BAX INHIBITOR-1 Is Required for Full Susceptibility of Barley to Powdery Mildew

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BAX INHIBITOR-1 (BI-1) is one of the few proteins known to have cross-kingdom conserved functions in negative control of programmed cell death. Additionally, barley BI-1 (HvBI-1) suppresses defense responses and basal resistance to the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* and enhances resistance to cell death—provoking fungi when overexpressed in barley. Downregulation of *HvBI-1* by transient-induced gene silencing or virus-induced gene silencing limited susceptibility to *B. graminis* f. sp. *hordei*, suggesting that HvBI-1 is a susceptibility factor toward powdery mildew. Transient silencing of *BI-1* did not limit supersusceptibility induced by overexpression of *MLO*. Transgenic barley plants harboring an *HvBI-1* RNA interference (RNAi) construct displayed lower levels of *HvBI-1* transcripts and were less susceptible to powdery mildew than wild-type plants. At the cellular level, *HvBI-1* RNAi plants had enhanced resistance to penetration by *B. graminis* f. sp. *hordei*. These data support a function of BI-1 in modulating cell-wall-associated defense and in establishing full compatibility of *B. graminis* f. sp. *hordei* with barley.

On the basis of preformed and induced defense mechanisms, plants are nonhosts to the majority of potentially pathogenic microbes (Thordal-Christensen 2003). Induced defense mechanisms are mounted in response to the perception of general elicitors, also known as pathogen-associated molecular patterns (PAMPs), by pattern recognition receptors (Altenbach and Robatzek 2007; Zipfel 2008). Nonhost resistance is characterized as being effective against a broad spectrum of potential pathogens and durable over time (Schweizer 2007; Niks and Marcel 2009). Plant colonization by a pathogen is the result of a sophisticated specialization process, in which a particular microorganism gained the ability to overcome preformed defense mechanisms and to deal with PAMP-triggered immunity (PTI), most likely through the delivery of pathogen-derived effector proteins into the host cell (Chisholm et al. 2006; Jones and Dangl 2006; Bent and Mackey 2007; Boller and Felix 2009).

Ascomycete powdery mildew fungi are obligate biotrophic pathogens that can colonize all aboveground parts of a huge

variety of land plants. In small-grain cereals, host species-specificity of powdery mildew fungi is high, so that a particular powdery mildew form *specialis* can colonize only one specific cereal species. The barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei*, for example, can infect barley (*Hordeum vulgare* L.) but does not grow on wheat or other cereal species (Schweizer 2007; Niks and Marcel 2009). This host species specificity might either be due to a lack of matching effector molecules on the side of the pathogen, or the lack of corresponding effector target proteins on the side of the host.

Basic compatibility can be achieved by the suppression or circumvention of resistance factor activity or the reprogramming of the host cell through targeting of host susceptibility factors. By definition, a susceptibility factor is a plant protein that is required for host susceptibility to a certain pathogen (Eckardt 2002). Consequently, loss of protein function should lead to enhanced resistance of the host plant to this particular pathogen, whereas its overexpression can support plant colonization by the intruder (Hückelhoven 2005). One of the most prominent susceptibility factors in a plant–pathogen interaction that fulfills these requirements is the barley seven transmembrane domain protein MILDEW LOCUS O (*MLO*). In barley, loss of *MLO* function in *mlo*-plants results in complete, race-nonspecific resistance to otherwise virulent barley powdery mildew fungi. This broad-spectrum resistance is associated with papilla formation and hydrogen-peroxide accumulation and stops the fungus at a prepenetration stage (Stolzenburg et al. 1984; Hückelhoven et al. 1999). However, it is not known whether *MLO* is a direct target of effector activity and which *MLO*-dependent signaling processes eventually result in accessibility of the host cell to powdery mildew haustoria (O’Connell and Panstruga 2006). Nevertheless, the requirement for *MLO* for susceptibility to powdery mildew fungi was found in dicotyledonous plant species as well (Consonni et al. 2006; Bai et al. 2008; Feechan et al. 2008), indicating a conserved pathway for defense suppression.

Among other barley proteins, which are required for full powdery mildew susceptibility, there are a WRKY transcription factor, the RHO-like GTPase RACB, the small peptide BLUFENSIN, and calmodulin (Kim et al. 2002; Schultheiss et al. 2002; Eckey et al. 2004; Meng et al. 2009).

BAX INHIBITOR-1 (BI-1) proteins are endoplasmic reticulum (ER)-resident negative regulators of stress-related cell death in plants and animals (Hückelhoven 2004; Watanabe and Lam 2009). Stable overexpression of this cell-death inhibitor in barley reduces susceptibility to the necrotrophic fungus *Fusa-*

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*The e-Xtra logo stands for “electronic extra” and indicates that Figure 5 appears in color online.

rium graminearum (Babaeizad et al. 2009). However, BI-1 not only negatively regulates cell death reactions but was also found to act as negative regulator of resistance to biotrophic powdery mildew fungi in barley. Transient or stable overexpression of *BI-1* strongly supported penetration of *B. graminis* f. sp. *hordei* into barley epidermal cells (Hückelhoven et al. 2003; Eichmann et al. 2004, 2006b; Babaeizad et al. 2009). Moreover, transient overexpression of *BI-1* even allowed *B. graminis* f. sp. *hordei* to break *mlo*-mediated penetration resistance and the nonadapted wheat powdery mildew fungus to penetrate epidermal cells of barley (Hückelhoven et al. 2003; Eichmann et al. 2004, 2006b). *BI-1* overexpression also suppressed a local defense-related hydrogen peroxide burst at sites of interaction with *B. graminis* f. sp. *hordei* (Eichmann et al. 2006b). However, transient knockdown experiments using an antisense construct or in vitro-synthesized double-stranded RNA were inconclusive (Hückelhoven et al. 2003). Here, we show that *BI-1* is differentially expressed in powdery mildew-targeted barley leaf epidermal tissue. We used improved RNA interference (RNAi)-based methods to show the possible role of BI-1 as a susceptibility factor. Transient and stable gene silencing experiments indicate that *BI-1* expression is required for full susceptibility of barley to *B. graminis* f. sp. *hordei*, and

that its transcriptional downregulation can influence penetration resistance to *B. graminis* f. sp. *hordei*.

RESULTS

HvBI-1 expression increases in epidermal tissue after *B. graminis* f. sp. *hordei* infection.

To evaluate powdery mildew-dependent gene expression reprogramming in barley plants, we inoculated first leaves of the susceptible barley cv. Ingrid and of its race-nonspecific powdery mildew-resistant back-cross line BCIngrid-*mlo5*, which does not express a functional MLO protein. We measured *B. graminis* f. sp. *hordei*-responsive gene expression by semiquantitative reverse-transcription polymerase chain reaction (RT-PCR). Expression of *PROTEIN DISULFIDE ISOMERASE (PDI)* and *PATHOGENESIS-RELATED PROTEIN 1b (PR1b)* were examined as positive controls for defense-associated gene expression (Bryngelsson et al. 1994; Eichmann et al. 2006a). In susceptible barley cv. Ingrid, a slight accumulation of *PDI* transcript was observed at 12 and 24 h after inoculation (HAI) with *B. graminis* f. sp. *hordei*, which preceded that of *PR1b* (Fig. 1A). Defense-associated expression of *PDI* and *PR1b* was somewhat stronger in the resistant BCIngrid-*mlo5* genotype when compared with the near-isogenic *MLO* wild type. *BI-1* transcript accumulated at a low level in noninoculated leaves regardless of the barley genotype. *BI-1* expression slightly increased from 24 HAI onward and was more prominent in resistant BCIngrid-*mlo5* (Fig. 1A).

