic *Hsp90* are likely to be targeted by the BSMV.HSP90as construct. As significant decreases in HSP90 protein level were observed, we can conclude that the VIGS response generated by the BSMV.HSP90as construct is likely to have targeted several members of the barley cytosolic *Hsp90* family and may be robust enough to characterize other genes present in multiple copy numbers.

BSMV fulfills several important characteristics for use as a reverse-genetic tool for functional characterization of genes associated with Blumeria resistance in barley. We identified *Mla* cultivars that tolerate BSMV accumulation and observed silencing without alteration or masking of the host response to pathogen challenge. We also demonstrated that BSMV challenge does not affect *Bgh* resistance as the percentage of relative penetration was similar in BSMV-uninoculated and BSMV.GFP- or BSMV.PDSas-inoculated plants. Consequently, BSMV infection is not likely to interfere with molecular mechanisms associated with secondary pathogen infection. In addition, the silencing response was robust enough in the epidermis to support *Bgh* colonization and was expressed in a sufficient number of cells to allow mycelial growth. Hyphae appear clustered on the leaf surface, possibly because of the nonuniform nature of the silencing response that could reflect the pattern of unloading BSMV in systemic leaves. Previous studies have highlighted the invasion pattern of a GFP-tagged BSMV-based vector (Haupt et al., 2001). Inoculation of source leaves of barley produced systemic symptoms on sink leaves within 4 to 5 d following inoculation. BSMV systemic movement mirrored the unloading pattern of phloem-mobile fluorescent solute (carboxyfluorescein). As in the case of carboxyfluorescein unloading, the exit of BSMV.GFP was discontinuous along the length of a single vein but covered the whole leaf. It was observed that the virus escapes first from major veins and then spreads cell to cell through the mesophyll and the epidermis.

The systemic nature of the VIGS response allows the rapid characterization of genes associated with local and systemic resistance in barley and other cereals. VIGS pervasiveness, in contrast to microprojectile-mediated RNAi approaches, which show phenotypes for a limited amount of time after bombardment (usually 4 d), allow characterization of genes whose corresponding proteins have a relatively slow turnover. In addition, all types of cells (both epidermal and mesophyll cells) and newly developing leaves displayed a significant systemic VIGS response. This opens up the possibility of conducting further studies of genes associated with a multilayered defense response in local and systemic tissues.

MATERIALS AND METHODS

BSMV Constructs and Cloning of cDNAs

For VIGS experiments, the γ RNA-based BSMV vector (Holzberg et al., 2002; Lacomme et al., 2003) was utilized to silence *Pds*, *Sgt1*, *Rar1*, and *Hsp90* genes in barley (*Hordeum vulgare*) as described previously (Lacomme et al., 2003). VIGS constructs were engineered by cloning barley *Sgt1*, *Rar1*, and *Hsp90* fragments using gene-specific primers harboring *NotI* and *PacI* restriction sites at their extremity. Primer sequences used to clone a 371-bp portion of *HvSgt1* (GenBank accession no. AF439974) were 5'-TTGCGCCG-CACATCAAGCTGGGCAGTTA-3' as a forward primer and reverse primer 5'-TATTAATTAAGGCTTGCTTGGCACTCTAC-3'. A 367-bp portion of *HvRar1* (GenBank accession no. AF192261) was amplified by RT-PCR using the forward primer 5'-GAGCGCCGCAAGCAAAGAAGCCATGAT-3' and the reverse primer 5'-GTTAATTAATCCTTGACGTTTTGCCACAT-3'. A fragment of 395 bp in length for *HvHsp90* (GenBank accession no. AY325266) was amplified from plasmid pCC194 harboring the full-length barley cytosolic HSP90 (*HvHsp90*) cDNA clone whose corresponding HvHSP90 protein interacts with *Sgt1* and *Rar1* (Takahashi et al., 2003) using the forward primer 5'-AAA-AGCGCCGCAAGCAGAAGCCTATC-3' and the reverse primer 5'-TATTTAATTAATCCTTGACAAGGTTTC-3'. PCR-amplified cDNA fragments were then digested with *PacI* and *NotI* and inserted into the γ .bPDS4-as (Holzberg et al., 2002) vector digested with *PacI*-*NotI* to generate constructs BSMV.SGT1as, BSMV.RAR1as, and BSMV.HSP90as. For the screening of barley cultivars with the strongest *Pds* VIGS response, the BSMV.GFP (Haupt et al., 2001), as a control of infection, and BSMV.hpPDS₄₆₀ (Lacomme et al., 2003) constructs were used. The BSMV.GFP construct and BSMV.PDSas (Holzberg et al., 2002; Lacomme et al., 2003) were used as control of BSMV infection for the Blumeria experiments. Generation of BSMV infectious RNAs from cDNA clones and inoculation procedures were as described previously (Holzberg et al., 2002).

