Expression of *BvGLP-1* Encoding a Germin-Like Protein from Sugar Beet in *Arabidopsis thaliana* Leads to Resistance Against Phytopathogenic Fungi

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Nematode (*Heterodera schachtii*) resistance in sugar beet (*Beta vulgaris*) is controlled by a single dominant resistance gene, *Hs1^{pro-1}*. *BvGLP-1* was cloned from resistant sugar beet. The *BvGLP-1* messenger (m)RNA is highly upregulated in the resistant plants after nematode infection, suggesting its role in the *Hs1^{pro-1}* mediated resistance. *BvGLP-1* exhibits sequence homology to a set of plant germin-like proteins (GLP), from which several have proved to be functional in plant basal or defense resistance against fungal pathogens. To test whether *BvGLP-1* is also involved in the plant–fungus interaction, we transferred *BvGLP-1* into *Arabidopsis* and challenged the transgenic plants with the pathogenic fungi *Verticillium longisporum* and *Rhizoctonia solani* as well as with the beneficial endophytic fungus *Piriformospora indica*. The expression of *BvGLP-1* in *Arabidopsis* elevated the H₂O₂ content and conferred significant resistance to *V. longisporum* and *R. solani* but did not affect the beneficial interaction with *P. indica* in seedlings. Microscopic observations revealed a dramatic reduction in the amount of hyphae of the pathogenic fungi on the root surface as well as of fungal mycelium developed inside the roots of transgenic *Arabidopsis* compared with wild-type plants. Molecular analysis demonstrated that the *BvGLP-1* expression in *Arabidopsis* constitutively activates the expression of a subset of plant defense-related proteins such as PR-1 to PR-4 and PDF1.2 but not PDF2.1 and PDF2.3. In contrast, the *PDF2.1* mRNA level was downregulated. These data suggest an important role of *BvGLP-1* in establishment of plant defense responses, which follow specific signaling routes that diverge from those induced by the beneficial fungus.

Plant genomes encode a subgroup of cupins called germin and germin-like proteins (GLP): water-soluble, protease-resistant, heat-stable, and sodium dodecyl sulfate-tolerant glycoproteins (Lane 1994; Woo et al. 2000), which assemble into homohexameric complexes in vivo (Zhang et al. 1995; Vallelia et al. 1998; Christensen et al. 2004). Several members of the germin family contain enzymes with oxalate oxidase (OxO) or superoxide dismutase (SOD) activities leading to hydrogen

peroxide (H₂O₂) production (Chiriboga 1966; Lane 1994; Zimmermann et al. 2006). In barley, six GLP subfamilies with 21 genes have been described (HvGER1 to 6), from which only HvGER1a proved to have OxO activity whereas HvGER4d and HvGER5a both showed SOD activity, and no any enzymatic activity could be detected from HvGER2a as well as from HvGER6a (Zimmermann et al. 2006). The *Arabidopsis* genome contains at least 12 unique GLP genes with diverse expression patterns. Of these, at least six are gene orthologs of barley (Druka et al. 2002; Wu et al. 2000) and rice. Thus far, no enzymatic activity has been reported from *Arabidopsis* GLP (Carter and Thornburgh 1999; Membré et al. 2000).

GLP have been proposed to play an important role in several aspects of plant development or stress tolerance and received considerable attention for their possible contribution to plant basal host resistance. GLP-encoding genes were found to be expressed differentially in various plant tissues and the expression pattern changed from developmental to conditional. Several genes were highly induced by fungal pathogen attack (Schweizer et al. 1999; Christensen et al. 2004; Zimmermann et al. 2006). It has been proposed that, through the generation of H₂O₂ due to their OxO or SOD activity, GLP may function as a cofactor for reinforcement of the cell wall by cross-linking of plant cell wall proteins in papillae at the infection site (Olson and Varner 1993; Thordal-Christensen et al. 1997; Wei et al. 1998), and as a signaling molecule inducing a range of defense responses in a direct or indirect manner (Lane 1994; Zhou et al. 1998). Transgenic plants ectopically expressing GLP-encoding genes have provided direct evidence for a defense role of GLP. Soybean, tobacco, sunflower, and rapeseed plants transformed with a wheat GLP with OxO activity showed enhanced resistance to *Sclerotinia sclerotiorum* (Zaghmout et al. 1997; Donaldson et al. 2001; Hu et al. 2003; Dong et al. 2008). Poplar and corn expressing the same gene were more resistant to *Septoria musiva* (Liang et al. 2001) and insect predation (Ramputh et al. 2002), respectively. On the other hand, the SOD activity of HvGER5a has been demonstrated to be required for resistance-enhancing activity (Zimmermann et al. 2006). Furthermore, epidermal barley cells transiently overexpressing barley GLP or transiently silenced for HvGER3 or HvGER5 all showed more resistance against *Blumeria graminis*, thus suggesting their crucial role in fine regulation of plant basal resistance against fungal pathogens (Schweizer et al. 1999; Christensen et al. 2004; Zimmermann et al. 2006).

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Hs1^{pro-1} confers nematode (*Heterodera schachtii*) resistance in sugar beet (*Beta vulgaris*) (Cai et al. 1997, 2005; Thureau 2003). Several candidate genes involved in the resistance have been cloned from the resistant sugar beet (Samuelian et al. 2004). The gene *BvGLP-1* (AY243465) is highly upregulated in the resistant plants by nematode infection. *BvGLP-1* exhibits sequence homology to a set of plant GLP, including the *Arabidopsis* GLP3 (AT5G20630) as well as the barley GLP HvGER2a (DQ647620) with 43 and 42% amino acid identity, respectively. To test the possible role of *BvGLP-1* in the plant–fungus interaction, we transferred *BvGLP-1* into *Arabidopsis* and challenged the transgenic plants with two pathogenic fungi, *Verticillium longisporum* and *Rhizoctonia solani* as well as with a beneficial endophytic fungus *Piriformospora indica*.

