The Barley ApoptosisSuppressor Homologue 
Bax Inhibitor-1 Compromises 
Nonhost Penetration Resistance of Barley to the 
Inappropriate Pathogen Blumeria graminis f. sp. tritici 

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BAX inhibitor-1 (BI-1) proteins have been characterized as suppressors of programmed cell death in mammals and plants. The barley BI-1 is a suppressor of nonspecific background resistance and mlo-mediated penetration resistance to the biotrophic fungal pathogen Blumeria graminis f. sp. hordei when overexpressed in epidermal cells of barley. We report here that BI-1 expression is also slightly up-regulated during interaction with the inappropriate wheat pathogen Blumeria graminis f. sp. tritici. Significantly, overexpression of BI-1 in single epidermal cells of barley by microprojectile-mediated transformation rendered cells susceptible to penetration by inappropriate B. graminis f. sp. tritici. The degree of transgene-induced accessibility to B. graminis f. sp. tritici was thereby similar to the effect achieved by overexpression of the defense suppressor gene Mlo and could not be further enhanced by double expression of both BI-1 and Mlo. Confocal laser scanning microscopy was used to locate a functional green fluorescing GFP:BI-1 fusion protein in endomembranes and the nuclear envelope of barley epidermal cells. Together, enhanced expression of barley BI-1 suppresses penetration resistance to B. graminis f. sp. tritici, linking barley nonhost resistance with cell death regulation. 

Additional keywords: caspase, hypersensitive cell death reaction. 

Nonhost resistance prevents plants from infection by pathogens that can propagate on other plant species and, hence, barley from the invasion by inappropriate formae speciales of the grass powdery mildew fungus Blumeria graminis (DC) Speer (syn. Erisiphe graminis DC) (Heath 2000a; Tosa et al. 1990). Nonhost resistance is the most common form of durable disease resistance in plants. Still, little is known about the genetic background and regulation of participating defense mechanisms, and the contribution of resistance (R) gene-like host factors to nonhost resistance may differ for each plant-microbe interaction (Heath 2000a). Recently, it was demonstrated that genetic loss of EDS1 function together with pharmacological inhibition of actin rearrangement is sufficient to induce susceptibility of the nonhost Arabidopsis to the wheat powdery mildew fungus (B. graminis f. sp. tritici). This demonstrated that active host defense rather than lack of basic compatibility factors determines Arabidopsis nonhost resistance to powdery mildew (Yun et al. 2003).

In inappropriate cereal–cereal powdery mildew interactions a couple of R gene like host factors appear to be functional since crosses of different formae speciales result in populations segregating for avirulence on former nonhost plants (Matsumura and Tosa 1995). Nonhost resistance of barley to B. graminis f. sp. tritici comes along with H2O2 accumulation, penetration resistance accompanied by formation of cell wall appositions (papillae) at sites of fungal penetration attempts, and a hypersensitive cell death reaction (HR) of single attacked cells (Hückelhoven et al. 2001a; Tosa and Shishiyama 1984). In contrast, basic compatibility is characterized by successful penetration and haustorium formation by appropriate, virulent B. graminis at the majority of interaction sites. Haustorium formation is the crucial step in the establishment of a compatible interaction. It leads to the formation of secondary hyphae, secondary penetration attempts, and eventually to the formation of new conidiophores and spores. Nevertheless, even susceptible hosts are not fully accessible to B. graminis but express different degrees of background resistance that is mainly achieved by penetration defense. Completely efficient defense mechanisms can be observed in mlo-mediated (Mlo mutant allele) broad-spectrum resistance or R gene-dependent resistance of barley to avirulent races of the barley powdery mildew fungus B. graminis f. sp. hordei that also trigger HR (Hückelhoven et al. 1999; Kita et al. 1981; Peterhansel et al. 1997). However, the importance of papilla formation for penetration resistance to powdery mildew fungi is unclear, since papillae are often inefficient, and surprisingly, lack of papilla-callose in pmr4 or glucan synthase 5 mutants leads to enhanced powdery mildew resistance in Arabidopsis (Jacobs et al. 2003; Nishimura et al. 2003; Zeyen et al. 2002).

