

Blufensin1 Negatively Impacts Basal Defense in Response to Barley Powdery Mildew^{1[W][OA]}

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Plants have evolved complex regulatory mechanisms to control the defense response against microbial attack. Both temporal and spatial gene expression are tightly regulated in response to pathogen ingress, modulating both positive and negative control of defense. BLUFENSIN1 (BLN1), a small peptide belonging to a novel family of proteins in barley (*Hordeum vulgare*), is highly induced by attack from the obligate biotrophic fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*), casual agent of powdery mildew disease. Computational interrogation of the *Bln1* gene family determined that members reside solely in the BEP clade of the Poaceae family, specifically, barley, rice (*Oryza sativa*), and wheat (*Triticum aestivum*). Barley stripe mosaic virus-induced gene silencing of *Bln1* enhanced plant resistance in compatible interactions, regardless of the presence or absence of functional *Mla* coiled-coil, nucleotide-binding site, Leu-rich repeat alleles, indicating that BLN1 can function in an *R*-gene-independent manner. Likewise, transient overexpression of *Bln1* significantly increased accessibility toward virulent *Bgh*. Moreover, silencing in plants harboring the *Mlo* susceptibility factor decreased accessibility to *Bgh*, suggesting that BLN1 functions in parallel with or upstream of MLO to modulate penetration resistance. Collectively, these data suggest that the grass-specific *Bln1* negatively impacts basal defense against *Bgh*.

The coevolution of plants and plant pathogens has generated a complex multilayered immune response (Jones and Dangl, 2006). Both temporal and spatial gene expression are regulated precisely in a system that balances both positive and negative control of defense. During the interaction of plants and plant pathogens, positive regulators potentiate defense by inducing genes involved in cell wall reinforcement, modification of the cytoskeleton, generation of toxic compounds (phytoalexins and peptides), formation of reactive oxygen species (ROS), and, potentially, programmed cell death in the form of the hypersensitive response (Schulze-Lefert and Panstruga, 2003; Brogden,

2005; Hüchelhoven, 2005; Jones and Dangl, 2006; Wise et al., 2007b; Graham et al., 2008). Of these resistance mechanisms, antimicrobial peptides have been found to be one of the most fundamentally conserved among vertebrates, invertebrates, insects, and plants. These peptides have a broad range of toxic activities that inhibit the progression of pathogen invasion, such as membrane destabilization, interfering with transport, and inhibition of protein function (Ganz, 2003; Brogden, 2005). Many of the identified plant antimicrobial peptides fall into well-characterized families such as the γ -thionins, defensins, knottins, and protease inhibitors (Yount and Yeaman, 2004; Graham et al., 2008). Of these, both defensins and knottins can exceed 100 family members within a species. This abundance, resulting from family expansion, divergence, and unequal recombination events, reflects the selection process driven by an ongoing arms race between host and pathogen in developing new offensive weaponry (Silverstein et al., 2007; Graham et al., 2008).

With the generation of hundreds of secreted peptides during the defense response (Kwon et al., 2008), to what degree could these peptides have developed roles other than their known or predicted toxic effect? Several peptides have been characterized recently that have roles in wounding (systemin; Pearce et al., 1991), self-incompatibility (SCR; Schopfer et al., 1999), stomatal patterning (EPF1; Hara et al., 2007), cellular proliferation and expansion (PSY1; Amano et al., 2007), abscission (IDA; Butenko et al., 2003), pollen

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formation (TPD1; Yang et al., 2003), root development (RALF; Pearce et al., 2001), shoot meristem development (CLAVATA3; Fletcher et al., 1999), and innate immunity (AtPep1; Huffaker et al., 2006). In the case of systemin, PSY1, SCR, and CLAVATA3, the corresponding peptide receptors have been identified, suggesting a general model of hormone activity via ligand-receptor pairing (Matsubayashi et al., 2001). Although many of these peptides may have evolved independently of those associated with antimicrobial activity, they are all relatively small, probably secreted to the apoplast, and typically subjected to extensive posttranslational processing and/or modification (Lindsey et al., 2002).

Negative regulators of plant defense are essential components that temper the severity of the immune response (Lam, 2004). Several gain-of-function and loss-of-function mutants have revealed genes associated with basal defense and effector-triggered immunity (Büsches et al., 1997; Frye et al., 2001; Hüeckelhoven et al., 2003; Behn et al., 2004; Jones and Dangl, 2006; Wang et al., 2006; Shen et al., 2007). Many of these genes have been shown to have important roles in overlapping pathways, suggesting a complex interconnected network of regulation. Potentially, negative regulators may control the intensity of programmed cell death, preventing excessive responses from damaging more than just the intended target while not compromising defense. Of the negative regulators cloned in plants, there exist two distinct classes, based on mechanistic similarity to existing defense pathways. *Edr1*, *AtWRKY58*, *HvWRKY1/2*, and *BAX inhibitor-1* (*BI-1*) are examples of well-characterized negative regulators in the known signal and transcriptional activation cascades. By contrast, genes such as the *Mlo* negative regulator of penetration resistance and several lesion-mimic mutants, such as *lsd1*, have only recently been characterized with regard to regulatory roles and importance during defense (Büsches et al., 1997; Frye et al., 2001; Hüeckelhoven et al., 2003; Wang et al., 2006; Shen et al., 2007). Although the former group of genes direct our attention to the complexity and redundancy of the regulatory network of plant defense, the latter set of genes are expanding our understanding of nonhost resistance, biotrophy, and the formation and/or progression of necrosis.

Over the past two decades, barley (*Hordeum vulgare*) powdery mildew, caused by *Blumeria graminis* f. sp. *hordei* (*Bgh*), has been developed as a model system to investigate host response to obligate fungal biotrophs (Bélanger et al., 2002; Panstruga, 2003, 2004; Schweizer, 2007). Pathogen recognition in barley-*Bgh* interactions is triggered in a pathogen race-specific manner by genes designated *Ml* (for *Mildew resistance locus*; Jørgensen, 1994). Approximately 30 distinct resistance specificities have been identified at the *Mla* locus; all cloned *Mla* alleles isolated so far encode coiled-coil, nucleotide-binding site, Leu-rich repeat (CC-NBS-LRR) resistance proteins (Wei et al., 2002; Shen et al., 2003; Halterman and Wise, 2004) that recognize, either directly or indirectly, corresponding fungal effector

(AVR) proteins (Ridout et al., 2006). Programmed cell death mediated by MLA proteins occurs after fungal penetration, when primordial haustoria are presumed to secrete AVR_a proteins. After recognition, MLA is translocated into the nucleus and binds the WRKY transcription factors HvWRKY1 and HvWRKY2, which instigate a signal cascade leading to the hypersensitive response (Shen et al., 2007). This MLA-AVR_a race-specific mechanism of resistance contrasts with the nonspecific penetration resistance mediated by loss-of-function mutations in the seven-transmembrane-protein MLO (Büsches et al., 1997). *mlo*-mediated resistance during papillae formation is extremely effective, although in the rare case when penetration does occur, *Bgh* colonization of the leaf tissue proceeds normally (Jørgensen, 1994). Similarly, overexpression of the barley homolog of BI-1 in epidermal cells generates supersusceptibility (Hüeckelhoven et al., 2003). BI-1 was found to negatively regulate the penetration resistance mediated by *mlo* and almost restored the penetration efficiency (PE) of *Bgh* to wild-type levels (Hüeckelhoven et al., 2003). Thus, negative regulators play a direct role in modulating the defense response of barley to *Bgh*.

We have characterized a novel family of small peptides, designated blufensins, which are induced during *Bgh* infection and resemble Cys-rich peptides. We show that one of these, BLUFENSIN1 (BLN1), negatively impacts plant defense during *Bgh* infection. BLN1 is predicted to be secreted and contains both structural and sequence similarities to the family of knottins. Our results establish a previously unrecognized role for small peptides as negative regulators of plant defense.

RESULTS

Identification of *Bln1* from Barley1 GeneChip Expression Profiles

Bln1 was initially identified from a time-course microarray experiment designed to discover genes that had differential patterns of expression associated with either incompatibility or compatibility in barley-powdery mildew interactions (Caldo et al., 2004). Using the MIXED procedure in SAS, a contrast statement was developed to test the expression levels in incompatible pairings specified by *Mla6-AVR_{a6}* and *Mla13-AVR_{a13}* compared with compatible interactions determined by *Mla6-avr_{a6}* and *Mla13-avr_{a13}*. *Bln1* (represented by Barley1 GeneChip probe set Contig12219_at) was one of 22 genes found to be differentially expressed at a threshold *P* value of <0.0001 and a false discovery rate of <7% (Fig. 1, experiment BB4; Caldo et al., 2004). To further characterize *Bln1* response in the scope of the barley transcriptome, we extended the analysis of Caldo et al. (2004) to another large expression-profiling experiment involving nearly isogenic lines C.I. 16151 (*Mla6*) and C.I. 16137 (*Mla1*) versus their respective loss-of-function mutants, *m1a6-m9472*

