

ROPGAPs of Arabidopsis limit susceptibility to powdery mildew

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Abbreviations: ROP, RHO of plants; GAP, GTPase activating protein; ADH, alcohol dehydrogenase; MAGAP1, microtubule associated GTPase activating protein 1

The barley ROP GTPase HvRACB is a susceptibility factor of barley to powdery mildew caused by the biotrophic fungus *Blumeria graminis* f.sp *hordei* (*Bgh*). In a recent publication, we reported about a MICROTUBULE-ASSOCIATED ROP GTPASE-ACTIVATING PROTEIN (HvMAGAP1) of barley. Transient-induced gene silencing or overexpression of HvMAGAP1 resulted in enhanced or reduced susceptibility to *Bgh*, respectively, indicating a possible HvRACB-antagonistic function of HvMAGAP1 in interaction with *Bgh*. HvMAGAP1 also influences the polarity of cortical microtubules in interaction with *Bgh*. In AtROPGAP1 and AtROPGAP4, Arabidopsis homologs of HvMAGAP1, knockout T-DNA insertions enhanced susceptibility of Arabidopsis to the virulent powdery mildew fungus *Erysiphe cruciferarum*, indicating functions of ROPGAPs in pathogen interaction of monocots and dicots. Here we discuss the role of AtROPGAP1 and AtROPGAP4 in Arabidopsis pathogenesis of powdery mildew in some more detail.

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Plant RHO-like ROP proteins are multifunctional switches involved in a variety of plant signaling processes, such as growth and development, cytoskeleton organization, secretion, hormone response, generation of reactive oxygen species (ROS) and susceptibility and resistance to plant pathogens.¹⁻⁴ As molecular switches, RAC/ROPs exist in a GDP-bound inactive form and a GTP-bound active form for targeting downstream effectors. The intrinsic GTPase activity of RAC/ROP proteins is low and requires specific activation by ROPGAPs (ROP GTPASE-ACTIVATING PROTEINS). ROPGAPs are typical members of the eukaryotic RHOGAP family and contain an arginine finger in their conserved catalytic GAP domain, which is supposed to reach into the GTP-binding pocket for stimulation of the GTPase activity of RAC/ROPs, resulting in hydrolysis of GTP and RAC/ROP shutdown.⁵

In the monocots barley or rice, several RAC/ROP proteins are involved in plant-pathogen interactions. In rice, OsRAC1 is a positive regulator of defense against the rice blast fungus *Magnaporthe grisea* by stimulating ROS production and cell death through interaction with an NADPH oxidase.^{6,7} In barley, HvRACB was identified as susceptibility factor to penetration by *Blumeria graminis* f.sp *hordei* (*Bgh*), influencing actin reorganization during pathogen attack.⁸⁻¹² Stable knock-down of *HvRACB* expression inhibits penetration success of *Bgh* and impairs establishment and expansion of fungal haustoria in barley epidermal cells. Furthermore, HvRACB is required for initiation and elongation of barley root hairs and affects adult plant height similar

to ROPs in Arabidopsis.^{13,14} HvRACB is hence a common factor of surface expansion both in epidermal root cells during formation of hairs and in epidermal leaf cells during formation of the haustorial complex. This includes formation of the extrahaustorial membrane, which is in continuum with the host plasma membrane, and of the extrahaustorial matrix a new apoplastic compartment with cell wall like constituents.