Although proof is lacking that cell death is essentially required for resistance, it is presumed that HR, which involves a programmed cell death (PCD) of one or a few attacked cells, is an effective mechanism to stop development of biotrophic pathogens. Additionally, the observation that Arabidopsis pen1 mutants impaired in nonhost penetration resistance to B. graminis f. sp. hordei show high frequencies of HR as a second line of defense underlines the importance of HR in resistance to powdery mildew fungi (Collins et al. 2003). Despite many differences between animal and plant PCD regulation, there are some common elements in both systems, such as the involvement of reactive oxygen intermediates (ROI), cysteine proteases, and DNA fragmentation (Heath 2000b; Lam et al. 2001). 

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expression was detected that slightly increased with leaf age. Upon inoculation with *B. graminis* f. sp. *tritici*, we observed a slightly increased *BI-1* transcript accumulation (Fig. 1B).

**BI-1 overexpression compromises penetration resistance of barley against *B. graminis* f. sp. *tritici***

To examine whether BI-1 was involved in defense mechanisms in the nonhost system barley–*B. graminis* f. sp. *tritici*, we performed transient *BI-1* overexpression in barley cv. Manchuria by biolistic transformation and subsequent microscopic evaluation of the interaction of *B. graminis* f. sp. *tritici* with epidermal cells cotransformed with *BI-1* and the green fluorescent protein reporter gene (GFP). Both genes were controlled by the cauliflower mosaic 3S promoter. In five independent experiments, overexpression of *BI-1* led to significantly (*P* < 0.001, Student’s *t* test) enhanced PE of *B. graminis* f. sp. *tritici* in transformed cells. The average PE increased from 2% in control cells transformed with GFP and empty vector to 24% in GFP and *BI-1*–transformed cells (Fig. 2A). This effect was less strong when we used the barley cv. Ingrid instead of cv. Manchuria. Since cv. Ingrid shows early cell death in nearly all cells penetrated by *B. graminis* f. sp. *tritici* (Peterhansel et al. 1997), we used the maize ubiquitin promoter-controlled *uidA* reporter gene and β-glucuronidase (GUS) activity-staining instead of the vital GFP reporter to identify transformed cells. In three independent experiments, *BI-1* overexpression shifted *B. graminis* f. sp. *tritici* PE from less than 1 to 5%. We found a similar level of *BI-1*-induced accessibility when we used the backcross line BCIngrid-mlo5 (I22) (Fig. 2B) that is completely resistant to penetration by appropriate *B. graminis* f. sp. *hordei* (Jørgensen 1994; Hückelhoven et al. 1999; Peterhansel et al. 1997). Interestingly, nontransformed cv. Manchuria is slightly accessible to penetration by *B. graminis* f. sp. *tritici* A95, whereas cv. Ingrid is almost completely resistant (Hückelhoven et al. 2001a; Trujillo et al. 2004).

**Simultaneous overexpression of *BI-1* and Mlo.**

To investigate potential synergistic effects of *BI-1* and MLO on the penetration rate of *B. graminis* f. sp. *tritici* to barley epidermal cells, *pBI-1* and *pMlo* were simultaneously delivered through biolistic transformation. We used the GUS activity-staining to identify transformed cells in cv. Manchuria. In these experiments, we observed a general tendency for higher

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**RESULTS**

**Expression of barley *BI-1* in response to the wheat powdery mildew fungus.**

In previous studies, we demonstrated that a barley *BI-1* homologue is differentially expressed in barley leaves upon attack by *B. graminis* f. sp. *hordei* in both susceptible *Mlo* and penetration-resistant mlo5 lines (Hückelhoven et al. 2001b; 2003). Here, we examined *BI-1* expression in the incompatible interaction of barley cultivar Manchuria with the wheat powdery mildew fungus (*B. graminis* f. sp. *tritici*) at early interaction stages. Expression was studied in first leaves densely inoculated with conidia of powdery mildew isolate *B. graminis* f. sp. *tritici* A95 during two days after inoculation. As a positive control for defense-related gene expression, we performed Northern analysis with the pathogenesis-related protein 1b (*PR-1b*) (Bryngelsson et al. 1994). *PR-1b* was expressed upon inoculation with *B. graminis* f. sp. *tritici* (Fig. 1A). The same RNA was used for one-step reverse-transcription-polymerase chain reaction (RT-PCR) to analyze *BI-1* expression. As demonstrated previously (Hückelhoven et al. 2003), a constitutive *BI-1* expression was induced in barley leaves upon inoculation with *B. graminis* f. sp. *tritici*. Upon inoculation with *B. graminis* f. sp. *tritici*, we observed a slightly increased *BI-1* transcript accumulation (Fig. 1B).