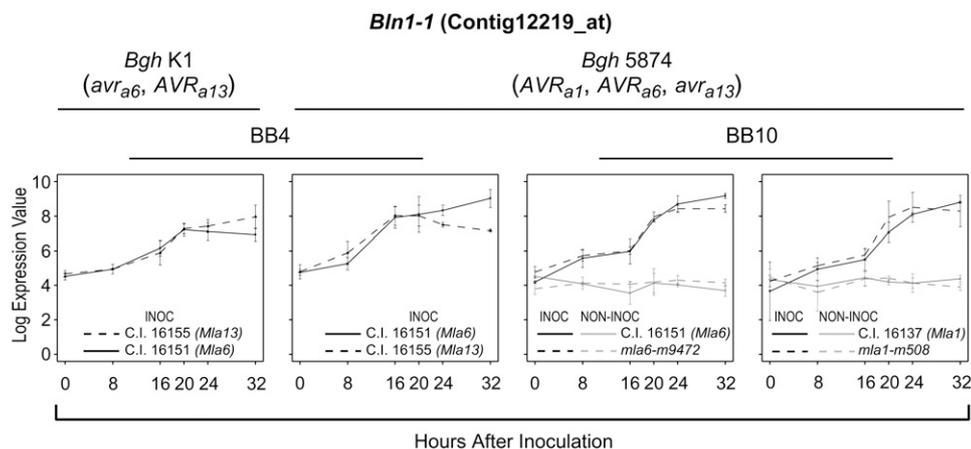


Figure 1. Time-course expression profiles of *Bln1-1* (Contig12219_at) in barley-*Bgh* interactions. The left two panels display data from BB4, an experiment described by Caldo et al. (2004), in which nearly isogenic barley lines harboring the contrasting *Mla* alleles, *Mla6* and *Mla13*, were challenged in pairwise combinations with the alternately virulent and avirulent *Bgh* isolates 5874 (AVR_{a1} , AVR_{a6} , avr_{a13}) and K1 (AVR_{a1} , avr_{a6} , AVR_{a13}). A mixed linear model analysis (Wolfinger et al., 2001) using the SAS mixed procedure was conducted to identify genes whose average pattern of expression in one host-pathogen interaction category (e.g. compatibility) differed significantly from its average pattern of expression in its contrasting category (e.g. incompatibility). Time-specific differences between the average expressions (0, 8, 16, 20, 24, and 32 hai) were tested for equality using an *F* statistic (Caldo et al., 2004). The BB10 experiment (shown in the right two panels) compared wild-type (*Mla*) plants and derived loss-of-function deletion mutants inoculated with *Bgh* isolate 5874 (AVR_{a1} , AVR_{a6}). Identical noninoculated plants were included for each treatment. Normalized average signal intensities and SE values were calculated based on three independent replications for both experiments. Derivations of SE values are shown for illustration, as each contrast uses pooled variances when testing for significant differences between incompatible versus compatible interactions.

and *m1a1-m508* (Fig. 1, experiment BB10). In addition to challenge with avirulent *Bgh* isolate 5874 (AVR_{a1} , AVR_{a6}), the BB10 experimental design included non-inoculated samples, which allowed us to observe, conclusively, significant *Bln1* induction over the 0- to 32-h time course in both incompatible and compatible pairings. The association with *Bgh* invasion (Caldo et al., 2004, 2006), in addition to strong induction by *Fusarium graminearum* (the causal agent of *Fusarium* head blight; Boddu et al., 2006, 2007), provided indirect evidence that *Bln1* plays a role in mediating defense responses to fungal pathogens.

Bioinformatic Classification of the Blufensin Family of Small Peptides

A BLASTn search using *Bln1* among the assembled ESTs used to create the Barley1 GeneChip (Harvest: Barley assembly 21; <http://138.23.191.142/hweb/>; Altschul et al., 1990; Close et al., 2004) identified the family member *Bln2*, represented by Barley1 probe set Contig26496_at. Strong induction of *Bln2* was observed after *Bgh* inoculation, but without a differential time-course expression pattern associated with incompatibility or compatibility, as was seen with *Bln1*. *Bln2* was induced, however, upon challenge with *Puccinia graminis* f. sp. *tritici*, causal agent of stem rust (Zhang et al., 2008), whereas *Bln1* was not. Conversely, it is possible that the observed noninduction of *Bln1* in response to *P. graminis* f. sp. *tritici* was due to

poor hybridization by allele-specific probes in Contig12219_at (see "Characterization of *Bln1* Transcripts" below).

Proteins encoded by both genes were then examined using the suite of motif recognition software orchestrated via InterProScan (Quevillon et al., 2005). Two matches were found: a localization signal peptide and a transmembrane domain, both positioned in the N-terminal region of the predicted protein. TargetP 1.1 and WoLF PSORT II were used for signal peptide prediction, with both predicting secretion and a cleavage site between amino acid residues 29 and 30 (Nielsen et al., 1997; Horton et al., 2006; Emanuelsson et al., 2007). The best match to the signal peptide was Pa-AMP-1 (for Antimicrobial Protein-1) from *Phytolacca americana* (common pokeberry), a member of the knottin family of antimicrobial peptides (Liu et al., 2000).

Next, we compared BLN1 and BLN2 to identify shared motifs or domains that may point to a known protein family. The use of InterProScan, BLAST, and the PANTHER database of motifs on all existing sequence information provided no information on the C-terminal regions of these two family members (Thomas et al., 2003; Mi et al., 2005). There were, however, several shared features between BLN1 and BLN2, including extensive amino acid conservation in the predicted signal peptide and cleaved peptide, the presence of only two Cys residues, and a single intron in a conserved position between the Cys residues. The

last two features are hallmarks of small Cys-rich antimicrobial peptides, which generally have an even number of Cys residues (for the formation of disulfide bonds) and a conserved intron approximately 150 nucleotides in length positioned near the signal peptide/cleaved peptide border (Graham et al., 2008). Antimicrobial peptides are conserved across all living organisms; therefore, a literature search was performed on all small peptides found in vertebrates and invertebrates, whether or not they have been shown to have antimicrobial activity. We found that small peptides with only two Cys residues are uncommon; in fact, only six have been identified to date, from cow, insects, and frogs (Zasloff, 2002). Among these, the number of amino acids between the Cys residues range from five to seven, compared with eight and nine found in BLN1 and BLN2, respectively. The conservation of Cys positions and signal peptide among BLN1, BLN2, and Pa-AMP-1 suggests a possible evolutionary connection between the *blufensin* and *knottin* gene families (Fig. 2A).

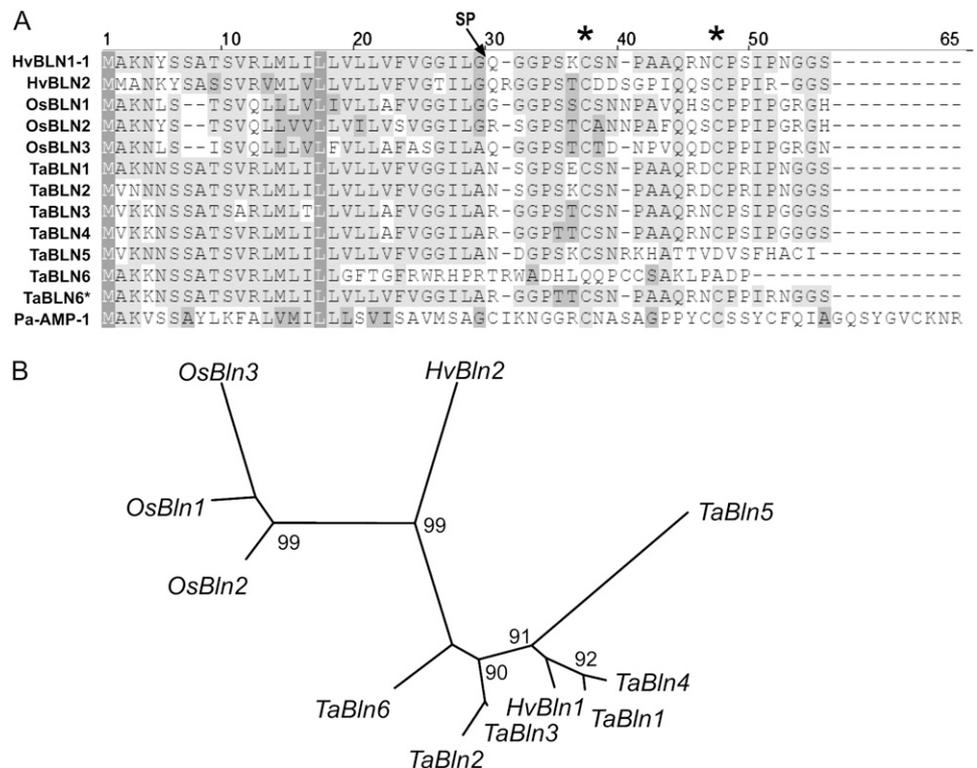
We next compared the blufensins with homologs in closely related species to determine the degree of residue conservation in this small peptide family. A tBLASTn search using BLN1 revealed three and six family members in rice (*Oryza sativa*) and wheat (*Triticum aestivum*), respectively (Fig. 2; Supplemental Table S1). No significant sequence similarity was found in available genomic sequences of species outside of the BEP clade of the Poaceae (grass) family, namely maize (*Zea mays*) and sorghum (*Sorghum bi-*

color). Moreover, within the BEP clade, no significant similarity was found in the 4× *Brachypodium* sequence (as available on October 1, 2008), suggesting that preservation of blufensins within this clade may be incomplete. Multiple sequence alignment revealed high similarity in the signal peptide region and conservation of specific residues in the cleaved peptide region (Fig. 2). The identification of two genes in diploid barley (*Triticeae* H genome) correlates with the six found in hexaploid wheat (*Triticeae* A, B, and D genomes), based on available EST data. As illustrated in Figure 2B, phylogenetic analysis of this family grouped the rice blufensins distinct from the wheat. Curiously, *HvBln1* grouped with its homologs in wheat, whereas *HvBln2* occupied a branch distinct from both rice and wheat. Several of the wheat blufensins are clustered together with barley blufensins, indicating significant sequence conservation.

