The Germinlike Protein GLP4 Exhibits Superoxide Dismutase Activity and Is an Important Component of Quantitative Resistance in Wheat and Barley

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Germinlike proteins (GLP) are encoded in plants by a gene family with proposed functions in plant development and defense. Genes of GLP subfamily 4 of barley (*HvGLP4*, formerly referred to as *HvOxOLP*) and the wheat orthologue *TaGLP4* (formerly referred to as *TaGLP2a*) were previously found to be expressed in pathogen-attacked epidermal tissue of barley and wheat leaves, and the corresponding proteins are proposed to accumulate in the apoplast. Here, the role of *HvGLP4* and *TaGLP4* in the defense of barley and wheat against *Blumeria graminis* (DC.) E. O. Speer, the cereal powdery mildew fungus, was examined in an epidermal transient expression system and in transgenic *Arabidopsis thaliana* plants overexpressing His-tagged *HvGLP4*. Leaf extracts of transgenic *Arabidopsis* overexpressing *HvGLP4* contained a novel His-tagged protein with superoxide dismutase activity and *HvGLP4* epitopes. Transient overexpression of *TaGLP4* and *HvGLP4* enhanced resistance against *B. graminis* in wheat and barley, whereas transient silencing by RNA interference reduced basal resistance in both cereals. The effect of *GLP4* overexpression or silencing was strongly influenced by the genotype of the plant. The data suggest that members of GLP subfamily 4 are components of quantitative resistance in both barley and wheat, acting together with other, as yet unknown, plant components.

Additional keywords: RNAi, transgene.

Attack of wheat and barley by *Blumeria graminis* (DC.) E. O. Speer, the causal agent of cereal powdery mildew and agronomically one of the most important pathogens of both crops, is accompanied by multiple defense responses. These include formation of cell-wall appositions (papillae) at sites of attempted penetration, hypersensitive cell death (HR) of attacked epidermal cells, as well as accumulation of a number of pathogenesis-related (PR) proteins and of low molecular-weight components (Schulze-Lefert and Vogel 2000; Thordal-Christensen et al. 2000). In many instances of disease resis-

tance mediated by single major genes of the host (*R* genes), the plant response is characterized by HR (Hückelhoven et al. 1999). On the other hand, quantitative or basal disease resistance has been found to be based on efficient papillae as well as on HR, to varying extent (Asher et al. 1983; Kmecl et al. 1995). Basal resistance is probably expressed in all susceptible wheat-*Blumeria* and barley-*Blumeria* interactions and defines the extent to which infection attempts fail.

Upon attack of barley and wheat by *B. graminis* or other pathogens, a number of defense-related or PR genes are induced, as in many other plant species (Gregersen et al. 1997; Schweizer et al. 1989). Some of these genes encoding, for instance, chitinases, β -1,3-glucanases, or thionins may play a role by directly attacking the invading pathogen or by building up new barriers against fungal ingress or spread. For others, the function is still unknown. Since the vast majority, if not all, of the PR genes are known to be induced in all types of incompatible as well as compatible interactions between wheat or barley and *B. graminis*, the defense-related relevance of these putative downstream effector genes remains unknown in these interactions (Thordal-Christensen et al. 2000).

In barley, the large and poorly understood superfamily of germinlike proteins (GLP) has recently been grouped into five subfamilies (Druka et al. 2002). Members of the barley and wheat GLP4 subfamily (*HvGLP4*, formerly referred to as *HvOxOLP*, and *TaGLP4*, formerly referred to as *TaGLP2*, respectively) appear to be expressed predominantly in *B. graminis*-attacked leaf epidermal tissue (Schweizer et al. 1999a; Wei et al. 1998). This expression pattern is supported by data-mining in the barley expressed sequence tag (EST) IPK database, in which 44 *HvGLP4* out of approximately 5,000 EST from pathogen-attacked epidermis compared with one of a total of approximately 106,000 ESTs from 21 other cDNA libraries. The deduced mature peptide sequences of *TaGLP4* and *HvGLP4* have residues 96% identical, which is clearly higher than identities between GLP of different subfamilies within one of those species, suggesting that the *HvGLP4* and *TaGLP4* genes are true orthologues. *HvGLP4* has been found to represent a cluster of at least nine duplicated genes (Druka et al. 2002; Wei et al. 1998), and the same may be true for *TaGLP4* in wheat, based on EST clustering analysis and hybridization patterns to wheat genomic DNA (data not shown). For convenience, the specific gene subfamily member of barley and wheat used in this study is simply named

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HvGLP4 and *TaGLP4*, respectively. Both have been found to be induced by *B. graminis* by using gene specific probes of the 5' or 3' untranslated region of the corresponding mRNAs (data not shown).

HvGLP4 exists in a heat-stable and sodium dodecyl sulfate (SDS)-resistant oligomeric form (Wei et al. 1998), similar to *TaGLP1* (oxalate oxidase). Crystallographic analysis of *TaGLP1* has demonstrated a homohexameric structure organized as a trimer of dimers with a molecular mass of approximately 100 kDa. While the monomer is stabilized by a single disulfide bond between two cysteine residues also present in GLP4, both the dimers and the trimer are stabilized by extensive hydrophobic interactions (Woo et al. 2000). The tight organization of the hexamer presumably accounts for the extreme heat-tolerance, the resistance to SDS, and the proteolytic stability known for GLP (Woo et al. 2000). Some GLP were identified as oxalate oxidases and superoxide dismutases (SOD) (Carter and Thornburg 2000; Woo et al. 2000; Yamahara et al. 1999), *HvGLP2* (formerly referred to as *HvGLP1*) was identified as an extra-cellular nucleotide-sugar pyrophosphatase/

phosphodiesterase (Rodriguez-Lopez et al. 2001), and a wheat GLP closely related to *HvGLP2* has recently been described as a serine protease inhibitor (Segarra et al. 2003). Besides known enzymatic activities for some germinlike proteins, data concerning their biological roles in plant development or stress responses are scarce. Only for wheat oxalate oxidase (*TaGLP1*) are functional data from transgenic plants overexpressing this gene available, and these suggest that the oxalate oxidase protein plays a role in warding off oxalate-producing pathogens as well as herbivores (Donaldson et al. 2001; Liang et al. 2001; Ramputh et al. 2002).