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**Fig. 1. *BI-1* expression in the nonhost interaction of barley with *Blumeria graminis* f. sp. *tritici*.** A. Gel blot analysis of pathogenesis-related gene 1b (*PR-1b*) transcript as control for defense related gene expression. Total RNA was isolated from barley (cv. Manchuria) first leaves inoculated with conidia of *B. graminis* f. sp. *tritici* and mock-inoculated control leaves 4, 12, 24, and 48 h postinoculation (hpi). Equal loading of RNA (5 μg) was checked by ethidium bromide staining of rRNAs. B. Using the same RNA, one-step reverse-transcription-polymerase chain reaction (RT-PCR) analysis was performed. An actin-like cDNA fragment was amplified under specific conditions during 25 PCR cycles as control. RT-PCR for *BI-1* was carried out with 30 cycles under specific conditions. Inverted pictures from ethidium bromide-stained gels are shown.
penetration rates in Mlo- or Bl-1-overexpressing cells, when compared with experiments with a GFP reporter construct. This might reflect a better coexpression of transgene and reporter when they are controlled by different promoters or detection of penetrated dead cells that might not be seen with the GFP reporter. To estimate individual effects of Bl-1 and Mlo, we bombarded barley first leaves with the respective construct together with empty pGY-1 vector. We found that both MLO and Bl-1 promoted fungal entry into barley epidermal cells but that coexpression of both genes did not further enhance accessibility achieved by individual expression (Fig. 3A). This appeared not to be a promoter competition effect, because we observed higher penetration rates when we enhanced amounts of either pMlo or pBI-1 plasmids coated on the microparticles for transformation. However, when we evaluated distinct and simultaneous MLO and Bl-1 effects seven days after inoculation, we detected improved fungal development in cells expressing both genes. More Bl. graminis f. sp. hordei germlings were able to establish haustoria and to develop elongated secondary hyphae (ESH) when Bl-1 and MLO were overexpressed together (38% of transformed cells) compared with single gene overexpression (approximately 25%) (Fig. 3B). Although Bl-1 performed similarly to MLO in inducing initial accessibility to Bl. graminis f. sp. hordei, the overexpression of Bl-1 and MLO together appeared to allow development of longer ESH than overexpression of MLO or Bl-1 alone. Occasionally, we detected formation of conidiophores and new conidia on cells overexpressing either Bl-1, MLO, or both. However, this occurred in less than 1% of transformed cells.

Localization of a GFP:Bl-1 fusion protein.

To monitor the intracellular localization of the barley Bl-1 protein, GFP was fused to the N-terminus of Bl-1. Subsequently, this construct was introduced into barley epidermal cells via particle bombardment. As a marker for protein localization in the cytosol and nucleoplasm, cells were cotransformed with DsRed (Dietrich and Maiss 2002). GFP:Bl-1 fluorescence was definitely distinguishable from that of DsRed. It appeared in a fine, net-shaped structure within the cytoplasm, indicative of endoplasmic reticulum (ER) (Fig. 4A through C). In single confocal sections, GFP:Bl-1 was clearly detectable in the nuclear envelope but did not enter the nucleus (Fig. 4D through F), which corresponds with findings of Kawai-Yamada and associates (2001) in Arabidopsis and yeast cells and of Bolduc and associates (2003) in tobacco BY-2 cells. To confirm proper localization, we controlled whether the GFP:Bl-1 fusion construct was functional in defense regulation of cv. Manchuria against Bl. graminis f. sp. tritici. Therefore, we performed transformation of barley epidermal cells with GFP:Bl-1 together with either GFP or GUS as additional reporters, because GFP:Bl-1 fluorescence was comparably