Characterization of *Bln1* Transcripts

We also investigated *Bln1* expression profiles in a third microarray data set (BB2) involving cv Sultan-5 (*Mla12*) as well as *m1a12* and *rar1* loss-of function mutants derived from the Sultan-5 genotype (Torp and Jørgensen, 1986; Freialdenhoven et al., 1994; Caldo et al., 2006; retrieved from BarleyBase/PLEXdb; <http://www.plexdb.org/>). Rather surprisingly, induced transcript accumulation was not observed in Sultan-5 or its mutant derivatives. We suspected that this was due to the divergence of *Bln1* sequences in

Figure 2. Multiple sequence alignment and phylogeny of the blufensin family. A, Alignment of protein sequences of blufensin family members in barley, rice, and wheat. *TaBln6* has a single nucleotide insertion that generates a frameshift. *TaBln6** represents the sequence that would result if the insertion were not present. SP designates the putative position of the signal peptide cleavage site, and asterisks at top designate the positions of the Cys residues. B, Unrooted phylogeny based on an alignment of the blufensin family coding sequences with 1,000 bootstraps. Support over 90% is shown at branch points in the phylogeny. The DNA alignment was used due to the poor bootstrap support generated from using the short protein sequence.



Sultan-5, which would interfere with efficient hybridization to Barley1 GeneChip probe sets. Therefore, additional *Bln1*-homologous cDNA and genomic clones were isolated from C.I. 16137 (*Mla1*), C.I. 16151 (*Mla6*), C.I. 16155 (*Mla13*), Sultan-5 (*Mla12*), and Golden Promise by reverse transcription (RT)-PCR and inverse PCR, respectively. Genomic DNA sequence analysis revealed that there are two copies of *Bln1* in C.I. 16137, C.I. 16151, and C.I. 16155, all of which are nearly isogenic derivatives of cv Manchuria (Moseman, 1972). These two copies were designated *Bln1-1* and *Bln1-2*. As illustrated in Figure 3A, *Bln1-1* is highly similar to *Bln1-2*, except that *Bln1-2* has three single nucleotide polymorphisms within the open reading frame (ORF), which generate three nonsynonymous changes in the C-terminal end of the predicted protein. A single-copy chimera of *Bln1-1* and *Bln1-2* is contained within Sultan-5 and Golden Promise, which was designated *Bln1-3* (Fig. 3A). Verification of copy number in each line was confirmed by Southern-blot analysis (data not shown). A conserved 124-nucleotide GT-AG-type intron was identified, with only two single nucleotide polymorphisms within the intron between *Bln1-1* versus *Bln1-2* and *Bln1-3* (Fig. 3B). The 3' untranslated regions (UTRs) of *Bln1-2* and *Bln1-3* were identical but highly dissimilar to *Bln1-1* (data not shown). The coding region, intron, and 3' UTR of *Bln1-3* were identical to those of *Bln1-2*, yet the promoter of *Bln1-3* was the same as that of *Bln1-1* (Fig. 3B; data not shown).

As the Barley1 GeneChip could not measure transcript accumulation of *Bln1-2* and *Bln1-3*, we designed primers (Supplemental Table S2) based on the newly discovered sequence polymorphisms to perform copy-specific RT-PCR of all three putative alleles or paralogs in response to *Bgh*. As illustrated in Figure 3C, *Bln1-3*-specific transcripts were amplified from RNA isolated from *Bgh*-inoculated leaves in all five cultivars, but no amplification product was detected from RNA isolated from noninoculated plants. However, when using primers specific for *Bln1-2*, no PCR product was detected from RNA isolated from either inoculated or noninoculated tissues. All sequenced ESTs in GenBank are identical to *Bln1-1* (Supplemental Table S1); therefore, our working hypothesis is that *Bln1-1/3* harbors a functional promoter, while *Bln1-2* may have a nonfunctional promoter or one not associated with leaf or *Bgh*-induced expression.

Promoter Analysis of *Bln1-1*

The 5' upstream regions of *Bln1-1*, *Bln1-2*, and *Bln1-3* were isolated using inverse PCR from genomic DNA of cv C.I. 16151 and cv Golden Promise. As shown in Figure 3B and Table I, several common motifs associated with defense (W-box, WRKY, MYB, P-box), and xylem- and root-specific expression were identified in the upstream region of *Bln1-1*. Of those associated with defense, three WRKY transcription factor-binding sites or W-boxes (TTTGACY) were found, at -602, -526,

and -391 bases from the TATA box (Rushton et al., 1996; Eulgem et al., 2000). Additionally, three MYB-binding sites (MACCWAMC) were found, at -92 and -45 nucleotides from the TATA box and within the 3' UTR of *Bln1-1* (Sablowski et al., 1994; Tamagnone et al., 1998). The xylem-specific expression elements ACII (CCACCAACCCCC) and XYLAT (ACAAAGAA) are located at -91 and -245 bases from the TATA box, respectively. ACII is an extended MYB-binding site motif with additional specific nucleotides that generate xylem-specific expression (Patzlaff et al., 2003; Gomez-Maldonado et al., 2004). Lastly, nine root-specific expression elements (ATATT) were found within -600 to -500 bases from the TATA box. The presence of these elements is consistent with EST evidence of expression in root tissue (Supplemental Table S1). All described motifs were highly oversampled with respect to prevalence in the rice genome (Table I). Specifically, the number of observed ACII and W-box motifs was only greater in less than 0.01% and 1.6% of all rice genes, respectively (quantile estimate based on rice V5).

Functional Analysis of *Bln1* via Barley Stripe Mosaic Virus-Induced Gene Silencing

A New DNA Bombardment-Based Silencing System for the Triticeae

In recent years, virus-induced gene silencing (VIGS) has emerged as a powerful reverse genetics tool for the functional analysis of gene candidates in both model and crop plant species. In monocots, *Brome mosaic virus* has been utilized for functional genomics studies in rice and maize (Ding et al., 2006), whereas *Barley stripe mosaic virus* (BSMV) has been used for barley and wheat (Holzberg et al., 2002; Lacomme et al., 2003; Hein et al., 2005; Scofield et al., 2005). In these previous studies, BSMV vectors were under the control of the T7 promoter, which requires *in vitro* transcription to make infectious RNA transcripts for plant inoculation. We developed a modified BSMV-VIGS system using particle bombardment of DNA into barley seedlings, which eliminates the *in vitro* transcription step and is more amenable to high-throughput studies. As illustrated in Figure 4A, the new BSMV-VIGS DNA vector set consists of independent BSMV: α , BSMV: β , and BSMV: γ clones under the control of the cauliflower mosaic virus 35S promoter. Silencing of *Phytoene desaturase* (*PDS*; Holzberg et al., 2002) was used to quantify the efficacy of silencing with this approach, resulting in approximately 80% of the newly inoculated plants exhibiting a photobleaching phenotype.

*Silencing of *Bln1* Enhances Plant Resistance in Compatible Interactions*

To examine the role of *Bln1* in the barley defense response to *Bgh*, we used the bombardment-based BSMV-VIGS approach to down-regulate *Bln1* gene expression. Figure 4A illustrates two *Bln1* cDNA frag-

