A Barley ROP GTPase ACTIVATING PROTEIN Associates with Microtubules and Regulates Entry of the Barley Powdery Mildew Fungus into Leaf Epidermal Cells

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Little is known about the function of host factors involved in disease susceptibility. The barley (Hordeum vulgare) ROP (RHO of plants) G-protein RACB is required for full susceptibility of the leaf epidermis to invasion by the biotrophic fungus Blumeria graminis f. sp hordei. Stable transgenic knockdown of RACB reduced the ability of barley to accommodate haustoria of B. graminis in intact epidermal leaf cells and to form hairs on the root epidermis, suggesting that RACB is a common element of root hair outgrowth and ingrowth of haustoria in leaf epidermal cells. We further identified a barley MICROTUBULE-ASSOCIATED ROP-GTPASE ACTIVATING PROTEIN (MAGAP1) interacting with RACB in yeast and in planta. Fluorescent MAGAP1 decorated cortical microtubules and was recruited by activated RACB to the cell periphery. Under fungal attack, MAGAP1-labeled microtubules built a polarized network at sites of successful defense. By contrast, microtubules loosened where the fungus succeeded in penetration. Genetic evidence suggests a function of MAGAP1 in limiting susceptibility to penetration by B. graminis. Additionally, MAGAP1 influenced the polar organization of cortical microtubules. These results add to our understanding of how intact plant cells accommodate fungal infection structures and suggest that RACB and MAGAP1 might be antagonistic players in cytoskeleton organization for fungal entry.

INTRODUCTION

Powdery mildew is a widespread disease to which many wild and crop plant species are susceptible. It is caused by ascomycete fungi of the Erysiphaceae family, which contains only obligate biotrophic plant parasites. Powdery mildew fungi show a high degree of adaptation to certain host species, resulting in species specialization, which can have, however, different degrees depending on the given parasite species (Micali et al., 2008; Ridout, 2009). The successful powdery mildew fungus invades living cells of aerial parts of the host epidermis for establishment of a haustorium as an essential organ that provides fungal nutrition. Haustoria are generated most likely with contribution of the host plant because the haustorial complex contains not only the fungal protrusion but also a surrounding extrahaustorial matrix and a neck band that seem to be built partly by the plant. The complex is further enveloped by an extrahaustorial host membrane, which is in continuum with the plasma membrane but of different constitution (Koh et al., 2005). This led to the postulation of a host cell developmental program that is involved in biogenesis of the haustorial complex for accommodation of the parasite (Parniske, 2000).

In barley powdery mildew interactions, pathogen race-nonspecific and race-specific resistance determine whether there is an outbreak and the severity of disease (Schulze-Lefert and Vogel, 2000). To overcome different layers of host resistance, pathogens evolved general pathogenicity factors and effectors (Thordal-Christensen, 2003). Manipulation of host cells by pathogen effectors requires specific effector targets in the host (DeWit et al., 2009). A host protein can be considered to be a susceptibility factor when its loss of function results in a reduced level of disease severity (O’Connell and Panstruga, 2006). This might be the case if the susceptibility factor is either a target of a pathogen effector or required to serve other demands of the pathogen. Additionally, negative regulators of host defense can act as susceptibility factors. The barley ROP (Rho of plants) protein RACB is a susceptibility factor to powdery mildew. ROP proteins are small monomeric G-proteins that constitute a plant-specific subfamily of the Rho family of signaling G-proteins. The Rho family consists of RHO, CDC42, and RAC, which are absent from the plant kingdom, and plant-specific ROPs (Brembu et al., 2006). Sequence similarity of ROPs to other Rho family proteins is highest to RAC; therefore, ROPs are often also called plant RACs. ROPs are involved in signaling for fundamental processes...
ROPs can be subdivided into type I ROPs that have a C-terminal CAAX box motif for posttranslational prenylation and type II ROPs that lack a functional prenylation motif but possess conserved C-terminal Cys residues for palmitoylation (Yalovsky et al., 2008). Furthermore, S-acylation is involved in the targeting and function of type I ROPs in subdomains of the plasma membrane (Sorek et al., 2010). ROP proteins are molecular switches and must be fine-tuned in activity. GTP-bound ROPs are the active form of the protein capable of downstream signaling, whereas nucleotide-free and GDP-bound ROPs are inactive. ROP partner proteins regulate subcellular localization, GTPase activity, and GDP/GTP nucleotide exchange of ROPs, which modulates ROP functions in a spatiotemporal manner. ROP-GDIIs are guanine nucleotide dissociation inhibitors supposed to bind C-terminal prenyl residues of GDP-bound RHO proteins and sequester RHO-GDP from the plasma membrane (Kieffer et al., 2000; Mucha et al., 2011). ROP-GEFs are guanine nucleotide exchange factors that facilitate the exchange of GDP for GTP and hence activate ROPs. In plants, there is a specific class of ROP-GEFs, also called plant-specific ROP nucleotide exchangers (Mucha et al., 2011). RHOGAPs are RHO GTPase activating proteins that build a complex with RHOs, in which a GAP Arg residue reaches into the GTP binding pocket of RHOs and establishes RHO GTPase activity, which is otherwise low (Rittinger et al., 1997a, 1997b). Hence, RHOGAP proteins induce hydrolysis of RHO-bound GTP and are therefore important for negative regulation of RHO signaling. In Arabidopsis thaliana, there are at least eight RHOGAP-like proteins that can be subdivided in two classes depending on whether they have a CDC42/RAC-interactive binding (CRIB) domain for G-protein binding or a pleckstrin homology domain for lipid binding (Wu et al., 2000; Eklund et al., 2010). RHOGAPs locally regulate ROP signaling in cell polarity by switching off active ROPs (Khare and Kost, 2006; Hwang et al., 2008, 2010).

ROPs are prominent regulators of cytoskeleton organization with respect to polarity and patterns of both filamentous F-actin and microtubules (MTs) (Yang, 2008). This puts ROPs at a pivotal position for polar cell growth, in which membrane delivery is a prerequisite for polar cell growth, in which membrane delivery is a prerequisite for polar cell growth, in which membrane delivery is a prerequisite for polar cell growth, in which membrane delivery is a prerequisite for polar cell growth, in which membrane delivery is a prerequisite for polar cell growth, and microtubules (MTs) (Yang, 2008). Furthermore, S-acylation is involved in the targeting and function of type I ROPs in subdomains of the plasma membrane (Sorek et al., 2010). ROP proteins are molecular switches and must be fine-tuned in activity. GTP-bound ROPs are the active form of the protein capable of downstream signaling, whereas nucleotide-free and GDP-bound ROPs are inactive. ROP partner proteins regulate subcellular localization, GTPase activity, and GDP/GTP nucleotide exchange of ROPs, which modulates ROP functions in a spatiotemporal manner. ROP-GDIIs are guanine nucleotide dissociation inhibitors supposed to bind C-terminal prenyl residues of GDP-bound RHO proteins and sequester RHO-GDP from the plasma membrane (Kieffer et al., 2000; Mucha et al., 2011). ROP-GEFs are guanine nucleotide exchange factors that facilitate the exchange of GDP for GTP and hence activate ROPs. In plants, there is a specific class of ROP-GEFs, also called plant-specific ROP nucleotide exchangers (Mucha et al., 2011). RHOGAPs are RHO GTPase activating proteins that build a complex with RHOs, in which a GAP Arg residue reaches into the GTP binding pocket of RHOs and establishes RHO GTPase activity, which is otherwise low (Rittinger et al., 1997a, 1997b). Hence, RHOGAP proteins induce hydrolysis of RHO-bound GTP and are therefore important for negative regulation of RHO signaling. In Arabidopsis thaliana, there are at least eight RHOGAP-like proteins that can be subdivided in two classes depending on whether they have a CDC42/RAC-interactive binding (CRIB) domain for G-protein binding or a pleckstrin homology domain for lipid binding (Wu et al., 2000; Eklund et al., 2010). RHOGAPs locally regulate ROP signaling in cell polarity by switching off active ROPs (Khare and Kost, 2006; Hwang et al., 2008, 2010).

ROPs are prominent regulators of cytoskeleton organization with respect to polarity and patterns of both filamentous F-actin and microtubules (MTs) (Yang, 2008). This puts ROPs at a pivotal position for polar cell growth, in which membrane delivery is a key step (Yalovsky et al., 2008). Patterning of Arabidopsis leaf epidermal pavement cells, width determination of hypocotyl cells, pollen tube growth, and initiation and maintenance of root hair growth all need proper ROP functions. Hence, cellular tip growth and diffuse growth both involve ROPs (Yang, 2008). In pavement cells of Arabidopsis, ROP6 regulates MT organization via RIC1 and restricts radial cell expansion. By contrast, Arabidopsis ROP2 inactivates RIC1 and promotes actin microfilament assembly and lobe formation by activation of RIC4 (Fu et al., 2005, 2009). Two Arabidopsis ROP signaling proteins can associate with MTs, the CRIB protein RIC1 (Fu et al., 2005, 2009) and RIP3, a member of the ICR/RIP family of ROP interactors (Lavy et al., 2007; Li et al., 2008), which additionally interacts on MTs with the kinesin-13 family member KINESIN-13A (Mucha et al., 2010).