Recently, we identified the barley MICROTUBULE-ASSOCIATED GAP 1 (HvMAGAP1) protein as potential regulator of RAC/ROPs in susceptibility to *Bgh*. HvMAGAP1 belongs to the family of ROPGAPs containing a CRIB domain for G-protein interaction.^{13,15} Transient-induced gene silencing of *HvMAGAP1* resulted in enhanced susceptibility of barley epidermal cells to *Bgh*. A similar effect was observed when a mutated version of HvMAGAP1, MAGAP-R185G, was expressed, which can still interact with HvRACB in planta but apparently exerts a dominant negative effect due to mutation of the catalytic residue. Similarly, Arabidopsis *Atropgap1* and *Atropgap4* knockout mutants display enhanced susceptibility in a compatible interaction with the powdery mildew fungus *Erysiphe cruciferarum*.¹³

Although many RAC/ROP-regulated processes have been identified in plants, only little is known about physiological processes controlled via RAC/ROP-regulating ROPGAP proteins. CRIB containing NtRHOGAP1 is an important factor for restriction of RAC/ROP activity to the apex of the growing pollen tube tip by lateral control of RAC/ROPs in tobacco. Overexpression of an inactive mutant NtRHOGAP1 protein version caused

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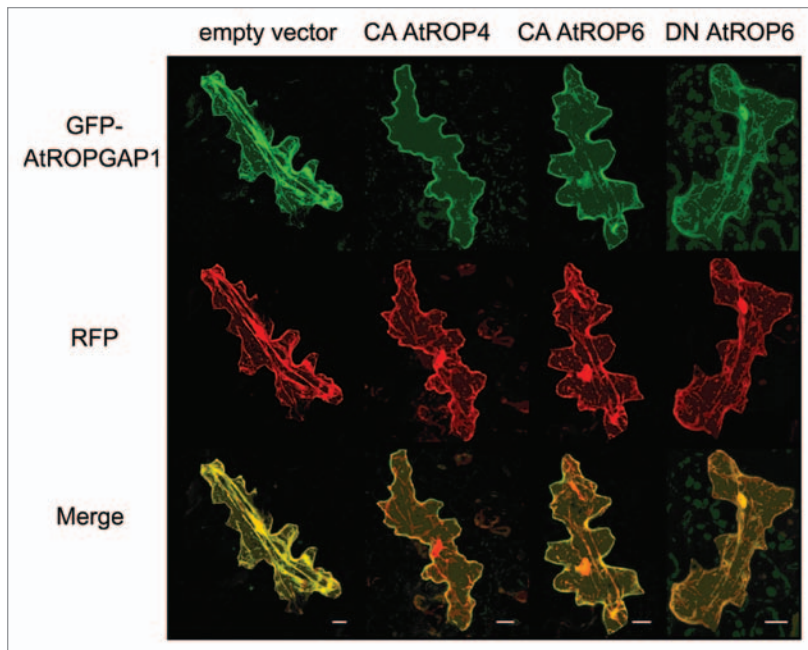


Figure 1. Subcellular localization of GFP-AtROPGAP1 in epidermal cells of Arabidopsis. Arabidopsis epidermal cells transiently transformed with *GFP-ROPGAP1* (first row) alone or together with CA *AtROP6*, CA *AtROP4* or DN *AtROP6* expression constructs. Soluble RFP (second row) was co-transformed to label the cytoplasm and nucleoplasm. GFP-AtROPGAP1 alone or co-expressed with unlabeled DN *AtROP6* displays cytoplasmic localization, whereas co-expression of unlabeled, membrane-associated CA *AtROP4* or CA *AtROP6* results in recruitment of GFP-AtROPGAP1 to the plasma membrane. Pictures are maximum projections of 20–30 optical sections at 2 μm increments. Scale bars represent 20 μm . In merged pictures signal co-localization is represented (third row). Similar results are obtained for GFP-AtROPGAP4 (online supplemental material of Hoefle et al.¹³).

depolarized growth similar to overexpression of NtRAC5.^{16,17} Another type of ROPGAP, a pleckstrin-homology domain-containing ROPGAP called REN1 also functions in regulation of pollen tube polarity in Arabidopsis.¹⁸ Depolarization of normally tip-growing root hairs also takes place when a constitutively activated (CA) version of barley HvRACB is stably expressed in barley.¹² The same CA *HvRACB* plants are super-susceptible to powdery mildew. However, super-susceptible *Atropgap1* or *Atropgap4* mutants do not display aberrant root hairs, suggesting function of other ROPGAPs in root hair growth (data not shown).