Fig. 2. Overexpression of Bl-1 abolishes nonhost penetration resistance. A, Penetration efficiency (PE) of Blumeria graminis f. sp. tritici to barley (cv. Manchuria) epidermal cells was evaluated at 48 h postinoculation in five independent experiments. M represents average values of independent experiments. PE of Bl. graminis f. sp. tritici was significantly enhanced in cells that were bombarded with pBI-1 compared with cells that were transformed with empty control pGY-1. Bars represent standard errors. GFP served as cotransformed reporter to identify transformed cells. B, Average PE of Bl. graminis f. sp. tritici on barley cv. Ingrid and BC Ingrid; mlo5 (I22). PE of Bl. graminis f. sp. tritici was enhanced significantly when epidermal cells were bombarded with pBI-1 compared with cells that were bombarded with empty control pGY-1. Columns represent average values from three independent experiments. Bars represent standard errors. The uidA gene served as a reporter to identify transformed cells by β-glucuronidase staining.

Fig. 3. Separate and simultaneous overexpression of Mlo and Bl-1 confer similar induction of defense suppression. Average penetration efficiency (PE) of Blumeria graminis f. sp. tritici in three independent experiments. Barley cv. Manchuria was bombarded with pMlo and pBI-1 both separately and simultaneously or with control empty vector. A, Overexpression of pMlo and pBI-1, respectively, induced enhanced susceptibility to Bl. graminis f. sp. tritici. Simultaneous overexpression of both genes further enhanced PE slightly but not significantly when evaluated at 48 h postinoculation. B, Overexpression of pMlo and pBI-1, respectively, induces enhanced susceptibility to Bl. graminis f. sp. tritici. Simultaneous overexpression of both genes further enhanced PE when evaluated at 7 days after inoculation. Columns represent average values. Bars represent standard errors.
weak and difficult to observe with standard fluorescence microscopy used to monitor susceptibility. Subsequent evaluation of interactions of transformed cells with germinated conidia of *B. graminis* f. sp. *tritici* revealed that, similar to the results obtained for overexpression of BI-1, the penetration efficiency of the fungus was clearly enhanced in GFP:BI-1 expressing cells compared with control cells. In two independent experiments, penetration efficiency increased from 7% to 23% in the GUS experiment and from 11 to 27% when GFP served as reporter (Fig. 4G).

**DISCUSSION**

In this study, we have shown that the potential cell death regulator BI-1 is involved in resistance during the interaction of barley with the inappropriate pathogen wheat powdery mildew fungus (*B. graminis* f. sp. *tritici*). We demonstrated that BI-1 expression is induced upon inoculation with *B. graminis* f. sp. *tritici*. A functional GFP:BI-1 fusion protein is localized in the nuclear envelope and, most likely, the ER of barley epidermal cells. Importantly, overexpression of BI-1 significantly compromised penetration resistance of barley against *B. graminis* f. sp. *tritici*, thus BI-1 besides MLO is a plant protein that breaks penetration resistance in a cereal-cereal powdery mildew nonhost interaction.

In animals, BAX protein can induce pore formation in the outer mitochondrial membrane, leading to cytochrome c release into the cytosol and subsequent activation of a cell death–inducing caspase cascade (Green and Reed 1998; Loeffler and Kroemer 2000). Although BAX and other proapoptotic members of the Bcl-2 family are apparently not present in plants, other factors regulating cytochrome c release, such as Ca**2+** and reactive oxygen intermediates, are involved in plant PCD. Cytochrome c release can be observed in plant PCD, but its relevance is not clear (Balk et al. 1999; Jones 2000; Yu et al. 2002).