Little is known about the role of ROPs in developmental processes of monocots (Schultheiss et al., 2005; Pathuri et al., 2008). However, function of ROP proteins in interactions of rice (Oryza sativa) and barley (Hordeum vulgare) with different pathogens is well established (Ono et al., 2001; Pathuri et al., 2008; Chen et al., 2010a; Lorek et al., 2010). For dicots, by contrast, a function of ROP signaling in authentic plant pathogen interactions is less well established and relies on heterologous expression of ROP mutant proteins (Moeder et al., 2005; Pathuri et al., 2009).

The rice ROP protein RAC1 is a crucial factor of both non-specific and race-specific immunity to the bacterial pathogen Xanthomonas oryzae pv oryzae and to the rice blast fungus Magnaporthe grisea (Ono et al., 2001). This involves a membrane-associated immune complex including RAC1, a ROPGEF, the NADPH oxidase RBOHB, the stress-related chaperones HOP/STI1, HSP70, and HSP90, RAR1, SGT1, and the potential scaffolding protein RACK1A. This complex is important for triggering downstream immune responses via mitogen-activated protein kinase 6 (Chen et al., 2010a and references therein). Rice RAC1-mediated immune responses include the entire arsenal of defense responses, such as the elicitor-triggered oxidative burst, defense gene expression, phytoalexin production, and the hypersensitive cell death (Kawasaki et al., 1999; Ono et al., 2001; Wong et al., 2007). Most recently, an association of rice RAC1 with a chitin pattern recognition receptor and with major RESISTANCE (R) proteins has been demonstrated (Chen et al., 2010b; Kawano et al., 2010). Apparently, rice RAC1 is activated by R proteins for triggering downstream responses in effector-triggered immunity (Kawano et al., 2010).

In contrast with rice RAC1, barley RACB as well as rice RAC4 and RAC5 support susceptibility to barley powdery mildew or rice blast, respectively (Schultheiss et al., 2002; Chen et al., 2010a). Genetic studies suggested that the function of RACB is linked to that of the major susceptibility factor MILDEW LOCUS O and a locus, ROR1, required for recessive mlo-specified resistance (Schultheiss et al., 2002, 2003). Transgenic barley expressing constitutively activated CARACB is more susceptible to penetration and haustorium formation by the barley powdery mildew fungus Blumeria graminis f. sp hordei and shows depolarized growth of root hairs (Schultheiss et al., 2005; Pathuri et al., 2008). Expression of CARACB also hinders polarization of F-actin toward the site of fungal attack in leaf epidermal cells of barley, which is otherwise seen during successful defense (Opalski et al., 2005). Additionally, CARACB can interact with the barley CRIB motif protein RIC171 in planta and recruits red fluorescent DsRED-RIC171 to the plasma membrane. DsRED-RIC171 concentrates at sites of fungal entry, and overexpression of RIC171 supports accommodation of fungal haustoria, suggesting that local activity of ROPs is involved in susceptibility to fungal invasion (Schultheiss et al., 2008). However, although RACB has been established as a susceptibility factor in the barley–powdery mildew interaction, little is known about the regulation and the mechanism of RACB function in organization of the cytoskeleton and in susceptibility to powdery mildew.

Here, we show that knockdown of RACB in stable transgenic barley limits the accommodation of haustoria in leaf epidermal cells and the initiation and maintenance of epidermal root hair tip growth. CARACB interacts in yeast and in planta with a previously unknown ROPGAP protein that decorates MTs. CARACB recruits the ROPGAP to the cell periphery where it regulates fungal penetration success. Genetic evidence suggests a function of ROPGAPs in limiting susceptibility of barley and Arabidopsis to...
powdery mildew. Our data suggest a role of ROP regulatory ROPGAPs and of polar MT organization in determining the success or failure of fungal infection structures invading intact plant cells.

RESULTS

Type I ROPs Are Required for Outgrowth of Tip-Growing Root Hairs

Barley RACB is described as potential susceptibility factor of barley to powdery mildew (Schultheiss et al., 2002, 2005). We introduced a RACB RNA interference (RNAi) cassette into the susceptible wild-type background barley cultivar ‘Golden Promise’ by Agrobacterium tumefaciens–mediated transformation to test whether knockdown of RACB limits powdery mildew success at the entire plant level. From 14 tested independent transgenic lines, we identified two lines, B15/1-16 and B16/2-4, that had \( \geq 50\% \) reduced amounts of RACB transcript in leaves as assessed by quantitative real-time PCR (qRT-PCR) (shown for B16/2-4 in Figure 1A). This rather modest decrease in RACB RNA might indicate that proper RACB function is essential for normal shoot development (Figure 1B), and plants with a stronger decrease are eliminated during regeneration in tissue culture (Hensel et al., 2008).

In barley, six homologous ROP proteins were identified, five of which are expressed in the epidermis of barley leaves (Schultheiss et al., 2003). To assess specificity of RACB RNAi in barley, transcript amounts of these ROPs were measured by qRT-PCR. The data revealed that both type I ROPs, RACB and RACD (the barley ROP most similar to RACB, with 77\% identity at the nucleotide level within the open reading frame), were cosilenced by the RNAi cassette, whereas the barley type II ROPs RAC1, RAC3, and ROP6 were only marginally affected (Figure 1A).

In all cases of a series of about 10 independent experiments, the knockdown of the type I ROPs negatively affected adult plant height (Figure 1B). In dicots, ROP proteins are described to be involved in establishment and maintenance of root hair tip growth (Molendijk et al., 2001; Jones et al., 2002; Tao et al., 2002). Microscopy inspection of root hair development in RACB knockdown lines revealed that initiation as well as maintenance of root hair growth was affected (Figure 1C). In multiple repeated experiments, all individuals of line B16/2-4 completely lacked root hairs when carrying the RACB RNAi construct, whereas corresponding individuals from line B15/1-16 showed sporadic and stunted root hairs. Azygous siblings, which lost the silencing cassette due to segregation in T1, developed root hairs normally (Figure 1C; see Supplemental Figure 1 online). To explain the different strengths of the root hair phenotype, we repeated qRT-PCR on RNA extracted from roots. This showed that silencing of type I ROPs was stronger in the roots when compared with leaves and reached a level of up to 90\% reduction of RACB in B16/2-4 lacking root hairs and 75\% in B15/1-16 having stunted root hairs (see Supplemental Figure 1 online). Hence, the strength

![Figure 1](image-url.png)

**Figure 1.** Transgenic Barley RACB Knockdown Plants Are Affected in Growth and Root Hair Development. **(A)** qRT-PCR of the five barley ROPs expressed in the epidermis of azygous controls and RACB knockdown (RACB KD) plants. Two independent biological replications led to very similar results. Error bars represent the SD. **(B)** Typical shoot development of transgenic RACB knockdown plants and azygous siblings 8 weeks after germination. **(C)** Root hair phenotypes of wild-type and RACB knockdown plants (T3 generation, B16/2-4). Pictures show roots of barley seedlings 2 d after germination. [See online article for color version of this figure.]
of the RACB silencing varied in a tissue-dependent way and correlated with the strength of the developmental phenotype in roots. The data show that knockdown of type I ROPs strongly affects outgrowth and elongation of root hairs in barley.

**RACB Knockdown in Barley Limits Accommodation and Expansion of Fungal Haustoria**

To examine the susceptibility to powdery mildew, RACB knockdown lines were inoculated with *B. graminis f. sp hordei*. Both lines displayed significantly reduced powdery mildew symptoms when compared with either azygous siblings or to the wild-type parent. On RACB knockdown barley, we counted ~50% fewer pustules than were observed on control plants (Figure 2A).

Impaired fungal growth can result from a reduced penetration success or from inhibition of postpenetration fungal development. To analyze the interaction phenotype of RACB knockdown barley on the single cell level, we microscopically recorded the penetration success of *B. graminis f. sp hordei*. On RACB knockdown barley, penetration efficiency was significantly reduced by 19% in average length of haustoria in cells of RACB knockdown barley (Figure 2C). Haustoria appearance was otherwise normal on RACB knockdown barley. Hence, knockdown of host type I ROPs hampers successful penetration, establishment, and expansion of fungal haustoria in barley epidermal cells.

**RACB Interacts with a MT-Associated ROPGAP in Barley**

Although RACB is characterized as a susceptibility factor in the barley–powdery mildew interaction, the mode of RACB action is not well understood. To identify interaction partners of barley ROP proteins, which are potentially involved in RACB function, yeast two-hybrid (Y2H) screenings with RACB, CARACB, and CARAC1 as bait proteins against cDNA libraries prepared from powdery mildew-infected barley leaves were performed. In these screenings, one partial cDNA clone that coded for a predicted RHOGAP domain with a CRIB motif was repeatedly isolated. BLAST searches identified a corresponding full-length protein (accession number AK371854) that shows strong similarity to known ROPGAPs of *Arabidopsis* and tobacco (*Nicotiana tabacum*; see Supplemental Figure 2 online). We later named the protein barley MICROTUBULE-ASSOCIATED GAP1 (MAGAP1; see below). Independent Y2H assays with barley ROPs demonstrated the potential of full-length MAGAP1 to interact with RACB and CARACB, but no interaction was observed for the combination of MAGAP1 with dominant-negative DN-RACB (Figure 3A).

