A Small GTP-Binding Host Protein Is Required for Entry of Powdery Mildew Fungus into Epidermal Cells of Barley[1]

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Small GTP-binding proteins such as those from the RAC family are cytosolic signal transduction proteins that often are involved in processing of extracellular stimuli. Plant RAC proteins are implicated in regulation of plant cell architecture, secondary wall formation, meristem signaling, and defense against pathogens. We isolated a *RacB* homolog from barley (*Hordeum vulgare*) to study its role in resistance to the barley powdery mildew fungus (*Blumeria graminis* f.sp. *hordei*). *RacB* was constitutively expressed in the barley epidermis and its expression level was not strongly influenced by inoculation with *B. graminis*. However, after biolistic bombardment of barley leaf segments with *RacB*-double-stranded RNA, sequence-specific RNA interference with *RacB* function inhibited fungal haustorium establishment in a cell-autonomous and genotype-specific manner. Mutants compromised in function of the *Mlo* wild-type gene and the *Ror1* gene (genotype *mlo5 ror1*) that are moderately susceptible to *B. graminis* showed no alteration in powdery mildew resistance upon *RacB*-specific RNA interference. Thus, the phenotype, induced by *RacB*-specific RNA interference, was apparently dependent on the same processes as *mlo5*-mediated broad resistance, which is suppressed by *ror1*. We conclude that an RAC small GTP-binding protein is required for successful fungal haustorium establishment and that this function may be linked to MLO-associated functions.

Complete resistance of barley (*Hordeum vulgare*) to the biotrophic, fungal pathogen *Blumeria graminis* f.sp. *hordei* (*Bgh*) is mediated by major resistance genes such as the *Mla* genes or by loss of MLO function in *Mlo*-mutant genotypes such as *mlo5*-barley (Jørgensen, 1994; Schulze-Lefert and Vogel, 2000). The latter is expressed exclusively via penetration resistance, which is characterized by formation of cell wall appositions and accumulation of phytoalexins, pathogenesis-related gene transcripts, and hydrogen peroxide (Stolzenburg et al., 1984; Zeyen et al., 1993; Peterhänsel et al., 1997; von Röpenack et al., 1998; Hückelhoven et al., 1999, 2000b). All of these characteristics are also found in susceptible barley, albeit to a lower extent, meaning that the *mlo* alleles confer a primed responsiveness for these defense reactions or the functional MLO is a control element of these fundamental resistance mechanisms (Büschges et al., 1997; Peterhänsel et al., 1997).

It is intriguing that *Bgh*-resistant *mlo* genotypes show hypersusceptibility to *Magnaporthe grisea* and to toxic culture filtrates of *Cochliobolus sativus* (Jarosch et al., 1999; Kumar et al., 2001). Thus, *Mlo* exerts an ambivalent role in controlling resistance to the biotroph *Bgh* and susceptibility to the hemibiotroph *M. grisea*. The MLO protein is a membrane-spanning protein reminiscent of a G-protein coupled receptor (Devoto et al., 1999). In animals, such proteins interact with heterotrimeric G-proteins and/or small GTP-binding proteins via different cytoplasmic domains (Naor et al., 2000). Small GTP-binding proteins such as those of the RAC family are cytosolic signal transduction proteins that often are involved in processing of extracellular stimuli. Plant RAC proteins are involved in regulation of plant cell architecture, secondary wall formation, meristem signaling, and defense against pathogens (Valster et al., 2000). Mammalian RAC1, in its GTP-binding form, is essential for stable assembly of an active NADPH oxidase complex in the plasma membrane of phagocytic and nonphagocytic cells. This complex is responsible for generation of superoxide radical anion ($O_2^{\cdot-}$) that is a signal molecule for cell proliferation in low concentrations, whereas it causes host cell death and pathogen killing in higher concentrations (Irani et al., 1997; Burstein et al., 1998; Irani and Goldschmidt-Clermont, 1998; Subauste et al., 2000).

Interaction of plant RAC homologs with the NADPH oxidase complex appears to regulate activity of NADPH oxidase that produces $O_2^{\cdot-}$ in response to pathogen attack (Hassanain et al., 2000; Ono et al., 2001). Rice (*Oryza sativa*) *Rac1*, when overexpressed in rice in its constitutive active form, leads to hypersensitive reaction (HR) at sites of attack by *M. grisea* and, therefore, to pathogen resistance. Expression of

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dominant negative forms of *Rac1* consistently results in enhanced susceptibility to *M. grisea* (Kawasaki et al., 1999; Ono et al., 2001).

