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ORIGINAL PAPER

Over-expression of the cell death regulator BAX inhibitor-1 in barley confers reduced or enhanced susceptibility to distinct fungal pathogens

Valiollah Babaeizad · Jafargholi Imani · Karl-Heinz Kogel · Ruth Eichmann · Ralph Hückelhoven

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Abstract BAX inhibitor-1 (BI-1) is a conserved cell death regulator protein that inhibits mammalian BAX-induced cell death in yeast, animals and plants. Additionally, HvBI-1 suppresses defense responses and resistance to the powdery mildew fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*) when over-expressed in single epidermal cells of barley. To test the potential of ectopic expression of BI-1 to influence fungal interactions with crop plants, we produced stable transgenic barley plants expressing a green fluorescing protein (GFP) fusion of HvBI-1 under control of the cauliflower mosaic virus 35S promoter. GFP-HvBI-1 plants were fertile and did not display obvious developmental alterations when compared to wild type parents. GFP-HvBI-1 plants were more resistant to single cell death induced by ballistic delivery of a mammalian proapototic BAX expression construct and more susceptible to biotrophic *Bgh*. Microscopic observation of the interaction phenotype revealed that enhanced susceptibility, i.e. a higher degree of successful establishment of haustoria in epidermal cells, was associated with a reduced frequency of hypersensitive cell death reactions. In contrast, young seedlings of GFP-HvBI-1 barley were more resistant to *Fusarium graminearum* than wild type or azygous controls. Hence the effect of GFP-HvBI-1 on the outcome of a particular plant–fungus interaction appeared dependent on the lifestyle of the pathogen.

V. Babaeizad and J. Imani contributed equally to this study.

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V. Babaeizad · J. Imani · K.-H. Kogel
Institute of Phytopathology and Applied Zoology,
Justus Liebig University of Giessen,
Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany

R. Eichmann · R. Hückelhoven (✉)
Lehrstuhl für Phytopathologie, Technische Universität München,
Am Hochanger 2, 85350 Freising, Germany
e-mail: hueckelhoven@wzw.tum.de

Introduction

Programmed cell death (PCD) is involved in plant development and in plant interaction with the environment. The ultimate nature of PCD requires strict control of mechanisms that trigger PCD or restrict its spreading. Mutations of genes that are involved in the control of PCD often result in spontaneous cell death and lesion mimic phenotypes. Lesion mimic mutants often show enhanced resistance to obligate biotrophic pathogens or enhanced susceptibility to necrotrophic pathogens (Dangl et al. 1996; Lam et al. 2001; Glazebrook 2005). This underscores that balanced regulation of PCD is critical for a plant to adapt to different environmental challenges.

In barley and *Arabidopsis*, the *mlo* (mildew resistance locus o)-mutation causes a lesion mimic phenotype in fully expanded elder leaves. Homozygous barley mutant *mlo*-plants are resistant to the obligate biotrophic powdery mildew fungus *Blumeria graminis* f.sp. *hordei*. In barley, a single *mlo* locus is sufficient to confer full recessively inherited race-nonspecific resistance to powdery mildew, whereas in *Arabidopsis* partial redundancy of *MLO* family genes causes a more quantitative nature of single *mlo*-mutations (Jørgensen 1992; Büschges et al. 1997; Consonni et al. 2006). Barley *mlo*-mutants show enhanced susceptibility to *Magnaporthe oryzae* or to toxic culture filtrate of *Bipolaris sorokinana* (teleomorph: *Cochliobolus sativus*) (Jarosch et al. 1999; Kumar et al. 2001). Additionally, when artificially inoculated on immature

caryopses, necrotrophic *F. graminearum* progresses deeper into the tissue of *mlo*-barley than of *MLO*-barley (Jansen et al. 2005). Accordingly, *Atmlo2/6* double and *Atmlo2/6/12* triple mutants are supersusceptible to necrotrophic *Alternaria* species and show an enhanced cell death reaction to hemibiotrophic *Phytophthora infestans* when compared with the wild type (Consonni et al. 2006).