In order to obtain functional data on defense-related genes in wheat and barley more rapidly than would be possible based on transgenic plants, a transient assay system was developed for overexpression of host or foreign genes as well as for gene silencing by RNA interference (RNAi) in single epidermal cells of wheat and barley (Nielsen et al. 1999; Schweizer et al. 1999b, 2000). The transient assay has proven valuable for revealing novel gene function in race-specific as well as race-nonspecific interactions between barley and *B. graminis* f. sp.

Fig. 1. The barley *HvGLP4* has superoxide dismutase (SOD) activity. **A**, Wild-type, N-, and C-terminally His-tagged versions of *HvGLP4* were expressed ectopically in *Arabidopsis thaliana* (Columbia). SP = wild-type signal peptide of *HvGLP4*; 6H = six histidine residues. **B**, Immunoblot, using an *HvGLP4*-specific antiserum, of denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels shows the monomers of the three versions of *HvGLP4* in crude *A. thaliana* leaf extract. Extract of *A. thaliana* transformed with the pCAMBIA3301 vector served as a negative control. **C**, Immunoblot, using an *HvGLP4*-specific antiserum, of semi-native SDS-PAGE gels loaded with crude leaf extract samples, which have not been boiled with a reducing agent prior to loading. **D**, In-gel SOD assay. I and II denote extracts from independent transgenic lines. **E**, Semi-native SDS-PAGE of recombinant C-terminally His-tagged *HvGLP4*, purified from transgenic *A. thaliana* by Ni-affinity chromatography, loaded onto three lanes, which were analyzed separately: as in C, D, and by Coomassie total protein staining. Numbers at the left borders of the gels or blots indicate molecular masses in kDa.

hordei (Azevedo et al. 2002; Hüchelhoven et al. 2003; Kim et al. 2002; Zhou et al. 2001). Using this assay, enhanced resistance against *B. graminis* f. sp. *tritici* of wheat epidermal cells transiently expressing *TaGLP1* (oxalate oxidase) and *TaGLP4* was obtained, suggesting that germinlike proteins play a role in pathogen defense of cereals (Schweizer et al. 1999a).

The race-nonspecific cell-wall penetration resistance exerted by leaf epidermal cells against the initial attack by many fungi is found to be accompanied by the accumulation of H_2O_2 in several plants (Heath 2001; Mellersh et al. 2002; Thordal-Christensen et al. 1997). Penetration resistance is believed to be the result of de novo development of a local cell wall fortification consisting of a number of structural polymers that become crosslinked by a proposed H_2O_2 -driven process. In cowpea, tomato, and plantain, removal of the penetration-associated H_2O_2 , using catalase, leads to susceptibility, suggesting that this H_2O_2 has a role in resistance (Mellersh et al. 2002). In barley and wheat, the temporal and spatial expression pattern of *GLP4*, together with sequence similarity to the H_2O_2 -generating oxalate oxidase (GLP1), led to speculations that this gene may be responsible for producing the penetration-associated H_2O_2 (Schweizer et al. 1999a; Wei et al. 1998). Here, we identify *HvGLP4* as a functional SOD, and by transient overexpression

and RNA interference experiments with *HvGLP4* and *TaGLP4*, we provide evidence that GLP4 contributes to disease resistance in wheat and barley.

RESULTS

HvGLP4 possesses SOD activity.

In order to characterize *HvGLP4* biochemically, we expressed this barley protein ectopically in *Arabidopsis thaliana*. Three gene constructs, all for 35S promoter-driven constitutive expression of *HvGLP4*, were generated: p35S-*HvGLP4* for the wild-type barley protein and p35S-N-His-*HvGLP4* plus p35S-*HvGLP4*-C-His for N- and C-terminal histidine (His)-tagged versions of the protein, respectively (Fig. 1A). *HvGLP4* has a predicted signal peptide directing the protein into the default secretory pathway. Therefore, the N-terminal His-tag was introduced to two amino acids C-terminal of the predicted signal peptide cleavage site. Transgenic plants with each of the three constructs expressed *HvGLP4* protein, as demonstrated by immuno-blotting of protein extracts using a *HvGLP4*-specific antiserum (Fig. 1B and C). *HvGLP4*, like other GLP, exists in an oligomeric, SDS-resistant form, and this oligomer can be revealed if run in a "seminative" way, i.e., on SDS gels without prior boiling with a reducing agent (Wei et al. 1998). After denaturing SDS-polyacrylamide gel electrophoresis (PAGE), the gel blot showed the N-terminally His-tagged monomers to be approximately 2 kDa larger than the wild-type and C-terminally His-tagged proteins (Fig. 1B), suggesting that the signal peptide has not been cleaved from the N-terminally His-tagged protein. This most likely explains why the oligomeric form of the N-terminally His-tagged protein did not assemble, as shown on the blot of the seminative gel (Fig. 1C). However, oligomers of the wild-type and the C-terminally His-tagged protein migrated to similar positions as the protein extracted from barley epidermal tissue (Fig. 1C).