The *R. solani* complex and *Verticillium* spp. represent two economically important groups of soilborne pathogens with a great diversity of host plants, including sugar beet and oilseed rape. *R. solani* infects primarily roots and stems of host plants (Keijer et al. 1997). Within the cortex, the rapid hyphal growth ultimately results in the collapse of the infected plants whereas *Verticillium* spp. invade the vascular system of the host plants through the root and systemically spread by conidia during the vegetation period (Zeise and Tiedemann 2002). The endophytic fungus *P. indica* colonizes roots of many plant species, including *Arabidopsis*, resulting in an increase in the biomass of roots and shoots (Varma et al. 1999; Peskan-Berghöfer et al. 2004). The beneficial fungus promotes nutrient uptake; allows plants to survive under water and salt stress; confers resistance to toxins, heavy metal ions, and pathogenic organisms; and stimulates seed production (Oelmüller et al. 2005).

Furthermore, both *R. solani* and *Verticillium* spp. as well as *P. indica* infect *Arabidopsis* plants (Keijer et al. 1997; Steventon et al. 2001; Veronese et al. 2003). Sugar beet is a recalcitrant crop species for transformation. Generation of transgenic sugar beet is still extremely labor intensive and time consuming. Therefore, *Arabidopsis* has been used as a model plant for studying sugar beet genes in regard to their role in plant–parasite interaction in our laboratory and worldwide (Thureau et al. 2003; Cai et al. 2005). Thus, transgenic *Arabidopsis* expressing *BvGLP-1* provides us unique tools for studying the function of the gene *BvGLP-1* and underlying molecular mechanisms.

In response to microbes, two distinct types of resistance are induced systemically throughout the plant: systemic acquired resistance (SAR) (Ross 1961) and induced systemic resistance (ISR) (Kloepper et al. 1992). Whereas SAR is induced by an infection with a necrotizing pathogen, ISR follows the colonization of the rhizosphere with selected nonpathogenic plant growth-promoting rhizobacteria (PGPR) (Pieterse et al. 1996). PGPR are present in large numbers on the root surface and protect plants from pathogen infection through induction of systemic resistance but without provoking any symptoms themselves. Specific recognition between protective ISR-inducing rhizobacteria and the plant activates the signaling cascade leading to ISR. The downstream signaling events in the rhizobacteria-mediated ISR pathway differ from those in the pathogen-induced SAR pathway. ISR is mainly regulated by the jasmonic acid (JA) and ethylene (ET) signaling routes (Glazebrook 2005; Van Baarlen et al. 2007). JA induces the expression of genes encoding defense-related proteins, such as thionins (Epple et al. 1995) and proteinase inhibitors (Farmer et al. 1992), whereas ET activates several members of the pathogenesis-related (PR) gene superfamily (Potter et al. 1993). Both regulators are involved in the activation of genes encoding plant defensins (Penninckx et al. 1996) and enzymes for phytoalexin biosynthesis (Gundlach et al. 1992). SAR is characterized by an early increase in salicylic acid (SA) (Malamy et al. 1990) and the concomitant activation of *PR-1* gene expression (Ward

et al. 1991) whereas plants expressing ISR did not, confirming that ISR and SAR are controlled by distinct signaling pathways that diverge in their requirement for SA. *PR-1* has been extensively used as a marker for SA-mediated SAR defense, whereas the expression level of *PDF1.2* and *PDF2.3* is not influenced by SA.

Here, we demonstrate that expression of *BvGLP-1* in transgenic *Arabidopsis* increases the H_2O_2 content in transgenic *Arabidopsis* plants and confers resistance to two fungal pathogens, *R. solani* and *V. longisporum*, but does not affect the beneficial interaction induced by *P. indica* in transgenic *Arabidopsis* seedlings even though the antifungal activity of *BvGLP-1* seems to restrict growth of *P. indica* in older plants. Furthermore, *BvGLP-1* specifically activates expression of a subset of defense-related proteins in *Arabidopsis*. These data suggest that *BvGLP-1* establishes plant resistance following a signaling route diverging from that induced by *P. indica*.

RESULTS

Generation and selection of transgenic *Arabidopsis* plants expressing *BvGLP-1*.

Twenty-two independent transgenic plants transformed with pAM194-*BvGLP-1* were generated. From these, six plants which carry a single copy of the transgene were chosen for generation of homozygous transgenic lines by a successive propagation (data not shown). The transcriptional activity of the transgene in six homozygous transgenic lines was determined by reverse-transcriptase polymerase chain reaction (RT-PCR) assay with *BvGLP-1*-specific primers. An RT-PCR fragment of 780 bp in size, as expected, was given in all six transgenic lines but not in the wild-type control (Fig. 1A), suggesting that *BvGLP-1* is transcriptionally active in transgenic plants. Because *BvGLP-1* may function as an enzyme with OxO or SOD producing H_2O_2 in plant cells, we expected for an elevated H_2O_2 level in transgenic plants expressing *BvGLP-1*. To confirm this, we colorimetrically determined the H_2O_2 content in transgenic *Arabidopsis* plants in comparison with that in wild-type C24 plants. A slight variability in H_2O_2 levels was observed in different transgenic lines but the levels are all significantly higher than those in the wild-type C24 control plants (Fig. 1B). Thus, the elevation of H_2O_2 content in transgenic plants expressing *BvGLP-1* suggests the OxO or SOD activity of *BvGLP-1*. Transgenic lines 1, 2, and 3 were chosen for further experiments in this study because of their relatively higher H_2O_2 content (Fig. 1B). Visually, no obvious differences in growth rate and morphology between transgenic and wild-type *Arabidopsis* plants were observed (data not shown).

Infection experiments of *Arabidopsis* plants with *R. solani* and *P. indica* on agar plates.