Human BAX inhibitor-1 (BI-1) was identified as a suppressor of mammalian proapoptotic BAX-induced cell death in yeast (Xu and Reed 1998). Plant BI-1 genes were later identified by targeted approaches and in transcriptome analyses of plant stress responses (reviewed in Hückelhoven 2004). Currently, plant BI-1 is described as a suppressor of plant cell death in interaction with fungal pathogens and endophytes, fungal toxins, salicylic acid, H_2O_2, endoplasmic reticulum stress and during perturbations of ion homeostasis (Kawai-Yamada et al. 2004; Deshmukh et al. 2006; Imani et al. 2006; Watanabe and Lam 2006; Ihara-Ohori et al. 2007; Watanabe and Lam 2008). Expression of barley BI-1 is responsive to powdery mildew inoculation in resistant and susceptible genotypes. Strong expression of HvBI-1 associates with both compatible interactions and with hypersensitive cell death responses (Hückelhoven et al. 2001, 2003). Transient over-expression of HvBI-1 in barley epidermal cells rendered cells susceptible to penetration by nonadapted *B. graminis* f.sp. *tritici* and supersusceptible to adapted *Bgh* (Hückelhoven et al. 2003; Eichmann et al. 2004). When *HvBI-1* is over-expressed in barley *mlo*-genotypes, *mlo* is heterologously complemented in a way that cells become accessible for penetration by *Bgh* and for fungal development. Thus, over-expression of BI-1 can functionally substitute for MLO in susceptibility to *Bgh* (Hückelhoven et al. 2003). MLO-like activity of BI-1 was further demonstrated by co-expression experiments in which *MLO* expression did not enhance BI-1 effects in induced susceptibility (Eichmann et al. 2004). Additionally, both MLO and BI-1 negatively control a *Bgh*-induced apoplastic H_2O_2 burst at sites of fungal attack (Hückelhoven et al. 1999; Eichmann et al. 2006).

In this study, we used stable transgenic barley plants expressing a green-fluorescing protein (GFP) fusion of HvBI-1 to generate evidence for the ability of HvBI-1 to alter interactions with fungi of different lifestyle, supporting that cell death regulators are potential keys to regulation of plant-microbe interactions.

Materials and methods

Generation of CaMV35S::GFP-HvBI-1 barley

Construction of binary vectors (pLH6000-CaMV35S::GFP-HvBI-1) for barley transformation, *Agrobacterium tumefaciens*-mediated transformation of immature embryos of barley cv. Golden Promise and regeneration was described previously (Deshmukh et al. 2006). Evaluation of the presence of the T-DNA was performed by genomic PCR with REDExtract-N-Amp plant PCR kit (Sigma-Aldrich Chemie GmbH, Munich, Germany) of *hygromycin* resistance gene (5′-primer 5′-CGTGCTTTCAGCTTCG ATGTAGG-3′ and 3′-primer 5′-AAGATGTTGGCGAC CTCGTATTG-3′) or *GFP-HvBI-1* (5′-primer 5′-CGTTCC AACCACGTCTTCAA-3′ and 3′-primer 5′-GGATCCT TGTAGAGCTCGTCCAT-3′), respectively. Confocal fluorescence images of GFP-HvBI-1 were recorded on a multichannel TCS SP2 confocal laser scanning microscope (Leica, Bensheim, Germany). GFP-HvBI-1 was excited with a 488 nm laser line and detected at 505–550 nm.

BAX-induced cell death in epidermal cells of barley

Ballistic delivery of pUC-derived vectors for expression of BAX and GFP as well as evaluation of BAX-induced cell collapse in epidermal cells of barley was performed as described previously (Eichmann et al. 2006). In brief, empty vector or CaMV35S::BAX constructs were co-precipitated with CaMV35S::GFP constructs on tungsten particles. Coated particles were delivered into epidermal cells of barley by a particle inflow gun (Schweizer et al. 1999). Cytoplasmic integrity and cytoplasmic streaming of GFP-expressing cells were observed at 10 h after inoculation by epifluorescence microscopy or CSLM, respectively.

Plant growth, inoculation and assessment of disease progress

Donor material and transgenic plants of the barley (*Hordeum vulgare* L.) cultivar ‘Golden Promise’ were grown in a growth chamber at 18°C, 60% relative humidity, and a photoperiod of 16 h (150 µmol m^{-2} s^{-1} photon flux density) with 60% relative humidity. The barley powdery mildew fungus *Blumeria graminis* (DC) Speer f.sp. *hordei* Em. Marchal, race A6, was maintained on barley cultivar ‘Siri’ under the same conditions. For macroscopic evaluation, *Bgh* was inoculated onto detached third leaves of 14 days old barley plants to give a density of 5 conidia mm^{-2}. Symptoms were evaluated 6 days after inoculation under a stereo microscope. For microscopic analysis, entire plants were inoculated with a density of 30 conidia mm^{-2}.

Induction of *F. graminearum* conidiation was carried out on SNA-agar plates (Nirenberg 1981) incubated at 18°C under near-UV and white light (TLD 36 W-08; TL 40 W-33 RS; Philips, Hamburg, Germany) with a 12 h

photoperiod for 7–14 days. Conidia were harvested from the plates with a sterile cheese cloth and sterile water.