Reactive oxygen intermediates (ROI) play multiple roles in plant pathogen interactions. $O_2^{\cdot-}$ or H_2O_2 induce defense mechanisms including pathogenesis-related gene expression and the HR. On the other hand, ROI are also signals that restrict cell death and lead to production of antioxidants. Spatial and quantitative differences in the occurrence of ROI are crucial for their mode of action (Levine et al., 1994; Tenhaken et al., 1995; Jabs et al., 1996). In barley, $O_2^{\cdot-}$ production takes place during attack by *Bgh* at sites of successful penetration of epidermal cells, but not at sites where fungal penetration is prevented (Hückelhoven and Kogel, 1998). In contrast, H_2O_2 accumulates subcellularly in barley at sites were penetration by *Bgh* is successfully prevented as well as in entire cells that undergo HR. Together, accumulation patterns of $O_2^{\cdot-}$ and H_2O_2 differ temporally and spatially in barley during attack by *Bgh* (Thordal-Christensen et al., 1997; Hückelhoven and Kogel, 1998; Kogel and Hückelhoven, 1999; Hückelhoven et al., 1999, 2000a, 2000b).

We show here that a barley RAC homolog is required for parasitic entry of the biotrophic powdery mildew fungus into epidermal host cells and, therefore, that this protein has a negative function in disease resistance of barley to *Bgh*.

RESULTS

Isolation of a Barley RACB Open Reading Frame

We recently isolated a partial coding sequence of a barley putative *Rac1* homolog (GenBank accession no. AJ290420; Hückelhoven et al., 2001). In this study, we isolated a complete open reading frame of the barley *Rac* homolog (see "Materials and Methods") that encodes a protein with more than 98% identity to RACB from rice and maize (*Zea mays*) and more than 55% identity to human RAC1 or RAC2. Therefore, the cDNA clone now is designated as barley *RacB* (GenBank accession no. AJ344223). The barley RACB homolog contains several conserved motifs that are essential for RAC function in animal systems. The CXXL motif is conserved at the C terminus. The Cys residue of this motif is the site of post-translational isoprenylation that directs active RAC proteins into the plasma membrane. The so-called effector loop of RAC protein can also be found in barley RACB (amino acids 28–48). This motif is responsible for interaction with target protein of RAC homologs such as NADPH oxidase. Barley RAC residues 127 to 140 resemble a specific effector loop that might be required for induction of $O_2^{\cdot-}$ generation via RAC (Hassanain et al., 2000). Motifs typically responsible for GTP binding and GTP hydrolysis, respectively, are also present in barley RACB. Together, the isolated barley cDNA encodes a protein that contains all typical motifs of small RAC GTP-binding proteins.

RacB Is Expressed in Epidermal Tissue

In our previous study, we described constitutive expression of the barley *RacB* homolog (designated as *Rac1*) in barley primary leaves. *RacB* expression was unaffected by inoculation with the powdery mildew fungus (*Bgh*; Hückelhoven et al., 2001). In this study, we wanted to know whether *RacB* is expressed in the epidermis of barley that is the only tissue attacked by *Bgh*. We analyzed tissue-specific expression of *RacB* in peeled abaxial epidermal strips and the residual part of primary leaves. Susceptible barley cultivars Pallas and resistant P10 were inoculated densely on the abaxial sides with *Bgh* race A6 by 24 h before sampling. As a positive control for epidermis-specific gene expression, an oxalate-oxidase like-protein gene (*OXLP*) was selected (Wei et al., 1998). Ubiquitin 1 (*Ubi*) was used as a marker for tissue-unspecific expression, and chloroplast-directed *BAS* (thioredoxin-dependent peroxide reductase) was selected as a marker for mesophyll expression. As shown in Figure 1, expression of *RacB* was stronger in peeled epidermal strips than in the rest of the leaves. Tissue specificity of *RacB* expression was similar to that of *OXLP* and different from that of *Ubi* and *BAS*.

We compared early expression of *RacB* in a highly resistant barley *mlo* line BCIngrid-*mlo5*, the respective susceptible near-isogenic parent Ingrid, and a susceptible mutant A89 (*mlo5 ror1*) between 0 and 24 h after inoculation (HAI). In Ingrid, about 50% to 60% of fungal penetration attempts lead to haustoria