The complete open reading frame of MAGAP1 encodes a protein of 484 amino acids. The protein possesses a conserved central region with high sequence similarity to known RHOGAP domains (amino acids 162 to 319). In this part of MAGAP1, the amino acid identity is >80% when compared with well-characterized ROPGAPs, such as *Arabidopsis* ROPGAP1 or tobacco ROPGAP1. Additionally, the GAP domain contains conserved amino acid residues described as being essential for binding of RHO proteins (Lys-219, Arg-223, Asn-285, Val-289; see Supplemental Figure 2 online) (Rittinger et al., 1997a) and for stimulation of GTP hydrolyzng activity of RHO-GTP (Arg-185) (Rittinger et al., 1997b; Klahre and Kost, 2006) (scanned at http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The G-protein binding domain (amino acids 103 to 140) is also sequence conserved and contains a CRIB motif with amino acids Ile-100, Pro-103, His-108, His-111, Asp-115, Phe-127, and Val-131, which are described as being responsible for binding active RHO-GTP (Burbelo et al., 1995; Morreale et al., 2000; Wu et al., 2000, 2001). In contrast with the highly conserved central region of the protein, the N and C termini of MAGAP1 are less well conserved. A BLASTP search against all protein sequences available at the National Center for Biotechnology Information (NCBI) and subsequent amino acid sequence alignments (Larkin et al., 2007) indicated similarity of amino acids 1 to 103 or amino acids 319 to 471 of MAGAP1 to sequences from monocots but not from dicots. However, the terminal 13 amino acids of MAGAP1 (amino acids 465 to 484) are again conserved in other ROPGAPs from both monocots and dicots. The N terminus of MAGAP1 further contains Glu-rich and Ser-rich stretches, which are also present in dicot ROPGAPs (see Supplemental Figure 2 online).

We analyzed interaction of MAGAP1 with RACB in barley by in vivo analysis of fluorescence resonance energy transfer (FRET). In FRET-based sensitized emission experiments, coexpression of the yellow fluorescent protein (YFP)-fused MAGAP1 and cyan fluorescent protein (CFP)-fused RACB resulted in a FRET efficiency of 15 to 18% at the cell periphery. FRET signals were also present at the nucleus. No FRET signal could be detected with the combination of YFP-MAGAP1 with CFP-DN-RACB (Figures 3B and 3C). This is consistent with the assumption that ROPGAPs target active ROPs. To validate these results, we used FRET measurement by fluorescence enhancement of CFP-CARACB after photobleaching of the YFP-MAGAP1 acceptor at the cell periphery, leading to similar results (Figure 3D; for details, see Methods). This supports that MAGAP1 interacts with CARACB but not, or weakly, with the DN form of RACB and suggests an interaction of the proteins at the cell periphery, where active RACB is localized (Schultheiss et al., 2003, 2008).

**MAGAP1 Is Associated with Barley MTs**

To investigate the subcellular localization of MAGAP1 in vivo, we fused MAGAP1 N-terminally to both green and red fluorescent protein (GFP and RFP). In transiently transformed barley cells, the GFP–MAGAP1 fusion protein displayed localization at cytoskeleton elements in the cell cortex and the perinuclear area but only weakly in transvacuolar cytoplasmic strands, where filamentous actin is observed (Figure 4A). These cytoskeleton elements resembled characteristic patterns of MTs. To reveal the identity of these cytoskeleton elements, we coexpressed GFP–MAGAP1 with barley actin depolymerization factor 3 (ADF3), which depolymerized F-actin in barley epidermis (Miklis et al., 2007). We observed that ADF3 caused characteristic fragmentation of the cytoplasm and stopped cytoplasmic streaming. MAGAP1–labeled cortical structures were loosened but not destroyed by overexpression of ADF3 (Figure 4B). The assumption that the MAGAP1-decorated cytoskeleton elements were...
MTs was further supported when we cotransformed barley cells with GFP-MAGAP1 and DsRED-MBD, a DsRED fusion with the MT binding domain of MICROTUBULE-ASSOCIATED PROTEIN4 (Marc et al., 1998). When expressed in barley epidermal cells, DsRED-MBD caused a mild disorganization of MTs. In particular, MTs had an unusual wavy appearance. However, DsRED-MBD and GFP-MAGAP1 colocalized at cytoskeleton elements, GFP-MAGAP1 was also distributed in the cytoplasm, albeit to a lesser extent (Figure 4C). We further characterized the MAGAP1-labeled cytoskeleton as MTs by treating leaves with the MT depolymerizing drug oryzalin (Figure 4E). The treatment completely abolished MAGAP1 association with the cytoskeleton and instead resulted in spherical aggregations of GFP-MAGAP1, whereas mock-treated controls appeared unaffected (Figure 4D). Together, this indicates that MAGAP1 decorates cortical MTs in barley and led us to name it MAGAP1.

Because the analysis of the MAGAP1 protein sequence revealed no common MT binding domain, we produced GFP fusions of amino acids 1 to 328 lacking the C terminus (MAGAP1 ΔCter) and of the C-terminal part (amino acids 319 to 484) of MAGAP1 lacking the CRIB and the GAP domains (Figure 5A). GFP-MAGAP1 and GFP-MAGAP1 Δter (amino acids 319 to 484) were associated with MTs (Figures 5B and 5D), whereas fusion of GFP to MAGAP1 ΔCter did not target the protein to MTs. Instead, the protein appeared in the cytoplasm and in transvacuolar cytoplasmic strands (Figure 5C). This demonstrates that the C-terminal amino acids 319 to 484 of MAGAP1 are required and sufficient for the association with MTs. Interestingly, the C terminus of MAGAP1 appears little conserved in dicot ROPGAPs (see Supplemental Figure 2 online).

Localization of GFP-MAGAP1 completely changed when we coexpressed CARACB, which is associated mainly with the plasma membrane (Schultheiss et al., 2003, 2008). With coexpression of CARACB, MT association of MAGAP1 was abolished. Instead, the protein appeared in the cytoplasm and in transvacuolar cytoplasmic strands (Figure 5C). This demonstrates that the C-terminal amino acids 319 to 484 of MAGAP1 are required and sufficient for the association with MTs. Interestingly, the C terminus of MAGAP1 appears little conserved in dicot ROPGAPs (see Supplemental Figure 2 online).

MTs Are Reorganized in Response to Fungal Attack

We used GFP-MAGAP1 and RFP-MAGAP1 to observe subcellular protein localization upon fungal attack from virulent B. graminis f. sp. hordei. GFP-MAGAP1–labeled MTs formed a radial pattern that strongly polarized to the site of fungal attack. Cell wall appositions (also called papillae), which barley cells build underneath fungal appressoria, were surrounded by a dense nest-like structure of cortical MTs, and the entire MT array was polarized to the site of fungal attack (Figures 6A and 6B). However, this was observed only when B. graminis f. sp. hordei

Figure 2. Interaction Phenotype of RACB Knockdown Barley and the Powdery Mildew Fungus B. graminis f. sp hordei.

(A) Bars present pustule numbers in one out of three experiments. In each experiment, at least three plants per line were evaluated with similar results. Pustule development of B. graminis f. sp hordei was significantly reduced on RACB knockdown lines B15/1-16 and B16/2-4 compared with the wild type (WT) and azygous controls (bars labeled with different characters represent means significantly different according to analysis of variance (ANOVA) followed by Duncan test, P < 0.05).

(B) Microscopy analysis of the B. graminis f. sp hordei interaction phenotype with barley 48 h after inoculation. Bars show fungal penetration efficiency as a percentage of all interactions. Penetration is significantly reduced on RACB knockdown lines B15/1-16 and B16/2-4 compared with the azygous and wild-type controls (bars labeled with different characters represent means significantly different according to analysis of variance (ANOVA) followed by Duncan test, P < 0.05). The graph shows data of one out of three independent experiments with similar results.

(C) Haustoria accommodation of B. graminis f. sp hordei in the epidermal cells of RACB knockdown lines B15/1-16 and B16/2-4 compared with the wild-type and azygous controls. Lengths of WGA-TMR–stained haustoria were measured 48 h after inoculation. Differences in haustoria length are significant (ANOVA, Duncan test, P < 0.001). Independent repetition of the experiment led to similar results. Error bars present the SD of the mean.
was hindered from penetration by basal resistance. By contrast, MTs were often organized transversely to the cell axis and did not show strong polarization in cells successfully penetrated by *B. graminis* f. sp *hordei*. At the site of fungal invasion and establishment of the haustorium, MTs were loosened when compared with cortical arrays in nonpenetrated cells (Figures 6C and 6D). Furthermore, fluorescence-tagged MAGAP1 often displayed diffuse localization at the cell periphery close to the collar and neck of the invading haustorium (Figure 6D).