To test for resistance to *F. graminearum* (Strain 1003) root rot, barley seeds were surface sterilized in 6% sodium hypochloride for 3 h, dehusked and washed three times with sterile water under a sterile laminar flow. Afterwards, seeds were laid on sterile water-soaked filter paper for germination. Three days old seedlings were used for inoculation with *F. graminearum* spore suspensions [5×10^4 spores mL^{-1} in 0.02% tween 20 (v/v) + 0.5% gelatine (w/v) aqueous solution] or mock inoculated for 2 h. Afterwards, inoculated seedlings were grown in a 2:1 mixture of expanded clay (Seramis®, Masterfoods, Nordhofen, Germany) and Oil Dri® (Damolin; Mettmann, Germany) in a growth chamber at 22°C/18°C day/night cycle, 60% relative humidity and a photoperiod of 16 h (240 µmol m^{-2} s^{-1} photon flux density), and fertilized weekly with 20 ml of a 0.1% Wuxal top N solution (N/P/K: 12/4/6). Afterwards, shoot length and weights were measured and root samples were subjected to DNA isolation for quantitative measurements of fungal DNA (Deshmukh and Kogel 2007).

For *F. graminearum* head blight assays, spikes were inoculated at anthesis as described by McCallum and Tekauz (2002) and placed under high humidity conditions in the greenhouse. After 3 weeks of incubation, seeds were harvested, surface-sterilized for one minute in 70% ethanol and 20 min in 3% sodium hypochloride and placed on 0, 5% potato dextrose medium. Seeds of plants were scored for resistance according to the rate of secondary infection originating from the kernels.

Microscopy of the interaction phenotype of barley and *B. graminis* f.sp. *hordei*

The outcome of the interaction of transgenic barley plants with *Bgh* was evaluated 30 h after inoculation. Penetration of attacked cells was ascertained by detection of haustoria formation. Whole cell staining with 3,3-diaminobenzidine (DAB) and auto-fluorescence were taken as reliable markers of cell death (Koga et al. 1990; Thordal-Christensen et al. 1997; Hückelhoven et al. 1999). Leaf fixation, bright-field and fluorescence microscopy was performed as described by Hückelhoven and Kogel (1998). Because the rate of fungal penetration into short and long epidermal cells is different, only short cells directly adjacent to stomata (cell type A) and short cells not directly adjacent to stomata (type B) were evaluated (for leaf topography see Koga et al. 1990). Detection of H_2O_2 was performed using the DAB-uptake method as described previously (Thordal-Christensen et al. 1997; Hückelhoven et al. 1999).

Quantitative measurement of fungal DNA via real time PCR

In the *F. graminearum* root rot assay the degree of root colonization was determined by using the $2^{-\Delta\Delta C_t}$ method for measurement of fungal DNA in relation to plant DNA (Livak and Schmittgen 2001). Cycle threshold (C_t) values were generated by subtracting the raw C_t values of *F.graminearum Fg16 N* (Nicholson et al. 1998) from the respective raw C_t values of plant-specific ubiquitin (Deshmukh et al. 2006). Genomic DNA was isolated from 100 mg of root tissue by DNeasy plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For quantitative PCR, 10 ng of total DNA were used. Amplifications were performed in 5 µl of SYBR green JumpStart Taq ReadyMix (Sigma-Aldrich, Munich, Germany) with 0.5 pmol oligonucleotides, using an Mx3000P thermal cycler (Stratagene, La Jolla, USA). After an initial activation step at 95°C for 7 min, 38 cycles (94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 82°C for 15 s) were performed. Respective melting curve was determined at the end of cycling to ensure amplification of only one PCR product. C_t values were determined with the Mx3000P V2 software supplied with the instrument. The following oligonucleotide pairs were used for the analyses: *HvUbiquitin* (GenBank accession No. M60175) 5′-primer 5′-ACCCTCGCCGA CTACAACAT-3′ and 3′-primer 5′-CAGTAGTGGCGG TCGAAGTG-3′; *Fg16 N* (Nicholson et al. 1998) 5′-primer 5′-ACAGATGACAAGATTCAGGCACA-3′ and 3′-primer 5′-TTCTTTGACATCTGTTCAACCCA-3′.

Results

Transgenic barley plants expressing GFP-HvBI-1 are less sensitive to BAX

To get a deeper understanding of the potential of BI-1 at the entire plant level, we produced transgenic barley expressing a green-fluorescing protein fusion of HvBI-1 under control of the cauliflower mosaic virus CaMV35S promoter (GFP-HvBI-1 barley, Deshmukh et al. 2006). Transgenic GFP-HvBI-1 barley developed like wild type in T_0 and following generations. Transgene expression was confirmed in all generations by transgene-specific genomic PCR, RT-PCR and by in vivo imaging of GFP-HvBI-1 by fluorescence microscopy. About half of the lines showed faint but visible fluorescence of GFP-HvBI-1 in leaves under a standard epifluorescence microscope. Confocal laser scanning microscopy revealed green fluorescence in a reticular pattern in barley cytoplasm and in the nuclear periphery. This was in accordance with where we expected

BI-1 to be localized when referred to literature data and to our previous results from transient expression of GFP-HvBI-1 (Kawai-Yamada et al. 2001; Eichmann et al. 2004; Hückelhoven 2004). Stable transgenic GFP-HvBI-1 barley expressed the protein in leaf tissue (Fig. 1a–e) as well as in root tissue (Fig. 1f, Deshmukh et al. 2006). Ubiquitous activity of the CaMV35S promoter in barley was further supported in CaMV35S::GFP barley plants (data not shown and Schultheiss et al. 2005). In all experiments, we worked with at least three independent segregating transgenic lines. Besides parent cultivar Golden Promise, azygous plants, which lost the transgene due to segregation, were included as controls in all experiments.