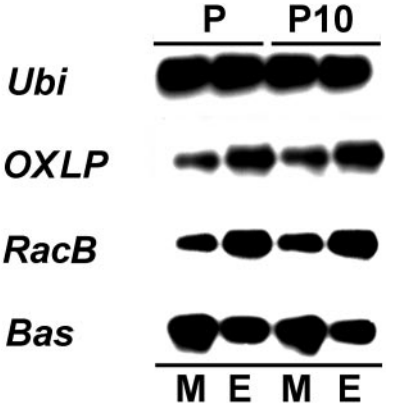

Figure 1. *RacB* is expressed in epidermal tissue. Reverse transcriptase (RT)-PCR with RNA from cv Pallas and cv BCP*Mla12* (P10) 24 HAI with *Bgh*A6. For extraction of total RNA, abaxial epidermal strips (E, inoculated site of the leaves) were separated from the mesophyll and adaxial epidermis (M). *Ubi* was selected as a marker for tissue-unspecific gene expression. *OXLP* was selected as a positive control for gene expression in the epidermal layer. *Bas* was selected as a positive control for gene expression in mesophyll cells. RT-PCR was carried out with 25 cycles under specific conditions. RT-PCR-products were denatured in gel, blotted, and detected by antisense RNA probes under stringent conditions.

formation between 12 and 24 HAI, whereas penetration rate in BCIngrid-*mlo5* was close to 0%. Cultivar A89, a *Mlo-Ror1* double-mutant line derived from BCIngrid-*mlo5*, is penetrated at 20% to 35% of the interaction sites by the *Bgh* isolate used (Hückelhoven et al., 2000b). *RacB* gene expression was slightly enhanced in response to *Bgh* inoculation as compared with *Ubi* expression that was taken as a constitutive marker. In the same RNA batch, the expression of *OXLP* as a positive control for *Bgh*-induced gene expression was enhanced from 8 HAI onward. At 14 HAI, when the first immature haustoria can be found in epidermal cells, *OXLP* expression was somewhat stronger in cv A89 and resistant BCIngrid-*mlo5* than in Ingrid (Fig. 2).

Sequence-Specific RNA Interference (RNAi) by *RacB*-double-stranded (ds) RNA Enhances Penetration Resistance

We addressed the question of whether RACB is involved in cellular accessibility or maintenance of basal resistance of barley to powdery mildew fungus. Host cell wall penetration and haustorium formation are the key steps in establishing host-pathogen compatibility. However, even susceptible barley cultivars such as Pallas or Ingrid prevent penetration at up to 50% of interaction sites, indicating a significant level of basal resistance. We used sequence-specific RNAi to induce gene silencing of *RacB*. RNAi produces phenotypes in plants that are very similar to those of knockout mutants (Waterhouse et al., 1998). It recently was shown that RNAi also functions transiently in barley if dsRNA is delivered into epidermal cells by biolistic bombardment (Schweizer et al., 2000). To test the efficiency of RNAi in induction of post-transcriptional gene silencing of RACB, we bombarded barley epidermal cells with *p*-green fluorescent protein (GFP):RACB that had been constructed for expression of a GFP:RACB fusion protein under control of the cauliflower mosaic virus 35 S promoter, together with *RacB*-dsRNA or heterologous control dsRNA (human thyroid hormone receptor dsRNA, *TR*), respectively. In four independent experiments, sequence-specific silencing of GFP:RACB led to a significant reduction of green fluorescing cells by 75% (Table I). This shows that dsRNA of *RacB* is suitable for inducing silencing of RACB in bombarded cells.

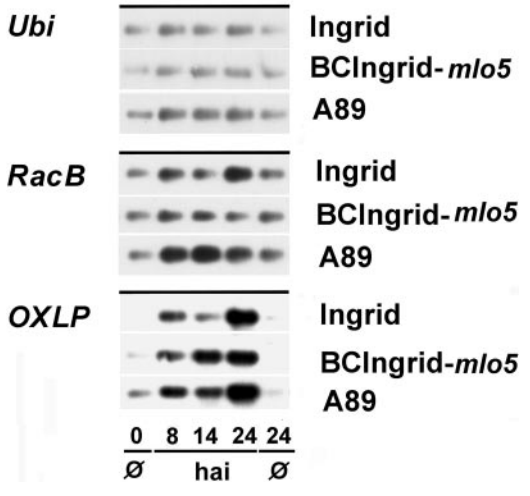

Figure 2. *RacB* expression in resistant and susceptible barley lines. RNA was isolated from cv Ingrid (*Mlo*, *Ror1*, susceptible), cv BCIngrid-*mlo5* (*mlo5*, *Ror1*, resistant), and cv A89 (*mlo5*, *ror1*, moderately susceptible) immediately before (0 Ø) inoculation at 8, 14, and 24 HAI with *Bgh* and 24 HAI from noninoculated control plants (24 Ø). *Ubi* was selected as a marker for constitutive gene expression. *OXLP* was selected as a positive control for *Bgh*-induced gene expression in the epidermal layer. RT-PCRs were carried out with 20 to 25 cycles under specific conditions. PCR products were denatured in gel, blotted, and detected by antisense RNA probes under stringent conditions.