To examine whether *BI-1* transcript accumulation increased in barley epidermal cells, the only tissue *B. graminis* f. sp. *hordei* infects, we densely inoculated the abaxial side of barley cv. Ingrid first leaves with *B. graminis* f. sp. *hordei* spores. At given time points after inoculation, we collected stripped abaxial epidermis and remaining leaf tissue separately. The *UBIQUITIN* gene was constitutively expressed regardless of tissue and treatment. Amplification of *RIBULOSE BISPHOSPHATE CARBOXYLASE (RUBISCO)* served as control to monitor mesophyll contamination. Hardly any *RUBISCO* transcript was detected in stripped epidermis, indicating proper tissue separation of the samples (Fig. 1B). In contrast, an epidermis-specific *OXALATE OXIDASE-LIKE PROTEIN (OXLP)*, also known as *GERMIN4* (Wei et al. 1998) gene showed *B. graminis* f. sp. *hordei*-specific transcript accumulation only in the epidermis (Fig. 1B). *PDI* was *B. graminis* f. sp. *hordei* responsively expressed in the epidermis and remaining leaf tissue, with the strongest signals at 16 and 24 HAI. *BI-1* was expressed in the epidermis and its transcript accumulated in response to *B. graminis* f. sp. *hordei*, showing enhanced levels in inoculated leaf samples by 16 and 24 HAI in both epidermal and mesophyll tissue (Fig. 1B). Taken together, *BI-1* is expressed in both epidermal and mesophyll tissue of barley leaves and its transcript level slightly increases in response to *B. graminis* f. sp. *hordei* infection.

Both virus- and transient-induced gene silencing of *BI-1* in barley enhance resistance to *B. graminis* f. sp. *hordei*.

To test whether BI-1 is required for susceptibility to the barley powdery mildew fungus, we employed two different transient RNAi-based gene silencing methods. Virus-induced gene silencing (VIGS) has been used to examine the function of genes associated with resistance of barley to powdery mildew (Hein et al. 2005). Transient-induced gene silencing (TIGS) is based upon particle bombardment-mediated transformation of single barley epidermal cells and has been optimized for investigation of cell autonomous gene function during cereal-powdery mildew interactions (Douchkov et al. 2005). BLAST-based searches against available barley sequences indicated that *HvBI-1* (Gen-

Fig. 1. Cultivar- and tissue-specific gene expression of *BAX INHIBITOR-1 (BI-1)* in the interaction of barley with *Blumeria graminis* f. sp. *hordei* (*Bgh*). **A**, Total RNA was isolated from *B. graminis* f. sp. *hordei*-inoculated barley cv. Ingrid and BCIngrid-*mlo5* leaves that were harvested at 12, 24, and 48 h after inoculation (HAI), and mock-inoculated control leaves that were harvested 0, 12, 24, and 48 HAI. Inverted gel photographs after ethidium bromide staining of two-step reverse-transcription polymerase chain reaction (RT-PCR) products are shown. Amplification of *UBIQUITIN* served as control for constitutive gene expression, and *PROTEIN DISULFIDE ISOMERASE (PDI)* and *PATHOGENESIS-RELATED PROTEIN 1b (PR1b)* as control for pathogenesis-related gene expression. **B**, Total RNA was isolated from *B. graminis* f. sp. *hordei*- or mock-inoculated barley cv. Ingrid leaves that were harvested at 0, 12, 24, and 48 HAI. At each time point, abaxial epidermal peels and remaining leaf tissue (mesophyll and adaxial epidermis) were collected separately. Inverted gel photographs after ethidium bromide staining of two-step RT-PCR products are shown. Amplification of *UBIQUITIN* served as control for tissue-unspecific constitutive gene expression, *RIBULOSE BISPHOSPHATE CARBOXYLASE (RUBISCO)* as control for mesophyll cell-specific gene expression, and *OXALATE OXIDASE LIKE PROTEIN (OXLP)* and *PDI* as control for epidermis-specific and general pathogenesis-related gene expression, respectively.

Bank accession number AJ290421) was the only target sequence of all RNAi constructs used in this study. For VIGS, we inoculated first leaves of barley cv. Clansman plants with a *Barley stripe mosaic virus* (BSMV) vector construct containing a *BI-1* antisense cDNA fragment (BSMV.BI-1as) (Fig. 2A) in order to silence the *BI-1* gene in systemic leaves. Control plants were either mock inoculated or inoculated with a BSMV green fluorescent protein (GFP) construct as a control (Hein et al. 2005). By 11 days after infection, asymptomatic systemic leaves were detached and inoculated with spores of *B. graminis* f. sp. *hordei* isolate CH4.8. Three days after inoculation, leaves were fixed and fungal structures were stained. To evaluate the level of resistance on *BI-1*-silenced and control leaves, we microscopically evaluated the percentage of spores that developed elongated secondary hyphae (ESH) after penetration of the host epidermis. Interestingly, approximately twice as many fungal spores produced ESH on systemic leaves of BSMV.GFP challenge-inoculated plants than on mock-inoculated plants (Fig. 2A). In contrast, fungal development was reduced on *BI-1* silenced leaves. Only 25% of the germinated fungi produced ESH on *BI-1*-

silenced plants compared with control plants (Fig. 2A), indicating enhanced resistance to fungal infection. Suppression of *BI-1* mRNA accumulation after BSMV.BI-1as challenge was confirmed by semiquantitative RT-PCR (Fig. 2B). *BI-1* expression was examined in the same systemic leaves of mock-, BSMV.GFP-, and BSMV.BI-1as-inoculated plants that were used for the *B. graminis* f. sp. *hordei* resistance test. *BI-1* expression was normalized against α -2 *TUBULIN* expression, which served as internal control, and then compared. *BI-1* transcript accumulation appeared to be slightly higher ($90 \pm 12\%$ standard error [SE] of the α -2 *TUBULIN* signal) in BSMV.GFP-challenged plants when compared with mock-inoculated plants ($67 \pm 9\%$ SE) but was significantly reduced in systemic leaves after infection with BSMV.BI-1as ($33 \pm 4\%$ SE) (Fig. 2B).