To check the role of *BvGLP-1* in pathogenic and beneficial plant–fungus interactions, we infected the transgenic *Arabidopsis* plants with *R. solani* (AG 2-1) and *P. indica* on agar plates in which the wild-type C24 plants served as a control. For infection with *R. solani*, wild-type and transgenic plants were transferred to agar plates, inoculated with fungal mycelium, and cocultivated in a growth chamber. Three days after infection, wild-type C24 plants showed clear disease symptoms, and $23 \pm 3\%$ ($n = 30$) of them suffered from the infection (Fig. 2), whereas only a few transgenic plants ($4 \pm 1\%$) showed visible symptoms. Twelve days after infection, wild-type plants suffered seriously from the fungal infection; they showed strong disease symptoms and $97 \pm 3\%$ ($n = 30$) of the infected plants died (Fig. 3B). In contrast, more than $50 \pm 3\%$ ($n = 30$) of the transgenic plants were still healthy and grew regularly. These data demonstrated that the expression of

BvGLP-1 in *Arabidopsis* interferes with *R. solani* infection, giving rise to significant resistance against the pathogen.

A similar experiment was performed with *P. indica*. Wild-type and transgenic plants were transferred to agar plates with fungal plaque (Fig. 4A). After 10 days of cocultivation, we observed a $24 \pm 4\%$ ($n = 60$ plates) increase in the fresh weight of wild-type seedlings cocultivated with *P. indica* relative to the uncolonized control. A similar result was obtained for the three transgenic *Arabidopsis* lines (Fig. 4B). We conclude that

processes leading to *P. indica*-mediated growth promotion of *Arabidopsis* seedlings on agar plates are independent of *BvGLP-1* expression and that higher H_2O_2 levels do not prevent the beneficial effects for the seedlings.

Infection experiments of *Arabidopsis* plants with *R. solani* and *V. longisporum* in soil.

To verify the data obtained from the agar plate experiments, we assessed the resistance of the *BvGLP-1*-expressing *Arabi-*

Fig. 1. Characterization of transgenic *Arabidopsis thaliana* lines transformed with pAM194-*BvGLP-1*. **A**, Reverse-transcriptase polymerase chain reaction analysis with six homozygous transgenic *A. thaliana* lines (*BvGLP-1*) using *BvGLP-1*-specific primers. *Arabidopsis* wild-type plants (C24) served as control. The *BvGLP-1* transcript of expected size is present in all transgenic lines but not in the control. The messenger (m)RNA levels for each cDNA probe were normalized with the *ubiquitin* mRNA level. **B**, Determination of the content of hydrogen peroxide (H_2O_2) in 7-day-old *BvGLP-1* transgenic *A. thaliana* (white) and the wild-type C24 (black). The H_2O_2 level was measured colorimetrically as described by Jana and Choudhuri (1982). Bars represent standard errors based on three independent experiments with 10 plants each. The assays show significant difference in H_2O_2 levels between transgenic lines and the wild-type *Arabidopsis* control as indicated by an asterisk. Significance tests were performed with Student's *t* test ($P < 0.05$).

Fig. 2. Survival rate of *Arabidopsis* plants after *Rhizoctonia solani* infection in agar test. Plants of three independent *BvGLP-1* transgenic *Arabidopsis thaliana* lines (white, light gray, and gray bars) and wild-type C24 (black) were infected with *R. solani* AG2-1. Seven-day-old seedlings of *A. thaliana* were transferred to agar plates for infection and the plant survival rate was scored 3, 6, 9, and 12 days postinoculation. Bars represent standard errors based on three independent experiments with 10 plants each. The data show significant differences in plant survival rates between transgenic lines and the wild-type control 9 and 12 days postinoculation. The asterisk indicates significance compared with the control (black bars) in each group according to the Student's *t* test ($P < 0.05$).

dopsis plants against *R. solani* in soil. For the *R. solani* infection assay, wild-type and transgenic *Arabidopsis* plants were directly transferred to pots filled with *R. solani*-infected soil and cocultivated in a growth chamber in which noninfected plants served as a control. No obvious growth and morphological differences between noninfected transgenic and wild-type *Arabidopsis* plants were visible (Fig. 3A). Seven days after cocultivation, clear differences between wild-type and transgenic plants were obvious (Fig. 5): strong symptoms appeared on most leaf surfaces of wild-type plants and more than $40\% \pm 3\%$ of them died after *R. solani* infection. In contrast, transgenic plants grew regularly even though slight disease symptoms were visible on a few leaves (Figs. 3A and 5). Twenty-one days after cocultivation, $87 \pm 3\%$ of the wild-type plants died after fungal infection while more than $76 \pm 3\%$ of transgenic plants survived (Fig. 5). These data are consistent with the results obtained from the agar plate assays and demonstrate that the expression of *BvGLP-1* in *Arabidopsis* confers significant resistance against *R. solani* infection.

A similar experiment was performed with *V. longisporum*. Wild-type and transgenic *Arabidopsis* seedlings were grown in small pots filled with soil for 14 days before infection. The infected plants were scored weekly and classified into the categories 1 to 9, based on the development of the disease symptoms (Table 1). Clear differences in the development of the disease symptoms were observed between transgenic and wild-type plants (Table 1; Fig. 3A). Fourteen days after infection, the first disease symptoms appeared on wild-type plants, in the form of chlorosis and dark-colored veins, mainly on older leaves but not yet on transgenic leaves, on which the first slight disease symptoms were only visible 21 days after infection (Table 1; Fig. 3A). After 21 days, noninoculated plants showed slight visual symptoms of senescence (score 1 to 2), wild-type plants suffered heavily from fungal infection, and more than 50% of the leaves showed severe disease symptoms (score 4 to 5) (Table 1). At 28 days after infection, more than 50% of the leaves of wild-type plants were dead (score 5 to 7), whereas only slight disease symptoms were detectable on the

Fig. 3. Examples of visual comparison between infected transgenic *Arabidopsis thaliana* expressing *BvGLP-1* and wild-type C24 plants in soil as well as on agar plates. **A**, *Arabidopsis* wild-type C24 and *BvGLP-1*-transgenic *Arabidopsis* plants infected with *Verticillium longisporum* and *Rhizoctonia solani* in soil 21 days postinoculation. Noninfected plants served as control. **B**, *Arabidopsis* wild-type C24 and *BvGLP-1*-transgenic *Arabidopsis* plants infected with *R. solani* on agar plate 12 days postinoculation.