The RFP-tagged C terminus of MAGAP1 did not influence the interaction outcome with *B. graminis* f. sp *hordei* (see below). Therefore, it was used as a MT marker to quantify differences in MT organization depending on fungal penetration success. This revealed that focusing of cortical MT arrays was the main difference between resistant and susceptible cells. More than 30% of cells that defended fungal penetration were strongly polarized, whereas <2% of penetrated cells showed MT polarization (Figures 7A and 7B). Penetrated cells instead showed parallel orientation of MTs at >30% of sites, whereas <10% of resistant cells showed parallel MTs (Figures 7A and 7B). The frequency of cells with disorganized MTs (either fragmented or randomized; Figures 7A and 7B) did not strongly depend on the outcome of the single cell interaction.
MAGAP1 in the interaction with the barley powdery mildew fungus. TIGS of MAGAP1 in epidermal cells resulted in a 50% increase of relative success of haustorium establishment by B. graminis f. sp hordei (Figure 7C). Off-target effects of the MAGAP1 TIGS construct are unlikely because the SiFi program (Nowara et al., 2010) did not identify further barley RNA sequences that would be targets of small interfering RNAs derived from double-stranded RNA of MAGAP1. The RNAi construct used also suppressed the expression of GFP-MAGAP1 driven from the cauliflower mosaic virus 35S promoter in 70% of cells when normalized by coexpression of mCherry, showing the efficacy of TIGS (see Supplemental Figure 3 online). Hence, abundance of MAGAP1 apparently limits penetration success of B. graminis f. sp hordei on barley.

Because TIGS of barley MAGAP1 influenced susceptibility to B. graminis f. sp hordei, we wondered whether ROPGAPs of the dicot model Arabidopsis could affect susceptibility to powdery mildew, too. We found ROPGAP1 (At5g22400) and ROPGAP4 (At3g11490) to be the Arabidopsis ROPGAPs with highest sequence similarity to MAGAP1 (see Supplemental Figure 2 online). To assess whether ROPGAP1 and ROPGAP4 are also able to interact with type I and type II ROPs, we performed Y2H analysis with leaf-expressed Arabidopsis ROPs. Both Arabidopsis ROPGAPs interacted with several ROPs, including RACB-like Arabidopsis type I ROPs 1-6 (see Supplemental Figure 4 online). GFP fusions of ROPGAP1 and ROPGAP4 revealed a cytoplasmic localization and colocalization with soluble nonfused RFP (see Supplemental Figure 5 online). By contrast, coexpression of Arabidopsis CAROP6 with GFP-ROPGAP1 or GFP-ROPGAP4 resulted in recruitment of GFP-ROPGAPs to the cell periphery (see Supplemental Figure 5 online). We then analyzed T-DNA insertion lines of ROPGAP1 and ROPGAP4 and identified for each gene two independent T-DNA insertion mutants. All four mutants of ROPGAP1 and ROPGAP4 may represent loss-of-function mutants because no corresponding transcripts could be amplified by RT-PCR from leaf tissue, whereas the parent Columbia-0 (Col-0) wild type expressed the gene under the same conditions (amplicons 3′ of the T-DNA insertion sites; see Supplemental Figure 6A online). After inoculation with the adapted powdery mildew fungus Erysiphe cruciferarum, enhanced disease symptoms were visible 7 d after inoculation on both ropgap1 and ropgap4 mutants when compared with the parent wild-type Col-0 (see Supplemental Figures 6B to 6D online). Faster development of the fungus was visible at the microscopic level at 48 h after inoculation (see Supplemental Figure 6E online). These data show a role for ROPGAP1 and ROPGAP4 in limiting susceptibility of Arabidopsis to E. cruciferarum and suggest a function for ROP signaling in the interaction of Arabidopsis with powdery mildew.

Overexpression of Variants of MAGAP1 Influences Fungal Success

Next, we used the RFP-MAGAP1 fusion protein and the nonfused RFP as a control in transient overexpression experiments. RFP-MAGAP1 significantly reduced relative penetration success of B. graminis f. sp hordei by ~20% (Figure 7D). RFP-MAGAP1ΔCter induced a similar but somewhat stronger effect
toward less susceptibility to fungal penetration. By contrast, RFP-MAGAP1 Cter, lacking the N terminus, and the CRIB and the GAP domains, did not influence the interaction outcome. This indicates that the MT-associated C terminus is neither required nor sufficient for the susceptibility-limiting effect of MAGAP1. To investigate further the importance of the GAP domain for the outcome of the powdery mildew interaction, we introduced a single amino acid exchange in the GAP domain, described to result in a diminished enzymatic activity of the protein and a dominant-negative effect (Wu et al., 2000; Klahre and Kost, 2006). The catalytic Arg, which was substituted by Gly (R185G) in the GAP domain, is highly conserved in RHOGAPs (Rittinger et al., 1997b; Mucha et al., 2011). In contrast with RFP-MAGAP1, transient overexpression of the RFP-MAGAP1 R185G caused a significant increase in penetration success of B. graminis f. sp. hordei of ~35% (Figure 7D). Hence, RFP-MAGAP1 R185G had the opposite effect of RFP-MAGAP1 Cter. RFP-MAGAP1 R185G caused a tendency toward more cells showing parallel orientation of MTs (Figure 7E). These data suggest a potential of MAGAP1 to regulate the polar organization of MTs in barley leaf epidemical cells.

**DISCUSSION**

Plant RHO-like ROP proteins are well known for having functions in polar and diffuse growth processes of plant cells. ROP signaling affects membrane trafficking and cytoskeleton organization. ROP activity is spatio-temporally fine-tuned by regulatory proteins that directly interact with ROPs. Here, we confirm a requirement for monocot barley ROP function in susceptibility to B. graminis f. sp hordei. Additionally, we show a function of barley type I ROPs in normal accommodation of haustoria in
epidermal cells of barley and in initiation and maintenance of root hair tip growth. This establishes barley type I ROPs as common factors of root hair outgrowth and ingrowth of a haustorial complex in leaf epidermal cells. We demonstrate that the barley type I ROP RACB can interact with MAGAP1, an MT-associated ROPGAP, in yeast and in planta. Barley MAGAP1 and Arabidopsis ROPGAP1 and ROPGAP4 limit susceptibility to powdery mildew and thus may negatively regulate ROP signaling in susceptibility. Data support that ROPs and ROP regulatory proteins are conserved elements of cell growth and cytoskeleton organization in monocots. Parasitic B. graminis f. sp hordei might co-opt ROP function in polar growth processes for accommodation of haustoria in epidermal cells of barley. By contrast, MAGAP1 may constitute an antagonist of barley ROPs at the cell periphery.

**RACB Is Involved in Establishment of Root Hairs and Haustoria**

Stable transgenic barley plants bearing a RACB RNAi cassette showed reduced levels of transcripts of type I ROPs, whereas transcripts of type II ROPs were not affected (Figure 1). RACB knockout plants showed fewer or no root hairs, depending on the level of ROP silencing. This supports a role of monocot type I ROPs in initiation and maintenance of root hair tip growth. For dicots, a function of ROP signaling in root hair growth is well
established (Yang, 2008; Yalovsky et al., 2008). Data on dicots also show a lack of root hairs upon RNAi-mediated knockdown of type I RAC1 in tobacco (Tao et al., 2002). Together, the data suggest that function of type I ROPs in tip growth of dicots is conserved in the monocot barley. This is further supported by ectopic expression of CARACB in barley and tobacco leading not only to enhanced susceptibility to powdery mildew but also to isotropic instead of polar growth of root hairs (Pathuri et al., 2008, 2009). Similar effects are typically seen in root hairs of dicots expressing CAROPs (Molendijk et al., 2001; Jones et al., 2002).

Figure 7. MT Organization and Interaction of Barley Cells with *B. graminis* f. sp hordei Is Influenced by MAGAP1.

(A) Example micrographs from categories of MT organization. Images show cells of barley leaves transiently expressing RFP-MAGAP1 24 h after inoculation with *Blumeria graminis* f. sp hordei. Arrows indicate the site of fungal attack. Pictures are maximum projections of 20 to 30 optical sections with 2-μm increments. Bars = 20 μm.

(B) Bars show MT organization in barley cells interacting with *Blumeria graminis* f. sp hordei. Epidermal barley cells transiently expressing RFP-MAGAP1 Cter as an MT marker and GFP for labeling of the cytoplasm and nucleoplasm. MT organization was evaluated 24 h after inoculation. Data present the mean of 10 independent experiments. In each experiment, at least 50 cells per construct were analyzed. The frequency of cells with MT focused to the site of attack is significantly higher when the fungus was stopped compared with the cells penetrated by the fungus, whereas the frequency of cells with parallel MT organization is significantly elevated in penetrated cells.

(C) Bars indicate the relative penetration success of *B. graminis* f. sp hordei after transient knockdown of MAGAP1. Data represent the means of six independent experiments with 100 cells each. Controls were transformed with the empty RNAi vector construct pPKTA30N and set as 100%. Penetration efficiency of *B. graminis* f. sp hordei was significantly enhanced.