Fig. 2. *Blumeria graminis* f. sp. *hordei*-induced superoxide dismutase in the barley leaf epidermis. SOD, In-gel assay of crude extracts of whole-leaf and epidermal tissue of cv. Ingrid, respectively, prepared 24 h postinoculation with *B. graminis* f. sp. *hordei* (+). – = noninoculated controls. The loaded samples were not boiled with reducing agent (seminative). Immunoblot, blot of a seminative gel developed with *HvGLP4*-specific antiserum.

Fig. 3. Constructs used for transient overexpression or RNA interference in wheat and barley leaf segments.

Fig. 4. Transient overexpression of *GLP4* enhances basal disease resistance in wheat and barley. Barley *HvGLP4* cDNA (construct pHvGLP4) was transiently overexpressed, followed by inoculation with *B. graminis* and scoring of the interaction phenotype 40 h postinoculation. Bombarded, successfully transformed cells were detected by β -glucuronidase-staining. As an exception, wheat cv. FAP75141 was bombarded with *TaGLP4* (construct pTaGLP4) and not with *HvGLP4*. As positive control, the WIR3 gene from wheat, encoding a putative peroxidase, was overexpressed (pWIR3, Schweizer et al. 1999b). Mean $\pm$ SE of at least four independent bombardments, with a total of approximately 2,000 observed interactions per cultivar and transgene. * = statistically significant (one-sample *t*-test against control value; *P* < 0.05).

Inspired by reports of SOD activity of other GLP (Carter and Thornburg 2000; Woo et al. 2000; Yamahara et al. 1999), the heterologously expressed *HvGLP4* proteins were subjected to an in-gel SOD assay. Crude extracts of plants producing the wild-type or the C-terminally His-tagged *HvGLP4* showed a catalytically active protein of approximately 100 kDa (Fig. 1D). Meanwhile, extracts of plants expressing the N-terminally His-tagged *HvGLP4* and of control plants transformed with an empty vector construct showed no activity (Fig. 1D and data not shown, respectively). In plants expressing oligomeric forms of *HvGLP4*, SOD activities were occasionally seen in several bands in the gel (Fig. 1D). Affinity purification on a Ni-NTA column of the C-terminally His-tagged *HvGLP4* directly from desalted, crude protein extracts was not successful. However, denaturation in 8 M urea allowed it to bind to the column. Following a gradual removal of the urea on the column using a 1-mM Mn^{++} buffer, an apparently pure protein oligomer was eluted, which had a mass of 72 kDa on the semipreparative gel. The same protein was recognized by the *HvGLP4*-specific antibody and exhibited SOD activity (Fig. 1E). On a denaturing gel, the protein had a mass of approximately 25 kDa (data not shown). The cause of the difference in size of the protein before and after affinity chromatography is currently not understood. GLP hexamers are believed to be organized in a very tight structure as a trimer of dimers stabilized by hydrophobic interactions (Woo et al. 2000). However, renaturation of the protein on the column may favor formation of a form that differs from the one assembled in the plant. Nevertheless, this experiment shows that *HvGLP4* has SOD activity and requires Mn^{++} , as omission of Mn^{++} in the buffer during renaturation resulted in loss of SOD activity (data not shown).

The transgenic *Arabidopsis* lines expressing active *HvGLP4* SOD under the control of the constitutive 35S promoter did not

exhibit an altered morphological phenotype. The susceptibility to *Erysiphe cichoracearum*, causing powdery mildew on *Arabidopsis*, was also not affected (data not shown).

Knowing that the barley epidermal *B. graminis* f. sp. *hordei*-induced *HvGLP4* possesses SOD activity, we wanted to confirm this response at the enzyme level. Figure 2 shows that, within the barley leaf, a SOD enzyme of approximately 100 kDa is concentrated in the epidermis and this activity increases in response to powdery mildew attack. The response is paralleled by an increase in abundance of a protein of the same size that is recognized by the *HvGLP4*-specific antibody.

GLP4 is important for quantitative resistance in wheat and barley.