Fig. 4. A, Cocultivation of wild-type (WT) and transgenic *Arabidopsis* seedlings with *Piriformospora indica* for 10 days. *Arabidopsis thaliana* seedlings were transferred to nylon discs placed on top of a modified PNM culture medium (Peřkan-Berghöfer et al. 2004). One seedling was used per petri dish. **B,** Increase in fresh weight (%) of WT seedlings (black bars) and seedlings of *BvGLP-1* transgenic lines (white, light gray, and gray bars) cocultivated with *P. indica* for 4 to 14 days compared with the uncolonized control. Bars represent standard errors based on three independent experiments with 60 seedlings each. The increase in fresh weight of WT seedlings (black) and seedlings of 3 *BvGLP-1* transgenic lines (white, light gray and gray bars) was significant between different time points (4 to 14 days) based on ANOVA analysis ($P < 0.05$). **C,** Increase in fresh weight (%) of WT (black) and *BvGLP-1* transgenic lines (white, light gray, and gray bars) cocultivated with *P. indica* for 4 to 14 weeks compared with the uncolonized control. Bars represent standard errors based on three independent experiments with 60 plants each. No significant differences in fresh weight of seedlings of *BvGLP-1* transgenic lines (white, light gray, and gray bars) were found between different time points (4 to 14 weeks) based on analysis of variance ($P < 0.05$). **D,** Colonization assays of the roots of 8- to 14-week-old WT and *BvGLP-1*-transgenic *Arabidopsis* plants grown in the presence (+) of *P. indica*; absence (–) served as control. The transcript levels of the fungal translation elongation factor 1 messenger (m)RNA (*cPitef1*) in the roots of colonized *Arabidopsis* seedlings were compared with the amount of the plant *actin* mRNA levels based on semiquantitative real-time polymerase chain reaction. RNA was isolated from the roots of the transgenic lines and the WT. After reverse transcription, *cPitef1* and *actin* were amplified. A significant reduction in the amount of fungal RNA was detected in the 14-week-old transgenic plants compared with the WT. **E,** Determination of the root colonization degree of WT and transgenic *Arabidopsis* plants. The transcript level of the fungal translation elongation factor 1 mRNA (*cPitef1*) and genomic DNA (*gPitef1*) in the roots of colonized *Arabidopsis* seedlings was compared with the amount of the plant *actin* nucleic acids resulting in the *cPitef1/actin* mRNA and *gPitef1/actin* mRNA ratios. The ratio of WT plants cocultivated with *P. indica* for 8 weeks was taken as 1.0 and the other values are expressed relative to it. A significant difference in the degree of root colonization was observed between WT and transgenic plants 14 weeks after cocultivation. Significance tests were performed with Student's *t* test ($P < 0.05$).

oldest leaves of the transgenic plants (score 1 to 2). These data suggest that the expression of *BvGLP-1* in *Arabidopsis* reduces the susceptibility of the plants to *V. longisporum* infection.

Microscopic observations of *Arabidopsis* roots infected with the phytopathogenic fungi.

For microscopic observation of the *R. solani* infection process, we stained the infected roots with lactophenol blue solution 3 and 7 days postinoculation. The lactophenol blue stained fungal hyphae. Washing the root system with the running tap water removed most of the nonattached mycelium. A dramatic reduction in the amount of fungal hyphae on the root surface (Fig. 6A and B) as well as of fungal mycelium normally developed inside the roots (data not shown) was observed in transgenic *Arabidopsis* when compared with wild-type roots. This result implies that the penetration as well as the development of the fungus in transgenic *Arabidopsis* roots was strongly inhibited. Accordingly, apart from the lower number of infections, the size of the lesions on the transgenic leaves was also reduced when compared with the wild-type plants (Fig. 6C and D). In conclusion, the microscopic observations support the finding from the infection experiments that *BvGLP-1* inhibits fungal infections on transgenic *Arabidopsis* plants.

Analysis of the transcript levels of selected defense related genes in transgenic plants.

PR proteins are involved in plant resistance to fungal infection. To define whether the resistance observed in this study is correlated with defense gene expression, we analyzed the *PR* and *PDF* transcript levels in noninoculated transgenic *Arabidopsis* plants and compared them with those in noninoculated wild-type plants by real-time PCR. All transgenic plants exhibited elevated levels of *BvGLP-1* transcripts (Fig. 1A). Even without pathogen attack, a strong transcriptional upregulation of *PR-1* to *PR-4* genes was observed in all selected transgenic lines compared with the wild-type control (Fig. 7) whereas no significant change in transcript level for *PDF 2.3* was visible. Furthermore, although the expression of *PDF1.2* was significantly upregulated in transgenic plants, the expression *PDF2.1* was downregulated when compared with the wild type (Fig. 7). Thus, *BvGLP-1* appears to regulate the expression of a subset of plant defense-related genes, leading to enhanced tolerance or resistance.

Cocultivation of *Arabidopsis* with the growth-promoting fungus *P. indica* in soil.

To evaluate the long-term impact of the *BvGLP-1* expression on a beneficial plant–fungus interaction, we performed a whole-life cocultivation assay of *Arabidopsis* with *P. indica* in soil. To this end, we transferred *Arabidopsis* plants cocultivated with or without the fungus from agar plates to pots. A growth promotion for the transgenic plants cocultivated with the fungus was still observed over a period of 4 to 8 weeks, and this promotion was comparable with the wild type (Fig. 4C). However, although the wild-type plants also responded to the fungus during later phases, flowered approximately 2 weeks earlier than the uncolonized control, and produced more seed, the beneficial effects for the transgenic plants became less (Fig. 4C). We observed only a slight but not significant increase in the fresh weight before the plants set flowers, the flowering time was only a few days earlier, and the seed yield was increased by only 5% ± 2% compared with the uncolonized transgenic plants.