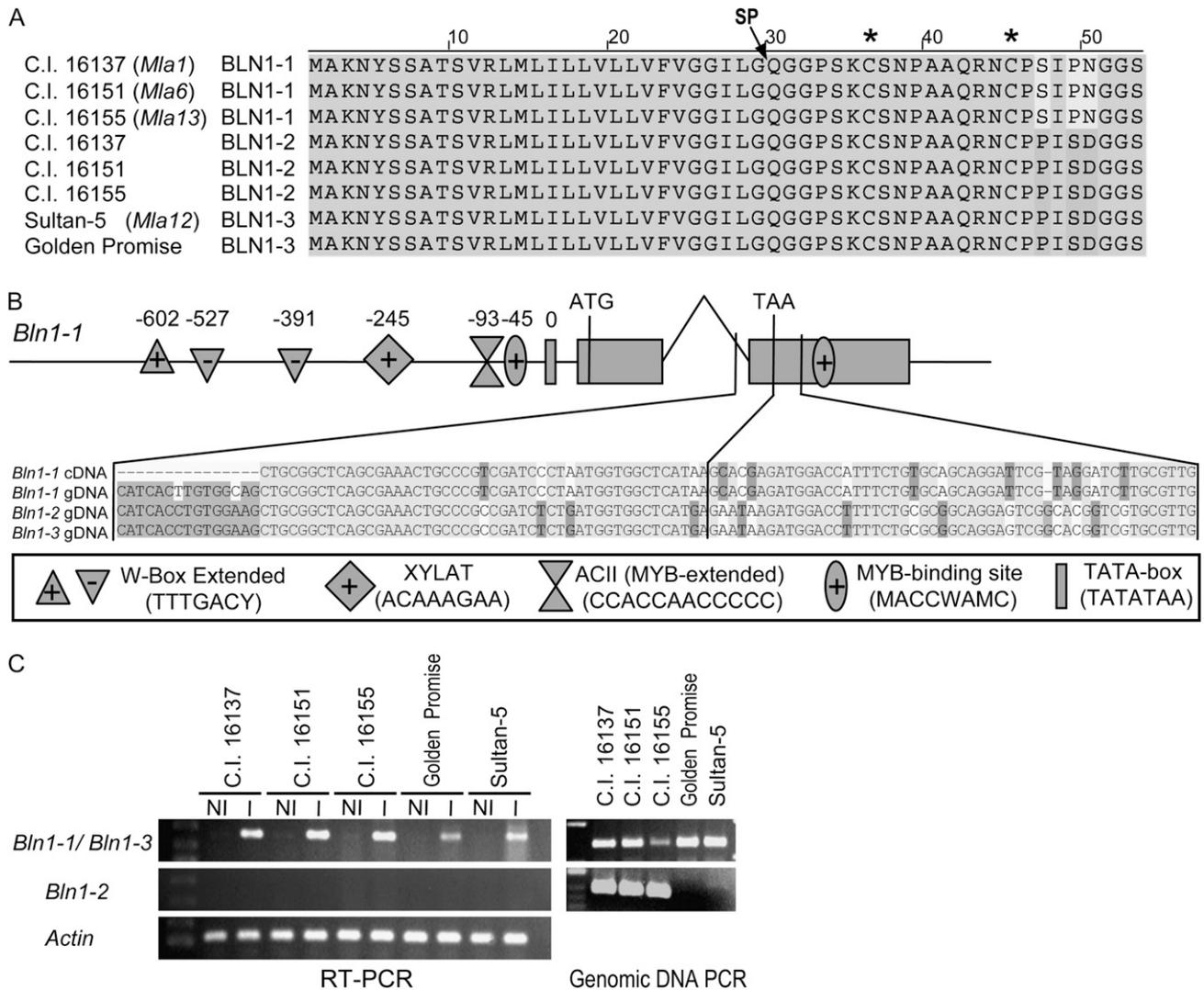


Figure 3. Comparison of *Bln1* sequences and transcript accumulation. A, Amino acid alignment of *Bln1-1*, *Bln1-2*, and *Bln1-3* alleles/paralogs. Gray shaded boxes indicate identity across all eight sequences. SP designates the putative position of the signal peptide cleavage site, and asterisks at top designate the positions of the Cys residues. B, *Bln1-1* gene model with promoter analysis of a genomic DNA fragment cloned via inverse PCR. Gray boxes indicate exons, with a single intron between exon 1 and 2. Symbols shown in the box at bottom indicate the positions of several promoter elements associated with defense (W-box, WRKY, MYB, P-box) and xylem- and root-specific expression. Alignment of genomic DNA of *Bln1-1* and *Bln1-2* and cDNA of *Bln1-1* shows the extensive nucleotide divergence between these paralogs, beginning near the end of the intron. C, Differential transcript accumulation of *Bln1* paralogs upon inoculation with *Bgh* isolate 5874. RT-PCR was performed on RNA isolated from seedling leaves 24 h after *Bgh* inoculation (I) or from noninoculated controls (NI). *Actin* was used as the internal control in all samples. The genomic DNA PCR results shown demonstrate the existence of different paralogs in different genotypes.

ments of different lengths inserted downstream of the stop codon of γb , designated BSMV:BlN1₂₄₈ and BSMV:BlN1₁₆₂. Wild-type γ -BSMV:00 was used as a negative control. After a survey of BSMV-bombarded cultivars, Clansman (*Mla13*) and C.I. 16151 (*Mla6*) were chosen for VIGS assays, since silencing of PDS in these genotypes resulted in fewer virus infection symptoms but significant photobleaching. Plants were inoculated with *Bgh* 5874 (*avr_{a13r}*, *AVR_{a6}*) 12 d after BSMV treatment, and third leaves were scored for *Bgh* infection type 7 d later.

Three independent experiments with Clansman infected with *Bgh* 5874 demonstrated that silencing *Bln1* visibly enhanced resistance. Microscopic inspection was carried out to determine PE, as calculated by the percentage of total conidiospores that produced haustoria and secondary hyphae. As shown in Figure 4B, BSMV:BlN1₂₄₈⁻ and BSMV:BlN1₁₆₂⁻ inoculated plants were significantly less susceptible at 7 d after inoculation (dai) than the inoculated BSMV:00 and non-BSMV-inoculated control plants (mock), resulting in a PE of 21% for BSMV:00, compared with 11% in BSMV:

Table 1. Analysis of sequence motifs in the *Bln1-1* promoter compared with rice

Sequence Motif	No. of Motifs		
	Statistically Expected ^a	Observed in Rice (Mean) ^b	Observed in <i>Bln1-1</i> Promoter
ACII (CCACCAACCCCC)	0.00011	0.00067	1 (99.9%) ^c
MYB (MACCWAMC)	0.22	0.35	2 (93.5%)
Root specific (ATATT)	1.78	4.01	9 (89.5%)
W-box (TTTGACY)	0.22	0.35	3 (98.4%)
XYLAT (ACAAAGAA)	0.028	0.068	1 (93.5%)

^aExpected number of motifs based on length of the *Bln1-1* promoter. ^bObserved number of motifs in the rice genome per gene. ^cPercentage of promoters in the rice genome that have a lower number of motifs compared with *Bln1-1*.

*Bln1*₂₄₈-silenced plants and 12% in BSMV:*Bln1*₁₆₂-silenced plants, respectively. In incompatible interactions, C.I. 16151 plants were fully resistant in BSMV:*Bln1*₂₄₈- and BSMV:*Bln1*₁₆₂-infected plants, with no significant difference observed between inoculated BSMV:00 and mock control plants. When C.I. 16151 test plants were inspected microscopically, no *Bgh* secondary hyphae were detected up to 7 dai in either silenced or control plants. Thus, the significant reduction in susceptibility in compatible interactions suggests that *Bln1* could function as a negative regulator of barley defense response to *Bgh* infection.

Semiquantitative RT-PCR of *Bln1* and *Bln2* mRNA from VIGS-Treated Plants

Transcript accumulation of *Bln1-1* was assayed to monitor the level of *Bln1* gene silencing. The third leaves of BSMV-treated plants were used for RT-PCR assays 24 h after *Bgh* inoculation. Barley *Actin* mRNA was used as an internal quantitative control for all samples (Halterman et al., 2003). Using *Bln1-1*-specific primers (Supplemental Table S2), semiquantitative RT-PCR revealed the reduction of *Bln1-1* transcripts in both BSMV:*Bln1*₂₄₈- and BSMV:*Bln1*₁₆₂-infected leaves compared with inoculated BSMV:00 and mock-inoculated plants (Fig. 4, B and C). There were no detectable amplicons at 20 cycles in *Bln1-1*-silenced plants. However, amplicons could be observed when using 25 cycles or more, indicating that silencing efficiency is not 100%. This is consistent with the observed heterogeneous silencing pattern observed in BSMV:PDS plants (Scofield et al., 2005). To check for off-target silencing of associated blufensin family members, *Bln2*-specific primers (Supplemental Table S2) were used for semiquantitative RT-PCR on the same RNA samples. *Bln2* mRNA levels were equivalent in BSMV:*Bln1*₂₄₈-, BSMV:*Bln1*₁₆₂-, BSMV:00-, and mock-inoculated control plants, indicating a low probability of cross-silencing with *Bln1-1* (Fig. 4, B and C). These results imply that the reduced susceptibility to *Bgh* in BSMV-VIGS-treated plants is due to the suppression of *Bln1* and not to silencing of its family member, *Bln2*.

Bln1-1 Is Highly Inducible in All Barley Genotypes Tested, But Silencing Consequences Differ

To further understand the role of *Bln1* in barley defense response to *Bgh*, we silenced *Bln1* in 12 additional barley genotypes and recorded the resulting infection types in compatible interactions with *Bgh* 5874 (Table II). Barley cv Black Hull-less seedlings were bombarded with BSMV:*Bln1*₂₄₈-, BSMV:*Bln1*₁₆₂-, and BSMV:00 constructs. Seven days after bombardment, BSMV-infected leaves that showed a visible stripe mosaic phenotype were utilized to recover recombinant virions, which, in turn, were uniformly applied to test plants by mechanical inoculation. After 12 d of silencing, plants were inoculated with the *Bgh* 5874 isolate. Seven days after *Bgh* inoculation, six genotypes, including Morex (*m1a*; Fig. 4C), exhibited a significant reduction in susceptibility in BSMV:*Bln1*₂₄₈- and BSMV:*Bln1*₁₆₂-transformed plants compared with BSMV:00 and mock plants, whereas the other seven were not significantly different from the BSMV:00 control (Table II). Moreover, silencing generated significantly reduced susceptibility in *Bln1*-silenced Ingrid (*M1o*) plants at 7 dai with *Bgh* (Table II), while silencing in *m1o-5* BC₇ Ingrid had no effect. When plants were inspected microscopically, we observed a reduction in PE from 38% in BSMV:00 plants to 22% in BSMV:*Bln1*₂₄₈-silenced and 28% in BSMV:*Bln1*₁₆₂-silenced *M1o* plants. Therefore, silencing of *Bln1* generates reduced susceptibility in the presence of wild-type *M1o*.