Using a recently developed transient assay system (Schweizer et al. 1999b; 2000), *HvGLP4* and *TaGLP4* were overexpressed as well as silenced by RNAi in a panel of susceptible wheat and barley cultivars. Leaf segments were bombarded with different constructs, as shown in Figure 3. Overexpression of *HvGLP4* and of *TaGLP4* significantly reduced the frequency of fungal haustoria formation in two of three tested wheat and in one of four tested barley cultivars (Fig. 4), whereas overexpression of *TaWIR3* encoding a putative peroxidase reduced haustorium formation in all tested lines. Overexpression of *HvGLP4* and of *TaGLP4* in wheat cv. Fidel reduced haustorium formation to $(65 \pm 10)\%$ and $(56 \pm 6)\%$ of control, respectively, indicating that both transgenes have a similar resistance-enhancing potential (Schweizer et al. 1999a) (Fig. 4). The trend for enhanced relative haustorium formation in wheat cv. Kanzler and barley cv. Golden Promise overexpressing *GLP4* genes was statistically not significant and will not be discussed further. Silencing of the *TaGLP4* gene in wheat and the *HvGLP4* gene in barley by RNAi revealed enhanced haustorium formation of *B. graminis* f. sp. *tritici* and *B. graminis* f. sp. *hordei* on one of the two tested wheat and barley cultivars, respectively (Fig. 5). In wheat cv. FAP75141 and in barley cv. Golden Promise, the effect was observed irrespective of whether RNAi was induced by microprojectile bombardment with double-stranded (ds)RNA-coated particles or by particles coated with RNAi constructs, giving rise to spliced hairpin dsRNA in vivo. Prolongation of the time between bombardment and challenge-inoculation considerably enhanced basal resistance in wheat, which prompted us to challenge-inoculate leaves as early as 4 h postbombardment. In barley, no effect of *pHvGLP4* hairpin was observed when leaves were challenge-inoculated 4 h after bombardment (data not shown). Therefore, the incubation time between bombardment and challenge-inoculation was prolonged to 48 h. This was possible because there was no dramatic increase in basal resistance of bombarded barley leaves over time, in contrast to wheat. Indeed, under these experimental conditions, there was a strong RNAi effect found in barley cv. Golden Promise (Fig. 5). Based on these results, it may be speculated that, in both wheat and barley bombarded with hairpin constructs, the RNAi effect took longer to become fully established than the available time between bombardment and attempted fungal penetration upon early challenge-inoculation (4 h). In barley, this may have resulted in no detectable effect, whereas in

Fig. 5. Transient silencing of *GLP4* by RNA interference (RNAi) induces hypersusceptibility to *Blumeria graminis* in wheat and barley. RNAi was induced by bombarding wheat and barley leaves with tungsten and gold particles, respectively, which were coated with double-stranded (ds)RNA or with hairpin constructs. Wheat was bombarded with dsRNA of *TaGLP4* or with construct *pTaGLP4*hairpin, whereas barley was bombarded with dsRNA of *HvGLP4* or with *pHvGLP4*hairpin. Note that barley leaves transformed with the *pHvGLP4*hairpin construct were challenge-inoculated 48 h postbombardment, whereas all other leaves were challenge-inoculated 4 h postbombardment. Mean $\pm$ SE of at least five independent bombardments, with a total of approximately 1,200 observed interactions per cultivar and transgene. * = statistically significant (one-sample *t*-test against control value; $P < 0.05$).

Table 1. *HvGLP4* double-stranded (ds)RNA specifically interferes with the accumulation of *HvGLP4::GFP* fusion protein

Constructs	Fluorescent cells/shot	
	Golden Promise	Ingrid
pHvPrx7 hairpin + pHvGLP4::GFP	370 ^a	342
pHvGLP4 hairpin + pHvGLP4::GFP	9	2
pHvPrx7 hairpin + pGFP	105	336
pHvGLP4 hairpin + pGFP	264	270

^a Mean values from two independent bombardment experiments.

wheat, it resulted in a weaker effect, compared with the one in barley that was challenge-inoculated 48 h after bombardment. By contrast, the RNAi effect of directly bombarded dsRNA may have been faster compared with hairpin constructs because no transcription and splicing was required within transformed cells but, also, more transient because it depended on limiting initial amounts of active principle (dsRNA) that reached the target cells (Schweizer et al. 2000).

The efficiency of RNAi was demonstrated in an experiment in which barley was cobombarded with a *HvGLP4::GFP* fusion construct plus the *HvGLP4* RNAi construct (Fig. 3 and Table 1). As control, an RNAi construct of barley peroxidase *Prx7* was cobombarded with the *HvGLP4::GFP* fusion construct. The *HvGLP4* RNAi construct inhibited the accumulation of *HvGLP4::GFP* fusion protein with an efficiency of approximately 98% in both cvs. tested (Ingrid and Golden Promise). The RNAi effect was gene specific, as the *pHvGLP4*hairpin RNAi construct had no inhibitory effect on the accumulation of green fluorescent protein (GFP) alone. These results indicate that the absence of an effect of *HvGLP4* RNAi on the interaction of barley cv. Ingrid with *B. graminis* f. sp. *hordei* was probably not due to a reduced RNAi capacity, as compared with cv. Golden Promise.

In an attempt to correlate *GLP4* expression to the observed effects of *GLP4* overexpression or silencing, we carried out Northern blot experiments in the four lines that were also used for RNAi studies (Fig. 6). The expectation might be that weak expressers would respond to *GLP4* overexpression with enhanced resistance, whereas strong expressers would respond to *GLP4* RNAi with enhanced susceptibility. Indeed, both lines showing enhanced susceptibility upon *GLP4* RNAi expressed the corresponding gene or genes to a high extent. However, in the case of the observed effect of *TaGLP4* overexpression in wheat cv. FAP75141 and the absence of an RNAi effect in barley cv. Ingrid, the expectation was not met by the results of the expression analysis.

***HvGLP4* may not be involved in localized H_2O_2 accumulation at sites of attempted penetration.**

HvGLP4 has been identified here as an extracellular SOD (Fig. 1), opening the possibility that this protein might be responsible for the localized microburst of H_2O_2 by converting

Fig. 6. Comparison of *GLP4* RNA abundance in wheat and barley genotypes. RNA was isolated from control (C) or inoculated leaves (24 h postinoculation), and blots of wheat and barley RNA were hybridized with probe *TaGLP4* and *HvGLP4*, respectively. The comparison of transcript abundance between wheat and barley was done by inoculating plants with *B. graminis* f. sp. *hordei* (h) or with *B. graminis* f. sp. *tritici* (t) and by probing parallel blots with *TaGLP4* or with *HvGLP4*. Ethidium bromide-stained gels prior to blotting are shown below the blots. RNA from genotype pairs to be compared comes from parallel inoculation experiments with comparable inoculum densities (around 30 to 50 conidia mm⁻²).