Penetration success into the first attacked epidermal cell determines the outcome of the barley-*B. graminis* f. sp. *hordei* interaction. This allows examination of the impact of gene over- or underexpression on susceptibility after ballistic transformation of barley leaf segments (Schweizer et al. 1999, 2000; Douchkov et al. 2005). Barley cv. Golden Promise leaf segments

Fig. 2. Virus-induced gene silencing (VIGS) or transient-induced gene silencing (TIGS) of *BAX INHIBITOR-1* (*BI-1*) enhance resistance in the barley-*Blumeria graminis* f. sp. *hordei* interaction. **A**, Schematic drawing of the *Barley stripe mosaic virus* (BSMV) BI-1as VIGS construct. A cDNA fragment of *BI-1* was cloned in antisense orientation behind the γ b gene of the γ subgenomic RNA. Barley cv. Clansman primary leaves were inoculated with BSMV.BI-1as or with BSMV green fluorescent protein (GFP), included as a control for BSMV infection, in addition to BSMV-noninfected (mock) plants. By 11 days after infection, asymptomatic systemic leaves were detached and inoculated with spores of *B. graminis* f. sp. *hordei* isolate CH4.8. Three days after *B. graminis* f. sp. *hordei* infection, the percentage of spores that developed elongated secondary hyphae (ESH) after penetration of the host epidermis was assessed. Data are from up to six independent plants corresponding to a total of 16 leaves. **B**, *BI-1* expression as measured by semiquantitative reverse-transcription polymerase chain reaction in the same systemic leaves of mock-, BSMV.GFP-, or BSMV.BI-1as-inoculated plants. Columns represent signal intensities of PCR products on gel photographs as measured with the ImageJ software relative to signal intensities of constitutively expressed α -2 *TUBULIN*. **C**, Schematic drawing of the RNA interference (RNAi) cassette of pIPKTA30N-BI-1 RNAi vector, which was used for TIGS of *BI-1*. Inverted repeats of a *BI-1* cDNA fragment are separated by intron 2 of the wheat *RGA2* gene. Expression of the construct is driven and terminated by the *Cauliflower mosaic virus* 35S promoter (CaMV35S_{prom}) and terminator (35S_T), respectively. Primary leaves of barley cv. Golden Promise were co-bombarded with pUbi- β -GLUCURONIDASE (GUS) and either pIPKTA30N-BI-1 (*BI-1* RNAi), pIPKTA36 (*MILDEW LOCUS O* [*MLO*] RNAi), or empty vector pIPKTA30N (control). Three days later, leaves were inoculated with *B. graminis* f. sp. *hordei* spores. The susceptibility index was calculated as the fraction of GUS cells containing at least one haustorium. Columns represent values relative to the empty-vector control. **D**, Elimination of GFP-BI-1 fluorescence after TIGS. Barley cv. Ingrid primary leaves were co-bombarded with pGY-1-DsRED, pGY-1-GFP-BI-1, and TIGS vector pIPKTA30N-BI-1 or empty pIPKTA30N. Two days after transformation, red fluorescing *DsRED*-expressing cells were examined for presence or absence of green GFP-BI-1 fluorescence. **A** through **D**, Bars represent standard errors; *, **, and *** indicate significance at $P < 0.05$, 0.005, and 0.0005, respectively, according to Student's *t* test when compared with controls.

were cobombarded with a β -GLUCURONIDASE (GUS) expression construct and either empty vector pIPKTA30N or *BI-1* RNAi vector pIPKTA30N-BI-1 (Fig. 2C). Similarly, *GUS* was coexpressed with an *MLO* RNAi construct (pIPKTA36) (Douchkov et al. 2005). Three days after transformation, leaf segments were densely inoculated with spores of *B. graminis* f. sp. *hordei* isolate CH4.8 and two days later inspected for GUS-expressing cells using a bright-field microscope. The susceptibility index was assessed as percentage of transformed, GUS-expressing cells that contained at least one haustorium (Douchkov et al. 2005). Average susceptibility indices relative to the control, which was set as 100%, are shown in Figure 2C. Fungal penetration into *BI-1*-silenced cells was clearly restricted and occurred in only $40 \pm 10\%$ SE of the transformed cells relative to control cells. Expectedly, reduction in penetration efficiency was even stronger in barley epidermal cells that expressed an *MLO* RNAi construct. Here, the relative susceptibility index was $22 \pm 8\%$ SE. We repeated the *BI-1* knockdown experiments but used GFP instead of GUS and *B. graminis* f. sp. *hordei* race A6 on barley cv. Ingrid. In these experiments, a shorter period (24 h) of gene silencing before inoculation led to a somewhat weaker but still significant reduction of fungal penetration success by approximately 30% (data not shown).

To further prove the efficiency of the *BI-1* RNAi construct, we examined its capacity to silence the expression of a *GFP-BI-1* translational fusion construct. To this end, we cobombarded barley cv. Ingrid leaf segments with pGFP-BI-1 and either empty vector pIPKTA30N or pIPKTA30N-BI-1. Additionally, we delivered *DsRED* as transformation marker (Dietrich and Maiss 2002). Two days after transformation, red fluorescing *DsRED* cells were examined for the presence or absence of green fluorescence of the *GFP-BI-1* fusion protein. Although $78 \pm 4\%$ SE of control cells displayed *GFP-BI-1* expression, green fluorescence was strongly suppressed after delivery of pIPKTA30N-BI-1 and could be detected in $31 \pm 10\%$ SE of the *DsRED*-positive cells only (Fig. 2D).

Together, VIGS and TIGS provided evidence for the contribution of *BI-1* to barley susceptibility to infection by *B. graminis* f. sp. *hordei*.

Fig. 3. Anthocyanin accumulation, indicative of cell viability, decreases in *BAX INHIBITOR-1* (*BI-1*)-silenced cells after *Blumeria graminis* f. sp. *hordei* inoculation. Quantitative analysis of the number of green fluorescent protein (GFP)- and anthocyanin-accumulating cells in the presence of empty pIPKTA30N or pIPKTA30N-BI-1 without (–*Bgh*) and with (+*Bgh*) powdery mildew inoculation. Barley cv. Golden Promise plants were transiently transformed with either empty vector or *BI-1* transient-induced gene-silencing construct pIPKTA30N-BI-1. Cells were co-bombarded with pGY-1-GFP as transformation marker and the *B-Peru/CI*-expression plasmid pBC17 for anthocyanin production. Note that anthocyanin accumulation is never seen in dead cells. Columns represent mean values of three independent experiments. Bars represent standard errors; * indicates significance at $P < 0.05$ according to Student's *t* test when compared with the respective empty vector control.

HvBI-1 has been shown to inhibit pathogen-induced cell death reactions in barley (Eichmann et al. 2006b; Babaeizad et al. 2009). Therefore, we assessed whether the enhanced resistance to *B. graminis* f. sp. *hordei* in *BI-1*-silenced cells was associated with cell death. The accumulation of anthocyanin pigments after transient expression of the maize genes *B-Peru* and *CI* in barley only occurs in intact vacuoles of viable cells. Therefore, we used cotransformation of RNAi vector pIPKTA30N-BI-1 together with the *B-Peru/CI*-expression plasmid pBC17 (Schweizer et al. 2000) to evaluate the potential effect of *BI-1* silencing on cell death. Cotransformation of pBC17 with empty vector pIPKTA30N served as control. GFP was used as an additional transformation marker. The experiment was performed with and without dense *B. graminis* f. sp. *hordei* inoculation (more than 50% of all epidermal cells were attacked by the fungus). If *BI-1* silencing would affect viability of the cells, the percentage of GFP- and anthocyanin-coaccumulating cells should be lower when compared with control cells. In the absence of powdery mildew conidia, approximately 60% of the GFP-expressing cells also displayed anthocyanin accumulation, regardless of whether *BI-1* was silenced or cells were transformed with empty vector (Fig. 3). However, upon inoculation with spores of *B. graminis* f. sp. *hordei*, the proportion of anthocyanin accumulating cells was lower after *BI-1* silencing ($41 \pm 2\%$ SE) when compared with control cells ($55 \pm 1\%$ SE) (Fig. 3), indicating that *B. graminis* f. sp. *hordei* can induce a certain degree of barley epidermal cell death when *BI-1* expression was reduced. However, no such effect was observed without pathogen inoculation.

Knockdown of *BI-1* expression by TIGS does not suppress supersusceptibility induced by *MLO* overexpression.