Table I. *Effect of RacB-dsRNA on transient expression of a RACB:GFP fusion protein*

	No. of Green Fluorescing Cells per Leaf[a]
Control-dsRNA	11.3 ± 2.0
RacB-dsRNA	2.9 ± 1.8

[a] Mean ± SE of four independent experiments.

To elucidate the role of small GTP-binding proteins in basal resistance or cellular accessibility, we bombarded Pallas leaf segments with *RacB*-dsRNA together with a GFP expression vector (pGFP; Schweizer et al., 1999). Leaves were subsequently inoculated with *Bgh*, and the outcome of the interaction was evaluated 48 h later by in vivo light and fluorescence microscopy (Nielsen et al., 1999). Penetration into GFP-expressing cells was confirmed by detection of haustoria in living cells and by judgment of fungal development on these cells by fluorescence and light microscopy (see "Materials and Methods").

In each of six independent experiments, bombardment of cv Pallas with *RacB*-dsRNA led to a reduced number of cells that were successfully invaded by *Bgh* as compared with leaf segments bombarded with heterologous *TR*-dsRNA. The resistance-inducing effect of *RacB*-dsRNA resulted in an average reduction of penetration efficiency (PE) of *Bgh* by 44% (Fig. 3).

Broad prehaustorial resistance in barley against *Bgh* is controlled negatively by the wild-type MLO protein. Barley *mlo5* genotypes without a functional MLO protein are race nonspecifically resistant to penetration by *Bgh* (Büschges et al., 1997; Jørgensen, 1994). Because *RacB*-dsRNA inhibited haustorium formation in cv Pallas that bears no functional resistance gene against *Bgh*A6, we speculated that *RacB* and *Mlo* might be functionally linked. To test this hypothesis, we selected a *mlo5* genotype (cv A89, *mlo5 ror1*, background Ingrid) that is moderately susceptible to *Bgh* due to the mutation in *Ror1* (Freialdenhoven et al., 1996). In this double-mutant genotype, we tested the impact of *RacB*-dsRNA in

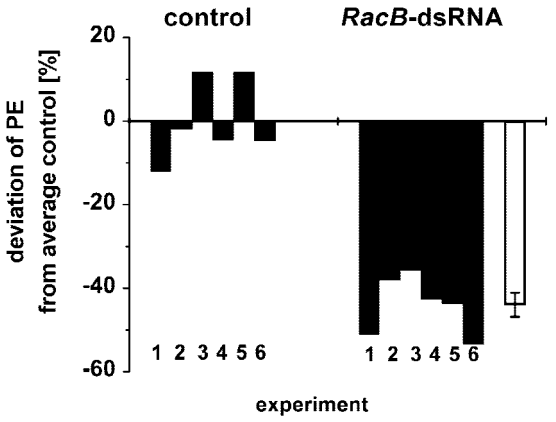

Figure 3. *RacB*-dsRNA interferes with the PE of *Bgh* in barley. Relative PE was evaluated in six independent experiments with *Bgh* on barley cv Pallas. PE of *Bgh* was reduced in cells that were bombarded with *RacB*-dsRNA compared with cells that were bombarded with control dsRNA (*TR*, human thyroid receptor-dsRNA). Negative and positive deviation of PE indicate reduced or enhanced PE, respectively, compared with average penetration frequency in six control experiments (adjusted to zero). Black columns, Relative PE at minimum 100 interaction sites in an independent experiment. White column, Average of the independent experiments with *RacB*-dsRNA. Error bar shows SE (relative PE of control and *RacB*-dsRNA are significantly different at $P = 0.000001$ level, Student's *t* test).