Plant BI-1 is predicted to be a transmembrane protein with seven transmembrane domains (Bolduc et al. 2003; Hückelhoven et al. 2003). In accordance with other reports, we found a GFP:BI-1 fusion protein expressed in barley epidermal cells to be predominantly localized to the nuclear envelope and a net-shaped structure within the cytoplasm, most probably representing the ER. Similar patterns of BI-1 distribution in plant and yeast cells were found by other researchers (Bolduc et al. 2003; Kawai-Yamada et al. 2001). Besides computer analyses already published (Bolduc et al. 2003; Hückelhoven et al. 2003), two findings support that the Bax Inhibitor-1 C-terminus is outside of the ER. First, Bolduc and associates (2003) found that digitonin digestion of tobacco cells, which permeabilizes the plasma membrane but not endomembranes, allowed immunodetection of BI-1-GFP (C-terminal GFP fusion) with GFP antibodies in the ER. Second, barley and other BI-1 proteins possess motifs close to the C-terminus related to RXR, KXXX, or both, representing structures that typically mediate ER retention of membrane proteins with the C-terminus in the cytoplasm (Hückelhoven et al. 2003; Shikano and Li 2003). Interestingly, the charged C-terminus of BI-1, which might be involved in protein-protein interaction, is important for cell death regulation (Yu et al. 2002). Since human BI-1 does not physically interact with BAX and no clear association with mitochondria could be demonstrated (Bolduc et al. 2003; Kawai-Yamada et al. 2001; Xu and Reed 1998), a physical interaction of BI-1 and mitochondria seems unlikely. However, BI-1 can interact with other human cell death regulators, e.g., Bcl-2 and Bcl-X**L**, and might affect other signaling molecules downstream of mitochondria as well, thereby monitoring mitochondrial functions without physical interaction with these

organelles. Taking into account that the ER functions as a calcium reservoir and that BI-1 shows a possible ion channel activity, it is possible that the protein could be implicated in the regulation of either cytosolic calcium (Bolduc et al. 2003), the redox status (Hückelhoven et al. 2003), or both.

The MLO protein is a modulator of defense responses to different pathogens and depends on calmodulin binding in *B. graminis* f. sp. *hordei* susceptibility (Kim et al. 2002). Functional comparison of BI-1 and MLO revealed strong similarities. The *Mlo* and BI-1 genes are similarly expressed in response to wounding, pathogen challenge, and reactive oxygen intermediates as well as during leaf aging (Hückelhoven et al. 2001b, 2003; Piffanelli et al. 2002; Sanchez et al. 2000). In barley, overexpression of both the BI-1 and the MLO genes confers supersusceptibility to *B. graminis* f. sp. *hordei*, and both genes, when overexpressed in *mlo*-genotypes, restore accessibility of epidermal cells to *B. graminis* f. sp. *hordei*. In breaking mlo-mediated penetration resistance, the effect of *Mlo* under control of the maize ubiquitin promoter appears to be about twice as strong (73%) as the effect of BI-1 under 35S (30%) (Hückelhoven et al. 2003; Kim et al. 2002). In our hands, expression of *Mlo* under control of 35S in *mlo*-barley results in about 60% susceptibility to penetration by *B. graminis* f. sp. *hordei* (R. Eichmann, H. Schultheiss, K.-H. Kogel, and R. Hückelhoven, unpublished data). Additionally, transient silencing of *Mlo* by dsRNA interference leads to 50% increased penetration resistance to *B. graminis* f. sp. *hordei* in susceptible barley

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**Fig. 4.** Localization of a GFP:BI-1 fusion protein. A through C, Confocal laser scanning whole cell projection of a barley epidermal cell transiently expressing GFP:BI-1 together with DsRed. The plasmids were coated on microprojectiles and were delivered into barley leaf epidermal cells. Scale bar = 12 μm. By 72 h after transformation, distribution of GFP:BI-1 (A and D) and DsRed fluorescence (B and E) was detected. D through F, Single confocal section of the nuclear region of a transformed cell. Scale bar = 6 μm. C and F represent the respective merged images. G, The GFP:BI-1 fusion protein is functional in defense suppression. Epidermal cells of barley cv. Manchuria were bombarded with pGFP:BI-1 or empty control pGY-1 plus either pGUS or pGFP as reporter gene constructs, respectively. Penetration efficiency of *Blumeria graminis* f. sp. *tritici* was evaluated in one experiment each.
(Schweitzer et al. 2000), whereas transient silencing of BI-1 is less effective (Hückelhoven et al. 2003). Together in appropriate interactions, MLO has an apparently stronger effect as compared with BI-1. However, these results need confirmation by stable transgenic plants expressing BI-1 dsRNA.