Because overexpression of HvBI-1 compromised *mlo5*-mediated resistance (Hückelhoven et al. 2003), we asked whether induction of susceptibility by *MLO* overexpression (Shirasu et al. 1999) can be reduced by simultaneous silencing of *BI-1*. Therefore, we ballistically transferred an *MLO* overexpression construct together with either the TIGS vector pIPKTA30N-BI-1 or the empty vector pIPKTA30N into epidermal cells of

Fig. 4. Knockdown of *BAX INHIBITOR-1* (*BI-1*) expression does not limit defense suppression by *MILDEW LOCUS O* (*MLO*) overexpression. Leaves of the *Blumeria graminis* f. sp. *hordei*-resistant barley backcross line Pallas-*mlo5* were transiently transformed by particle bombardment. Expression construct pGY-1-MLO was co-expressed with either *BI-1* RNA interference construct pIPKTA30N-BI-1 or empty vector pIPKTA30N. Control cells were co-bombarded with pIPKTA30N-BI-1 and empty vector pGY-1. Two days after transformation, leaves were densely inoculated with *B. graminis* f. sp. *hordei* conidia. Microscopic determination of *B. graminis* f. sp. *hordei* penetration efficiency into transformed cells was assessed 2 days later. Columns represent mean values of three independent experiments. Bars represent standard errors. Averages highlighted with the same letter are not statistically different at $P < 0.05$ according to Student's *t* test.

the *B. graminis* f. sp. *hordei*-resistant barley backcross line BCPallas-*mlo5*. Control cells were cobombarded with empty pGY-1 vector and pIPKTA30N-BI-1. GFP served as transformation marker. Two days after bombardment, leaf segments were densely inoculated with *B. graminis* f. sp. *hordei* conidia. After another 2 days, powdery mildew penetration efficiency (i.e., the percentage of attacked transformed cells that contained at least one haustorium) was assessed. As expected, penetration efficiency was very low ($2 \pm 2\%$ SE) in control *mlo5* cells without *MLO* overexpression (Fig. 4). Overexpression of *MLO* strongly increased fungal penetration efficiency to $65 \pm 4\%$ SE (Fig. 4). *MLO*-mediated increase in susceptibility was not limited by simultaneous knockdown of *BI-1* expression. Penetration efficiency here was $69 \pm 5\%$ SE (Fig. 4).

Stable knockdown of *BI-1* expression reduces *B. graminis* f. sp. *hordei* colonization on barley plants.

Stable transgenic barley plants, expressing a *BI-1* RNAi cassette under the control of the strong constitutive maize *UBIQUITIN1* promoter (Fig. 5A), were produced. Altogether, transgenic plants of 18 different lines of the T1 generation were tested in inoculation experiments for enhanced resistance to colonization by *B. graminis* f. sp. *hordei*. We identified two lines, BG37/2E10 and BG37/2E11, that showed a consistently decreased colonization by *B. graminis* f. sp. *hordei* in repeated experiments. The first leaf was used to prove the presence of the transgene by either PCR or a hygromycin-resistance test. Leaf segments of the second leaf were then placed on water agar plates and inoculated with *B. graminis* f. sp. *hordei* conidia. After 5 to 7 days, powdery mildew colonies became visible. Representative examples of reduced powdery mildew colonization in individual plants of the segregating T1 population, which coincided with suppressed *BI-1* expression, are shown in Figure 5B and C. Here, total RNA was extracted from third leaves of individual plants and semiquantitative RT-PCR was performed to examine *BI-1* expression. Constitutive expression of the *UBIQUITIN* gene was used to demonstrate equal amounts of RNA template. *BI-1* expression was down-regulated in plants of *BI-1* RNAi lines BG37/2E10 (E10) and

BG37/2E11 (E11) but not of line BG37/2E05 (E05) (Fig. 5B). *BI-1*-silenced plants showed a clearly reduced number of powdery mildew pustules when compared with either wild-type (WT GP) or nonsilenced plants (Fig. 5C). In all experiments, transgenic plants of lines BG37/2E10 and BG37/2E11 showed a reduction in pustule development after inoculation whereas line BG37/2E05 behaved like the wild type. One of the repeated experiments is summarized in Figure 5D. Taken together, data from stable transgenic *BI-1* RNAi plants further support results from *BI-1* TIGS and VIGS experiments, hereby confirming that silencing of *BI-1* restricts colonization by *B. graminis* f. sp. *hordei*.

Enhanced resistance in stable, transgenic *BI-1* RNAi plants is associated with papilla formation but not constitutive upregulation of defense-related genes.

Transmission of *BI-1* silencing and corresponding resistance into the T2 and T3 transgenic generation was inconsistent, although the T-DNA was inherited as expected (data not shown). We performed quantitative real-time PCR to assess residual *BI-1* expression in pooled RNA extracted from T1 plants of line BG37/2E10 (E10), which consistently showed reduced colonization by *B. graminis* f. sp. *hordei*. Expression of *BI-1* in BG37/2E10 was strongly decreased and corresponded to $26 \pm 0.4\%$ standard deviation of that in Golden Promise wild type (GP WT) plants (Fig. 6A). In order to investigate the mechanism underlying enhanced resistance in T1 *BI-1* RNAi plants, second leaves of intact plants of line BG37/2E10 and GP WT, as well as individuals that lost the T-DNA due to segregation (azygous controls), were inoculated with *B. graminis* f. sp. *hordei* spores. At 30 HAI, inoculated leaves were detached and transferred to 3,3-diaminobenzidine (DAB) solution for the detection of hydrogen peroxide (H_2O_2). Leaves were inspected microscopically for the frequency of successful penetration by the fungus into barley epidermal cells, as indicated by haustorium formation and the development of elongated secondary hyphae, the frequency of papilla formation (nonpenetrated living cells), and the occurrence of the hypersensitive response (HR) as indicated by whole-cell DAB staining (Fig. 6C through

Fig. 5. BAX INHIBITOR-1 (*BI-1*) silencing in stable transgenic barley plants reduces colonization by *Blumeria graminis* f. sp. *hordei*. **A**, Schematic drawing of the RNA interference (RNAi) cassette of the binary pLH6000-BI-1 RNAi vector that was used for stable transformation of barley. Inverted repeats of a *BI-1* cDNA fragment are separated by intron 2 of the wheat *RGA2* gene. Expression of the RNAi cassette is driven by the maize *UBIQUITIN* promoter (ZmUBI_{prom}) and terminated by the *NOPALINE SYNTHASE* terminator (NOS_T). **B** and **C**, Pustule development on individual *BI-1*-silenced and nonsilenced plants. **B**, Total RNA of individual plants of *BI-1* RNAi line BG37/2E05 (numbers 8 and 9), BG37/2E10 (numbers 7 and 9), and BG37/2E11 (numbers 5 and 6) and one wild-type Golden Promise plant (GP WT) was extracted from third leaves and *BI-1* expression was analyzed by semiquantitative reverse-transcription polymerase chain reaction (RT-PCR). RT-PCR analysis of constitutively expressed *UBIQUITIN* (*UBI*) and ethidium bromide-stained total RNA were used to show similar concentrations of template RNA. Inverted gel photographs are shown. **C**, Development of powdery mildew pustules on the same plants that were analyzed in **B**. The second leaf of each plant was inoculated with *B. graminis* f. sp. *hordei* conidia. Pustule development was allowed for 7 days and then photographed. **D**, Second leaves of GP WT and transgene-positive plants of *BI-1* RNAi lines BG37/2E05 (E05), BG37/2E10 (E10), and BG37/2E11 (E11) were inoculated with spores of *B. graminis* f. sp. *hordei*. Seven days after inoculation, pustules on a 5-cm leaf segment were counted. Columns represent mean numbers of pustules per centimeter of leaf segment. Bars represent standard errors of the means. One representative experiment is shown.