O_2^- radicals, which are produced by e.g., NAD(P)H oxidases, to H_2O_2 at sites of attempted penetration by primary or appressorial germ tubes of *B. graminis* f. sp. *hordei* or *B. graminis* f. sp. *tritici*. If this hypothesis is true, then RNAi targeted to *HvGLP4* in bombarded cells should prevent or at least significantly reduce the localized H_2O_2 microburst, as detected by the oxidation of 3,3-diaminobenzidine (DAB) in bombarded leaves previously loaded with DAB. For this purpose, DAB oxidation associated with attempted appressorial penetration was assessed in cells transformed with the β -glucuronidase (GUS) reporter gene together with the RNAi control construct *pZmA1*hairpin (targeting the maize *A1* gene) or together with *pHvGLP4*hairpin. Figure 7 shows that RNAi of *HvGLP4* in barley cv. Golden Promise, which showed strong hypersusceptibility upon silencing of this gene, did not significantly reduce the percentage of transformed cells with detectable DAB oxidation. The same was true in neighboring, nontransformed cells, indicating that the amounts of DAB that reached the transformed leaf areas and that directly influenced staining intensities were comparable between control and test leaves bombarded with *pZmA1* hairpin and *pHvGLP4*hairpin, respectively.

DISCUSSION

The identification of *HvGLP4* as functional Mn-SOD puts this barley epidermis-expressed protein together with a group of GLP with known SOD activity (Carter and Thornburg 2000; Woo et al. 2000; Yamahara et al. 1999). These GLP-SOD are unrelated to previously characterized intracellular plant SOD (Alscher et al. 2002). Plant SOD, proposed to be involved in scavenging of reactive oxygen species together with catalase but also generating H_2O_2 , have been found to be up-regulated

Fig. 7. RNA interference (RNAi) of *HvGLP4* does not reduce local accumulation of H_2O_2 underneath fungal appressoria. Leaves bombarded with *pHvGLP4*hairpin were challenge-inoculated with *B. graminis* f. sp. *hordei* and were loaded with 3,3-diaminobenzidine (DAB) from 16 to 24 h postinoculation, followed by β -glucuronidase (GUS)-staining and microscopic inspection of DAB-staining underneath appressoria in GUS-stained cells. Control leaves were bombarded with an RNAi construct directed against the *A1* gene of maize. As additional control, DAB-staining underneath appressoria in cells immediately adjacent to GUS-stained cells attacked by *Blumeria* spp. was scored. Mean $\pm$ SE from four independent experiments.

in incompatible plant-pathogen interactions (Babitha et al. 2002; Delledonne et al. 2001; Gay and Tuzon 2000). Some of these are intracellular SOD (Delledonne et al. 2001), but often, the SOD type is not analyzed further. The presence of an N-terminal signal peptide suggests *HvGLP4* to be extracellular. Based on this and the expression patterns of *HvGLP4* and of the wheat orthologue *TaGLP4*, we speculated that this SOD is responsible for the localized H_2O_2 production that is a general response observed at the site of penetration in barley and wheat attacked by powdery mildew (Schweizer et al. 1999a; Thordal-Christensen et al. 1997). The SOD substrate, the superoxide anion, might be produced by NADPH oxidase, an enzyme reported to be activated upon pathogen attack (Keller et al. 1998; Sagi and Fluhr 2001; Yoshioka et al. 2001). The functional data from overexpression and RNAi of *HvGLP4* and *TaGLP4* do support a role of these germinlike proteins in quantitative resistance of cereals against *B. graminis*, although the importance of SOD enzymatic activity in the plant-pathogen interactions studied here remains open.

The resistance-enhancing effect of *HvGLP4* overexpression and the hypersusceptibility-inducing effect of *TaGLP4* and *HvGLP4* silencing was genotype dependent, in contrast to the resistance-enhancing effect of *WIR3* overexpression. This suggests a complex mode of action of GLP4, requiring one or several other host components that might be more or less limiting in a genotype-dependent manner. The fact that, in one of the strong GLP4 expressers, cv. FAP75141 (Fig. 6), overexpression as well as silencing affected resistance, whereas in the other, cv. Ingrid, neither overexpression nor RNAi had an effect also argues for a complex situation involving not just GLP4 protein. Theoretically, genotype dependence of the GLP4 effect might have reflected different expression levels of individual members of the GLP4 subfamily in the different wheat and barley cvs. together with incomplete silencing of the whole subfamily of approximately nine genes by the hairpin constructs used. However, this appears unlikely for the following reasons. First, the chosen hairpin constructs were targeted against strongly expressed subfamily members (formerly referred to as *HvOxOLP* and *TaGLP2a*, respectively). Second, nucleotide-sequence identity in the coding region targeted by the RNAi constructs was found to be higher than 90% among subfamily members (G. Zimmermann and P. Schweizer, unpublished data). This degree of sequence similarity was found to be sufficient for efficient silencing by RNAi, as revealed by cross-silencing experiments of *HvMlo* in barley by a hairpin construct targeted against *TaMlo* (Elliott et al. 2002; P. Schweizer, unpublished data). On the other hand, the effect of *WIR3* overexpression was not dependent on host genotype, demonstrating the general competence of all tested cvs. to respond to transgene expression with enhanced resistance.