To confirm function of the GFP-HvBI-1 fusion in entire plants, we transiently expressed proapoptotic mammalian BAX in epidermal cells of GFP-HvBI-1 barley and wild type parents. In wild type barley epidermal cells, expression of BAX after ballistic transformation leads to cessation of cytoplasmic streaming and fragmentation of the protoplast. Effects on vital movement and cytoplasmic integrity were observed by means of co-expression of soluble GFP, which strongly labeled the cytoplasm and nucleoplasm. BAX induced epidermal cell collapse started about 6 h after delivery of BAX and was completed by 24 h in wild type barley. When we compared cellular integrity by 10 h after delivery of plasmids, most empty vector-transformed cells of wild type were viable, whereas about 60% of BAX-transformed cells displayed lack of cytoplasmic movement or fragmentation of the protoplast, respectively. In contrast, only 20% of GFP-HvBI-1 barley cells (average of three lines; E4L3, E8L1 and E14L1) were affected by expression of BAX (Fig. 1g). Azygous controls were indistinguishable from wild type (not shown).

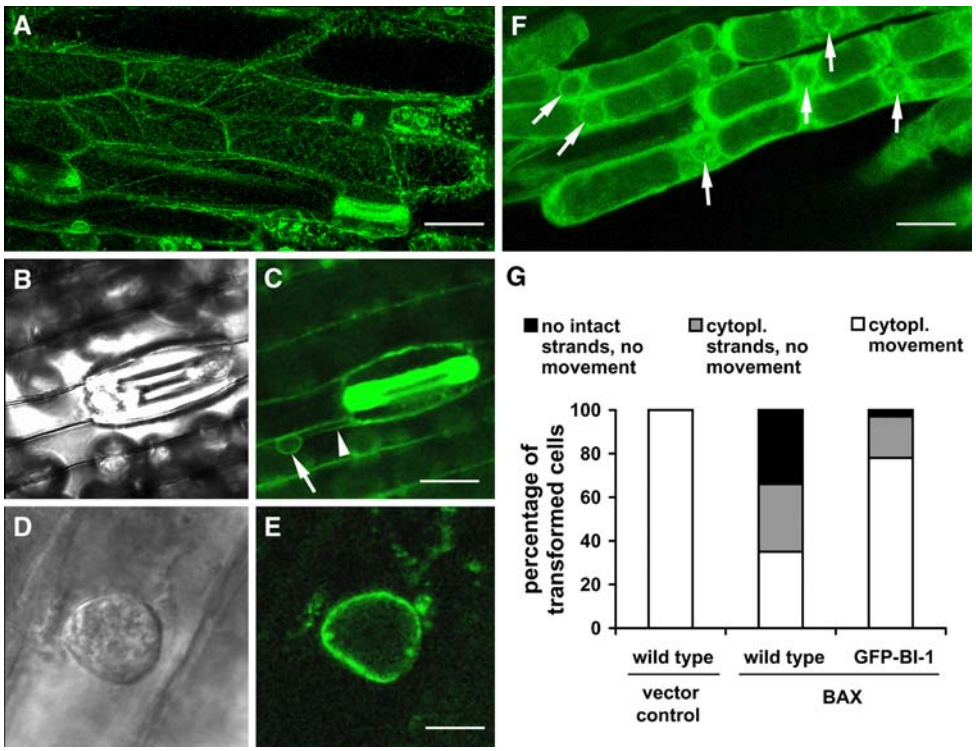

Fig. 1 Validation of expression and cell death controlling effects of GFP-HvBI-1 in leaves of transgenic barley plants (line E4L3). Subcellular localization of GFP-HvBI-1 by CLSM. **a** Projection of five optical sections (2 µm increments) through the epidermis of GFP-HvBI-1 barley. GFP-HvBI-1 emission is visible in a reticular endomembrane system. *Bar* 50 µm. **b**, **c** Pictures represent transmission and GFP-HvBI-1 channels of the same sample of GFP-HvBI-1 barley. Note the perinuclear signal (*arrow*) of GFP-HvBI-1 and a potential endoplasmic reticulum strand (*arrow head*). *Bar* 30 µm. **d**, **e** Single optical section through a nucleus in the epidermis of GFP-BI-1 barley. *Bar* 10 µm. **f** Projection of five optical sections (2 µm increments) through detached epidermal cells of GFP-HvBI-1 barley roots grown on water agar. GFPHvBI-1 emission is visible in a reticular endomembrane system and around nuclei (*arrows*). *Bar* 30 µm. **g** Effects of GFP-HvBI-1 on controlling cell death induced by mammalian pro-apoptotic BAX in epidermal cells of barley. *Stacked columns* represent the frequencies of cellular responses to expression of either GFP alone or GFP and BAX in wild type or GFP-HvBI-1 genetic backgrounds. GFP-expressing cells showed rapid vital movement (*white columns*), cessation of vital movement (*grey columns*) or cessation of vital movement combined with discontinuity of cytoplasmic strands (*black columns*), depending on BAX expression and on the genetic background. *Each column* summarizes the frequencies of events in 300 GFP-expressing cells evaluated on 6 leaves of wild type or three GFP-HvBI-1 barley lines, respectively