E). Azygous controls did not differ from wild-type plants in the frequency of interaction types. In both cases, penetration frequency was approximately 37%, while 60% of the attacked cells were nonpenetrated living cells and approximately 3% underwent HR (Fig. 6B). In contrast, penetration efficiency of *B. graminis* f. sp. *hordei* was significantly lower in leaves of *BI-1* RNAi plants ($17 \pm 4\%$ SE, Student's *t* test $P < 0.05$, 46% of the wild-type control). The drop in penetration efficiency was accompanied by a corresponding increase in the occurrence of nonpenetrated living cells ($81 \pm 5\%$ SE) showing DAB staining in papillae, whereas the frequency of HR was unchanged when compared with that in wild-type and azygous control plants ($3 \pm 1\%$ SE) (Fig. 6B). Microscopic data indicate that penetration resistance of BG37/2E10 largely explains enhanced resistance to powdery mildew at the symptom level. Furthermore, semiquantitative RT-PCR analysis indicated that resistance in *BI-1* RNAi plants was not accompanied by constitutive upregulation of the defense-associated genes *PATHOGENESIS-RELATED PROTEIN 1b* and 5 (*PR1b*; *PR5*), *CALRETICULIN 3* (*CRT3*), and *BARLEY CHEMICALLY INDUCED GENE 3* (*BCI-3*) (data not shown).

DISCUSSION

Little is known about plant proteins involved in susceptibility to fungal pathogens. Here, we present evidence that HvBI-1 is required for full accessibility of barley epidermal cells to

penetration by *B. graminis* f. sp. *hordei* and subsequent symptom formation. This establishes BI-1 as a susceptibility factor involved in fungal accommodation in intact plant cells.

The intimate interaction of plants with biotrophic, haustoria-forming fungal microorganisms presumably requires a reprogramming of the host cell in favor of the pathogen. This seems to be necessary in order to support fungal accommodation in an intact plant cell and the subsequent biotrophic life style (O'Connell and Panstruga 2006). The HvBI-1 protein was a candidate susceptibility factor for powdery mildew because its transient or stable overexpression supports both pathogen entry into host cells and pustule development on barley leaves (Hückelhoven et al. 2003; Eichmann et al. 2004; Babaeizad et al. 2009). In addition, a GFP-BI-1 fusion protein strongly accumulates at sites of fungal contact with the host cell (i.e., underneath attacking appressoria or at sites of successful penetration). *BI-1* overexpression also suppressed a local oxidative burst that is normally associated with penetration resistance (Hückelhoven et al. 1999; Eichmann et al. 2006b). However, direct proof for the requirement of BI-1 for pathogenesis was lacking, due to the fact that previous RNAi-based transient *BI-1* knockdown experiments using either in vitro transcribed double-stranded RNA or an antisense construct did not reliably influence the interaction with *B. graminis* f. sp. *hordei* (Hückelhoven et al. 2003) and, thus, evidence was based on overexpression only. Here, we used improved RNAi-based methods to downregulate *BI-1* expression in the context of *B.*

Fig. 6. Microscopic inspection of the interaction of *BAX INHIBITOR-1* (*BI-1*)-silenced and wild-type (WT) barley plants with *Blumeria graminis* f. sp. *hordei* at 36 h after inoculation. **A**, Analysis of *BI-1* expression in *BI-1* RNA interference (RNAi) line BG37/2E10 (E10) by quantitative real-time polymerase chain reaction. Total RNA was extracted from the third leaf of four randomly selected plants that were positive for the presence of the transgene, then pooled. *BI-1* expression level was normalized against barley *UBIQUITIN CONJUGATING ENZYME* and is presented relative to the expression level in WT Golden Promise (GP WT), which was set as 100%. Columns represent mean values of three technical repetitions. Bar represents standard deviation. **B**, Second leaves of *BI-1* RNAi line BG37/2E10 (*BI-1* RNAi E10, eight leaves) as well as corresponding azygous controls that had lost the transgene due to segregation (three leaves) and parental line GP WT (five leaves) were inoculated with spores of the barley powdery mildew fungus and, 30 h later, stained with 3,3-diaminobenzidine (DAB) for 6 h for detection of hypersensitive response (HR) by whole-cell H_2O_2 staining. Successful fungal penetration was indicated by haustorium formation (Hau) and development of elongated secondary hyphae. Nonpenetrated cells either survived fungal attack (nonpenetrated living cells [NPLC]) or displayed HR without visible haustorium formation. Bars represent standard errors; * indicates significance at $P < 0.05$ according to Student's *t* test when compared with GP WT. **C** through **E**, Exemplary pictures of the *B. graminis* f. sp. *hordei* interaction types evaluated in **B**. Extracellular fungal structures appear dark due to staining with acetic ink. Asterisks mark fungal spores. Size bars = 30 μ m. **C**, Successful fungal penetration into a barley epidermal cell as indicated by haustorium formation (hau). **D**, Nonpenetrated living cell. The fungus could not penetrate the attacked and still-intact cell, possibly due to papilla (pap) formation. **E**, Barley epidermal cell that responded with an HR to fungal attack. Note that the whole cell appears dark due to dark precipitates after DAB staining of H_2O_2 .

graminis f. sp. *hordei* infection and showed its impact on the outcome of the interaction.

HvBI-1 expression increases

in epidermal tissue after *B. graminis* f. sp. *hordei* infection.

The interaction of the barley powdery mildew fungus with its host plant takes place at the epidermal cell layer. A powdery mildew-induced increase in *BI-1* expression was reported previously, using whole leaves (Hückelhoven et al. 2001; Eichmann et al. 2004). However, previous studies did not clearly show whether or not *BI-1* transcripts are present in the epidermis (Hückelhoven et al. 2003). Here, we analyzed pathogen-dependent *BI-1* expression in the epidermis in more detail and found that weak constitutive expression of *BI-1* in the epidermis slightly increased at 16 and 24 h after *B. graminis* f. sp. *hordei* inoculation, which coincides with attempts for host cell penetration and haustorium establishment under the conditions applied (Hückelhoven et al. 1999; Eichmann and Hückelhoven 2008). *BI-1* expression in epidermal leaf tissue and its *B. graminis* f. sp. *hordei*-induced transcript accumulation were also evident from global gene-expression studies using barleyPGRC1 10K cDNA arrays (Schweizer 2008) (P. Schweizer, unpublished data). Epidermis expression of *BI-1* was also the prerequisite for the analysis of gene function by TIGS upon ballistic transformation.

Both VIGS and TIGS of *BI-1*

in barley enhanced resistance to *B. graminis* f. sp. *hordei*.