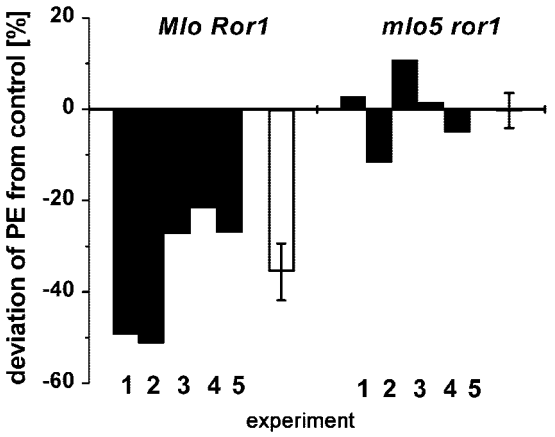

Figure 4. The influence of *RacB*-dsRNA on the PE of *Bgh* is dependent on the barley genotype. Relative PE was evaluated in five independent experiments with *Bgh* on barley lines Pallas, Ingrid, or A89. The PE of *Bgh* is reduced in cv Pallas (*Mlo Ror1*, experiments 1 and 2) or cv Ingrid (*Mlo Ror1*, experiments 3–5) cells that were bombarded with *RacB*-dsRNA compared with cells bombarded with control dsRNA (not shown). Penetration of susceptible double-mutant A89 (*mlo5 ror1*, experiments 1–5) was not affected by *RacB*-dsRNA. Black columns, Relative PE in an independent experiment. White columns, Average of five independent experiments with *RacB*-dsRNA. Error bars show SEs (influence of *RacB*-dsRNA on PE in *Mlo Ror1* and *mlo5 ror1* genotypes, respectively, is significantly different at $P < 0.002$, Student's *t* test).

comparison with wild-type *Mlo* genotypes. In five independent experiments, *RacB*-dsRNA did not prevent haustoria establishment in cv A89, whereas in the same experiments, PE was reduced by *RacB*-dsRNA in cv Pallas and cv Ingrid (*Mlo Ror1* genotypes; Fig. 4). Thus, resistance induced by *RacB*-dsRNA such as *mlo*-mediated resistance does not work in cv A89. It is remarkable that the *RacB*-dsRNA effect was stronger in cv Pallas than in cv Ingrid (Fig. 4, experiments 1 and 2 or 3–5, respectively). Absolute PEs are shown in Table II.

To rule out the possibility that *RacB*-dsRNA influences the transformation rate or the survival rate of attacked cells, we compared the number of GFP-expressing cells on control and *RacB*-dsRNA bombarded leaves (Table III). Microscopic evaluation showed that *RacB*-dsRNA did not influence the number of total or attacked GFP-expressing cells in any genotypes used. This demonstrates that RNAi by *RacB*-dsRNA strongly affects processes linked to successful establishment of the fungus but not cell death of host cells.

DISCUSSION

We have shown that *RacB*-dsRNA specifically interferes in barley epidermal cells with haustorium establishment by the plant parasitic, biotrophic powdery mildew fungus. Delivery of *RacB*-dsRNA into epidermal cells induced resistance with a similar efficiency as *Mlo*-dsRNA (Schweizer et al., 2000). Therefore, our results tag an RAC small GTP-binding protein as a host element that is required for successful invasion by *Bgh*.

Several lines of evidence could exclude nonspecific effects of *RacB*-dsRNA. First, in all experiments, the effect of *RacB*-dsRNA was compared with that of nonspecific *TR*-dsRNA, which has no plant homologs. An effect of *TR*-dsRNA was excluded in several experiments (data not shown). Second, the effect of *RacB*-dsRNA was genotype specific (Fig. 4). Third, *RacB*-dsRNA did not influence the number of nonattacked or attacked GFP-expressing cells (Table III). Fourth, when we bombarded barley with pGFP:RACB for expression of a GFP:RACB fusion protein together with *RacB*-dsRNA, the number of cells showing GFP fluorescence was reduced by 75% compared with experiments with heterologous *TR*-dsRNA. This shows that *RacB*-dsRNA induced gene silencing of the *RacB:GFP*-transgene. Thus, the biological effects of *RacB*-dsRNA are most likely a result

Table II. *Penetration frequencies of Bgh on barley leaves bombarded with dsRNA*

Line	Penetration Frequency[a]	
	Control-dsRNA	*RacB*-dsRNA
	%	
Pallas (*Mlo Ror1*)	57.0 ± 2.3	31.8 ± 1.6
Ingrid (*Mlo Ror1*)	53.8 ± 6.5	39.0 ± 4.0
A89 (*mlo5 ror1*)	27.4 ± 0.6	27.5 ± 1.6

[a] No. of penetrated cells divided by no. of attacked cells multiplied by 100 (mean ± SE).

Table III. *Transformation rates on barley leaves bombarded with dsRNA*

Line	No. of GFP-Expressing Cells per Shot[a,b]				n[c]
	Control-dsRNA		*RacB*-dsRNA		
	Total	Attacked	Total	Attacked	
Pallas (*Mlo Ror1*)	34.3 ± 4.6	16.0 ± 2.2	33.9 ± 4.8	15.5 ± 1.4	6 (21)
Ingrid (*Mlo Ror1*)	51.0 ± 8.9	27.6 ± 8.7	49.9 ± 5.6	31.5 ± 7.8	3 (11)
A89 (mlo5 ror1)	34.4 ± 5.4	18.1 ± 4.0	34.1 ± 5.5	16.7 ± 3.8	5 (22)

[a] Four leaves were bombarded per shot. [b] Mean ± SE. [c] No. of independent experiments (shots in *n* experiments each for control and *RacB*-dsRNA).

of post-transcriptional gene silencing of endogenous *RacB*. In barley, high sequence identities of dsRNA and target genes are necessary for RNAi (Schweizer et al., 2000). However, because *RacB* is probably very similar to other barley *Rac* genes, we cannot exclude that we might have affected the expression of RAC proteins other than RACB by *RacB*-dsRNA.