Transgenic barley expressing GFP-HvBI-1 is more susceptible to powdery mildew

We next challenged GFP-HvBI-1 barley by inoculation with virulent *Bgh*. We used corresponding azygous segregants from independent transformation events and/or the parent Golden Promise as controls. Azygous controls were always very similar to parent Golden Promise. When we used CaMV35S::GFP plants as controls for potential effects of GFP they behaved as wild type Golden Promise or were slightly more resistant in some experiments (not significantly different from wild type at $P < 0.05$, Student's *t* test; data not shown). In contrast, GFP-HvBI-1 barley showed enhanced susceptibility to powdery mildew when compared to either wild type parent or to azygous controls at 6 days after inoculation. Depending on the line used, we obtained up to 47% (line E15L7) more powdery mildew colonies when compared to controls. However, also lower levels of enhanced susceptibility proved statistically significant (Student's *t* test, Fig. 2) and could be reproduced in independent experiments (data not shown). A meta-analysis of all lines and siblings that contained the GFP-HvBI-1 construct (GFP-BI-1 positives in Fig. 2) revealed highly significant differences in pustule formation when compared to azygous controls. In average, GFP-HvBI-1 barley plants displayed about 20% more pustules than controls. This meta-analysis may under-estimate the GFP-BI-1 effect on susceptibility, because it included also several lines, which expressed GFP-BI-1 at a microscopically non-visible level. In experiments with further independent GFP-BI-1 lines, similarly about half of the lines proved super-susceptible to powdery mildew (data not shown).

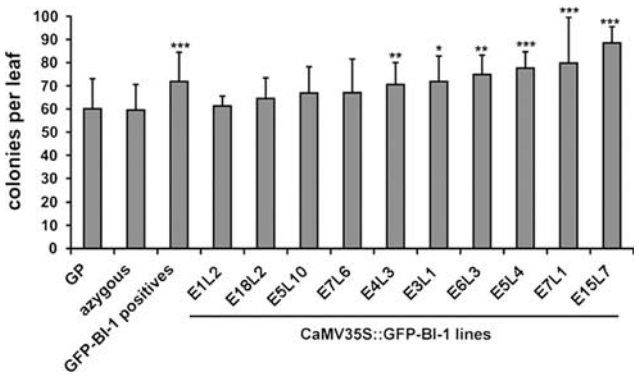

Fig. 2 GFP-HvBI-1 barley lines display enhanced susceptibility to fungal invasion and to colony formation. Number of fungal colonies at 6 days after inoculation on excised leaves of the parent cv. Golden Promise, azygous segregants, the GFP-HvBI-1-positive segregants (average of all lines) and the GFP-HvBI-1-positive segregants from 10 individual transgenic lines (10 leaves each). Six out of ten lines and the average of all GFP-HvBI-1-positives are significantly more susceptible to colony formation of *Bgh*. *Error bars* represent standard deviations of the mean. *, **, *** indicate *P* values of <0.05, 0.01 or 0.001 (Student's *t* test) when compared to azygous controls. Data have been confirmed in two independent biological repetitions with similar results