VIGS and TIGS have been used in cereals to demonstrate the function of different proteins in barley resistance or susceptibility to powdery mildew fungi (Douchkov et al. 2005; Hein et al. 2005). Our transient gene-silencing experiments described here provide evidences that *BI-1* knockdown limits susceptibility of barley epidermal cells to *B. graminis* f. sp. *hordei* haustorium establishment. This effect was observed with two different fungal isolates and in three different barley genetic backgrounds, suggesting a perhaps general requirement of *BI-1* for full susceptibility to *B. graminis* f. sp. *hordei*. In barley, several genes have been shown by TIGS to be involved in susceptibility to powdery mildew. Among them are the small RHO-like G-protein RACB, a WRKY transcription factor, BLUFENSIN1, calmodulin, and MLO (Kim et al. 2002; Schultheiss et al. 2002; Eckey et al. 2004; Meng et al. 2009). However, only in the case of MLO was this function demonstrated at the whole-plant level because *mlo*-null mutants are completely resistant to *B. graminis* f. sp. *hordei* (Jørgensen 1992; Büschges et al. 1997). It is not understood to which extent genes that contribute to susceptibility operate in the same or overlapping pathways. For calmodulin and RACB, an MLO-cooperative function has been suggested based on protein–protein interaction studies or indirect genetic evidence, respectively (Kim et al. 2002; Lorek et al. 2010). It has also been hypothesized that *BI-1* could function downstream of or in parallel with MLO, because overexpression of *BI-1* partially substituted for MLO function in susceptibility to *B. graminis* f. sp. *hordei* in an *mlo*-null mutant background (Hückelhoven et al. 2003; Eichmann et al. 2006b). Additionally, both MLO and *BI-1* share the ability to break resistance to the nonadapted wheat powdery mildew fungus *B. graminis* f. sp. *tritici* when transiently overexpressed (Elliott et al. 2002; Eichmann et al. 2004). Coexpression of MLO and *BI-1* did not result in further increase in susceptibility to *B. graminis* f. sp. *tritici* when compared with individual overexpression of the genes (Eichmann et al. 2004). Our gene-silencing experiments in *MLO* or *mlo* genotypes now show that knockdown of *BI-1* expression restricts susceptibility in *MLO* genotypes but cannot limit super-susceptibility induced by overexpressed *MLO* in an *mlo5*-

mutant background (Fig. 4). This suggests that *BI-1* acts in parallel to rather than downstream of MLO.

Enhanced resistance in stable, transgenic *BI-1* RNAi plants is associated with papilla formation but not constitutive upregulation of defense-related genes.

BI-1 has the ability to suppress cell death in plants induced by diverse triggers, including biotic stress (Watanabe and Lam 2009). The knockout of cell death regulator proteins often results in a lesion mimic phenotype, including spontaneous or abiotically induced leaf cell death and stress-related gene expression (Lorrain et al. 2003). Interestingly, MLO controls salicylic acid-independent penetration resistance to powdery mildew in *Arabidopsis*, whereas spontaneous defense reactions and cell death in *mlo* mutants require salicylic acid (Consonni et al. 2006). TIGS of *BI-1* did not lead to spontaneous death of cells, as assessed from the number of transformed cells that expressed a GFP marker protein and accumulated anthocyanin pigments as a result of *B-Peru/CI*-expression on detached leaves. Additionally, transgenic *BI-1* RNAi plants had a wild-type phenotype and did not show spontaneous lesion formation or defense-associated gene expression. Hence, partial knockdown of *BI-1* did not influence the viability of nonstressed barley cells.

The induced death of attacked but nonpenetrated cells is part of the basal defense reaction of barley to *B. graminis* f. sp. *hordei*. When inoculated with *B. graminis* f. sp. *hordei*, TIGS of *BI-1* moderately but significantly lowered the number of transformed single cells that showed simultaneous GFP-fluorescence and anthocyanin accumulation on leaf segments, perhaps indicating *B. graminis* f. sp. *hordei*-induced cell death when *BI-1* expression is down. Consistently, stable overexpression of GFP-*BI-1* lowered the frequency of *B. graminis* f. sp. *hordei*-induced cell death on detached leaves (Babaeizad et al. 2009). However, intact, stable transgenic *BI-1* RNAi-plants did not show more frequent *B. graminis* f. sp. *hordei*-induced cell death but, exclusively, penetration resistance associated with formation of DAB-stained papilla (Fig. 6B). We have observed before that detached leaves show a higher frequency of *B. graminis* f. sp. *hordei*-induced cell death when compared with intact plants (Pathuri et al. 2008; unpublished data). Together, *BI-1* seems to influence *B. graminis* f. sp. *hordei*-induced cell death on detached leaves only but influences penetration resistance on both detached leaves and intact plants. Hence, *BI-1* might be involved in cell-death control and cell-wall-associated prepenetration defense, albeit in potentially distinct pathways. Together, data support that silencing of *BI-1* did not simply suppress biotrophy but limited accessibility to fungal penetration.

Similar to stable knockdown of *BI-1* in barley, an *Arabidopsis BI-1* (*AtBI-1*) null mutant did not show spontaneous cell death but did show enhanced sensitivity to a fungal toxin and to an avirulence factor of *Pseudomonas syringae* (Watanabe and Lam 2006; Kawai-Yamada et al. 2009). Transient ballistic overexpression of *Arabidopsis BI-1* (AT5G47120) under control of the *Cauliflower mosaic virus* (CaMV) 35S promoter in barley leaf segments significantly enhanced susceptibility to penetration by *B. graminis* f. sp. *hordei*, indicating a conserved ability of monocot and dicot *BI-1* proteins to support accessibility to *B. graminis* f. sp. *hordei* (C. Weis, unpublished results).

Collectively, our transient and stable gene-silencing assays demonstrate a contribution of the *BI-1* protein to susceptibility of barley to the powdery mildew fungus *B. graminis* f. sp. *hordei*.

Little is known about the mode of *BI-1* action. The protein is involved in the ER-stress response (Watanabe and Lam 2009) and was suggested to build a membrane pore that may be involved in cross-membrane transport of ions (e.g., Ca^{2+}) (Ihara-Ohori et al. 2007). A role in Ca^{2+} homeostasis of the ER is sup-

ported because BI-1 is a membrane-resident ER protein and knockout or overexpression of BI-1 modulate the amount of ER-releasable Ca^{2+} (Chae et al. 2004). The fact that AtBI-1 interacts with calmodulin further supports a role in Ca^{2+} signaling (Ihara-Ohori et al. 2007). Very recently, it was shown that AtBI-1 could interact with *Arabidopsis* fatty acid hydroxylases via Cytochrome b5 in planta, and that overexpression of *AtBI-1* increases the level of very long chain fatty acids, which are typically incorporated into sphingolipids (Nagano et al. 2009). Sphingolipids are involved in cell death activation in plants and are components of lipid raft-like detergent resistant membrane microdomains, which are thought to be important for processes such as signal transduction, membrane trafficking, cytoskeletal organization, and pathogen entry (Bhat et al. 2005; Raffaele et al. 2009). How far these possible modes of action are also involved in BI-1 function in powdery mildew susceptibility in barley needs to be examined in future experiments.

MATERIALS AND METHODS

Plants, pathogen, and inoculation.

The barley (*H. vulgare* L.) cv. Ingrid and its corresponding backcross line BCIngrid-*mlo5* (I22) as well as Pallas backcross line BCPallas-*mlo5* (P22) were obtained from L. Munk (Royal Veterinary and Agricultural University, Copenhagen, Denmark). Plants were grown in a growth chamber at 18°C with 60% relative humidity and a photoperiod of 16 h (60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density). Barley powdery mildew fungus *B. graminis* f. sp. *hordei*, race A6, was inoculated on the adaxial side of primary or secondary barley leaves, giving a density of 5 conidia mm^{-2} for macroscopic evaluation of pustule development, 20 conidia mm^{-2} for microscopic analysis, or 100 conidia mm^{-2} for gene-expression studies. For tissue-specific gene-expression studies, the abaxial side of primary leaves was inoculated and later peeled off. For gene-expression analysis as well as microscopic and macroscopic inspection, intact barley plants were inoculated.