(D) Bars indicate the relative penetration success of *B. graminis* f. sp hordei after transient overexpression of the RFP-MAGAP1, RFP-MAGAP1 ΔCter, RFP-MAGAP1 Cter, or RFP-MAGAP1 R185G construct. Data represent the means of five independent experiments. Controls were transformed with the nonfused RFP construct and set as 100%. Penetration efficiency of *B. graminis* f. sp hordei was significantly reduced when RFP-MAGAP1 or RFP-MAGAP1 ΔCter were overexpressed, whereas transient overexpression of RFP-MAGAP1 R185G enhanced the penetration efficiency. No significant differences from the control were observed when RFP-MAGAP1 Cter was expressed.

(E) MT organization during pathogen attack is influenced by the expression of MAGAP1 variants. For the evaluation of the MT organization, epidermal barley cells transiently expressed RFP-MAGAP1, RFP-MAGAP1 R185G, or RFP-MAGAP1 Cter as MT markers and GFP for labeling of the cytoplasm and nucleoplasm. In each experiment, MT organization of at least 50 cells per construct were analyzed 24 h after inoculation. Data present the mean of 10 independent experiments. Overexpression of RFP-MAGAP1 led to a significant increase of cells with focused MT. Error bars represent confidence intervals at P < 0.05. Two-tailed Student’s t test: *P < 0.05, **P < 0.01, and ***P < 0.001.
2002). The same RACB RNAi lines that lacked root hairs developed significantly less powdery mildew disease. At the level of single-cell interactions, this is explained by the fact that less fungi established haustoria in epidermal cells. Additionally, accommodation of haustoria appeared to be hampered because at 2 d after inoculation, when haustoria normally are fully expanded, haustoria were significantly smaller on RACB RNAi plants than on wild-type or azygous siblings (Figure 2C). In summary, the data suggest that type I ROPs constitute common elements of outward growth of epidermal root hairs and inward growth of haustorial complexes. The data further support the hypothesis that haustorium accommodation reflects an inverted tip growth program of barley potentially co-opted by B. graminis f. sp. hordei (Schultheiss et al., 2003). Many plant tip growth processes are regulated by ROP proteins that show a high structural conservation (Mucha et al., 2011). High structural conservation of ROPs might also provide a basis for targeting by pathogen effectors because the host may lack the potential for evolutionary diversification.

Most recently, it was demonstrated that the receptor-like kinase FERONIA functions upstream of ROPs in Arabidopsis root hair growth (Duan et al., 2010). The same kinase is required for both the successful accommodation of sperm in the female gametophyte and for the full susceptibility of leaves to powdery mildew (Kessler et al., 2010). Thus, ROP GTPases and upstream receptor-like kinases may constitute common elements that mediate polarity in outward cell growth and in communication with invading cells of self or nonself.

**RACB Interacts with MAGAP1**

MAGAP1 interacted with RACB and CARACB, but not with DNRACB, in yeast and in planta (Figure 3). Additionally, GFP-MAGAP1 was associated with cortical and perinuclear MTs when expressed alone but located at the cell periphery when coexpressed with CARACB (Figure 5E). Because CARACB is associated with the plasma membrane (Schultheiss et al., 2003, 2008) and FRET signals were strongest at the cell periphery, data suggest that active RACB recruited MAGAP1 to the cell periphery. MAGAP1 is thus a previously undiscovered type of ROPGAP that associates with MTs when ROPs are inactive but is apparently released upon ROP activation for negative regulation. Similar mechanisms of RHO regulation by MT-associated regulatory proteins are known from metazoans. For instance, RacGAP50C recruits β-tubulin at the minus ends of MTs for polar orientation in polynuclear myotubes of Drosophila melanogaster (Guerin and Kramer, 2009). Mammalian RHOGEF-H1 is associated with MTs, and upon depolymerization of MTs from the plus end, it is released for activation of RHO (Birkenfeld et al., 2008). This is involved in many processes, from normal cell functions to cell death control and pathological processes. MT organization represents a target of RHO effectors. Hence, MT association of RHO regulatory proteins might provide a basis for feedback regulation of RHO activity, which is controlled via the physiological effect of RHO on MTs. In Arabidopsis, the ROP2-kinesin-like MRH2 pathway, the ROP6-RIC1 pathway, and the ROP2-RIP3-kinesin13A pathway are examples of signaling toward organization and stability of MTs (Yang et al., 2007; Fu et al., 2009; Mucha et al., 2010, 2011). For monocots, however, nothing is known about potential ROP downstream signaling toward MTs. MAGAP1 may thus provide a starting point for analysis of ROP-mediated organization of MTs in monocots.

**ROPGAPs Modulate Fungal Success on Barley and Arabidopsis**

ROPGAPs are negative regulators of ROP downstream signaling. At the mechanistic level, this is explained by stimulation of the otherwise low intrinsic GTPase activity of RHO-like ROPs (Wu et al., 2000; Klahre and Kost, 2006). RHOGAPs possess a conserved Arg residue that reaches into the GTP binding pocket of RHO proteins and stimulates hydrolysis of GTP, thereby inactivating RHOs (Rittinger et al., 1997a, 1997b). Overexpression of MAGAP1 lowered fungal success in establishing haustoria. This is consistent with the assumption that MAGAP1 may switch off GTP-bound ROPs, such as RACB, involved in susceptibility. Consequently, RNAi directed against MAGAP1 resulted in enhanced susceptibility to fungal penetration. We thus conclude that abundance of MAGAP1 contributes to the quantitative success or failure of B. graminis f. sp. hordei to penetrate. When overexpressed, the single amino acid mutant MAGAP1 R185G had the opposite effect of MAGAP1. The single amino acid exchange at position Arg-185 should abolish catalytic activity of MAGAP1. Position Arg-185 should abolish catalytic activity of MAGAP1. Overexpression of MAGAP1 lowered fungal success in establishing haustoria. This is consistent with the assumption that MAGAP1 may switch off GTP-bound ROPs, such as RACB, involved in susceptibility. Consequently, MAGAP1 R185G has a dominant-negative effect when binding to RACB-GTP but lacking GAP activity. In agreement with this, the tobacco ROPGAP1 R183A mutant has a dominant-negative effect and causes aberrant growth when expressed in pollen tubes (Klahre and Kost, 2006). The data thus suggest that the effect of MAGAP1 on fungal penetration success depends on GAP activity.

The question arose whether the MT association of MAGAP1 has a direct function on MTs or the protein is perhaps sequestered by MT. We favor the latter explanation because activated CARACB interacted with MAGAP1 at the plasma membrane rather than at MTs. Additionally, the C terminus of MAGAP1 was sufficient to associate fused GFP or RFP to MTs but did not influence the interaction with B. graminis f. sp. hordei. By contrast, cytoplasmic GFP-MAGAP1ΔCter was recruited by CARACB and influenced the interaction outcome. The potential of MAGAP1 to modulate the interaction outcome was thus independent of MT association and was mediated by the part of the protein containing the GAP domain. Association of MAGAP1 with MTs might hence not reflect a direct function of MAGAP1 in MT organization but a sequestered status of the protein, which is released and recruited upon ROP activation.

Ectopic expression of rice DNRAC1, barley CARACB, or barley CARAC3 in tobacco influenced immunity to viral, bacterial, and fungal pathogens (Moeder et al., 2005; Pathuri et al., 2009). However, little evidence exists for function of endogenous ROP signaling in the interactions of dicots with pathogens. We found that Arabidopsis mutants lacking expression of ROPGAP1 or ROPGAP4 support earlier development of E. cruciferarum and more disease symptom formation on the plants. This is similar to what we observed after silencing of MAGAP1 in barley epidermis. ROPGAP1 and ROPGAP4 are the Arabidopsis proteins.
most similar to barley MAGAP1. For both proteins, it was previously shown that they regulate the activity of Arabidopsis ROPs in vitro and in planta (Wu et al., 2000; Baxter-Burrell et al., 2002). Because ROPGAP1 and ROPGAP4 seem to have a relatively broad binding activity to leaf-expressed ROPs, it is difficult to predict the ROPs that might be responsible for modulating the interaction outcome in Arabidopsis. This difficulty is further enhanced by redundant functions of plant ROPs, with single mutants often showing only mild or no phenotypes. Additionally, ectopic expression of constitutively activated or dominant negative ROPs often influences plant development. However, the lack of single ROPGAPs in Arabidopsis was sufficient to cause a disease phenotype without causing pleiotropic effects on the development of the shoot or of epidermal pavement cells. Hence, the data suggest that ROP regulatory GAPs in both monocot and dicot plants limit susceptibility to powdery mildew fungi. Arabidopsis ROPGAP1 and ROPGAP4 do not possess high similarity to MAGAP1 at the MT-associating C terminus (see below). This might support that in both barley and Arabidopsis the GAP function is responsible for the regulatory role of ROPGAPs in pathogenesis of powdery mildew.