The above results contrast with the level of *Bln1-1* transcript accumulation at 24 h after inoculation (hai) in all 13 genotypes, as demonstrated by RT-PCR analysis (Fig. 5). It is possible that the ability of the host plant to tolerate virus accumulation interfered with the efficiency of BSMV-induced gene silencing, since the seven genotypes with no significant reduction in susceptibility to *Bgh* exhibited significant necrosis along the mid and lateral veins in the upper half of the second leaf, a strong BSMV infection symptom. Specific cultivars must be utilized that provide a suitable genetic background to tolerate the substantial levels of BSMV accumulation that are required to elicit a significant VIGS response (Hein et al., 2005). Thus,

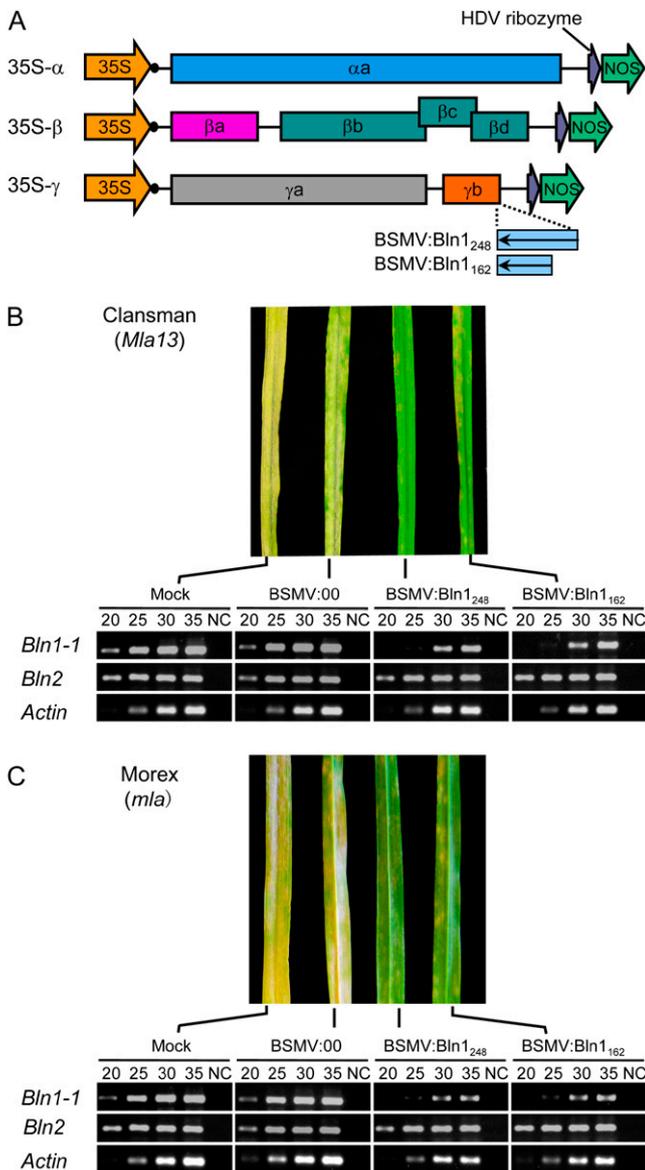


Figure 4. BSMV-VIGS of *Bln1* in Clansman (*Mla13*) and Morex (*mla*). **A**, Schematic representing the DNA-based BSMV-VIGS constructs. The three subgenomes of BSMV are under the control of the cauliflower mosaic virus 35S promoter. Resulting transcripts are cleaved at the 3' terminus by the HDV ribozyme. **B**, Clansman (*Mla13*) plants were subject to four treatments: mock (carborundum phosphate buffer), empty vector (BSMV:00), and test constructs (BSMV:Bln1₂₄₈ and BSMV:Bln1₁₆₂). Plants were inoculated with *Bgh* at 12 d after treatment and photographed at 7 dai. Semiquantitative RT-PCR was performed with *Bln1-1*- and *Bln2*-specific primers to detect mRNA degradation of targeted transcripts. The lanes designated 20, 25, 30, and 35 indicate the number of amplification cycles performed for each sample. For each sample, lane NC shows the results of 35 cycles of PCR without RT, as a negative control. *Actin* transcripts served as a quantitative control for each sample. **C**, BSMV-VIGS of *Bln1* in Morex. Protocols were as described for **B**.

although *Bln1-1* is highly expressed in all genotypes upon inoculation of *Bgh*, there were diverse phenotypic effects of attempted *Bln1* silencing in different genotypes.

Overexpression of *Bln1* Results in Hypersusceptibility to *Bgh*

In light of the enhanced resistance to *Bgh* in compatible interactions due to *Bln1* silencing, we hypothesized that overexpression of *Bln1* should render comparable epidermal cells supersusceptible. To test this, we utilized single-cell-transient overexpression of *Bln1* in barley epidermal cells (Shirasu et al., 1999). The full-length *Bln1-1* ORF was cloned into the vector pUbi:Nos to create the expression construct pUbi:Bln1. The pUbi:Bln1 plasmid was then cobombarded with the pUGN GUS expression vector (Nielsen et al., 1999) into Clansman (*Mla13*) epidermal cells and subsequently challenged with the virulent *Bgh* isolate 5874 (*avr_{a13}*). Control bombardments were performed with the pUGN reporter construct alone. Fungal PE was calculated as the ratio of GUS-marked cells exhibiting elongating secondary hyphae to the total transformed cells attacked by *Bgh*.

As shown in Table III, generalized linear mixed-model analyses for three independent experiments revealed that the formation of elongating secondary hyphae in compatible interactions (an indicator of PE) was significantly more likely for constructs pUGN + pUbi:Bln1 than for construct pUGN alone ($P = 0.0028$). Overexpression of *Bln1* in C.I. 16151 (*Mla6*) cells did not compromise resistance in incompatible interactions with *Bgh* isolate 5874 (*AVR_{a6}*). Combined with the BSMV-VIGS experiments above, results from the overexpression experiments provide additional support for the hypothesis that *Bln1* negatively regulates basal defense but does not compromise effector-triggered *Mla6*-mediated race-specific resistance.

DISCUSSION

BLN1 Plays a Key Role for Powdery Mildew Susceptibility in Barley

We have shown here that BLN1, a small peptide of the novel blufensin gene family, negatively impacts the defense response to barley powdery mildew. Based on the expression profiling results of Caldo and colleagues (2004), *Bln1* was one of several genes that exhibited an equivalent pattern of transcript accumulation in both incompatible and compatible interactions during germination of *Bgh* conidiospores and formation of appressoria (Fig. 1). However, during establishment of the periahaustorial interface between penetrating *Bgh* and host epidermal cells, divergent expression of these transcripts occurred, in which compatible interactions led to lower accumulation of transcripts compared with paired incompatible interactions.

In gene-for-gene-mediated incompatible interactions, the increase in *Bln1* transcript accumulation could be interpreted to imply that *Bln1* transcript accumulation is intimately associated with *Bgh* defense. However, lower *Bln1* transcript accumulation in

Table II. *Bgh* 5874 infection types after BSMV-VIGS of *Bln1* in 13 compatible barley-*Bgh* interactions

No.	Genotype ^a	Mock	BSMV:00	BSMV: <i>Bln1</i> ₂₄₈	BSMV: <i>Bln1</i> ₁₆₂
1	Clansman (<i>Mla13</i>)	3–4 ^b	3	2	2
2	Morex (<i>m1a</i>)	3–4	3–4	2	2
3	Ingrid (<i>Mlo</i>)	4	4	2	2
4	Harrington	3–4	3–4	1–2	1–2
5	Stephoe	3–4	3–4	2	2
6	HOR11358 (<i>Mla9</i>)	4	3–4	1–2	1–2
7	C.I. 16147 (<i>Mla7</i>)	3	3	3	3
8	C.I. 16149 (<i>Mla10</i>)	3	3	3	3
9	C.I. 16139 (<i>Mlg</i>)	4	4	4	4
10	C.I. 16141 (<i>Mlh</i>)	4	4	4	4
11	C.I. 16143 (<i>Mlk</i>)	3	3	3	3
12	C.I. 16145 (<i>Mlp</i>)	3	3	3	3
13	OWB rec	4	4	4	4

^aGenotypes in rows 1 to 6 exhibited significantly reduced susceptibility, while genotypes in rows 7 to 13 were not significantly different after silencing. Order of genotypes is identical to that in Figure 5. ^bPlants were inoculated with *Bgh* isolate 5874 (*AVR_{a1}*, *AVR_{a6}*). The rates of severity of *Bgh* infection are presented as 0 to 4, indicating levels of sporulation from completely resistant (0) to completely susceptible (4).

compatible interactions would suggest that its expression was influenced by *Bgh* invasion and that its reduction is correlated with increased susceptibility (Caldo et al., 2004). In fact, we observed the opposite. Decreased susceptibility in compatible interactions was observed via BSMV-VIGS-mediated suppression of *Bln1*, whereas susceptibility was enhanced after *Bln1* overexpression. *Bgh*-induced *Bln1* transcript accumulation was evident in all 18 barley genotypes tested, implicating a conserved mechanism of regulatory control. *Bln1* silencing enhanced plant resistance in compatible interactions, regardless of the presence or absence of *Mla* CC-NBS-LRR alleles, indicating that BLN1 can function in a *R*-gene-independent manner. Based on the phenotypic observations described above, we propose two hypotheses that model the function of BLN1: (1) BLN1 is a negative regulator of penetration defense, resulting in the attenuation of host defenses that retard fungal infection, similar to *mlo*-mediated resistance; and (2) BLN1 is a susceptibility factor that is required for promoting fungal establishment, penetration, and/or colonization. Indeed, these hypotheses are not mutually exclusive, because the difference could be considered semantic in that a negative regulator of defense could be considered one class of susceptibility factors.