RNA extraction and expression analysis.

Total RNA was extracted from frozen plant material using TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. For semiquantitative two-step RT-PCR, 2 to 5 μg of total RNA from each sample was reverse transcribed into cDNA using the QuantiTect reverse transcription kit (Qiagen GmbH, Hilden, Germany). Semiquantitative two-step RT-PCR reactions were performed with cycling conditions that were selected to avoid overcycling (Table 1).

VIGS.

A 450-bp cDNA fragment of *BI-1* (GenBank accession number AJ290421) was amplified by PCR using specific primers 5'-CAGCGGCCGCCACGACTCCCTCAAGAACT-3' and 5'-CATATTAATTAACCAGAGCAGGATCGACA-3' and then introduced in anti-sense orientation into a γ -RNA-based BSMV vector (Holzberg et al. 2002; Lacomme et al. 2003; Hein et al. 2005) to produce BSMV.BI-1as (Fig. 2A). BSMV.GFP (Haupt et al. 2001; Hein et al. 2005) was used as control of BSMV infection. In vitro transcription from cDNA clones and preparation of BSMV infectious RNA as well as virus inoculation of barley cv. Clansman plants was performed as described by Holzberg and associates (2002) and Hein and associates (2005). BSMV noninfected plants (mock) served as an additional control.

For *B. graminis* f. sp. *hordei* microcolony development assessment after VIGS, 5-cm segments of third, fourth, or fifth leaves of BSMV- and mock-inoculated barley plants (cv. Clansman) were detached and inoculated with spores of a virulent *B. graminis* f. sp. *hordei* isolate CH4.8, giving a density of 15 to 20 conidia cm^{-2} as previously described (Hein et al. 2005). Remaining segments of the same leaves were sampled in order to measure the endogenous *BI-1* mRNA levels. RNA was extracted from each individual sample using the RNeasy plant mini-kit (Qiagen, Crawley, U.K.). Semiquantitative RT-PCR was performed to monitor *BI-1* expression levels as previously described (Lacomme et al. 2003; Hein et al. 2005). Constitutively expressed α -2 *TUBULIN* (GenBank accession number Y08490) served as internal control and was amplified using specific primers 5'-CAGGCTTGTTTCTCAGGTCA-3' and 5'-ATTCAGAGCACCGTCAAACC-3', giving a fragment of 64 bp. Primers 5'-ATGGACGCCTTCTACTCGACCTC-3' and 5'-CAAAGATGGACGTGACAAACTGC-3' were used to amplify a *HvBI-1*-specific fragment of 502 bp. After gel-electrophoretic separation, ethidium bromide-stained gels were photographed and pixel intensities were measured using the software ImageJ, which is available online from the National Institutes of Health. Mean intensities of *BI-1* signals were normalized against mean intensities of α -2 *TUBULIN* signals and displayed as percentage of *BI-1* expression relative to α -2 *TUBULIN* expression.

TIGS.

A 402-bp *BI-1* cDNA fragment was amplified by PCR using gene-specific primer 5'-TAGTTCCGACACAAGCGAGC-3' and universal primer MVR-26 5'-CTCACTAAAGGGAACAAAGCTGGAG-3' and inserted in antisense orientation into

Table 1. Primers and polymerase chain reaction (PCR) conditions for gene expression analyses by semiquantitative reverse-transcription PCR

Identity (accession no.)	5' and 3' primer sequences	Annealing temperature (°C)	Cycle number	Product size (bp)
UBIQUITIN (M60175)	ACCCTCGCCGACTACAACAT CAGTAGTGGCGGTCTGAAGTG	...	...	...
RUBISCO (U43493)	TCCTTTCCAGGGGCTCA GAGGCAAGGCACCCACT	60	26	263
PR1b (Z26333)	AAGCTGCAAGCGTTCGCC AGGTGTTGGAGCCGTAGTC	...	...	...
PDI (L33252)	GAGCCAAGGGGCTTCCAGT TGCTCAGAGCTCATCTTCA	58	17	223
BI-1 (AJ290421)	GTCCACCTCAAGCTCGTTT ACCCTGTCACGAGGATGCTT	...	...	...
OXLP (X93171)	GGCCGACATGCATTACACAG CATCTGATATTGCTGGGTCTG	60	25	184
BCI3 (AJ628169)	GTCATCGACCAAGCCATC GTACGCGTCGAAGCTCGT	...	...	...
CRT3 (AK248906)	TGCGATGACCCGGAAT TCCGTCGCTCTCCTTCC	58	27	269
PR5 (AJ276225)	CGGCGGTGCTCTTCC GGCGGGCACGTTGAT	...	...	...
		57	25	506
		58	24	165
		55	35	154
		60	25	164

the pIPKTA38 Gateway compatible entry vector (Douchkov et al. 2005). The *BI-1* cDNA fragment was then introduced as inverted repeats into the pIPKTA30N destination vector (Douchkov et al. 2005) by a standard Gateway LR reaction. The resulting RNAi construct pIPKTA30N-BI-1 thus contained inverted repeats of BI-1 separated by the second intron of the wheat *RGA2* gene under the control of the strong constitutive CaMV 35S promoter (Fig. 2C).

Ballistic transformation of epidermal cells of barley cv. Golden Promise leaf segments was performed using a PDS-1000/He plant transformation gun with hepta-adaptor (Bio-Rad Laboratories GmbH, Munich), as described previously (Douchkov et al. 2005). For each shot, 1-µm gold particles were coated with 7 µg of *pUbiGUS* (*uid*a, *GUS* gene from *Escherichia coli* under control of the maize ubiquitin1 promoter with first intron) as transformation marker, together with 7 µg of empty pIPKTA30N vector, pIPKTA30N-BI-1, or pIPKTA36 (*MLO* RNAi). Leaf segments were bombarded with coated gold particles 72 h prior to inoculation with spores of *B. graminis* f. sp. *hordei* isolate CH4.8. At 40 HAI, GUS activity staining was performed. For this, leaves were vacuum infiltrated with a GUS-staining solution containing the GUS-substrate 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid (Biosynth AG, Staad, Switzerland) and 1.4 mM K-hexacyanoferrate(II) and K-hexacyanoferrate(III) each, and then incubated overnight at 37°C as described by Schweizer and associates (1999). Leaves were destained in 7.5% (wt/vol) trichloroacetic acid and 50% methanol for 5 min and then examined under the light microscope. The susceptibility index was calculated as number of penetrated GUS cells divided by the total number of GUS cells multiplied by 100. In each individual experiment, between 100 and 340 GUS-expressing cells were evaluated per variant. All together, six independent experiments were conducted.

Simultaneous overexpression of *MLO* and knockdown of *BI-1*.

For simultaneous *MLO* overexpression and *BI-1* TIGS, we ballistically transferred 0.5 µg per shot of an *MLO* overexpression construct (pGY-1-MLO) (Eichmann et al. 2004) together with 1 µg per shot of either TIGS vector pIPKTA30N-BI-1 or empty vector pIPKTA30N into epidermal cells of the *B. graminis* f. sp. *hordei*-resistant barley backcross line BCPallas-*mlo5*. Control cells were cobombarded with 0.5 µg per shot of empty pGY-1 vector and 1 µg per shot of pIPKTA30N-BI-1. GFP served as transformation marker. Three independent experiments were conducted.