The mlo-mediated defense mechanism seems also to be active in the nonhost interaction with B. graminis f. sp. tritici, because some isolates of B. graminis f. sp. tritici are restricted earlier on mlo-barley as compared with Mlo-barley, in which these isolates paradoxically trigger a cell death response (Hückelhoven et al. 2001a; PeterhänSEL et al. 1997). This is explainable because functional MLO acts as a suppressor of penetration resistance and developmental cell death but not as a suppressor of R gene-mediated HR that might be involved in postpenetration defense (Matsumura and Tosa 1995; PeterhänSEL et al. 1997). Vice versa, overexpression of functional MLO suppresses penetration resistance in the nonhost interaction of barley and wheat with inappropriate formae speciales of B. graminis (Elliott et al. 2002). As demonstrated here, the same holds true for BI-1 when it is overexpressed in barley epidermal cells attacked by B. graminis f. sp. tritici. When expressed from its endogenous promoter however, expression of BI-1 induced by B. graminis f. sp. tritici (Fig. 1B) is either misplaced or insufficient to induce accessibility to the fungus. The slightly enhanced penetration efficiency of B. graminis f. sp. tritici detected 48 h after inoculation in cells simultaneously overexpressing Mlo and BI-1, compared with the discrete effects of the single genes, does not argue for a synergistic effect in initial cellular accessibility. Rather, both proteins appear to target a similar mechanism. We speculate that BI-1 interferes with the MLO pathway downstream of MLO (Hückelhoven et al. 2003). This is supported by the fact that BI-1 overexpression weakens penetration resistance to B. graminis f. sp. tritici even in the mlo5 genotype, which does not express MLO. BI-1 expression in BCIgrid-mlo5 induced more than 20% susceptibility to B. graminis f. sp. hordei (Hückelhoven et al. 2003) but only 7% susceptibility to B. graminis f. sp. tritici (Fig. 2B), demonstrating that BI-1 is more efficient when an appropriate fungus attacks. However, the fact that simultaneous overexpression of BI-1 and MLO slightly enhanced fungal development at late-infection time-points (corresponding roughly to 6 days after penetration) indicates a possible teamwork of both proteins in postpenetration defense suppression and maintenance of single-cell compatibility (Fig. 3B). However, we observed little sporulation of B. graminis f. sp. tritici on MLO or BI-1 overexpressing cells. This shows that both proteins, either alone or together, were insufficient to abolish postpenetration defense completely. Since postpenetration defense can be assumed to be mediated by R gene-like factors, one should consider that BI-1 does not suppress R gene-dependent resistance to B. graminis, such as Mla-mediated resistance. This is supported by the observation that BI-1 mRNA levels strongly increased during Mlg- and Mla12-dependent HR to B. graminis f. sp. hordei and that overexpression of BI-1 does not seem to induce susceptibility of Mla12-barley to avirulent B. graminis f. sp. hordei (Hückelhoven et al. 2001b, 2003; and R. Eichmann, H. Schultheiss, K.-H. Kogel, and R. Hückelhoven, unpublished data). Interestingly, both barley mlo-mediated and Arabidopsis nonhost resistance but not barley Mla-mediated resistance depend on ROR2/PEN1, a syntaxin-like protein that appears to be involved in apoplastic defense as part of the membrane dynamic machinery (Collins et al. 2003; PeterhänSEL et al. 1997).

The observation that BI-1 affects penetration resistance more strongly in barley cultivar Manchuria than in cv. Ingrid might be explained by a lower background penetration resistance of cv. Manchuria to B. graminis f. sp. tritici and B. graminis f. sp. hordei that we detected microscopically after inoculation of 7-day-old plants (Trujillo et al. 2004).