Host Accessibility and Susceptibility Factors in Plant Defense

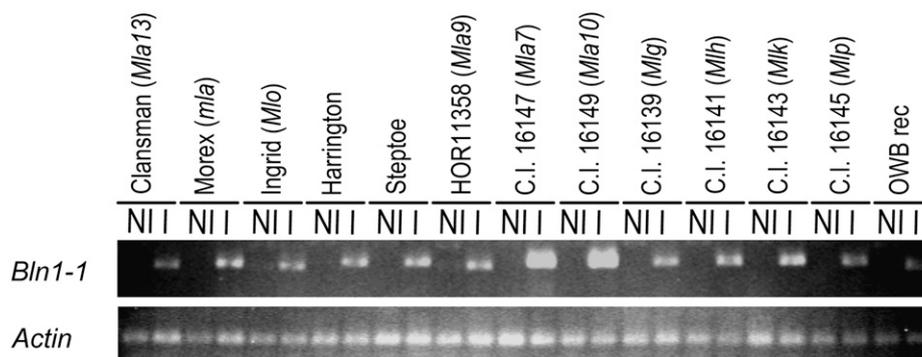
The possibility that BLN1 has been recruited by *Bgh* to take advantage of host factors normally utilized for basic metabolism and defense is not without reason. To acquire nutrients from host cells, obligate biotrophic fungi have evolved mechanisms to secrete effectors to suppress host defenses (Dodds et al., 2004; Catanzariti et al., 2006; Wang et al., 2007) and to induce host susceptibility genes (Schulze-Lefert and Panstruga,

2003; Hüchelhoven, 2005). In *Arabidopsis* (*Arabidopsis thaliana*) and barley, several host susceptibility factors have been identified for powdery mildew, but how pathogens utilize these host genes remains unclear (Schultheiss et al., 2002, 2003; Hüchelhoven et al., 2003; Hüchelhoven, 2005). The observation of proteins similar to the plant blufensin family within many Ascomycota species may indicate functional mimicry (Abramovitch et al., 2006). To determine if convergent coevolution of the blufensin family with host pathogens might exist, both BLAST and pattern matching using regular expression identified conserved family members (M. Moscou and R. Wise, unpublished data). Interestingly, matches were found in the genomic sequences of the two grass fungal pathogens, *Magnaporthe grisea* and *Blumeria graminis* f. sp. *hordei*, but no significant homology was found outside of the Ascomycota phylum. If these fungal proteins are expressed in hyphae and at the periahaustorial interface, they may suggest a role in plant susceptibility, the establishment of feeding structures, and/or biotrophic interactions between the plant and the pathogen (Dodds et al., 2004). An example of functional mimicry is provided by a root-knot nematode-secreted protein found to have high similarity to the peptide hormone CLAVATA3, which binds to CLAVATA1 to stimulate root formation (Huang et al., 2006). Alternatively, the possibility exists that *Bgh* induces a gene in barley that acts as a stimulant to fungal growth. This notion of the induction of host susceptibility factors and/or functional mimicry of plant and pathogen signaling peptides presents a coevolutionary model of selection for and against factors that mediate this biotrophic interaction.

Negative Regulators in Plant Defense

Our early understanding of disease defense came via studies involving *R* gene-mediated resistance, also

Figure 5. RT-PCR to detect *Bln1* transcript accumulation in 13 barley genotypes. RNA was isolated from seedling leaves at 24 h after *Bgh* inoculation (I) or from noninoculated controls (NI). *Actin* transcripts served as a quantitative control for each sample. Order of genotypes is identical to that in Table II.



known as effector-triggered immunity, in which a rapid and evolutionarily adapted response is generated after recognition of an invading pathogen. This is in contrast to pathogen-associated molecular patterns-triggered immunity or basal defense, which expresses a nonspecific and broader type of resistance response. Negative regulation of the basal defense pathway prevents unchecked potentiation of the response and deleterious effects on normal cell functions (Alexander and Hilton, 2004; Ge et al., 2007). As we demonstrated that *Bln1* did not require a functional effector-triggered resistance, we surmise that *Mla*-mediated postpenetration resistance is epistatic to the negative regulation of *Bln1*-mediated suppression.

MLO, as a negative regulator of penetration resistance, but not *Mla*-mediated hypersensitive response, is essential for compatibility to all known *Bgh* isolates (Büschges et al., 1997; Piffanelli et al., 2002). Hypothesized to be a host susceptibility factor, it is believed that MLO is recruited by *Bgh* to diminish the plant defense response (Büschges et al., 1997; Devoto et al., 1999). A small GTP-binding protein of the barley RAC family is associated with MLO-mediated suppression of *Bgh* defense (Schultheiss et al., 2002), and RACs can

regulate subcellular gradients of Ca^{2+} (Schultheiss et al., 2003). A domain that mediates a Ca^{2+} -dependent interaction with calmodulin has been identified in MLO, and loss of calmodulin binding inhibits the capacity for MLO to negatively regulate *Bgh* defense (Kim et al., 2002). Like *HvCaM3*, silencing of *Bln1* also enhanced resistance to *Bgh* in plants harboring wild-type *Mlo* but not in *mlo-5* mutants, suggesting that BLN1 functions in parallel with or upstream of MLO to modulate penetration resistance. Preliminary experiments using a BLN1-GFP fusion construct bombarded into barley epidermal cells indicated that BLN1 was undetectable in the nucleus and located mainly in the cytoplasm and the cell periphery. By contrast, the GFP control was mainly localized in the nucleus and the cytoplasm (Y. Meng and R. Wise, unpublished data). Computational analysis of the BLN1 signal peptide predicts that BLN1 is secreted into the apoplast, which is consistent with these early fusion assays. MLO is localized to the plasma membrane (Devoto et al., 1999). Indeed, if *Bln1* is secreted into the apoplast, it may act as a ligand to generate a signal transduction cascade, influencing *Bgh* accessibility.

Table III. Results of overexpression of *Bln1* in cultivars Clansman (compatible) and C.I. 16151 (incompatible) after inoculation with *Bgh* 5874

Cultivar (<i>R</i> Gene)	Construct	<i>Bgh</i> 5874 (<i>AVR_{36r}</i> , <i>avr₃₁₃</i>)			
		Total No. of GUS Cells with Conidiospore ^a	Total No. of GUS Cells with Elongating Secondary Hyphae ^a	PE ^b	<i>P</i> (Control Versus <i>Bln1</i>) ^c
Clansman (<i>Mla13</i>)	Ubi:GUS	281	84	29.9	
Clansman (<i>Mla13</i>)	Ubi:GUS + pUBI:BlN1	505	273	54.1	0.0028
C.I. 16151 (<i>Mla6</i>)	Ubi:GUS	263	0	0	
C.I. 16151 (<i>Mla6</i>)	Ubi:GUS + pUBI:BlN1	372	1	0.3	n.s.

^aRaw numbers indicate the combined results of three independent experiments. ^bPE represents GUS-stained cells with secondary hyphae among the total number of GUS-stained cells with spores attached. ^c*P* values were obtained using a generalized linear mixed model to test for significant differences in secondary hyphae formation by comparing the test constructs versus the empty vector negative control. n.s., Not significant.

Alternative Modes of Action of *Bln1* Function

Several functional models can account for the process by which *Bln1* mediates the balance between susceptibility and resistance. These are based on a specific tissue or compartment in which BLN1 functions. If BLN1 were localized to the cytoplasm, it may act as an oxidation sensor (Cumming et al., 2004). Normally, the reductive environment of the cytoplasm does not permit stable disulfide bonds. But with the formation of ROS, disulfide binding is known to alter the structures of proteins involved in several pathways, including the master regulator of defense, NPR1, which loses intermolecular disulfide bonds after being catalyzed by thioredoxins (Cumming et al., 2004; Tada et al., 2008). In this scenario, *Bln1* could activate a negative regulatory response due to the formation of a disulfide bond after exposure to ROS formed at any of several stages of the defense response.