Transient knockdown of *GFP-BI-1* expression.

Barley cv. Ingrid epidermal cells were transiently transformed by particle bombardment with 1 µg per shot of the RNAi vector pIPKTA30N-BI-1 or empty vector pIPKTA30N together with 1 µg of the pGY-1-GFP-BI-1 (GFP-BI-1 under the control of the constitutive CaMV 35S promoter) construct. As reporter gene, 0.5 µg of pGY-1-DsRED was simultaneously cotransformed. Two days after bombardment, *DsRED*-expressing cells were inspected for presence or absence of GFP-BI-1 fluorescence by microscopy; 100 cells per variant were examined in three independent experiments.

Cell-death assay.

For the examination of cellular viability after TIGS, we used particle bombardment to deliver 7 µg per shot of RNAi vector pIPKTA30N-BI-1 or empty vector pIPKTA30N together with 7 µg of the *B-PeruCl1*-expression plasmid pBC17 (Schweizer et al. 2000) and pGY-1-GFP into epidermal cells of barley cv. Golden Promise primary leaves. Five days after

transformation, GFP-expressing cells were examined for the presence or absence of red anthocyanin pigments (Schweizer et al. 2000) using fluorescence and bright-field microscopy, respectively. Three independent experiments were conducted with and without inoculation, respectively. Inoculation was conducted 3 days after transformation and 2 days prior to microscopic inspection. In each individual experiment, between 120 and 340 GFP-expressing cells were evaluated per variant.

Examination of basal powdery mildew resistance on transgenic *BI-1* RNAi barley plants.

Binary pLH6000-BI-1 RNAi vector was constructed by introducing a *SalI* fragment containing inverted repeats of the full-length *BI-1* cDNA, which are separated by the second intron of the wheat *RGA2* gene, into the vector pUbi-ABM (DNA Cloning Service, Hamburg, Germany) between the promoter of the maize *UBIQUITIN1* gene with first intron and the terminator of the *Agrobacterium tumefaciens* *NOPALINE SYNTHASE* gene. The resulting *Ubi1p-BI-1-Nost* RNAi cassette was then transferred into the pLH6000 vector (GenBank accession number AY234328) (DNA Cloning Service) using *SfiI* restriction sites, resulting in pLH6000-BI-1 RNAi (Fig. 5A), which was introduced into *Agrobacterium* strain AGL-1. Stably transgenic barley lines were then generated using immature embryos of cv. Golden Promise according to Hensel and co-workers (2008).

Per experiment, 10 T1 plants of each independent transgenic line were grown along with control plants of the nontransgenic parental line Golden Promise. First leaves were used to test for the presence of the transgene by genomic PCR after DNA extraction with the REDExtract-N-Amp plant PCR kit (Sigma-Aldrich Chemie GmbH, Munich) (Babaeizad et al. 2009), using primers 5'-GGATAGCCCTCATAGATAGAGTACTAAC TAA-3' (*RGA2* intron-specific) and 5'-ACCCTGTCACGAGG ATGCTT-3' (*BI-1*-specific). Alternatively, segments of first leaves were tested for their ability to stay green after 7 days on hygromycin-containing (hygromycin B at 200 mg liter⁻¹) 0.5% water agar plates, which is conferred by the *HYGROMYCIN PHOSPHOTRANSFERASE* gene on the T-DNA. Second leaves were then used for *B. graminis* f. sp. *hordei* resistance assays. For this, leaf segments were placed on 0.5% water agar plates and then inoculated with 3 to 5 conidia mm⁻². At 5 to 7 days after inoculation, the number of pustules per centimeter of leaf segment was assessed.

Analysis of *BI-1* expression in RNAi barley plants using semiquantitative one-step RT-PCR.

For semiquantitative analysis of *BI-1* expression, total RNA of plants of pLH6000-BI-1 RNAi lines BG37/2E05, BG37/2E10, BG37/2E11, and GP WT was extracted (described above). To check for equal RNA concentrations, 2 µg of total RNA was loaded onto a denaturing agarose gel (Fig. 5B). Semiquantitative one-step RT-PCR was performed using the OneStep RT-PCR Kit (Qiagen GmbH), according to the manufacturer's instructions. Primers 5'-GTCCACCTCAAGCTCG TTT-3' and 5'-ACCCTGTCACGAGGATGCTT-3' were used to examine *BI-1* expression and 5'-ACCCTCGCCGACTACA ACAT-3' and 5'-CAGTAGTGCGGGTCAAGTG-3' were used for the analysis of constitutively expressed *UBIQUITIN* (GenBank accession number M60175).

Analysis of *BI-1* expression in RNAi barley plants using quantitative real-time PCR.

Quantitative real-time PCR was performed to analyze *BI-1* expression in stably transgenic pLH6000-BI-1 RNAi plants. Total RNA of four plants of pLH6000-BI-1 RNAi line BG37/2E10 and GP WT was extracted (described above) and then

pooled. Total RNA (1 µg) of each barley line was converted to cDNA template using the QuantiTect reverse transcription kit (Qiagen GmbH) according to the manufacturer's instructions. Control reactions confirmed that no interfering genomic DNA was present. Primers 5'-GTCCACCTCAAGCTCGTTT-3' and 5'-ACCTGTACGAGGATGCTT-3' were used to amplify a *BI-1* cDNA fragment (GenBank accession number AJ290421) (Deshmukh et al. 2006). Primers 5'-TCTCGTCCCTGAGATTGCCACAT-3' and 5'-TTTCTCGGGACAGCAACAATCTTCT-3' were used to amplify a barley *UBIQUITIN CONJUGATING ENZYME 2* (*UBC2*, GenBank accession number AY220735) (Jensen et al. 2007) cDNA fragment, which served as endogenous control to normalize variations in quantity of cDNA. Primer pairs were tested for similar amplification efficiency. Triplicate quantitative RT-PCR analyses were then performed using SsoFast EvaGreen Supermix (Bio-Rad Laboratories GmbH) with 100 ng of cDNA each. PCR was carried out on the Mx3005P Real-Time PCR System (Stratagene, La Jolla, CA, U.S.A.) under the following conditions: 95°C for 10 min and 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. All products were subjected to melting curve analysis. Relative gene expression differences were calculated using the comparative cycle threshold (C_T) method ($\Delta\Delta C_T$) (Libault et al. 2007).

Histological staining and microscopic analysis of stably transgenic BI-1 RNAi plants.

Second leaves of PCR-tested *BI-1* RNAi plants of line BG37/2E10 as well as corresponding azygous controls that had lost the transgene due to segregation (PCR negative plants) and parental line Golden Promise were inoculated with spores of *B. graminis* f. sp. *hordei* and, 30 h later, stained with DAB for 6 h to detect whole-cell H_2O_2 accumulation (Thordal-Christensen et al. 1997; Hükelhoven et al. 1999). Leaves of intact plants were inoculated. Leaves were destained and fixed as described previously (Hükelhoven and Kogel 1998). For bright-field microscopy, fungal structures were stained with acetic ink (10% [vol/vol] blue ink in 25% acetic acid). Successful fungal penetration was indicated by haustorium formation and development of elongated secondary hyphae. Nonpenetrated cells either survived fungal attack (nonpenetrated living cells) or displayed HR, as indicated by whole-cell DAB staining without visible haustorium formation.

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