Table 1. Disease scores on *Arabidopsis thaliana* plants inoculated with *Verticillium longisporum* in soil^a

Line	No. of seedlings	Disease scores at days postinoculation			
		7	14	21	28
Noninfected C24	30	1	1	1 to 2	1 to 2
Infected C24	30	1	2	4 to 5	5 to 7
Noninfected BvGLP-1	30	1	1	1 to 2	1 to 2
Infected BvGLP-1	30	1	1	1 to 2	2
Noninfected BvGLP-1-2	30	1	1	1	1 to 2
Infected BvGLP-1-2	30	1	1	2	2
Noninfected BvGLP-1-3	30	1	1	1	2
Infected BvGLP-1-3	30	1	1	1	2

^a Plants of three independent *BvGLP-1* transgenic *A. thaliana* lines and wild-type C24 were infected with *V. longisporum*. Assessment for scoring disease symptoms induced by the *Verticillium* sp. was performed according to Zeise (1992): 1, no symptoms; 2, slight symptoms on oldest leaf (yellowing, black veins); 3, slight symptoms on next younger leaves; 4, approximately 50% of leaves show symptoms; 5, >50% of leaves show symptoms; 6, up to 50% of leaves are dead; 7, >50% of leaves are dead; 8, only the apical meristem is still alive; 9, plants are dead. Disease scores were based on three independent experiments with 10 plants each. Noninfected plants served as control.

Fig. 5. Survival rate of *Arabidopsis* plants infected with *Rhizoctonia solani* AG2-1 in soil. Plants of three independent *BvGLP-1* transgenic *Arabidopsis thaliana* lines (white, light gray, and gray bars) and wild-type C24 (black) were infected with *R. solani* AG2-1. Seven-day-old seedlings of *A. thaliana* were transferred to soil for infection and the plant survival rate was scored 7, 14, and 21 days postinoculation. Bars represent standard errors based on three independent experiments with 10 plants each. The data show significant differences in plant survival rates between transgenic lines and the wild-type control 7, 14, and 21 days after inoculation based on Student's *t* test ($P < 0.05$), as indicated by an asterisk for each group.

Fig. 6. Light microscopic observation of *BvGLP-1* transgenic and wild-type *Arabidopsis* plants infected with *Rhizoctonia solani* after washing and staining with lactophenol blue solution 3 days (roots) and 7 days (leaves) postinoculation, respectively. **A**, *R. solani* growing in an undirected manner on the root surface of *BvGLP-1* transgenic *Arabidopsis* without firm attachment and **B**, attachment and directed-growth of *R. solani* hyphae over the root of *Arabidopsis* wild-type plants and formation of dome-like infection cushions. **C**, Intact leaf of *BvGLP-1* transgenic *Arabidopsis* and **D**, overgrowing of *R. solani* in *Arabidopsis* wild-type plants leading to leaf and tissue maceration. Symbols: r = root, m = mycelium, h = hyphae.

Fig. 7. Transcript analysis of *BvGLP-1*-activated defense related genes in transgenic *Arabidopsis* plants. Each pathogenesis-related (*PR*) and *PDF* gene expression level was determined by real-time polymerase chain reaction with three independent *BvGLP-1*-transgenic *Arabidopsis* lines. Wild-type *Arabidopsis* C24 plants served as control. Transcript levels for each gene were normalized with the *ubiquitin* messenger RNA level. Bars represent standard errors based on three independent experiments with 10 plants each. Transcript levels of PR-1, PR-2, PR-3, PR-4, and PDF1.2 but not of PDF2.3 were significantly higher in transgenic lines than in the wild-type control. In contrast, transcription of PDF2.1 in transgenic lines was significantly suppressed. Significance determined with the Student's *t* test ($P < 0.05$) was indicated by an asterisk in each group.

Because root colonization is a critical parameter for the beneficial interaction between *P. indica* and *Arabidopsis*, we checked the degree of root colonization of the transgenic plants and compared it with the wild type. We extracted RNA or DNA from the roots of 8- to 14-week-old plants cocultivated with *P. indica* for determination of root colonization by real-time PCR analysis. In both cases, the ratios were not significantly different (Fig. 4E), indicating that the *Pitef1* messenger (m)RNA level is proportional to the amount of the *Pitef1* DNA during our experimental conditions. No significant difference in the degree of root colonization was detected for wild-type and transgenic plants cocultivated with the fungus for 8 weeks (Fig. 4D and E) but we observed a significant reduction in the amount of fungal DNA or RNA in the 14-week-old transgenic plants when compared with the wild type (Fig. 4D and E). In two independent experiments with 30 plants each, root colonization was reduced by approximately 65% compared with the wild type. These results indicate that BvGLP-1 expression restricts root colonization during later growth phases, which subsequently leads to a decrease in benefits to the plant.

DISCUSSION

Plant GLP genes are members of large multigene families exhibiting diverse patterns of expression (Bernier and Berna 2001). Transgenic approaches make use of ectopic expression of GLP gene-enhanced resistance or tolerance to various pathogenic organisms, including *Sclerotinia sclerotiorum*, *Sep-toria musiva*, *Ostrinia nubilalis*, and *Blumeria graminis* (Thompson et al. 1995; Schweizer et al. 1999; Donaldson et al. 2001; Liang et al. 2001; Ramputh et al. 2002; Christensen et al. 2004). We extended these studies by using soilborne fungal pathogens and demonstrated that BvGLP-1 confers broad resistance to the two phytopathogenic fungi *R. solani* and *V. longisporum* when transferred into *Arabidopsis*. We found that BvGLP-1 expression in *Arabidopsis* stimulates the H₂O₂ production in plant cells, thus strongly suggesting its OxO or SOD activity, even though its enzymatic activity still remains to be determined. In sugar beet, the expression of BvGLP-1 is induced by nematode infection in resistant plants; therefore, it is reasonable to speculate that BvGLP-1 may represent a key point regulating plant defense responses, although the mechanism by which BvGLP-1 confers resistance to these pathogens is unclear thus far.