High-Throughput DNA-Based BSMV-VIGS Promotes Functional Analysis of Genes Associated with Defense

Recently, a DNA-based *Bean pod mottle virus* (genus *Comovirus*) was developed as an efficient tool for a wide range of applications in soybean (*Glycine max*) functional genomics (Zhang et al., 2009). Accordingly, functional analysis of barley genes associated with resistance to *Bgh* was facilitated by the development of a similar, DNA-based BSMV-VIGS system. Relative to the commonly used RNA-based BSMV-VIGS, which uses mechanical inoculation of in vitro-generated transcripts (Hein et al., 2005; Scofield et al., 2005), the biolistic-based delivery system is easier to handle and cost efficient. Since the experimental substrate is DNA, as opposed to RNA, constructs are more stable and increase the probability of obtaining silenced plants. Using biolistic transfer of wild-type BSMV constructs to barley cv Black Hull-less, 80% to 100% of the plants normally display a BSMV infection phenotype at 7 d after bombardment.

BSMV-based VIGS constructs can be passaged through the barley host, thus inexpensively amplifying recombinant virions. Since we usually test the effect of silencing on multiple plants from one cultivar or multiple cultivars, utilization of this traditional "plant pathology" step makes this system more amenable to high-throughput applications. An intermediate mechanical infection step has also been adopted to infect *Arabidopsis* using the sap of *Nicotiana benthamiana* infected with *Tobacco rattle virus* (Lu et al., 2003) and to infect rice using sap from barley infected with *Brome mosaic virus* (Ding et al., 2006). In our hands, recombinant virions from one infected Black Hull-less plant could be used to test approximately 30 additional plants of different cultivars. The one drawback is that the additional 7 d required for the secondary BSMV infection can result in instability of the recombinant inserts during viral replication (Bruun-Rasmussen

et al., 2007). To verify the stability of our constructs, RT-PCR was conducted on RNA isolated from BSMV-VIGS-treated leaf tissue at 24 hai with *Bgh*. About 50% of BSMV:*Bln1*₂₄₈- and BSMV:*Bln1*₁₆₂-derived transcripts contained the *Bln1* inserts (data not shown). Even so, these plants displayed significant reduction in susceptibility (Fig. 4).

The BSMV vector was useful in many different cultivars. Since BSMV has a broad host range among the grasses (e.g. oat [*Avena sativa*], maize, and wheat), we anticipate that this system could be used as a powerful tool for functional studies in a wide range of economically important plant species. In this study, this effective reverse genomics tool was used to characterize a novel Blufensin family member, *Bln1*, which negatively impacts barley basal defense response to *Bgh*. Other *Bln* family members in barley, rice, and wheat may also have associated functions in crop defense response to biotrophs. Functional identification of this novel gene family may shed light on mechanisms that are required for the regulation of grass disease resistance.

CONCLUSION

The development of new technology that translates primary research in model systems to agronomic traits of interest in crop species is now feasible. The high-throughput silencing assay permitted our investigation of the negative regulatory role of BLN1 during disease defense, implicating another protein in addition to MLO, BI-1, and RACB. As these and new regulators are identified, our understanding of the delicate balance between resistance and susceptibility will broaden to a spectrum of quantitative regulatory network responses.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Fungal Isolates

For functional analysis, seedlings of barley (*Hordeum vulgare*) lines C.I. 16151 (*Mla6*), C.I. 16137 (*Mla1*), C.I. 16155 (*Mla13*), Clansman (*Mla13*), Sultan-5 (*Mla12*), Golden Promise, C.I. 16147 (*Mla7*), C.I. 16149 (*Mla10*), HOR11358 (*Mla9*), C.I. 16143 (*Mlk*), C.I. 15229 (Steptoe), Ingrid (*Mlo*), Harrington, C.I. 16139 (*Mlg*), OWB rec, C.I. 16145 (*Mlp*), C.I. 16141 (*Mlh*), *mlo-5 BC7* Ingrid, and C.I. 15773 (Morex) were grown in a temperature-controlled greenhouse with supplemental lighting. Following BSMV-VIGS bombardment/mechanical inoculation, plants were transferred to a temperature-controlled growth chamber with a 16-h photoperiod with light intensity ranging from 400 to 1,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$, a daytime temperature of 24°C, and a dark temperature of 20°C. Subsequent to *Blumeria graminis* f. sp. *hordei* (*Bgh*) inoculation, plants were kept in the *Bgh* chamber. *Bgh* isolates 5874 (*AVR_{a1}*, *AVR_{a6}*, *avr_{a7}*, *avr_{a9}*, *avr_{a10}*, *AVR_{a12}*, *avr_{a13}*, *avr_{gt}*, *avr_{kr}*, *avr_{kr}*, *avr_p*), K1 (*AVR_{a1}*, *avr_{a6}*, *AVR_{a13}*), and CC148 (*AVR_{a1}*, *avr_{a6}*, *AVR_{a13}*) were propagated on Manchuria barley (C.I. 2330) in separate controlled-growth chambers at 18°C (16 h of light/8 h of darkness).

Isolation of Fast-Neutron-Derived, *mLa6* Loss-of-Function Mutants

The C.I. 16151 line was obtained by introgression of the *Mla6* gene into the universal susceptible cv Manchuria (Moseman, 1972). Seeds of C.I. 16151 were

treated with fast neutrons at 4 Gy Nf at the International Atomic Energy Agency. M1 seeds were space planted at the U.S. Department of Agriculture-Agricultural Research Service Small Grains Laboratory in Aberdeen, Idaho. Single spikes from each individual M1 plant were harvested to represent the M2 family, which was screened for mutant segregants by sowing intact spikes consisting of 25 to 40 seeds in potting soil following the method of Wise and Ellingboe (1985). Each of 40 M2 families as well as the susceptible control (cv Manchuria, C.I. 2330) were sown per flat. When the first leaves were completely unfolded (approximately 10 cm high), plants were inoculated with *Bgh* isolate 5874 (*AVR_{ab}*), and families were scored for infection type 7 d after inoculation. Seedlings that produced cell death symptoms or sporulating *Bgh* colonies were selected for rescue. Putative mutants deemed as homozygous by a 1:3 mutant:wild-type segregation ratio were advanced to the M3 generation and then retested with *Bgh* 5874. Selected mutants that displayed sporulating *Bgh* colonies were crossed pairwise among each other as well as to *m1a1-m508*, *m1a1-m600* (Zhou et al., 2001), and *rar1-1* (Torp and Jørgensen, 1986; Freialdenhoven et al., 1994; Jørgensen, 1996). *m1a6-m9472* was confirmed by genetic complementation, Southern-blot (Halterman et al., 2001), and Barley1 GeneChip (Caldo et al., 2004) analyses.

Expression Profiling and Analysis

The Barley1 GeneChip probe array (part no. 900515) is distributed by Affymetrix. The array includes 22,792 probe sets derived from 350,000 ESTs clustered from 84 cDNA libraries, in addition to 1,145 barley gene sequences from the National Center for Biotechnology Information (NCBI) nonredundant database (Close et al., 2004). Total RNA was isolated using a hot (60°C) phenol/guanidine thiocyanate method as described by Caldo et al. (2004). Probe synthesis and labeling were performed at the Iowa State University GeneChip Core facility (<http://www.biotech.iastate.edu/facilities/genechip/Genechip.htm>). All detailed protocols can be accessed online within the BarleyBase/PLEXdb parallel expression database for plants and plant pathogens (<http://barleybase.org/>; <http://plexdb.org/>; Shen et al., 2005; Wise et al., 2007a).

Plants harboring *Mla6* (*Rar1* dependent) and *Mla1* (*Rar1* independent) both exhibit rapid and absolute resistance responses when challenged by *Bgh* isolates that carry cognate *AVR_{ab}* and *AVR_{al}* genes, respectively (Wise and Ellingboe, 1983; Boyd et al., 1995). *m1a6-m9472* is a fast-neutron-derived, *Mla6* deletion mutant derived from C.I. 16151 (see above). *m1a1-m508* is a γ -radiation-derived, *Mla1* deletion mutant derived from C.I. 16137 (Zhou et al., 2001). Time-course GeneChip expression profiling was used to compare barley lines that harbor *Mla6* and those with *m1a6-m9472* as well as the nearly isogenic line harboring *Mla1* and those with *m1a1-m508*. The experiment (designated BB10) was based on a split-split plot design described for BB2 by Caldo et al. (2006), with barley first leaves harvested at 0, 8, 16, 20, 24, and 32 h after inoculation with *Bgh* isolate 5874 (*AVR_{al}*, *AVR_{ab}*). Identical noninoculated plants were included for each treatment. The BB10 study consisted of 144 Barley1 GeneChip hybridizations (4 genotypes \times 6 time points \times 2 inoculation treatments \times 3 biological replications) and the BB4 (Caldo et al., 2004) study consisted of 108 hybridizations (3 genotypes \times 6 time points \times 2 isolates \times 3 biological replications), resulting in a total of 84 treatment combinations for the two experiments. Both studies were conducted under identical conditions, except that inoculations for BB4 were performed in 2002 and inoculations for BB10 were conducted in 2004. Interpretation of results was based on gene expression data within each experiment (Stevens and Doerge, 2005).

Normalization and Data Analysis

Normalization, data transformation, and mixed linear model analysis (Wolfinger et al., 2001) for the BB10-derived microarray data were patterned after the methods used by Caldo et al. (2004). The mixed linear model analysis was performed using the SAS MIXED procedure. Contrast statements in SAS were made to compare mRNA expression over time in noninoculated and inoculated plants for the individual genotypes.