RNA Extraction, cDNA Synthesis, and Semiquantitative RT-PCR

Barley endogenous plant gene cDNA fragments were obtained from total RNA extracted from frozen barley cultivar Clansman leaves using the Qiagen RNeasy plant mini kit (Qiagen, Crawley, UK) as described previously (Lacomme et al., 2003). DNaseI treatment (DNA-free kit; Ambion, Austin, TX) and first-strand cDNA synthesis by oligo(dT) priming using SuperScript II RNase H⁻ reverse transcriptase (Invitrogen Life Technologies, Paisley, UK) were as described previously (Lacomme et al., 2003).

For semiquantitative RT-PCR analysis, primers that anneal outside the region of the cDNA cloned into BSMV to trigger silencing were used to ensure that only the endogenous mRNA was amplified. Ubiquitin cDNA was used as an internal constitutively expressed control. First-strand cDNA was used as a template for PCR amplification through 30, 35, 40, 45, and 50 cycles. As 30 cycles (*ubiquitin*, *Hsp90*), 40 cycles (*Pds*, *Sgt1*), or 50 cycles (*Rar1*) of amplification corresponded to the log-linear phase of PCR product amplification in the nonsilenced control samples, these conditions were selected for comparison of relative accumulation of both target *Pds*, *Sgt1*, *Rar1*, *Hsp90* and control *ubiquitin* mRNAs in all samples. Primers for amplifying the 97-bp *ubiquitin* PCR fragment (GenBank accession no. X04133) were 5'-GCAAGTAAGTG-CCTGGTCATGA-3' as forward primer and 5'-ACAACCAGACATGCTCCA-ACCT-3' as reverse primer. Primers for amplifying the 113-bp *Pds* PCR fragment (GenBank accession no. AY062039) were 5'-TGGAGCTTATCC-CAATGTACAGAA-3' as forward primer and 5'-TGTATCCCCTGGCTTG-TTTG-3' as reverse primer. Primers for amplifying the 613-bp *Sgt1* PCR fragment (GenBank accession no. AF439974) were 5'-TCGGATCTGGAGAG-CAAGGCCAAGGAGG-3' as forward primer and 5'-ATCTGTTCACCAAA-GTCAACAACCACGC-3' as reverse primer. Primers for amplifying the 426-bp *Rar1* PCR fragment (GenBank accession no. AF192261) were 5'-TGATGGCA-TGAAAGAGTGGAGCTGTTG-3' as forward primer and 5'-ATGCAGC-ATCATGGTTATCTCTCC-3' as reverse primer. Primers for amplifying the 664-bp *Hsp90* PCR fragment (GenBank accession no. AY325266) were 5'-AATTCTGACGATGAAGACGAGGAGGA-3' as forward primer and 5'-AGAAGCTCAGCAATCTTGCTCTGTTCTGG-3' as reverse primer.