Transgenic barley expressing GFP-HvBI-1 undergoes less hypersensitive cell death

In order to find out why GFP-HvBI-1 barley displayed enhanced susceptibility to powdery mildew, we undertook a microscopic inspection of the interaction of second leaf segments with *Bgh*. We selected four lines that had been found to be macroscopically super-susceptible to *Bgh*, showed GFP-BI-1 fluorescence under blue light excitation and/or were more resistant to BAX-induced cell death (Figs. 1, 2 and data not shown). We chose 30 h after inoculation for evaluation of the interaction phenotype, because at this time successful fungal germlings had already developed visible haustoria in penetrated cells and short elongated secondary hyphae on the leaf surface. In contrast, hypersensitive cell death reaction (HR) was obvious from whole cell H_2O_2 accumulation, that was histochemically evidenced by staining with 3,3-diaminobenzidine (DAB, Thordal-Christensen et al. 1997). HR was further evident from discontinuity of cytoplasmic strands and from whole cell autofluorescence of dead cells. Parent Golden Promise wild type leaves showed a background resistance phenotype with many attacked but non-penetrated living cells or attacked cells that underwent a single cell HR. About 20% of attacked cells allowed for fungal penetration and for development of elongated secondary hyphae on the leaf surface. In contrast, up to 40% of GFP-HvBI-1 cells allowed for fungal penetration and for development of elongated secondary hyphae. This effect was stronger in lines E14L1 and E15L7, when compared to lines E4L3 and E8L1. This was in accordance with stronger super-susceptibility at the macroscopic level (Fig. 2 and data not shown) and may explain enhanced colony development at 6 days after inoculation. Hence, GFP-HvBI-1 barley was more susceptible to establishment of fungal infection structures within living cells of barley. Non-penetrated cells either survived or displayed HR. The frequency of HR was strongly reduced in GFP-HvBI-1 barley (Fig. 3).

GFP-HvBI-1 barley seedlings are less susceptible to *F. graminearum*

Susceptibility to biotrophs is often accompanied by resistance to necrotrophs and vice versa. This provoked us to challenge three days old GFP-HvBI-1 seedlings and corresponding controls with spore suspensions of necrotrophic *F. graminearum*. Wild type and azygous segregants showed browning of roots and coleoptiles from 4 to 5 days after inoculation onwards. Additionally, a strong growth reduction was observed in plants that were infected with

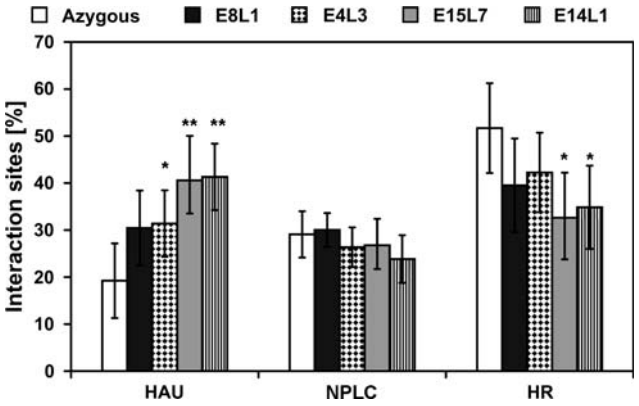

Fig. 3 Interaction phenotype of intact plants with *Bgh* at 30 h after inoculation. Lines E8L1, E4L3, E14L1 and E15L7 as well as corresponding azygous controls were inoculated and 26 h later stained with 3,3-diaminobenzidine for 4 h for detection of HR by whole cell DAB staining. Accessible cells allowed for fungal haustorium formation (HAU) and development of elongated secondary hyphae. Non-penetrated cells either survived fungal attack (NPLC, non-penetrated living cells) or displayed HR without visible haustorium formation. *Error bars* show 95% confidence intervals. *, ** indicate significance at $P < 0.05$ or $P < 0.01$ according to Student's *t* test when compared to azygous controls. Repetition of the experiments led to similar results

F. graminearum. In contrast, GFP-HvBI-1 barley showed less browning of roots and coleoptiles and did not display fungus-induced growth reduction (Fig. 4a, b). To distinguish whether GFP-HvBI-1 barley tolerated the presence of *F. graminearum* or restricted fungal development, we assessed fungal biomass in roots by quantitative PCR of fungal DNA and normalized data by the amount of plant DNA in each sample. This revealed that *F. graminearum* developed on GFP-HvBI-1 roots only about 10% of the amount of DNA that it developed on wild type barley (Fig. 4c). Hence, GFP-HvBI-1 barley seedlings have transgene-mediated partial resistance to *F. graminearum*.

To assess the potential of BI-1 to protect spikelets from infection by *F. graminearum*, we spray-inoculated spikes of wild type and GFP-HvBI-1 barley during flowering. After ripening, kernels of inoculated plants were harvested, surface-sterilized and put on diagnostic agar plates to assess seed borne infection during germination. According to this assay, much less kernels of the next generation of GFP-HvBI-1 harbored *F. graminearum* than kernels of wild type or azygous segregants. Kernel infection rates of two GFP-HvBI-1 lines were reduced by 36 or 45% when compared to azygous or wild type parent plants, respectively (Table 1).

Discussion

Our data show that BI-1 modulates cell death reactions and supports susceptibility to powdery mildew in barley. In turn, over-expression of GFP-HvBI-1 mediates partial resistance to cell death provoking *F. graminearum*. Together with literature data this suggests that BI-1 can function as an interface to modulate cell death in interaction with plant pathogens of different lifestyles.