We tried to answer the question of whether the SOD activity of *HvGLP4* is directly responsible for localized H_2O_2 formation at sites of attempted penetration. For this purpose, we analyzed localized DAB oxidation in bombarded and *B. graminis* f. sp. *hordei*-attacked, epidermal cells with silenced *HvGLP4* by using the DAB-uptake method of Thordal-Christensen and associates (1997). For these experiments, we used cv. Golden Promise, which showed strong hypersusceptibility upon RNAi of *HvGLP4*. However, RNAi of *HvGLP4* did not significantly reduce the fraction of cells with detectable localized DAB oxidation at sites of attempted fungal penetration, compared with control cells that expressed an RNAi construct targeted against the maize *A1* gene. These experiments suggest that there may exist one or more GLP4 (SOD)-independent mechanisms of H_2O_2 production in barley epidermis cells attacked by *Blumeria* spp. Production of H_2O_2 due to residual *HvGLP4* expression in cells bombarded with the *HvGLP4* RNAi construct

appears unlikely, because the same construct silenced cobombarded *HvGLP4::GFP* very efficiently. Moreover, basal levels of *HvGLP4* expression in the material used for the RNAi experiments usually was very low (Fig. 6), which makes it unlikely that the residual DAB oxidation was due to preformed not-yet-turned-over *HvGLP4* protein in cell walls. Most importantly, although the fraction of transformed cells with detectable DAB oxidation did not decline in cells with silenced *HvGLP4*, there was induced hypersusceptibility. This suggests that the resistance-enhancing effect of *HvGLP4* was not dependent on its contribution to localized H_2O_2 accumulation as reflected by DAB oxidation. Extracellular peroxidases have recently been identified as alternative candidates for the production of H_2O_2 (Bolwell et al. 2002). Moreover, in several plant-pathogen interactions, localized DAB oxidation was found to occur independently from transcription or translation (Mellersh et al. 2002), which argues against an inducible component like *HvGLP4* as sole source of H_2O_2 and suggests a second mechanism based on preformed peroxidase. However, in contrast to the documented existence in barley epidermis of preformed peroxidase activity capable of oxidizing DAB at the expense of H_2O_2 (Thordal-Christensen et al. 1997), no information on extracellular peroxidases capable of generating H_2O_2 is currently available in barley.

If GLP4 proteins are not primarily involved in localized H_2O_2 accumulation in epidermis cells attacked by *Blumeria* spp., what might be the mechanism of their resistance-enhancing effect? Some GLP have been found to be associated with the cell wall (Bernier and Berna 2001). Upon stress treatments of barley, *HvGLP2* (formerly referred to as *HvGLP1*) became insolubilized (Vallélian et al. 1998), and covalent cross-linking of transiently expressed oxalate oxidase (*TaGLP1*) at sites of attempted penetration by *B. graminis* f. sp. *tritici* was observed in wheat (Schweizer et al. 1999a). Moreover, domain-swap experiments between *TaGLP1* and *HvGLP2* resulting in proteins that had lost oxalate-oxidase activity but still retained at least partially their resistance-enhancing potential, support a structural role of *TaGLP1* (Schweizer et al. 1999a). Therefore, GLP might also have a function as structural cell-wall proteins reinforcing the extracellular macromolecular network in response to attempted fungal ingress. *HvGLP4* has been found to be expressed in several stressed and unstressed tissues of barley (G. Zimmermann and P. Schweizer, unpublished data). Therefore, it may be speculated that the SOD activity of the protein or proteins is essential for their function in one or several tissues other than leaf epidermis or in stresses imposed onto leaves other than attack by *Blumeria graminis*. Further studies on structure-function relationships in proteins of the GLP4 subfamily, including mutated forms without SOD activity, as well as the search for a putative H_2O_2 -generating peroxidase and cross-linking substrates will be required to further elucidate the basis of penetration resistance in cereals.

MATERIALS AND METHODS

Plants and fungi.

A. thaliana ecotype Columbia-0 was transformed according to the floral dip method of Clough and Bent (1998). Transgenic lines were selected on soil after spraying with Basta (Bayer, Leverkusen, Germany).

Wheat seedlings and *Blumeria graminis* DC. Speer f. sp. *tritici* Em. Marchal were grown and maintained as described (Schweizer et al. 1999b). Barley seedlings were grown in a growth chamber (16-h light from metal halogen lamps; 8-h dark, 70% relative humidity, 18°C constant temperature). *B. graminis* f. sp. *hordei* (Isolate 4.8 carrying *AvrMla9*) was maintained by weekly transfer to fresh barley cv. Golden Promise.

Plasmids.