Animal and plant PCD have long been subject to intense studies. Comparative analysis revealed clear differences between the kingdoms but also some common features. Importantly, mammalian cell death regulators when expressed in plants accomplish a death-promoting or -repressing function analogous to that in animals, indicating evolutionarily conserved pathways of cell death control in eukaryotes (Dickman et al. 2001; Lacomme and Santa Cruz 1999; MitsuHARA et al. 1999). Especially in regard to the control of necrotrophic pathogens, strong interest in the regulatory mechanisms of plant cell death has emerged. It seems that pathogens take advantage of signal transduction pathways of their host plant. For example, Sclerotinia sclerotiorum establishes a successful pathogenesis in tobacco by activating plant PCD. Conversely, expression of anti-apoptotic proteins that inhibited this cell death pathway protected tobacco plants from fungal infection (Dickman et al. 2001). Moreover, expression of the baculovirus apoptosis-inhibitor gene p35 in tomato protects against cell death induced by the Alternaria alternata f. sp. lycopersici mycotoxin and against disease caused by Alternaria alternata, Colletotrichum coccodes, and Pseudomonas syringae pv. tomato (Lincoln et al. 2002). Vice versa, it is conceivable that biotrophic microbes utilize plant cell-survival mechanisms to support their lifestyle. In this regard, it is interesting that expression of p35 in resistant NN tobacco delayed HR to tobacco mosaic virus inoculation and provoked systemic spreading of the virus (del Pozo and Lam 2003).

It has been discussed that the biotrophic barley powdery mildew fungus might target the barley MLO protein in order to suppress plant defense (Panstruga and Schulze-Lefert 2002). Barley mlo genotypes are characterized by a more frequent occurrence of both spontaneous and B. graminis f. sp. hordei–induced secondary mesophyll cell death (Jorgensen 1994; PeterhänSEL et al. 1997; Piffanelli et al. 2002; Schulze-Lefert and Vogel 2000). Although mlo-mediated resistance prevents penetration and does not rely on epidermal HR, it is inhibited by overexpression of the potential cell death suppressor BI-1 in epidermal cells. This provides further evidence for a link between susceptibility to biotrophs and cell survival (Hückelhoven et al. 2003). Together, cell death regulation appears to be crucial in penetration resistance and establishment of basic compatibility of plants with both biotrophs and necrotrophs. Thereby, plant survival strategies limiting progression of necrotrophs might support biotrophic lifestyles and vice versa. Support for this hypothesis comes from the fact that B. graminis f. sp. hordei–resistant mlo mutants are extremely susceptible to the rice blast fungus Magnaporthe grisea and to toxic culture filtrates of Cochliobolus sativus (Jarosch et al. 1999; Kumar et al. 2001).

MATERIALS AND METHODS

Plants, pathogen, and inoculation.

The barley (Hordeum vulgare L.) cultivar Ingrid and its corresponding backcross line BCIgrid-mlo5 (I22) were obtained from L. Munk (Royal Veterinary and Agricultural University, Copenhagen, Denmark). Cultivar Manchuria was provided by J. Pons-Kühnemann (Justus-Liebig University, Giessen, Germany).

Plants were grown in a growth chamber at 18°C with 60% relative humidity and a photoperiod of 16 h (60 μmol m⁻² s⁻¹ photon flux density). Barley powdery mildew fungus Blumeria graminis f. sp. tritici isolate A95 was inoculated on barley leaves giving a density of 80 conidia mm⁻² for gene expression studies and 150 conidia mm⁻² for gene function assessment on
transformed leaf segments. *B. graminis* f. sp. *tritici* was maintained on *Triticum aestivum* cv. Kanzler under the same conditions.

RNA extraction and expression analysis.

Total RNA was extracted from 8 to 10 primary leaf segments (5 cm long), using RNA extraction buffer (AGS, Heidelberg, Germany) according to the manufacturer’s instructions. For Northern blots, 5 µg of total RNA from each sample were separated in agarose gels and were blotted by capillary transfer to positively charged nylon membranes. Detection of mRNAs was performed according to the DIG system user’s guide (Roche, Mannheim, Germany) with fluorescein-labeled antisense RNA probes (Hülckelhoven, et al. 2001b). Before immunodetection, blots were washed stringently two times for 20 min in 0.1% (wt/vol) sodium dodecyl sulfate, 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 68ºC.

To detect low level transcripts, we used the OneStep RT-PCR kit (Qiagen, Hilden, Germany) for semiquantitative RT-PCR, following manufacturer’s instructions. To maintain different transcript levels, 25 cycles were used during the exponential amplification phase for *actin* and 30 cycles for *BI-1*. cDNAs were separated in agarose gels. Primers used were: 5’-ctgtaggaagaagctgtagacgg-3’ (5’ primer) and 5’-tcggatcactgcctgacccatc-3’ (3’ primer) for a 758-bp *actin* cDNA fragment (GenBank accession number AJ234400) and 5’-atggacgccttctactcgacctcgg-3’ (5’ primer) and 5’-gcagcactgagcggatcagc-3’ (3’ primer) for a 478-bp *BI-1* cDNA fragment (GenBank accession number AJ290421).