A possible explanation for the antifungal function of BvGLP-1 in *Arabidopsis* could be the generation of H₂O₂ through its enzymatic activity (OxO or SOD) in plant cells. H₂O₂ is important for reinforcement of the cell wall by cross-linking of plant cell wall proteins in papillae at the infection sites. Consequently, this leads to the protection of the cell against fungal penetration. In support of this, in our microscopic observations we found a drastic reduction in the amount of attached *R. solani* hyphae on the root surface as well as of developed hyphae within root cells in transgenic plants compared with the wild type, suggesting a strongly reduced penetration and development of the fungus in transgenic *Arabidopsis* roots. Local accumulation of H₂O₂ has been found in several barley-*B. graminis* interactions to be correlated with mlo-mediated resistance (Piffanelli et al. 2002). Furthermore, an enhanced resistance by transient overexpression of HvGER5a in epidermal barley cells was found to be dependent on its SOD activity (Zimmermann et al. 2006). Similar observations have been also reported by Olson and Varner (1993), Thordal-Christensen and associates (1997), Wei and associates (1998), and Christensen and associates (2004).

On the other hand, H₂O₂ produced by BvGLP-1 may function as a signal molecule activating plant defense responses. Sig-

naling pathways required for plant defense responses are complex, and even members from the same gene family that are induced by a single pathogen may require different signal molecules or combinations of signaling pathways for their expression (Ferrari et al. 2003). SA, JA, and ET are hormones involved in the regulation of resistance against different pathogens. SA is a key regulator of pathogen-induced SAR (Gaffney et al. 1993), whereas JA and ET regulate a largely distinct set of genes and are required for ISR. Both types of induced resistance are effective against a broad spectrum of pathogens, and several lines of evidence demonstrate cross talk between the pathways (Glazebrook 2005). It appears that defense genes that are activated against necrotrophic fungi are regulated primarily by the ET and JA signal transduction pathways, whereas biotrophic pathogens are countered more efficiently by SA-controlled defense mechanisms (Thomma et al. 1998). To test whether *Arabidopsis* plants overexpressing BvGLP-1 induce defense genes from SAR or other defense pathways, we analyzed the expression of PR and PDF genes, representative for two different signaling pathways (Thomma et al. 1998). In *Arabidopsis*, induction of PR-1, PR-2, and PR-5 follows an SA-dependent pathway, whereas the induction of the plant defensin PDF1.2, the basic chitinase gene PR-3, and the hevein-like protein gene PR-4 depends on a pathway involving at least JA as a signal molecule (Li et al. 2004). When BvGLP-1 was overexpressed in *Arabidopsis*, transgenic plants exhibited enhanced transcript levels for PR1-4 and PDF1.2. Hence, BvGLP-1 expression in *Arabidopsis* leads to the activation of both the SA- and JA/ET-dependent pathways, because the SA-dependent PR-1 and PR-2 genes and the JA/ET-dependent PR-3, PR-4, and PDF1.2 genes are upregulated in the transgenic lines. Because activation of the respective genes in the transgenic lines occurs even in the absence of the pathogens, the plants appear to be better protected. This strongly suggests that BvGLP-1 may be functional as a key signaling component of plant resistance mechanisms by specifically regulating the expression of a set of plant defense-related genes prior to pathogen attack. In this context, transgenic sugar beet overexpressing BvGLP-1 will provide the final evidence of the gene function.

Oxalic acid (OA) represents an important virulence factor and acts as an elicitor inducing programmed cell death that is required for pathogenicity as well as for disease development of various fungal pathogens (Bateman and Beer 1965; Stone and Armentrout 1985; Godoy et al. 1990). It is reasonable to speculate that degradation of fungal OA may represent a possible mechanism of the BvGLP-1-mediated resistance against the two fungal pathogens studied here, *V. longisporum* and *R. solani*. Evidently, overexpression of an oxalate decarboxylase from *Collybia velutipes*, an OA-degrading enzyme, was found to confer resistance to infection caused by the OA-producing fungus *Sclerotinia sclerotiorum* in transgenic tobacco and tomato (Kesarwani et al. 2000). Similarly, soybean, tobacco, sunflower, and rapeseed plants transformed with a wheat OxO showed enhanced resistance to *S. sclerotiorum* (Zaghmout et al. 1997; Donaldson et al. 2001; Hu et al. 2003; Dong et al. 2008). In this scenario, an enhanced OxO activity leads to OA degradation (and, thus, an inhibition of toxin-induced cell death) on the one hand and H₂O₂ production (and, thus, a stimulation of the hypersensitive response) on the other hand. Further analyses are required to understand the enzymatic reaction of BvGLP-1 which leads to enhanced tolerance or resistance against *R. solani* and *V. longisporum*.

Interestingly, the presence of BvGLP-1 does not primarily affect the beneficial interaction between *P. indica* and *Arabidopsis* (Fig. 4). This clearly indicates that different signaling processes are required for the activation of the resistance against the two tested necrotrophs and the beneficial interaction with

P. indica. However, in long-term cocultivation experiments (i.e., in older plants which were exposed to *P. indica* during their whole life), the expression of *BvGLP-1* in plants appears to have an impact on growth and development of the beneficial fungus as well, because root colonization by *P. indica* is significantly reduced. We propose that, in older plants, *BvGLP-1*-induced defense gene activation and H_2O_2 production restricts fungal growth and, thus, diminishes root colonization and the benefits for the plants. It has been proposed for barley that programmed cell death is required for the colonization of the roots by *P. indica* (Deshmukh et al. 2006); however, we do not have any evidence that this plays a role in this scenario. It is worth noting that, in spite of the reduced growth promotion during later stages of the interaction, we did not observe any harm to the plants, and the biomass and seed production was not lower for plants cocultivated with *P. indica* when compared with the uncolonized control.

Our results showed that the transgenic plants were not completely protected against infection by *R. solani* and *V. longisporum*, because they became infected and necrotic after a longer period of infection. It has been reported that overexpression of PR proteins such as chitinases (Broglie et al. 1991, Datta et al. 2000) or a ribosome-inactivating protein (Maddaloni et al. 1997) resulted in enhanced resistance to *R. solani*, primarily through a delay in the development of disease symptoms. However, the observed delay of the disease may give the transgenic plants enough time to induce defense mechanisms to ward off the pathogen systemically. Because of a broad resistance to various pathogens, *BvGLP-1* provides a promising candidate gene for genetic engineering for improving crop resistance to different pathogens.

MATERIALS AND METHODS

Plant material and fungal strains.