Three constructs for *HvGLP4* overexpression in *A. thaliana* (Fig. 1) were generated in a modified pCambia3301 binary expression vector (Cambia, Canberra, Australia). This vector confers expression under control of the cauliflower mosaic virus 35S promoter and has a *Bar* gene for plant selection. The *N-His-HvGLP4* coding sequence was prepared by amplifying the barley *HvGLP4* cDNA pBH6-903 (accession number X93171) in pBluescript (Stratagene, La Jolla, CA, U.S.A.), using the 75-mer primer 5'-AGCGTTGGTCTCATGCGAGGCAACTTCCTCCGACCCTCATCATCATCATCATGACCCTAGCCCACTCCAAGA-3' and the vector primer 5'-CACACAGGAAACAGCTATGACC-3', followed by trimming of the fragment using *BsaI* and *EcoRV* and recloning of the fragment back into pBH6-903 digested with the same enzymes. The 75-mer primer introduced six histidine residues, two amino acids C-terminal of the predicted signal peptide cleavage-site. The *HvGLP4-C-His* coding sequence was prepared by amplifying the barley cDNA pBH6-903 using the 55-mer primer 5'-CACCAACACGTGACTTAATGATGATGATGATGATGATGATGTTGTTCTCCAG-3' and the vector primer 5'-CGCTCTAGAACTAGTGGATC-3'. The 55-mer primer introduced six histidine residues at the C-terminal end of the protein. The fragment was cloned into the pCRscript vector (Stratagene). In order to modify pCambia3301, its GUS start codon (ATG) was destroyed by an *NcoI* digest followed by a treatment with *Taq* polymerase in the presence of only dCTP. Subsequently, the GUS-coding region was removed by a *PmlI/BglII* digest that left the *nos* terminator intact. The *HvGLP4* coding sequence in the pBH6-903 cDNA and the *N-His-HvGLP4* and *HvGLP4-C-His* coding sequences were all cut from their vectors using *BamHI* and *PmlI* and were ligated into the *BglII/PmlI*-digested modified pCambia3301 vector.

Constructs for transient overexpression and RNAi studies (Fig. 3) were based on a wheat *TaGLP4* cDNA clone (accession number AJ237942) and the barley *HvGLP4* cDNA clone pBH6-903. Expression construct pTaGLP4 (35S_{prom}-*TaGLP4*-35S_{term}; formerly referred to as pGLP2) (Schweizer et al. 1999a) and pHvGLP4 (35S_{prom}-*HvGLP4*-35S_{term}) was used for *GLP4* overexpression in wheat and barley. Construct pHvGLP4 was obtained by cloning a *SalI/PstI* fragment of pBH6-903 between the *SalI/PstI* sites of the expression vector pGY1 (Schweizer et al. 1999b). The *TaGLP4* cDNA clone (accession number AJ237942) and pPcrGLP4 were used for in vitro synthesis of dsRNA (Schweizer et al. 2000) and contained *TaGLP4* near full-length cDNA in pBluescript (Stratagene) and a 630-bp polymerase chain reaction (PCR) fragment encompassing the open reading frame of *HvGLP4* in pCR-TOPOII (Invitrogen, Karlsruhe, Germany), respectively. Primers 5'-GCTGATGACTTCTTCAAGGC-3' and 5'-ATTACATATGCTCCTTTTAT-3' were used for PCR amplification of *HvGLP4* from genomic barley DNA (cv. Atlas). Constructs pTaGLP4-hairpin and pHvGLP4-hairpin were used for hairpin RNAi and contained inverted repeats (IR) of either the *TaGLP4* or *HvGLP4* genes, separated by the 313-bp second intron of the wheat *RGA2* gene (accession no. AF326781). These give rise to dsRNA in vivo with the intron loop between IR1 and IR2 being spliced out. The RNAi expression vector pJP26 was made by cloning the wheat *RGA2* intron in pGY1 between *XbaI* and *SalI* sites of the multiple cloning site. Construct pTaGLP4-hairpin (35S_{prom}-*TaGLP4*_{IR1}-*RGA2*_{intron}-*TaGLP4*_{IR2}-35S_{term}) was obtained by adaptor PCR and cloning of one PCR fragment between the *SmaI* and *XbaI*-sites of pJP26 (upstream of the intron), followed by cloning the other fragment between the *SpeI* and *PstI* sites of the intermediary product (downstream of the intron). The PCR fragments were obtained from pGLP2 by using primers 5'-ACGTCTTCAAAGCAAGTGG-3'

and 5'-GCTCTAGATTCACTCATTACAGACACAA-3' (IR1, ends cut with *SmaI* and *XbaI*) or 5'-ACGTCTTCAAAGCAAGTGG-3' and 5'-GGACTAGTTCACTCATTACAGACACAA-3' (IR2, ends cut with *SpeI* and *PstI*). Construct pHvGLP4-hairpin (35S_{prom}-*HvGLP4*_{IR1}-*RGA2*_{intron}-*HvGLP4*_{IR2}-35S_{term}) was used for hairpin RNAi of *HvGLP4* and was obtained by adaptor PCR and cloning of the one PCR fragment between the *BamHI* and *XbaI* sites of pJP26 (upstream of intron), followed by cloning the other fragment into the *SalI/SphI* sites of the intermediary product. The PCR fragments were obtained from pPB-903 by using adapter primers 5'-TTATTAGGATCCATGACTTCTTCAAGGCAGCC-3' and 5'-TTATTATCTAGAGGATGCATATCTAGGAACTC-3' (IR1, sites *BamHI* and *XbaI*) or 5'-ATTATTGCATGCATGACTTCTTCAAGGCAGCC-3' and 5'-TTATTAGTTCGACGGATGCATATCTAGGAACTC (IR2, sites *SalI* and *SphI*). The GFP-fusion construct encoding *HvGLP4* fused with GFP (pHvGLP4::GFP) was obtained by in-frame cloning of a *HvGLP4* PCR fragment upstream of GFP between the *SmaI* and *BstXI* sites of pGermin::GFP (Schweizer et al. 1999a). This replaced Germin gf2.8 with *HvGLP4*. The *HvGLP4* PCR fragment was obtained using primers 5'-TTATTACCCGGGGAAACACAAGACAAGGAA-3' and 5'-TTATTACCACGACCCTGGTGTGGTTGTTCTCCAGA-3', followed by cutting of the ends with *SmaI* and *BstXI*. The RNAi control constructs pZmA1-hairpin and pPrx7-hairpin contain inverted repeats of the maize *A1* (encoding dihydroflavonol-4-reductase, accession number X05068) and barley *Prx7* (encoding pathogen-induced vacuolar peroxidase accession number AJ003141 [Kristensen et al 1999]) genes, respectively, and were obtained essentially as described for pHvGLP4 hairpin.