Transient transformation and evaluation of penetration efficiency.

We transformed barley via biolistic delivery of expression vectors into epidermal cells of leaf segments, using a transient transformation protocol originally developed for wheat (Schweizer et al. 1999). Each shot consisted of 312 µg of 1.1-µm tungsten particles with 0.5 µg of *pGFP* (GFP under control of CaMV 35S promoter) or *pUbiGUS* (*uidA*, the GUS gene from *Escherichia coli* under control of the maize ubiquitin promoter) together with 0.8 µg of empty *pGY1* vector or *pBI-1* containing *BI-1* under control of CaMV 35S (Hülckelhoven et al. 2003; Schweizer et al. 1999). In coexpression experiments, 0.5 µg of both *BI-1* and *Mlo* plasmid (both genes under control of CaMV 35S promoter) together with 0.5 µg of *pUbiGUS* as reporter gene were employed for each shot. To check for individual overexpression effects, *BI-1* and *Mlo* were coexpressed with 0.5 µg of empty *pGY1* vector.

Leaf segments were bombarded with coated particles 4 h before inoculation with *B. graminis* f. sp. *tritici* isolate A95. Inoculation with 200 µl conidia mm⁻² led to the attack of approximately 50% of the transformed cells. The outcome of the interaction, i.e., the penetration frequency of germinated *B. graminis* f. sp. *tritici* conidia on transformed epidermal cells, was evaluated subsequently, using fluorescence and bright field microscopy as previously described by Schultheiss and associates (2002). For GUS activity staining, leaves were vacuum-infiltrated with a solution of the substrate for GUS, X-gluc, and were incubated overnight at 37ºC, as described by Schweizer and associates (1999). For each individual experiment, a minimum of 100 interaction sites was evaluated. Penetration efficiency was calculated as the number of penetrated cells divided by the number of attacked cells multiplied by 100 and was used as a measure for resistance of bombarded cells.

Construction of the pGFP:BI-1.

The *BI-1* open reading frame was amplified by PCR, using the primers 5’-gtgtgacgcgctagctgacgagc-3’ (5’ primer, containing a *BamHI* site) and 5’-gtgtgacgcgctagctgacgagc-3’ (3’ primer, containing a *SalI* site) and, subsequently, were cloned into the pGEM-T vector (Promega, Mannheim, Germany). After sequence confirmation, the *GFP*/*BI-1* fragment was cloned into the expression vector pGY1 (Schweizer et al. 1999). The GFP-coding fragment was amplified using the oligonucleotides 5’-gtgtgacgcgctagctgacgagc-3’ (5’ primer, containing a *BamHI* site) and 5’-gtgtgacgcgctagctgacgagc-3’ (3’ primer, containing a *BamHI* site), which eliminates the stop codon, and was inserted in-frame into pGY1-BI-1, using the internal *BamHI* site of the BI-1 forward primer (i.e., at the N-terminal end of BI-1).

For functional examination of the GFP:BI-1 fusion protein, 0.8 µg of *pGFP:BI-1* fusion construct per shot together with 0.5 µg of *pGFP* or 0.5 µg of *pUbiGUS* as reporter gene plasmids were biolistically delivered into barley epidermal cells as described above.

Localization of GFP:BI-1.

Barley leaf segments were transiently transformed via particle bombardment as described above, with each shot delivering 0.5 µg of *DsRed* (pe35AscOptRed; *DsRed*-C1 under control of CaMV 35S promoter, obtained from E. Maiss, University of Hannover, Germany) plasmid together with 0.8 µg of *pGFP:BI-1* fusion construct. *DsRed* was used as control for localization in the cytosol and nucleus (Dietrich and Maiss 2002). Leaf segments were analyzed three days after transformation. Fluorescence of GFP, *DsRed*, and GFP:BI-1 was detected at 505 to 530 nm. *DsRed* was excited by a 488-nm laser line and was detected at 580 to 650 nm.

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LITERATURE CITED