Microarray Data Access

All detailed data and data from expression profiling have been deposited in BarleyBase/PLEXdb (<http://barleybase.org/>; <http://plexdb.org/>), a MI-AME-compliant expression database for plant GeneChips (Shen et al., 2005). Files are categorized under accession numbers BB4 for the 108 GeneChip

Caldo et al. (2004) study and BB10 for the 144 GeneChip, *Mla* genotypes and derived mutant experiment. Data files have also been deposited in ArrayExpress (<http://www.ebi.ac.uk/arrayexpress>) with accession numbers E-MEXP-142 for the Caldo et al. (2004) study and E-TABM-142 for the BB10 investigation. Barley1 GeneChip data files for the *Fusarium graminearum* (Boddu et al., 2006) and *Puccinia graminis* f. sp. *tritici* (Zhang et al., 2008) experiments are categorized under PLEXdb accession numbers BB9 and BB49, respectively.

Identification of the Blufensin Family

Unigene numbers used refer to those originally assigned in assembly 21 from Close et al. (2004), which was the template used for designing the Affymetrix Barley1 GeneChip. BLAST version 2.2.13 from the NCBI (www.ncbi.nlm.nih.gov) was used for all sequence database queries. The Web site interface of InterProScan (www.ebi.ac.uk/interpro/; July 2008) was utilized for domain and structure prediction. Databases used for identifying family members were Gramene (www.gramene.org) for rice (*Oryza sativa*), PlantGDB (www.plantgdb.org) for all other plant species, and both NCBI (www.ncbi.nlm.nih.gov) and UniProt (www.uniprot.org) for targeting all other organisms.

Multiple Sequence Alignment and Phylogeny of the Blufensin Family

The VectorNTI program AlignX was used to align the unigenes, ORFs, and peptides of the blufensin family. As the sequences are short, visual inspection of the alignment was used to correct any misalignments. The phylogeny was generated using the software package Phylip, using *dnajpars* and *protpars* for DNA and protein sequence, respectively. Bootstrap support was performed with 1,000 replications, with only support values above 90% shown.

Promoter Analysis of Barley and Rice Blufensins

Promoters were subjected to motif search using the Plant Cis-Acting Regulatory DNA Elements database with the release version of February 2007 (Higo et al., 1999). As an extensive barley promoter sequence is currently unavailable, rice promoter sequence was used to determine if predicted occurrences of motifs were similar to those observed biologically. A Python script was developed to parse the promoter elements of the rice genome (version 5) using regular expressions to determine the occurrence of different motifs in both the forward and reverse strands of gene promoters using the same amount of sequence available for *Bln1-1*.

Biostic-Based BSMV Vector Construction

The DNA-based BSMV constructs used in this study were modified from in vitro transcription-based BSMV clones (Scofield et al., 2005). Full-length cDNAs of BSMV α -, β -, and γ -subunits were amplified using high-fidelity Platinum *Taq* DNA polymerase (Invitrogen); primers are listed in Supplemental Table S2. The BSMV-Rev universal reverse primer was used in combination with each of the specific forward primers to amplify cDNA of BSMV α -, β -, and γ -subunits. PCR products were then inserted into 35S expression vector SMVNVEC (provided by Dr. Alan Eggenberger, Iowa State University) between *StuI* and *SmaI* sites. The 3' hepatitis delta virus (HDV) ribozyme will self-cleave to generate an authentic 3'-end BSMV genome RNA.

Silencing Constructs

Total RNA was extracted from C.I. 16151 (*Mla6*) plants at 20 hai with *Bgh* isolate 5874 (*AVR_{ab}*) according to the method of Caldo et al. (2004). First-strand cDNA was synthesized using 2 μ g of total RNA, oligo(dT)₂₀ primer, and SuperScript III reverse transcriptase (Invitrogen). Subsequently, first-strand cDNA was used as the template to amplify two independent fragments with lengths of 248 and 162 bp, respectively. Primers were designed according to the *Bln1* EST sequence (GenBank accession no. BE216690) and are listed in Supplemental Table S2. Positions of the two fragments on the EST sequence were from 28 to 275 bp and from 39 to 200 bp. Amplified PCR fragments each contained introduced *PacI* and *NotI* recognition sites at the 5' and 3' ends, respectively, and were inserted into the *PacI* and *NotI* sites of BSMV: γ . The resulting vectors were designated BSMV:Bln1₂₄₈ and BSMV:Bln1₁₆₂, respectively.

Microprojectile Bombardment

All constructs were screened in at least three independent experiments. Biolistic bombardment of barley plants was carried out according to Halterman and Wise (2004) using a biolistic PDS-1000/He system (Bio-Rad) with minor modifications. Gold particles (Bio-Rad) were coated with plasmid BSMV: α , BSMV: β , and BSMV: γ (or the recombinant BSMV:Bln1₂₄₈ or BSMV:Bln1₁₆₂) at a molar ratio of 1:1:1, and the mixture was delivered to leaves using 900-psi rupture discs using a Hepta adaptor microcarrier. Eight 7-d-old Black Hull-less barley seedlings (susceptible to BSMV) were used per bombardment. Subsequently, plants were transferred to 7.5- × 7.5-cm pots for 7 to 10 d for viral replication and systemic infection. Virus-infected barley was maintained in a growth chamber (Percival Scientific) at 24°C with 16 h of light (550 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and 8 h darkness at 20°C.

Mechanical Infection of BSMV and Powdery Mildew Inoculation

Seven to 10 d after bombardment, plants displaying a BSMV infection phenotype (brown streak on the first leaf and chlorotic mosaics on the second leaf) were selected. Leaves from the infected plant were ground with 2 to 5 volumes of 0.05 M phosphate buffer (pH 7.2) in an ice-cold mortar. Carborundum (0.05 g; Sigma-Aldrich) was added to the buffer for optimal grinding. Seven-day-old healthy barley seedlings were then infected with the appropriate recombinant virions by rubbing the first leaf with crude virus extract four to six times between thumb and index finger, with new gloves used for each construct to prevent contamination. Twelve days after mechanical infection, plants displaying a BSMV infection phenotype (brown stripe on the first leaf) were inoculated with fresh *Bgh* conidiospores and placed in an 18°C growth chamber (16 h of light/8 h of darkness). *Bgh* infection types were scored at 7 dai.

Staining and Microscopy

The staining process was performed according to Hein et al. (2005) with minor modifications. 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:water) and stained with Coomassie Brilliant Blue R-250 stain (0.05% [w/v] Coomassie Brilliant Blue in 50% methanol and 10% acetic acid). A Zeiss Axio Imager M.1 microscope was used for observation.

Semiquantitative RT-PCR

Primers for semiquantitative RT-PCR are listed in Supplemental Table S2. Third leaves from BSMV-VIGS-treated plants 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 (Ambion). Two micrograms of RNA was transcribed into cDNA with an oligo(dT)₂₀ 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 for 20, 25, 30, 35, and 40 cycles. *Actin* was used as an internal constitutive expression control for cDNA quantitative normalization. The intensities of PCR-generated fragments were analyzed and quantified using Gel Doc 2000 and Quantity One version 4.2.1 (Bio-Rad).

Bln1 Transient Overexpression

The full-length ORF of *Bln1* was amplified from vector BSMV:Bln1₂₄₈ using with both sense (5'-TCAAAGCTTACGAGGATATGGCAAAGAAGTAC-3') and antisense (5'-AGTGATATCTTATGAGCCACCATTAGGGATCG-3') primers. *EcoRV* and *HindIII* were used to double digest the PCR product, which was inserted into the expression vector pUbi:Nos, which was also digested with the same enzymes. The newly constructed vector, pUbi:Bln1, was cobombarded with pUGN (Nielsen et al., 1999) into barley epidermal cells in three independent experiments. A generalized linear mixed model was fit to the data from the three experiments. The model assumed a binomial response for each leaf. The logit of the binomial success probability (probability of hyphae formation) was modeled as a linear function of an overall mean, fixed construct effects, random experiment effects, and random effects for leaves within experiments and constructs.

Inverse PCR

Inverse PCR was performed according to Meng et al. (2007) with minor modifications. One microgram of genomic DNA sample was subjected to overnight digestion with 5 units of *AflIII*, *MspI*, and *NcoI*. The primers used are listed in Supplemental Table S2. The conditions used for PCR were as follows: 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 3 min. A final extension step was performed at 72°C for 10 min.

Accession Numbers

Accession numbers are FJ156737 (C.I. 16151), FJ156738 (C.I. 16155), and FJ156739 (C.I. 16137) for *Bln1-1* genomic sequences; FJ156740 (C.I. 16151), FJ156741 (C.I. 16155), and FJ156742 (C.I. 16137) for *Bln1-2* genomic sequences; and FJ156743 (Sultan-5) and FJ156744 (Golden Promise) for *Bln1-3* genomic sequences. Accession numbers for *Bln2* genomic sequences are FJ156745 (C.I. 16151), FJ156746 (C.I. 16155), FJ156747 (C.I. 16137), FJ156748 (Sultan-5), and FJ156749 (Golden Promise).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Annotations of Blufensin family members in barley, rice, and wheat.

Supplemental Table S2. Primers used for inverse PCR, *Bln1* transcript detection, BSMV-VIGS vector construction, *Bln1* silencing constructs, and semiquantitative RT-PCR.

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