Protein analyses.

Crude protein was extracted from leaves of 4-week-old Basta-resistant transgenic lines of *A. thaliana* in 100 mM CaCl₂ (1:2, fresh weight plant material/buffer). Ni-NTA affinity purification of the recombinant His-tagged protein was preceded by a heat treatment of 80°C for 3 min, followed by a 10,000 × *g* centrifugation for 5 min in order to remove heat-unstable proteins. The supernatant was supplied with urea to a final concentration of 8 M in order to denature *HvGLP4* and facilitate proper binding of the His-tag. Four column volumes (CV) of extract was loaded on to a 5-ml Ni-NTA column (Qiagen, Hilden, Germany) at 0.5 ml/min. Bound protein was washed sequentially with i) 8 M urea in 20 mM Tris, pH 8.0; ii) 8 M urea in 20 mM Tris, pH 6.3; iii) 8 M urea in 20 mM Tris, pH 8.0, 1 mM Mn⁺⁺. Bound *HvGLP4*-C-His was renatured on the column by the removal of urea in a 4-CV gradient to 20 mM Tris, pH 8.0, 1 mM Mn⁺⁺. A final 6-CV wash in 20 mM Tris, pH 8.0, preceded the elution, subsequently performed by adding imidazole to 60 mM into the 20 mM Tris, pH 8.0, buffer.

SDS-PAGE and blotting onto Immobilon-P membranes (Millipore, MA, U.S.A.) were performed according to Sambrook and associates (1989) and Harlow and Lane (1988), respectively. The SDS-resistant *HvGLP4* protein was electrophoresed under semisensitive conditions on standard SDS-PAGE. "Semisensitive" denotes the fact that the samples are made without reducing agent in the sample buffer and are not boiled prior to loading. Antiserum, raised against denatured *HvGLP4* expressed in *Escherichia coli* (Wei et al. 1998), was used for immunodetection. In order to ascertain recognition of the oligomeric form of *HvGLP4* on semisensitive gel blots, the proteins on these blots were denatured after blotting according to Wei and associates (1998). In-gel assaying for SOD activity was performed according to Beauchamp and Fridovich (1971).

Proteins of barley leaf epidermal and mesophyll tissues were prepared according to Wei and associates (1998).

Transient gene expression in wheat and barley.

For transient RNAi in wheat, segments of first leaves of 7-day-old seedlings were bombarded with DNA- or dsRNA-coated tungsten particles as described (Schweizer et al. 1999b, 2000). For transient overexpression in wheat and for all experiments in barley leaf segments of 7-day-old seedlings were bombarded with gold particles (diameter 1 µm) in a PDS-1000/He system (Bio-Rad, München, Germany), using the hepta adaptor with a helium pressure of 900 psi. The particle coating was done according to Schweizer and associates (1999b), except that the particle supernatant was completely removed after coating and particles were finally resuspended in pure ethanol (21 µl containing 1,050 mg gold microcarrier per shot). For all experiments in wheat and barley, leaf segments were inoculated with *B. graminis* f. sp. *tritici* or *B. graminis* f. sp. *hordei* 4 h postbombardment, except for RNAi experiments with the pGLP4 hairpin construct in barley, in which leaf segments were inoculated either 4 or 48 h postbombardment. For wheat and barley, pUbiGUS containing GUS was used as reporter gene for transformed epidermal cells, and leaf segments were GUS-stained 40 h postinoculation, followed by staining of epicuticular fungal structures by Coomassie blue R-250 and scoring of interaction phenotypes by light microscopy (Schweizer et al. 1999b). The efficiency of RNAi was tested in barley by cobombarding pHvGLP4::GFP with pHvGLP4 hairpin, followed by determination of the number of GFP cells 48 h later.

Statistics for transient gene expression.

Frequencies of haustorium formation by *B. graminis* were calculated relative to the corresponding controls (GUS reporter gene plus empty vector or GUS reporter gene plus control RNAi hairpin construct) accompanying each transformation experiment. These relative values were found to be normal distributed ($\chi^2 = 2.35$) and could, therefore, be tested for significant difference to the control value (100%) by using a one-sample *t*-test.

Detection of localized H₂O₂ production by DAB-staining.

H₂O₂ was detected in situ by the DAB-uptake method (Thordal-Christensen et al. 1997). Leaves of 7-day-old barley seedlings were bombarded by placing entire seedlings (including root) into the PDS-1000/He system. Bombarded seedlings were grown further in liquid culture for 48 h, followed by inoculation with *B. graminis* f. sp. *hordei*. Sixteen hours postinoculation, leaves were cut and allowed to take up DAB as described (Thordal-Christensen et al. 1997). Leaves were stained for GUS activity (24 h postinoculation), and absence or presence of detectable DAB-staining in GUS-stained transformed cells was analyzed by light microscopy. Attention was paid to analyze only GUS-stained cells surrounded by cells that showed DAB-staining at sites of attempted penetration.

RNA blot analysis.

Total RNA was isolated and blotted onto nylon membranes, followed by hybridization with germin probes as described (Vallelian et al. 1998).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

The Institute of Plant Genetics (IPK) Crop EST database: pgrc.ipk-gatersleben.de/cr-est/login.php