Western-Blotting Experiments

Silenced and control leaves were ground in liquid nitrogen and mixed with two volumes of grinding buffer supplemented with Complete Protease

Inhibitor Cocktail (Roche Diagnostics, Lewes, UK) as described by Bieri et al. (2004). Proteins were separated on 12% SDS-PAGE gels and blotted to Immobilon-P membrane (Millipore, Bedford, MA) using the Mini-Protein II system (Bio-Rad, Hercules, CA). Anti-SGT1 and anti-RAR1 antibodies have been previously described (Azevedo et al., 2002). The HSP90 antibody is an affinity-purified polyclonal antibody raised against a 17-amino acid peptide mapping within amino acids 1 to 50 at the amino terminus of HSP90 from *Arabidopsis thaliana*. This region is conserved between HSP90 in dicots (such as *Nicotiana benthamiana* GenBank accession nos. AY368904 and AY519499; *Lycopersicon esculentum* GenBank accession no. AAR12195) and monocots (such as barley GenBank accession no. AY325266 targeted for silencing; *Triticum aestivum* GenBank accession no. X98582; *Oryza sativa* GenBank accession nos. AB111810 and AY077617; *Zea mays* GenBank accession nos. S59780 and A48426). This antibody recognizes the N-terminal conserved region of HSP90 (HSP90- α aE-17; Santa Cruz Biotechnology, Heidelberg), as described previously (Lu et al., 2003; Liu et al., 2004). Blots were incubated with primary antibodies against SGT1 (rat polyclonal, 1:2,000), RAR1 (rabbit polyclonal, 1:5,000), and HSP90 α (goat polyclonal, 1:1,000; HSP90- α aE-17, Santa Cruz Biotechnology) followed by incubation with respective secondary antibodies anti-rat, anti-rabbit, or anti-goat IgG-peroxidase conjugates (Amersham Biosciences, Little Chalfont, UK), and then chemiluminescent autoradiography (ECL Plus; Amersham Biosciences). Molecular weight standards Broad Range (Bio-Rad) or DualVue Markers (Amersham, Uppsala) were run alongside.

Plant Growth

All work involving virus-infected material was conducted in containment glasshouses under Scottish Executive Environment and Rural Affairs Department license GM/243/2005.

Barley cultivar Golden Promise (susceptible to *Bgh*), and cultivars harboring the *Mla13 R* gene, such as Clansman, Spire, Tyne (harboring *Ml [La]* and *Mla13*), Pallas near-isogenic line P11, and Digger (harboring *Mla13*), were grown, as described previously (Hein et al., 2004), in controlled environment chambers at 22°C with a 16-h photoperiod in natural light supplemented with sodium lights (light intensity ranging from 400–1,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

Mildew Growth and Barley Leaf Inoculation

BSMV-infected and control barley leaves originating from the main stem (leaves 2, 3, and 4) or from axillary shoots (leaves 1, 2, and 3) were harvested after 7, 14, or 21 d post challenge with BSMV constructs. Leaf segments of about 5 cm length were cut and immediately placed in boxes (to provide containment, SEERAD GM/243/2005) containing 0.5% (w/v) distilled water agar and 1 mM benzimidazole. The leaf segments were then inoculated with an avirulent strain of powdery mildew (*Bgh*), 139 (*Avr13*; obtained from NIAB), to give approximately 15 to 20 sporulating colonies per cm^2 and incubated at 15°C in continuous light (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

Staining and Microscopy

Leaves were fixed, cleared, and stained to allow observation of both fungal development and plant responses to attempted infection. A modified method was used for this process (Carver et al., 1991). Leaves were fixed for 24 h on filter paper soaked with 1:1 (v/v) ethanol:acetic acid and for 48 h on filter paper soaked with lactoglycerol (1:1:1 [v/v] lactic acid:glycerol:H₂O), and stained with aniline blue 0.1% (w/v) in 0.1 M K₃PO₄. Excess dye was carefully rinsed off with distilled water. Leaves were mounted onto microscope slides with mounting media Histomount (VWR, Poole, UK) prior to microscopy. Samples were observed under light microscope Nikon Optiphot I (Nikon, Kingston, UK), and pictures were taken using a Color Video Camera KY-F55B (Photonic Science, Robertsbridge, UK) using software ImageProPlus 4.1 (Media Cybernetics, Silver Spring, MD).

Availability of Biomaterials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all parts of the material. Obtaining any permission will be the responsibility of the requester.

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