The resistance inducing effect of *RacB*-dsRNA effect was somewhat stronger in cv Pallas than in cv Ingrid. Because RACB apparently plays a negative role in broad resistance to *Bgh*, different levels of broad resistance in cv Pallas and cv Ingrid might influence RACB activity. In the barley double-mutant A89 (*mlo5-ror1*), *RacB*-dsRNA did not interfere with resistance. Therefore, it appears that the function of a RAC protein is linked to elements of the MLO/ROR network. Because MLO and ROR1 are involved in broad resistance against *Bgh*, this finding suggests that *RacB*-dsRNA interferes with race-unspecific penetration resistance of barley against *Bgh*, and that the same processes underlying *mlo*-mediated resistance limit this effect. Because RACB and MLO are required for fungal entry in barley epidermal cells, we speculate that they might be linked functionally. It is interesting that functional RACB and functional MLO play negative roles in resistance to *Bgh*, whereas losses of RAC1 or MLO function lead to hypersusceptibility to the fungal parasite *M. grisea* (Jarosch et al., 1999; Ono et al., 2001). Thus, MLO and RAC G-proteins are signal transduction elements that play ambivalent roles in resistance to biotrophic *Bgh* and hemibiotrophic *M. grisea*.

The mechanism by which RAC interferes with penetration resistance needs to be elucidated. One possibility might be that RAC interacts with the cytoskeleton. In mammals, RAC activation is triggered by bacterial pathogens that invade nonphagocytic cells and in phagocytes during phagocytosis. Thereby, RAC is involved in actin reorganization processes during plasma membrane ruffling or bacterial engulfment (Knodler et al., 2001). Both processes appear to resemble the process of plasma membrane invagination during establishment of a fungal haustorium in a plant cell. If barley RAC is needed for plasma membrane invagination, loss of RAC function should lead to inhibition of haustorium formation, as shown here. We speculate that the *Bgh* triggers a RAC small GTP-binding protein and that this process depends on MLO allowing plasma membrane invagination as a prerequisite for establishment of compatibility. Also, active RAC could be involved in cytoskeleton organization processes that antagonize formation of cell wall appositions. Cytoskeleton reorganization appears to be required for penetration resistance of barley coleoptiles to nonhost pathogens such as *Erysiphe pisi* (Kobayashi et al., 1997).

RAC proteins are involved in activation of the $O_2^{\cdot-}$-generating NADPH oxidase complex (Bokoch, 1995; Hassanain et al., 2000). In previous studies, we have shown that enhanced $O_2^{\cdot-}$ generation in barley cells attacked by *Bgh* temporally and spatially coincided with successful penetration and haustorium formation, but not with processes resulting in penetration resistance. Resistant *mlo5* genotypes did not produce $O_2^{\cdot-}$ during the period of attempted penetration (Hückelhoven and Kogel, 1998; Kogel and Hückelhoven, 1999). Thus, it is tempting to speculate that barley RACB functions via activation of NADPH oxidase and that $O_2^{\cdot-}$ generation influences penetration resistance to *Bgh* negatively. In contrast to $O_2^{\cdot-}$, H_2O_2 accumulates at sites of formation of cell wall appositions in which *Bgh* sticks (Thordal-Christensen et al., 1997; Hückelhoven et al., 1999, 2000b). Thus, H_2O_2 is strictly associated with barley defense reactions. Together, the balance of $O_2^{\cdot-}$ and H_2O_2 might be crucial for accessibility of epidermal cells.

MATERIALS AND METHODS

Plant Materials, Pathogen, and Inoculation

The barley (*Hordeum vulgare*) lines Ingrid, Pallas, and the backcross line BCIngrid-*mlo5* were obtained from Lisa Munk (Royal Veterinary and Agricultural University, Copenhagen). Their generation was described previously (Kølster et al., 1986). The mutant A89 was obtained from Paul Schulze-Lefert (Max-Plank-Institute for Plant Breeding Research, Köln, Germany). Plants were grown in a growth chamber at 18°C with 60% relative humidity and a photoperiod of 16 h (60 μmol m^{-2} s^{-1} photon flux density). The barley powdery mildew fungus, *Blumeria graminis* (DC) *Speer* f.sp. *hordei* Em. Marchal, race A6 (Wiberg, 1974) was inoculated onto barley primary leaves to give a density of 50 conidia mm^{-2}. *Bgh* was maintained on barley cv Siri under the same conditions.