Arabidopsis thaliana C24 (Lehle Seeds, Round Rock, TX, U.S.A.) was used for generation of transgenic plants. *R. solani* AG2-1 was grown on potato dextrose agar (PDA) for 7 days at 24°C with a 12-h photoperiod according to Keijer and associates (1997). *V. longisporum* isolate 43, provided by E. Diederichsen (Institut für Biologie, Angewandte Genetik, FU Berlin), was cultured on PDA for 14 days in the dark and further cultivated every 2 to 3 weeks according to Eynck and associates (2007).

Generation of transgenic *A. thaliana* plants.

BvGLP-1 (GenBank accession number AY243465) was cloned into the binary vector pAM194 (KWS Saat AG, Einbeck, Germany) under the transcriptional control of the 35S promoter, resulting in the plant expression construct pAM194-*BvGLP-1*. The recombinant binary vector was transformed into *Agrobacterium tumefaciens* GV3101 (Koncz and Schell 1986). Transgenic *Arabidopsis* plants were generated by using the root transformation protocol (Valvekens et al. 1988). Transgenic plants were propagated under selective condition to the T3 generation. The homozygous phenotype was selected by kanamycin resistance. The presence of the transgene was confirmed by transgene-specific PCR amplification.

Infection assay of *Arabidopsis* plants with *Rhizoctonia* spp. on agar plates.

Transgenic as well as wild-type *Arabidopsis* seed were sterilized with 5% $Ca(OCl)_2$ for 10 min and then in 70% ethanol for a further 5 min. After washing with sterile water, seed were germinated on 0.2× KNOP medium (Knop 1860) for 5 days in a plant growth chamber (22°C, photoperiod = 16 h of light and 8 h of darkness). Seedlings were transferred to new 0.2× KNOP agar plates for infection experiments in which the non-

transgenic *Arabidopsis* seedlings served as a control. The seedlings were incubated with 1-cm-diameter mycelium plaques from 7-day-old potato dextrose *R. solani* culture. The plates were incubated in the growth chamber (22°C, photoperiod = 16 h of light and 8 h of darkness). At 3, 6, 9, and 12 days after inoculation, the plant survival ratio was scored.

Infection assay of *Arabidopsis* plants with *Rhizoctonia* spp. in soil.

Sterile soil was infected by placing a 1-cm-diameter disc of mycelium isolated from 7-day-old potato dextrose broth culture of *R. solani* at a 1-cm depth in each pot 10 days before planting. *Arabidopsis* seed were sterilized as described before and grown at 22°C with a photoperiod of 16 h of light and 8 h of darkness on agar plates containing 0.2× KNOP medium for 7 days. One-week-old *Arabidopsis* seedlings were transferred to infected pots and grown in a growth chamber at 22°C with a photoperiod of 16 h of light and 8 h of darkness. Based on the symptoms, scored 7, 14, and 21 days after planting, plants were classified either to healthy (no or scarce disease symptoms) or diseased (severe wilting or dead plants) groups as described by Broglie and associates (1991) and Perl-Treves and associates (2004).

Infection assay of *Arabidopsis* plants with *Verticillium* spp. in soil.

Arabidopsis plants were cultivated in soil in small pots (diameter = 35 mm) under short-day conditions (photoperiod = 16 h of light and 8 h of darkness). Spores of *V. longisporum* were obtained by gently incubating in liquid CZAPEK-DOX medium for 14 days at 25°C on the rotary shaker. The resulting suspension was filtered through sterile gauze. Spore concentration was determined with a Fuchs-Rosenthal counting chamber. Infections were made by direct application of spore suspensions to the soil as described by Zeise and von Tiedemann (2002). Direct inoculation was conducted 2 weeks after germination. The pots were inoculated by infection of 1 ml of conidia suspension (4×10^7 conidia ml^{-1}) per plant near roots. Plants were scored weekly (7, 14, 21, and 28 days postinoculation) based on the developed disease symptoms and classified into nine classes (Zeise and von Tiedemann 2002) with the following modifications: 1, no symptoms; 2, slight symptoms on oldest leaf (yellowing, black veins); 3, slight symptoms on next younger leaves; 4, approximately 50% of leaves show symptoms; 5, >50% of leaves show symptoms; 6, up to 50% of leaves are dead; 7, >50% of leaves are dead; 8, only the apical meristem is still alive; and 9, plants are dead.

Cocultivation experiments with *P. indica*.

Arabidopsis seed were surface sterilized and placed on petri dishes containing Murashige and Skoog (MS) nutrient medium (Murashige and Skoog 1962). The plates were incubated for 7 days at 22°C under continuous illumination ($100 \mu mol m^{-2} s^{-1}$). *P. indica* was cultured as described previously (Peškan-Berghöfer et al. 2004). After inoculation with the fungus, the plates were kept at room temperature in the dark for 1 to 2 weeks. Nine-day-old *Arabidopsis thaliana* seedlings were transferred to nylon discs (mesh size: 70 μm) placed on top of a modified PNM culture medium (Peškan-Berghöfer et al. 2004). One seedling was used per petri dish. After 24 h, fungal plugs of 5 mm in diameter were placed at a distance of 1 cm from the roots. Plates were then incubated at 22°C under continuous illumination from the side. Plants were assayed 4, 6, 8, 10, 12, and 14 days after cocultivation. The experiments on soil were followed the protocol described by Peškan-Berghöfer and associates (2004). *Arabidopsis* seedlings were germinated on MS medium before transfer to sterile soil mixed with the