The C Terminus Associates MAGAP1 to MTs

Expression of truncated versions of MAGAP1 demonstrated that the C terminus of the protein is required and sufficient for association of the protein with MTs. Removal of the C terminus completely abolished MT association. By contrast, removal of the N terminus, the CRIB, and the GAP domain did not hinder the MT association of the residual C terminus. Conversely, RFP-MAGAP1ΔCter was recruited to the cell periphery by CARACB but not RFP-MAGAP1Cter. Hence, the MT association and the ROP binding are established by different parts of MAGAP1. Interestingly, the C terminus of MAGAP1 seems not to be conserved in dicot ROPGAPs. Accordingly, Arabidopsis ROPGAP1 and ROPGAP4 were not associated with MTs when tagged by GFP. This further supports that the C terminus of MAGAP1 contains previously unknown structural elements for MT association. However, it remains to be elucidated how exactly MAGAP1 associates to MTs.

Host MTs in the Interaction with B. graminis f. sp hordei

MTs are reorganized in barley epidermal cells upon attack by powdery mildew fungi (Baluska et al., 1995; Kobayashi et al., 1997). By contrast, Takemoto et al. (2003) did not observe strong differences of MT organization in compatible versus incompatible couples of three different Arabidopsis–oomycete interactions. We observed qualitative and quantitative differences in MT arrays when we compared penetrated and resistant cells (Figures 6 and 7). In cells that successfully defended fungal penetration attempts and built cell wall appositions, MTs often formed a strongly polarized pattern. In this, MTs concentrated to the site of attack and built circular concentric arrays around the cell wall apposition. This might have a role in trafficking of defense-associated compounds or positioning of glucan synthases in the plasma membrane, as typically seen in secondary cell wall formation (Paradez et al., 2006). This view is supported by the observation that application of oryzalin limited penetration resistance of barley coleoptiles to the nonadapted powdery mildew fungus Erysiphe pisi (Kobayashi et al., 1997). MT polarization toward the site of interaction was absent or weak in cells that contained an expanding haustorium. MTs often showed a parallel nonpolarized orientation in penetrated cells. This was similar to what was observed for F-actin in a compatible interaction between barley and B. graminis f. sp hordei (Opalski et al., 2005). Additionally, MTs often loosened at the subcellular point of pathogen entry. This likely reflects an influence of the fungus on host MT organization or on local ROP activity. Similar to plant cell tip growth, ingrowth of the haustorial complex and formation of the extrahaustorial host membrane may require a zone of membrane delivery, where MTs are usually absent and ROP activity is high (Smith and Oppenheimer, 2005; Yang, 2008). Accordingly, cortical MTs were only occasionally found at the haustorial neck or body. Instead, signals from fluorescent MAGAP1 were diffuse at the cell periphery where the fungus invaded (arrow in Figure 6D). This could indicate release of MAGAP1 from MTs, when they locally loosen for fungal invasion (see Supplemental Figure 8 online for a model). It might even be that the fungus actively triggers local loosening of cortical MTs to get access to the cell. This might involve ROP activity. This idea is supported by the fact that knockdown of RACB limits haustorial invasion. ROP activity at sites of haustorial invasion is further indicated by recruitment of both MAGAP1 and the barley ROP downstream effector RIC171 to the site of fungal entry (Schultheiss et al., 2008). In this context, it is interesting that Arabidopsis-activated ROPs have the potential to loosen cortical arrays of MTs during polar cell growth (Molendijk et al., 2001; Fu et al., 2005, Mucha et al., 2011). In root trichoblasts, ROPs are further localized to the sites of root hair initiation, where MTs are randomized or loosened during outgrowth of the root hair (Molendijk et al., 2001; Jones et al., 2002; Van Bruaene et al., 2004). ROP activity may also be key to local membrane delivery. This is well established in plant cell tip growth (Yakovlevsky et al., 2008; Yang, 2008). Hence, ROPs might signal for membrane delivery in formation of the extrahaustorial host membrane.

During the interaction of plants with biotrophic fungi that form haustoria or arbuscules in living plant cells, the plant cell architecture has to be reorganized for accommodation of fungal infection structures (Parniske, 2000). This is accompanied by rearrangements of F-actin, MTs, endomembranes, and the plasma membrane (Genre and Bonfante, 2002; Genre et al., 2005; Koh et al., 2005; Opalski et al., 2005; Eichmann and Hückelhoven, 2008). Our data support that ROPs and ROP regulatory proteins are involved in organization of the cytoskeleton for accommodation of fungal structures within intact plant cells. This might involve further components that are also required for tip growth of plant cells.

METHODS

Plant Growth, Pathogens, Inoculation, and Macroscopy Assessment of Disease Progression

Wild-type and transgenic barley (Hordeum vulgare) plants of the cultivars ‘Golden Promise’, ‘Pallas,’ and the backcross (BC) lines BCPallasm1o5,
BCPallasMla12, and BCPallasMlg were grown in a growth chamber at 18°C with 60% relative humidity and a photoperiod of 16 h with 150 μmol m⁻² s⁻¹. BC lines (Keister et al., 1986) were obtained from Lisa Munk (Royal Veterinary and Agricultural University, Copenhagen, Denmark). For harvesting of barley roots, seeds were grown for 7 d on wet filter paper in a growth chamber under the conditions described. *Blumeria graminis* f. sp. *hordei*, race A6, the barley powdery mildew fungus, was maintained on the cultivar ‘Golden Promise’ under the above-described conditions.

For the analysis of transgenic plants, the second leaf of 14-d-old plants was inoculated with *B. graminis* f. sp. *hordei* to give a density of 5 conidia mm⁻² for macroscopy analysis, whereas for microscopy evaluation a density of 20 conidia mm⁻² was used. In transient expression experiments, detached primary leaf segments of barley were placed on 0.5% water-agar 7 d after germination and inoculated with >100 conidia mm⁻². Macrocscopy analysis of disease progression of *B. graminis* f. sp. *hordei* haustoria was assessed by counting the developing powdery mildew pustules per cm² leaf area 6 d after inoculation.

*Arabidopsis thaliana* Col-0 plants and *Arabidopsis* T-DNA insertion mutants in the Col-0 background were grown in a growth chamber at 22°C and a 10-h photoperiod with 120 μmol m⁻² s⁻¹ light and 65% relative humidity. *Arabidopsis* SALK T-DNA insertion lines of ROPGAP1 (SALK_049590 and SALK_114884) and ROPGAP4 (SALK_038694 and SALK_068684) were ordered from the Nottingham Arabidopsis Stock Centre. Genotyping of the *Arabidopsis* insertion lines was performed by PCR with the insert specific primer LBb1.3 and the mutant-specific primers of ropgap1-1 (LPSALK_049590 and RPSALK_049590), ropgap1-2 (LPSALK_114884 and RPSALK_114884), ropgap4-1 (LPSALK_038694 and RPSALK_038694), and ropgap4-2 (LPSALK_068684 and RPSALK_068684) (see Supplemental Table 1 online).

Arabidopsis powdery mildew *Erysiphe cruciferarum* was grown on *Arabidopsis* Col-0 plants and for increased conidia production on *Arabidopsis* pad4 mutant plants (Glazebrook et al., 1996) at the same conditions. Five-week-old Arabidopsis plants were inoculated with *E. cruciferarum* for macroscopy and microscopy evaluation of disease progression with a density of 3 to 5 conidia mm⁻². *Arabidopsis* susceptibility to *E. cruciferarum* was scored by visual examination 7, 9, and 11 d after inoculation. Plants were distributed in three categories of susceptibility with 0 to 30%, 30 to 60%, and >60% diseased leaf area.

**Production and Genotyping of Transgenic Plants and Mutants**

To produce the binary pLH6000-RCAB RNAi vector, a *BamHl/XbaI* and a *Spel/SalI* fragment encoding the full-length *RCAB* were inserted as inverted repeat in the pLH6000 vector (GenBank accession number AL234328). Inverted repeats are separated by the second intron of the wheat (*Triticum aestivum*) *RG2* gene and are located between the promoter of the maize (*Zea mays*) *UBQUITIN1* gene with the first intron and the *terminator* of the *Arabidopsis thaliana* *Nopaline SYNTHASE* gene. The construct was transferred into the *Agrobacterium* strain *Agl-1* for production of stable transgenic barley plants. Stably transgenic barley plants were generated by *Agrobacterium*-mediated transformation using the pLH6000-RCAB RNAi construct as described by Hensel et al. (2008).

The presence of the *RCAB* RNAi construct in the transgenic barley plants was verified by PCR with the maize *UBQUITIN1* promoter-specific primer Ubi-Prom-5’ and the *RCAB*-specific primer RacB-5’ (see Supplemental Table 1 online).

**Histological Staining and Microscopy Analysis of Powdery Mildew Development**

To evaluate the interaction outcome of barley with *B. graminis* f. sp. *hordei* on the single-cell level, leaf samples were harvested 48 h after inoculation. For bright-field microscopy, leaves were cleared in ethanol acetic acid solution (6:1 [v/v]), and fungal surface structures were stained with acetic ink (10% blue ink [v/v]) in 25% acetic acid. Penetrated cells bearing a haustorium and nonpenetrated cells were distinguished. For each of three independent experiments, each three leaves with each 100 interaction sites were evaluated.