Isolation of epidermal tissue for expression analysis was performed by scribing adaxial sides of leaf tips with a scalpel without harming the abaxial epidermis. Leaf tips were folded back and taken as a handle to peel off epidermal strips that were cut off the leaf tips and frozen in liquid nitrogen immediately.

Isolation of Barley *RacB*, Cloning, Sequencing, and Probe Generation

We isolated cDNA fragments by the use of one-step RT-PCR kits (Invitrogen, Carlsbad, CA or Qiagen, Hilden, Germany). A complex RNA pool out of barley seedlings was used as a template. RNA was isolated from cv Pallas at 3, 5, and 7 d after germination. In addition, RNA was isolated from cv Pallas and backcross lines bearing *mlo5*, *Mlg*, or *Mla12* at 1, 2, and 5 d after inoculation with *Bgh*A6 at the 7th d after germination. All isolated RNAs were diluted to a concentration of 1 μg μL^{-1} and they were pooled. Primers were designed using GenBank or expressed sequence tag database information for specific barley sequences or rice (*Oryza sativa*) sequences. To amplify a putative barley *RacB* cDNA, we designed primers from rice and barley sequences. Primers 5′-GGATCCGATGAGCGCGTCCAGGTT-3′ (from GenBank accession no. AF250327) and 5′-GTCGACCTTCGCCCTTGTTCTTTGTC-3′ (from GenBank accession no. BF260616) were suitable to generate a 642-bp RT-PCR product including 618-bp barley-specific sequence (GenBank accession no. AJ344223). We isolated cDNAs from gels and cloned them into pGEM-T-Vektor (Promega, Mannheim, Germany). cDNAs were sequenced from plasmids by use of the Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing kit (Amersham Biosciences, Freiburg, Germany) and were analyzed for similarities in the GenBank database using the BLAST algorithm (Altschul et al., 1997). Because the 5′ end of the isolated complete *RacB* open reading frame contained primer-derived sequences, we carried out RACE. First-strand cDNA synthesis and RACE were carried out as suggested by the manufacturer (GeneRacer; Invitrogen, Karlsruhe, Germany). First strand cDNA synthesis started from mRNA that was isolated from total RNA using the Dynabeads mRNA Purification kit (Dynal, Hamburg, Germany) according to the manufacturer's instructions. Hot-start touch-down RACE-PCR included the GeneRacer 5′primer and the *RacB*-specific primer 5′-GGATCCGATGAGCGCGTCCAGGTT-3′. Touch-down PCR was carried out with initial denaturation (5 min at 94°C), five cycles at a 70°C annealing temperature, five cycles at 68°C, and 28 cycles at 66°C. Each annealing was followed by a 1-min primer extension at 72°C and a 30-s denaturation at 94°C. The final extension time at 72°C was 10 min. The resulting RACE product of approximately 400 bp was reamplified with the gene-specific primer and the 5′GeneRacer nested primer, and was then isolated, cloned, and sequenced as already described.

For probe generation, plasmids were amplified in *Escherichia coli*, isolated, and used for in vitro transcription using T7 or SP6 RNA polymerases and digoxygenin- or fluorescein-labeled nucleotides (DIG-Luminescence Detection kit; Roche Molecular Biochemicals, Mannheim, Germany).

RNA Extraction and RT-PCR

Total RNA was extracted from eight to 10 primary leaf segments (5 cm long) or from 20 epidermal strips (mentioned before) using RNA extraction buffer (Applied Genetechnology Systems, Heidelberg) according to the manufacturer's instructions. RNA contents of the extracts were measured by UV photometry and were adjusted after checking in ethidium bromide-stained gels taking rRNA bands as a measure.

The OneStep RT-PCR kit (Qiagen) was used for semiquantitative RT-PCR following the manufacturer's instructions. To estimate template amounts, the RT-PCR reaction was stopped during the exponential phase of amplification, maintaining initial differences in target transcript amounts. PCR products were separated in agarose gels, denatured, blotted on nylon membranes, and detected with specific nonradioactively labeled RNA probes according to the DIG System user's guide (Roche Molecular Biochemicals). Prior to immunodetection of DNA-RNA hybrids, blots were washed stringently two times for 20 min in 0.1% (w/v) SDS and 0.1× SSC (15 mM sodium chloride and 1.5 mM sodium citrate, pH 7.0) at 68°C.