Table 2. Primers used in the gene expression experiments

AGI number	Primer	Primer sequence 5'-3'
AT2G14610	PR-1 f/r	GGAGCTACGCAGAACAACTA/AGTATGGCTTCTCGTTCACA
AT3G57260	PR-2 f/r	CTACAGAGATGGTGTCA/AGCTGAAGTAAGGGTAG
AT3G12500	PR-3 f/r	TGGATGGGCTACAGCACC/CTAAATAGCAGCTTCGAGGAGG
AT3G04720	PR-4 f/r	GGCCGGACAACAATGCGGTCGTCAAGG/CAAGCATGTTTCTGGAATCAGGCTGCC
AT5G44420	PDF1.2 f/r	ATGGCTAAGTTTGCTTCCA/TTAACATGGGACGTAACAGATAC
AT2G02120	PDF2.1 f/r	GATGGGTCCAGTCACGGTC/TTCAAGAACACACTAAACACGC
AT2G02130	PDF2.3 f/r	CACACACAACGTGCAACG/CGGAAACACACAAACCAATG
AY243465	BvGLP-1 f/r	CTCCTAGCCTCTGTGAATTCTAGC/GAATGGAAACAAGCAACATATGATATC
AT4G05320	Ubiquitin	ACTCTACCGGAAAGACAATC/TGACGTTGTGTCGATGGTGTCAG
At3G53750	Actin	GGTCGTGACCTCACAGATGCTCGCTCCTGCTCATAGTCAAGAGC

fungus (0.6%, wt/wt). Cultivation occurred in multi-trays with Aracon tubes in a plant growth chamber at 22°C under long-day conditions. For the exposure of *Arabidopsis* seedlings to a loan of *P. indica*, the fungus was inoculated in liquid KM media (Vadassery et al. 2009) until an optical density at 650 nm of 0.5, and 3.5 ml of this suspension was distributed on top of a petri dish containing 20 ml of the modified PNM culture medium. The plates were then incubated at 22°C under continuous illumination ($80 \mu\text{mol m}^{-2} \text{s}^{-2}$) for 72 h. During this time, the fungal hyphae started to develop a loan. Then, 9-day-old *Arabidopsis* seedlings were transferred to the loan.

Determination of the degree of root colonization.

Roots of sterile seedlings or plants in pots cocultivated with *P. indica* were used for the determination of colonization. The colonized (and control) roots were removed from the soil, intensively rinsed with an excess of sterile water (50 ml each) to remove the soil and the loosely attached fungal hyphae, and then frozen in liquid nitrogen for RNA or DNA extraction. *P. indica* was monitored with a primer pair for the translation elongation factor 1 (*Pitef1*) (Bütehörn et al. 2000): ACCGTC TTGGGGTTGTATCC and TCGTCGCTGTCAACAAGATG. The degree of the root colonization was determined based on the ratios of *Pitef1* mRNA (*cPitef1*) and *Pitef1* genomic DNA (*gPitef1*) of the fungus relative to the *actin* RNA from the plant.

Staining assays and light microscopy observations.

Lactophenol blue staining was used to analyze host-pathogen interaction (Perl-Treves et al. 2004). To monitor fungal hyphae on or in *Arabidopsis* roots, seedlings were removed from agar plates and intensively washed with running tap water for 2 min to remove nonattached mycelium. The lactophenol blue staining was carried out in small petri dishes with gentle shaking, and seedlings were cleared using hot 10% (wt/vol) KOH for 10 min. The seedlings were subsequently stained using lactophenol blue solution for 5 min and destained with water for at least 20 min until no more blue color came out. Stained seedlings were examined and documented under the bright-field microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

Semiquantitative- and real-time RT-PCR.

Semiquantitative and real-time RT-PCR were used to assay the expression patterns of the genes of interest. Noninoculated transgenic as well as control *Arabidopsis* roots were removed from the soil or plates, rinsed 12 times with an excess of sterile water (50 ml each), and frozen in liquid nitrogen for RNA extraction. Extraction of total RNA from *Arabidopsis* roots was performed following the trizol protocol (Gibco BRL Life Technologies). The total RNA was treated with RNase-free DNase (Fermentas, St. Leon-Rot, Germany) for 60 min at 37°C. Synthesis of cDNA was carried out using a Superscript III First-Strand Synthesis System (Invitrogen, Karlsruhe, Germany) according to the instructions of the manufacturer. RT-PCR was performed in 50- μl reactions consisting of 2.5 μl of cDNA at 10

ng/ μl , 5 μl of 10 $\times$ buffer, 0.5 μl of 10 mM dNTPs, 5 μl each of primer at 10 pmol/ μl , 2.5 units of Taq polymerase (Invitrogen), and 31.5 μl H₂O under a PCR program of 94°C for 50 s, 54°C for 1 min, and 72°C for 1 min for 25 cycles, followed by 10 min at 72°C. Amplicons, separated on a 1% (wt/vol) agarose gel, were visualized under UV light. The housekeeping *ubiquitin* gene served as a control and the mRNA levels for each cDNA probe were normalized to the *ubiquitin* message RNA level. The real-time PCR was performed using the iCycler iQ Detection System (Bio-Rad Laboratories GmbH, Munich). The iQ SYBR Supermix (Bio-Rad Laboratories GmbH) was used for PCR reactions according to the manufacturer's instructions in a final volume of 20 μl . The iCycler was programmed to 95°C for 2 min; 35 cycles of 95°C for 30 s, 55°C for 40 s, and 72°C for 45 s; and 72°C for 10 min, followed by a melting curve program (55 to 95°C in increasing steps of 0.5°C). The mRNA levels for each cDNA probe were normalized with respect to the *actin/ubiquitin* message level. Fold induction values were calculated with the $\Delta\Delta\text{CP}$ equation of Pfaffl (2001).

Primers used in the described gene expression experiments are shown in Table 2.

H₂O₂ measurements.

The H₂O₂ level was measured colorimetrically as described by Jana and Choudhuri (1982). H₂O₂ was extracted by homogenizing plant material with 2 ml of phosphate buffer (50 mM, pH 6.5), including 10 mM 3-amino-1,2,4-triazole (Sigma, Germany). The homogenate was centrifuged at $6,000 \times g$ for 25 min at 4°C. Extracted solution (600 μl) was mixed with 200 μl of 0.1% titanium sulfate (Sigma) in 20% H₂SO₄ (vol/vol) and the mixture was then centrifuged at $6,000 \times g$ for 15 min at 4°C. Fluorescence was measured at 410 nm by using a TECAN SPECTRA fluorescence spectrophotometer (TECAN, Germany). Because the generated H₂O₂ is expected to correlate with the amount of BvGLP-1 expressed in plant cells, the H₂O₂ level was calculated on the basis of total protein quantified according to Bradford (1976).

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