Visualization of fungal haustoria in plant cells was performed by wheat germ agglutinine-tetramethylrhodamin staining (WGA-TMR). For WGA-TMR-staining, leaves were incubated for 20 min in PBS buffer (8 g NaCl, 2.8 g Na2HPO4 · 7 H2O, 0.24 g K2HPO4, and 0.2 g KCl in 1 liter water, pH 7.4) and transferred to WGA-TMR-staining solution (1 × PBS, 10 μg/mL BSA, and 10 μg/mL WGA-TMR). After vacuum infiltration, leaves were left in the staining solution at least overnight at 4°C in the dark. The lengths of *B. graminis* f. sp. *hordei* haustoria were measured after confocal laser scanning microscopy (CLSM; Leica TCS SP5; Leica Microsystems). WGA-TMR was exited by a 561-nm laser line and the emission detected at 571 to 610 nm. For each of three independent experiments, each three leaves with each 50 haustoria were evaluated. To analyze the interaction of *Arabidopsis* with *E. cruciferarum*, leaf samples were harvested 48 h after inoculation.

For evaluation of the root phenotype from RACB knockdown plants, roots of 2-d-old seedlings were fixed and stained with modified pseudo-Schiff propidium iodide staining as described by Truernit et al. (2008).

**Isolation of MAGAP1 and Introduction of Mutations**

The complete coding sequence of barley MAGAP1 was identified in the nucleotide database of NCBI (AK250639; http://blast.ncbi.nlm.nih.gov/). The coding sequence was amplified from a barley cDNA pool with the primers MAGAPISmal-5’ and MAGAPIXbal-3’ introducing restriction sites for cloning behind the cauliflower mosaic virus 35S promoter (P3S) of the plant expression vector pGY-1 (Schweizer et al., 1999; see Supplemental Table 1 online).

Point mutations were introduced into MAGAPI by PCR techniques. The 3’ and the 5’ part of the cDNA were amplified separately with complementary primers containing the modified triplets. The substitution of Arg versus Gly at position 185 (R185G) in the GAP domain was achieved with the primers R185G. To identify the domains responsible for MT binding of MAGAPI, leaf samples were transformed to pGY-1, resulting in the construct pGY1-MAGAPI R185G.

**Protein Localization in Planta**

For localization studies, leaves of barley plants were transformed to transiently express GFP- or RFP-MAGAPI fusion constructs under the control of P3S promoter via particle bombardment (see below). Fusion constructs of MAGAPI with GFP were achieved by amplification of the coding sequence with the primers MAGAPIXbal-5’ and MAGAPISal-3’ (see Supplemental Table 1 online) and insertion as N-terminal fusions behind and in frame with GFP lacking the stop codon into the expression vector pGY-1 by the Xbal and SalI restriction sites. N-terminal RFP fusions of MAGAPI were obtained by insertion of RFP lacking the stop codon into the Smal restriction sites of pGY1-MAGAPI and pGY1-MAGAPI R185G. To identify the domains responsible for MT binding of MAGAPI, the N-terminal (amino acids 1 to 328) part and the C-terminal (amino acids 319 to 484) part of the protein were fused N-terminally to GFP individually. Amplification was performed with the primers MAGAPIXbal-5’ and MAGAPInter for the N-terminal part, and MAGAPCter and MAGAPISal-3’ (see Supplemental Table 1 online) for the C-terminal part. As described above, introduced restriction sites Xbal and SalI were used for ligation into pGY-1 in frame with GFP. The
RFP fusions from MAGAP1Cter and MAGAP1Cter were obtained by replacement of GFP by a RFP coding sequence lacking the stop codon.

Depending on the fusion construct, the plasmids pGY1-GFP (Schweizer et al., 1999) or pGY1-RFP encoding soluble cytoplasmic and nucleoplasmic GFP or RFP in pGY-1 under the control of the P35S promoter were cotransformed as transformation marker. The construct pGY1-RFP was obtained by insertion of RFP in the Smal restriction sites of pGY1. In the localization studies, each shot delivered 1 μg of each fusion construct and 0.5 μg of the transformation marker. In transient coexpression experiments, each shot delivered 1 μg GFP- or RFP-MAGAP1 fusion construct together with 1.2 μg of pGY-1-CARACB or 0.8 μg of pGY-1-ADF3 (Miklis et al., 2007). Leaves were inspected 24 or 48 h after bombardment by CLSM. In the colocalization experiments, leaves were cotobombarded with 1 μg per shot of the MT marker DsRED-MBD (Marc et al., 1998) and pGY1-GFP-MAGAP1. Localization was analyzed 26 h after bombardment by CLSM. Pictures were generated by sequential scanning to avoid channel crosstalk. GFP was excited by a 488-nm laser line and detected at 500 to 550 nm, while RFP was excited by a 561-nm laser line and detected at 571 to 610 nm.

For imaging of Arabidopsis ROPGAP1 and ROPGAP4 localization in planta, the respective cDNA fragment was amplified from an Arabidopsis cDNA pool and fused in frame with GFP into the plant expression vector pGY-1 using the introduced restriction sites Sal/I and RsaI (ROPGAP1) or Sal/I/Xhol (ROPGAP4). Per shot, 0.8 μg of the respective fusion construct was transferred by particle bombardment into epidermal cells of Arabidopsis. In coexpression experiments, 0.8 μg of full-length CAROP6 in the plant expression vector pGY-1 and 0.8 μg of the GFP-ROPGAP1 or GFP-ROPGAP4 fusion construct were cotransformed into Arabidopsis leaf epidermal cells by particle bombardment. Imaging was done 24 h after bombardment by CLSM.

**FRET Analysis**

Interaction of RACB and MAGAP1 in planta was verified by FRET analysis. Fusion of MAGAP1 with YFP was achieved by insertion of the Xbal/Sal fragment of MAGAP1 into the pGY-1 vector containing YFP. CFP fusion constructs of mutated CARACB and DNFACB were obtained by replacement of the GFP in RACB fusion constructs via the BamHI restriction sites (Schultheiss et al., 2003). Leaves of 7- to 8-d-old barley plants were cotransformed by particle bombardment with 1.2 μg pGY-1-CFP-CARACB or pGY-1-CFP-DNFACB together with 1 μg pGY-1-YFP-MAGAP1. To calculate the correction factors in the FRET analysis, indicated amounts of each construct were also transformed alone (donor and acceptor references). In all FRET experiments, cells with medium fluorescence of CFP and YFP fusion constructs were selected. In the FRET analysis with the acceptor photobleaching method, first a cell expressing one of the respective CFP-RACB variants and YFP-MAGAP1 was imaged. For bleaching, a region at the cell periphery was defined, where both acceptor and donor were detected. In the bleaching process, the defined area was scanned 30 times with 100% relative laser intensity of the 514-nm laser line (20% of 20-mW laser power at 514 nm). Afterwards, a picture was taken with the identical settings and position as before bleaching. The fluorescence intensity of the CFP-RACB donor variants was measured in the bleached region before (Dpre) and after bleaching (Dpost), and the FRET efficiency (FRETeff) was quantified following the equation: FRETeff = (Dpost − Dpre)/Dpost. FRET measurements with the sensitized emission method were executed by detection of the fluorescence signal of donor CFP-RACB variants, FRET, and acceptor YFP-MAGAP1 in a line by line sequential scan acquisition. Calibration coefficients to correct for excitation and emission crosstalk were calculated with the donor-only and acceptor-only references. In brief, measuring parameters were first adjusted with a cell expressing the YFP and CFP fusion constructs. These parameters remain constant in the entire experiment. To calculate calibration factors, pictures were taken from the donor only and acceptor only references. Calibration factor β corrects for donor crosstalk and was calculated from the donor only reference by division of the CFP donor emission (excited 458-nm laser) in the YFP channel by the CFP donor emission (excited 458-nm laser) in the CFP channel. The factor γ corrects for acceptor cross-excitation and was calculated from the acceptor only reference by division of the YFP channel emission excited by the donor (excited 458-nm laser) with the YFP channel emission of YFP (excited 514-nm laser).

Finally, a region for FRET measurement was defined in the FRET picture and was automatically inserted by the software at the identical position in the donor and the acceptor channel. The apparent FRET efficiency EA was calculated after Wouters et al. (2001) by the equation: \[ EA = \frac{B - A^* - C^* - \gamma}{B} \]. FRET analysis was performed 24 h after bombardment by the sensitized emission and the acceptor photobleaching method using the Leica Application Suite, Advanced Fluorescence 1.8.0 software (Leica Microsystems). In all experiments, CFP was excited by a 458-nm laser line and detected at 465 to 500 nm, YFP was excited by a 514-nm laser line and detected at 524 to 600 nm.

**Analysis of MT Dynamics**

To evaluate MT dynamics in pathogen-attacked cells, barley leaves were transiently transformed via particle bombardment with the respective Hv MAGAP1 overexpression construct pGY1-Hv MAGAP1, pGY1-Hv MAGAP1 R185G, or pGY1-Hv MAGAP1 Cter and a GFP overexpression construct as transformation marker (as described above). The leaves were inoculated 16 h after bombardment with conidia of Blumeria graminis f. sp hordei and evaluated 24 h after inoculation by CLSM as described above. Fungus-attacked cells were distinguished as penetrated or nonpenetrated, and each cell was categorized according to its MT organization as focused, parallel, randomized, or fragmented.