In constant contact with potential pathogenic microbes, plants need to control their non-specific elicitor-triggered responses to restrict defense to a level that allows for plant growth and survival. Nonspecific stress reactions and plant defense reactions share a great overlap of physiological events such as the accumulation of reactive oxygen species. Hence, plant survival factors may sense stress signals and may have functions in controlling defense reactions that when reaching a certain threshold culminate in cell death (Jones and Dangl 2006). Our data support that enhanced expression level of BI-1 weakens defense and background resistance to *Bgh* in barley (Fig. 2 and 3). Expression of BI-1 is triggered in both compatible and incompatible interactions. We assume that BI-1 is a general survival factor that directly or indirectly senses defense-associated stress to locally control cell death where defense is triggered (Hückelhoven et al. 2001; Hückelhoven 2004). We further speculate that *Bgh* directly or indirectly targets BI-1 to suppress defense and to support survival of haustoria-containing cells during fungal establishment and reproduction. As an obligate biotrophic parasite, *Bgh* may require host targets involved in cell survival in order to gain control over plant cell death mechanisms. In susceptibility to *Bgh*, HvBI-1 might thus function as a survival factor and as a suppressor of defense reactions or in accommodation of fungal haustoria. Indeed, GFP-HvBI-1 accumulates at sites of penetration by *Bgh* and over-expression of HvBI-1 in single epidermal cells weakened the apoplastic oxidative burst at sites of attack by *Bgh* in barley (Eichmann et al. 2006). In this study we show that stable expression of GFP-HvBI-1 restricted the frequency of HR and the development of necrotrophic *F. graminearum.* All GFP-BI-1 lines that were more susceptible to powdery mildew than wild type controls displayed faint but visible green fluorescence under blue-light excitation, whereas lines that contained GFP-BI-1 mRNA but appeared dark under the microscope usually were not significantly more susceptible. Together this indicates that a certain threshold of BI-1 might be required for cellular accessibility to *Bgh*. However, it is not yet clear whether HvBI-1 is required for survival of cells attacked by *Bgh*. Transient knock-down of HvBI-1 did not result in spontaneous cell death (unpublished results of R.E. and Patrick Schweizer, IPK Gatersleben; Germany). Hence, the molecular mechanism of HvBI-1-modulated susceptibility to *Bgh* remains subject to further investigations.

Loss of MLO function enhances susceptibility to cell death-provoking fungal pathogens (Jarosch et al. 1999;