The primers were: 5′-GTTCATCAAGTGCGTCACCGTG-3′ (5′ primer) and 5′-TTAGCTTCCTCAGTTCTTCCCTG-3′ (3′ primer) for a 387-bp *RacB* cDNA fragment; 5′-CGCGCCGCAGCCGAGTACGAC-3′ (5′ primer) and 5′-GTCACAAAAACA-CATGTAACC-3′ (3′ primer) for a 674-bp barley *BAS* cDNA fragment (GenBank accession no. Z34917); 5′-GGC-CGACATGCATTCACCAG-3′ (5′ primer) and 5′-CATCT-GATATTGCTGGGTCTG-3′ (3 ′ primer) for a 506-bp *OXLP* cDNA fragment (GenBank accession no. X93171); and 5′-CCAAGATGCAGATCTTCGTGA-3′ (5′ primer) and 5′-TTCGCGATAGGTAAAAGAGCA-3′ (3′ primer) for a 513-bp *Ubi* cDNA fragment (GenBank accession no. M60175).

Construction of pGFP:RACB

For expression of a GFP:RACB fusion protein, cDNAs of *GFP* (GFPemd-b in pGFP; Schweizer et al., 1999) and *RacB* were amplified from plasmids by PCR using primers with attached restriction sites. PCR products were cloned into pGEM-T, amplified in *E. coli*, digested using primer-specific restriction enzymes, isolated from gels, and cloned one after another in pGY1 (Schweizer et al., 1999). Primers were designed in a way that allowed cloning of *GFP* upstream of the *RacB* 5′ end under elimination of the *GFP* stop codon. The primers used were 5′-GGATCCATGGTGAGCAAGGGCGAG-3′ and 5′-GGATCCTTGTACAGCTCGTCCAT-3′ for *GFP* and the *RacB* primers already mentioned. Orientation of the inserts was checked by PCR.

Transient Transformation, RNAi, and Evaluation of Fungal Development

A transient transformation protocol originally developed for wheat (*Triticum aestivum*) to assess gene function

in the interaction with powdery mildew was used to induce RNAi via biolistic delivery of dsRNA into epidermal cells of barley leaf segments as described by Schweizer et al. (1999) and Schweizer et al. (2000; compare also Nielsen et al., 1999). For the transient transformation assay, plants were grown in a growth chamber at 24°C (20°C in the dark) with 60% relative humidity and a photoperiod of 16 h (240 μmol m^{-2} s^{-1} photon flux density). In principle, 312 μg of 1.1-μm tungsten particles was coated with dsRNA (2 μg) together with pGFP (1 μg; GFP under control of cauliflower mosaic virus 35S promoter) as a transformation marker for each shot. ds*RacB* RNA was obtained by annealing of sense and antisense RNA synthesized in vitro (Schweizer et al., 2000). Leaf segments were bombarded with coated particles 4 h before inoculation with *Bgh*, race A6. Inoculation with 100 conidia mm^{-2} led to an attack rate of approximately 50% on transformed cells. Interaction outcome was judged subsequently by fluorescence and light microscopy. For each individual experiment, at least 100 interaction sites were evaluated. Transformed GFP-expressing cells were identified under blue light excitation. Three different categories of transformed cells were distinguished: (a) penetrated cells, which contained an easily visible haustorium; (b) cells that were attacked by a fungal appressorium but did not contain a haustorium; (c) and cells that did not contain a haustorium and were not attacked by *Bgh*. Cells that contained more than one haustorium were scored as one penetrated cell independent of the number of fungal penetration attempts. Cells with multiple attacks from *Bgh* without a haustorium were scored as one nonpenetrated cell. Stomata cells and stomata guard cells were excluded from the evaluation. Surface structures of *Bgh* were detected by light microscopy or by fluorescence staining of the fungus with 0.1% calcofluor (w/v in water) for 30 s.

Deviation of PE referring to average control PE was used as a measure for susceptibility of cells that were bombarded with *RacB*-dsRNA compared with those bombarded with control *TR*-dsRNA (human thyroid receptor-dsRNA; Fig. 3). In five independent experiments, *TR*-dsRNA did not change the PE of *Bgh* compared with water. Deviation of PE was calculated for each experiment as the number of penetrated cells divided by the total number of attacked cells (PE) minus average PE in the controls divided by average PE of the controls multiplied by 100.

Deviation of PE referring to individual control PE was used to compare the impact of RNAi in different genotypes (Fig. 4). Therefore, PE in each experiment with *RacB*-dsRNA was divided by PE of individual controls, normalized by subtraction of one and multiplication by 100.

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owner of all or parts of the material. Obtaining any permission will be the responsibility of the requestor. No restrictions or conditions will be placed on the use of any novel materials described in this paper that would limit their use in noncommercial research purposes.

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