**Y2H Screening and Targeted Y2H**

Y2H screening and transformation of yeast was performed according to the yeast protocols handbook and the Matchmaker library construction and screening kits manual (Clontech) using Saccharomyces cerevisiae strains Y187 and AH109. Y2H vectors pGBK7T and pGAD77 were used to express fusions with the GAL4 DNA binding domain (GAL4 DNA-BD) and the GAL4 activation domain (GAL4 AD). For screening, RACB, constitutively activated CARACB lacking the C-terminal CAAX motif and constitutively activated CARAC1 lacking the terminal 14 amino acids, including Cys residues that might be palmitoylated, were fused to the GAL4 DNA binding domain in pGBK7T (amino acids 1 to 193; Schultheiss et al., 2008). The Y2H library was generated from barley leaves consisting of a pool of Blumeria graminis f. sp hordei inoculated plants harvested at 0, 4, 8, 12, 24, and 52 h after inoculation from near-isogenic backcross lines (mlo5, Mlo Mlg, and Mla12) of the cultivar ‘Pallas.’ The Y2H library was used to screen 5 million mating events with RACB, 5 million mating events with CARACB, and 10 million mating events with CARAC1. Transformed yeast cells were selected on SD medium lacking Leu and Trp. Selection of yeast diploids expressing interacting proteins was performed on SD medium lacking Leu, Trp, His, and adenine.

For targeted Y2H assays, barley and Arabidopsis RAC/ROPs lacking their C-terminal residues responsible for membrane binding (see Supplemental Table 2 online) were fused with the GAL4 DNA binding domain in pGBK7T and cotransformed with the respective barley and Arabidopsis GAP constructs in pGAD77 into yeast.

**Transient Overexpression and TIG5**

Transformation of 7- to 8-d-old barley leaves was performed as described earlier (Douchkov et al., 2005; Eichmann et al., 2010) with the PDS-1000/He System (Bio-Rad Laboratories) plant transformation gun with
hepta-adaptor. In all overexpression experiments, each shot delivered 1-μm gold particles (25 mg/mL) coated with 7 μg of the respective expression construct pGY-1-RFP-MAGAP1, pGY-1-RFP-MAGAP1 R185G, pGY-1-RFP-MAGAP1 3Cter, pGY-1-RFP-MAGAP1 3Cter, or the empty vector control pGY-1-RFP, and 3.5 μg of the reporter plasmid pGY-1-GFP.

To produce the pIPKTA30N-MAGAP1 RNAi construct for TIGS, the full-length cDNA fragment of MAGAP1 was inserted as a Smal/SalI fragment in the antisense orientation into the Gateway-compatible entry vector pIPKTA38. Then, the cDNA fragment was transferred as inverted repeats into the pIPKTA30N destination vector by a standard Gateway LR reaction (Douchkov et al., 2005).

Seven micrograms of the RNAi construct pIPKTA30N-MAGAP1, the empty vector pIPKTA30N, and 3.5 μg of the reporter plasmid pGY-1-GFP were used in TIGS experiments. Microscopy evaluation of B. graminis f. sp. hordei penetration success was performed according to Eichmann et al. (2010).

Functionality of the pIPKTA30N-MAGAP1 RNAi construct was evaluated by cotransformation of barley leaves with 1 μg of the GFP-MAGAP1 fusion construct and 1 μg of the empty vector control pIPKTA30N or the pIPKTA30N-MAGAP1 RNAi construct per shot. In each shot, 0.5 μg of the soluble mCherry as transformation marker expressed from a P3SS promoter was included. Red fluorescent, mCherry expressing cells were inspected for green fluorescence indicating expression of the GFP-MAGAP1 fusion 48 h after bombardment. In each experiment, 100 cells per variant were evaluated.

Drug Treatment

Influence of MT depolymerizing drugs on the integrity of GFP-MAGAP1–labeled MTs was tested by transient expression in barley leaves (see above). The epidermis on the abaxial side of the leaves was removed 6 h after bombardment, and the leaves were floated with the abaxial side on the drug-containing solution. The solution consisted of 0.25% (v/v) DMSO and 32.5 μM MT-depolymerizing drug oryzalin (30 mM stock in ethanol). In the mock treatment, equal amounts of ethanol and DMSO were added. Inspection of the leaves by CLSM was performed after 13 h of incubation.

Gene Expression Analysis

Total RNA from leaves of 3-week-old barley plants or 4-week-old whole plants of Arabidopsis or roots of 7-d-old barley seedlings was extracted from frozen plant material using TRIzol reagent (Invitrogen). From each sample, 1 μg of total RNA was reverse transcribed into cDNA using the QuantiTect reverse transcription kit (Qiagen). To measure ROP transcript amounts in the barley RACB knockdown lines B16/2-4 and B15/1-16, qRT-PCR was performed. RNA was extracted and reverse transcribed into cDNA from a pool of three plants of both lines each and azygous control plants as described above. Triplicate qRT-PCR analyses with the SsoFast EvaGreen Supermix (Bio-Rad Laboratories) were conducted with 100 ng cDNA of each sample. PCR was performed on the Mx3005P Real-Time PCR system (Stratagene). PCR conditions and primers were used as described previously (Schultheiss et al., 2003). The UBQUITIN transcript of barley was amplified in parallel as endogenous control and to normalize variations in cDNA quantity with specific primers Ubi-5' and Ubi-3' (see Supplemental Table 1 online).

Knockout of ROPGAP1 and ROPGAP4 in Arabidopsis was verified by two-step RT-PCR. The constitutively expressed 18S/25S RNA served as quality control of the cDNA synthesis and was amplified with the primers At18S-5' and At18S-3' (see Supplemental Table 1 online). Presence of the ROPGAP1 and ROPGAP4 transcripts was tested in the T-DNA insertion mutants with gene-specific primers as indicated in Supplemental Table 1 online.

Hv RACB and Hv RACD expression in roots was evaluated by qPCR. PCR conditions and primers were used as described previously (Schultheiss et al., 2003). RNA was extracted at least from three transgenic or azygous plants per line. Triplicate reactions were performed with the Maxima SYBR Green qPCR Master mix (Fermentas). Per reaction, 200 ng cDNA of each sample was used. As an endogenous control, UBQUITIN CONJUGATING FACTOR2 (UBC2) was amplified simultaneously with the specific primers HvUBC2fwd and HvUBC2rev (see Supplemental Table 1 online) and used for normalization of cDNA quantity variations.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: barley: RACB (AJ344223), RACD (AJ439334), RAC1 (AJ518933), RAC3 (AJ518932), ROP6 (AJ439333), MAGAP1 (AK371854), UBQUITIN (M60175), and UBC2 (AY220735); Arabidopsis ROPGAP1 (At5g22400, NM_122145), Arabidopsis ROPGAP4 (At3g11490, NM_111982), Arabidopsis 18S (X16077), Nicotiana tabacum ROPGAP1(ABG85154), Sorghum bicolor ROPGAP (XP_00248226), Zea mays ROPGAP (AC29275.1), and Oryza sativa ROPGAP (NP_001065778.1).

Author Contributions

C.H. performed research, analyzed data, and wrote the article. C.H., H.S., and G.H. performed research. F.B. contributed new analytic tools, J.K. designed the research, and R.H. designed the research, analyzed data, and wrote the article.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Root Hair Phenotype and Expression Analysis of RACB and RACD in Roots of RACB Knockdown Plants.

Supplemental Figure 2. Amino Acid Alignment of Barley MAGAP1 and Related Plant ROPGAPs.

Supplemental Figure 3. Determination of TIGS Efficiency of the MAGAP1 RNAi Construct.

Supplemental Figure 4. Interaction of ROPGAP1 and ROPGAP4 with Arabidopsis ROPs in Yeast.

Supplemental Figure 5. Localization of ROPGAP1 and ROPGAP4 in Epidermal Cells of Arabidopsis.

Supplemental Figure 6. Development of E. cruciferarum Is Enhanced on Arabidopsis ropgap1 and ropgap4 Knockout Plants.

Supplemental Figure 7. Localization of RFP-MAGAP1 Variants in Barley Epidermal Cells.

Supplemental Figure 8. Schematic Drawing of Subcellular ROP, MAGAP1, and MT Organization in Interaction with B. graminis f. sp. hordei.

Supplemental Table 1. Oligonucleotides Used for Verification of Transgenic Plants, Cloning, and qRT-PCR.

Supplemental Table 2. Amino Acids of Barley and Arabidopsis ROPs Fused to the GAL4 Binding Domain in the Yeast Two-Hybrid Vector pGBKKT7.

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A Barley ROP GTPase ACTIVATING PROTEIN Associates with Microtubules and Regulates Entry of the Barley Powdery Mildew Fungus into Leaf Epidermal Cells

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