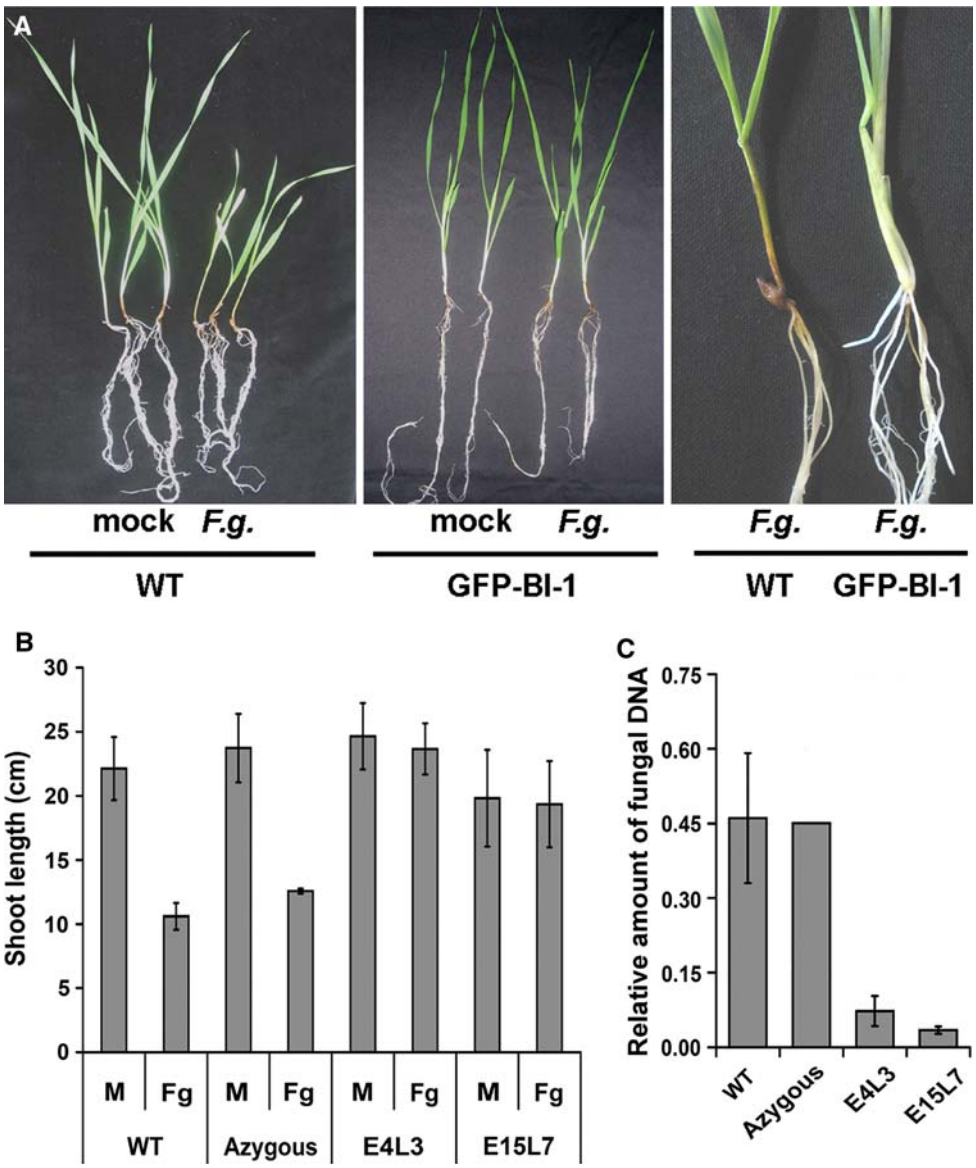

Fig. 4 GFP-HvBI-1 shows seedling resistance to *F. graminearum*. **a** Macroscopic appearance of wild type and GFP-HvBI-1 barley 10 days after seedling inoculation with *F. graminearum* or mock inoculation, respectively. Note growth retardation and necrosis in wild type, which is hardly visible in GFP-HvBI-1 barley. **b** Evaluation of average shoot length of wild type barley, azygous segregants and GFP-HvBI-1-positive segregants of two GFP-HvBI-1 barley lines (E4L3 and E15L7). Shoot length was measured in 13-day-old plants 10 days after inoculation with *F. graminearum* (Fg) or mock treatment (M), respectively. *Error bars* represent standard deviations of the mean of three biological repetitions. **c** Quantitative PCR of fungal DNA in relation to plant DNA in roots at 10 days after inoculation with *F. graminearum*. Fungal DNA was measured in the same genotypes as in B with DNA pooled from two (azygous) or three independent biological repetitions. *Error bars* represent standard deviations

Table 1 Relative kernel infection rates of GFP-HvBI-1 barley after spike infection of the previous generation

Plant material	Relative infection rate ± SD[a]
Golden Promise	100
Azygous segregants of line E15L7	92.7 ± 7.4
GFP-HvBI-1 E15L7	54.8 ± 7.8
GFP-HvBI-1 E4L3	56.1 ± 6.8

[a] Percentage of kernels with outgrowth of *F. graminearum* relative to the infection rate of Golden Promise (set as 100%). Data were confirmed in two independent experiments

Kumar et al. 2001; Jansen et al. 2005; Consonni et al. 2006). Therefore, recessive *mlo*-mutations conferring resistance to powdery mildew in different plant species may be evolutionary short-lived due to potential fitness costs (Bai et al. 2008). Accelerated cell death and higher levels of basic defense reactions to powdery mildew fungi in *mlo*-genotypes (Peterhänsel et al. 1997; Zierold et al. 2005; Consonni et al. 2006) apparently do not affect these pathogens but support necrotrophic growth. This provoked the idea that over-expression of BI-1 may restrict growth of necrotrophic pathogens (Hückelhoven et al. 2003). Indeed ectopic expression of HvBI-1 in carrots protects leaves and roots from spreading of *Botrytis cinera* or *Chalara elegans*, respectively (Imani et al. 2006). Additionally, *Piriformospora indica,* a root-colonizing beneficial fungus that is related to disease-causing *Rhizoctonia* and prefers dead tissue for proliferation, develops much less biomass in roots of GFP-HvBI-1 than in wild type (Deshmukh et al. 2006). In contrast, *Arabidopsis bi-1* mutants show accelerated cell death when confronted with the fungal toxin fumonisin B1 (Watanabe and Lam 2006).

F. graminearum is one of the most important pathogens on wheat, and it also infects barley. It causes root rot, crown rot and head blight and produces an arsenal of toxic metabolites that harm human and livestock health (Goswami and Kistler 2004). In temperate zones, *F. graminearum* is important as the causal agent of head blights whereas in subtropical zones *Fusarium* root and crown rot greatly affect stability of cereal crops. Both foot and head diseases are difficult to control by chemical means. Our results indicate that *F. graminearum* may not develop well on plants that strictly control cell death due to over-expression of the cell death suppressor GFP-HvBI-1. However, enhanced susceptibility of GFP-HvBI-1 barley to *Bgh* alerts for carefulness in regard to undesired effects of ectopic expression of cell death regulators. There may be a general relationship between resistance to necrotrophic pathogens and susceptibility to biotrophs and vice versa (Glazebrook 2005). However, data suggest that ectopic cell death regulation might help to control plant diseases without undesired effects if expression would be specifically regulated. For *Fusarium* on cereal crops this asks for promoters that are tissue-specific and pathogen-responsive.

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