AtROPGAP4 is involved in tolerance to oxygen deprivation.¹⁹ Oxygen deprivation is suggested to cause ROS production via AtROP-mediated activation of an NADPH oxidase. Elevated levels of cellular hydrogen peroxide cause induction of *ADH* (*ALCOHOL DEHYDROGENASE*) expression, which is important for alcoholic fermentation and cell survival under oxygen deprivation. In parallel to expression of *ADH*, expression of *AtROPGAP4* increases to attenuate AtROP signaling via a negative feedback loop to balance the survival process.¹⁹

Similar to HvMAGAP1, AtROPGAP1 and AtROPGAP4 belong to the CRIB domain-containing ROPGAPs. In former studies it was shown that both Arabidopsis proteins regulate activity of AtROPs in vitro and in planta.^{15,19} Targeted yeast-two

hybrid (Y2H) assays revealed a broad binding capacity for the AtROPGAPs to leaf-expressed AtROPs.¹³ Heterologous Y2H assays with barley HvRAC/ROPs revealed also interaction of AtROPGAPs with several barley RAC/ROPs in yeast with AtROPGAP4 interacting with HvRACB and CA HvRACB (data not shown). This may support conserved functions of AtROPGAPs and HvMAGAP1 although they are differently localized in epidermal leaf cells.¹³ In contrast to HvMAGAP1, which is associated with microtubules in barley epidermal cells, GFP-fusions of AtROPGAP1 and AtROPGAP4 show mainly cytoplasmic and nuclear localization in Arabidopsis epidermal cells.¹³ This can be explained because the C-terminus of HvMAGAP1, responsible for association of the protein with microtubules, greatly differs in sequence from that of Arabidopsis ROPGAPs. However, similar to barley HvMAGAP1, AtROPGAP1 and AtROPGAP4 translocate to the cell periphery or plasma membrane when corresponding membrane associated CA RAC/ROPs are co-expressed. This is evident for CA *AtROP4* and CA *AtROP6*, but dominant negative *AtROP6* does not recruit GFP-AtROPGAPs supporting interaction of AtROPGAPs with the active form of AtROPs in planta (Fig. 1).¹⁵ Hence AtROPGAPs might be readily recruited to the cell periphery when ROPs are activated thereby warranting a tight spatial control of ROP activity.

In contrast to the enhanced susceptibility of *Atropgap1* and *Atropgap4* mutants to the adapted powdery mildew fungus *E. cruciferarum*, inoculation experiments with *Botrytis cinerea* revealed no alteration of basal resistance to this necrotrophic fungus (data not shown). This might indicate a specific role for AtROPGAPs in limiting susceptibility to powdery mildew rather than in regulation of general or hormone-regulated defense pathways. Accordingly, expression of defense-related genes like *PR1* (indicator for salicylic acid (SA)-dependent defense²⁰) and *PDF1.2* (marker for jasmonate (JA)-dependent defense processes²¹) was unchanged in *Atropgap* mutants inoculated with *E. cruciferarum* when compared with wild type. Additionally, *Atropgap1* and *Atropgap4* did not show an obvious developmental phenotype (data not shown). Microscopic inspections showed that *E. cruciferarum* succeeds better in establishment of haustoria in epidermal pavement cells of *Atropgap4* (SALK_038694) than on the wild type and develops more epiphytic hyphae 48 h after inoculation (Fig. 2). The effect appears comparatively weak, when considering the effect of knocking down *HvMAGAP1* in barley.¹³ However, this may be due to a higher redundancy of ROPGAPs in Arabidopsis than in barley. The same *Atropgap4* mutant, however, is fully resistant to penetration by the non-adapted biotrophic barley powdery mildew fungus *Bgh* just like the wild type (data not shown). This shows that defense to direct penetration at the cell wall is not greatly affected in this mutant. Together this suggests that functional AtROPGAPs limit compatibility with the powdery

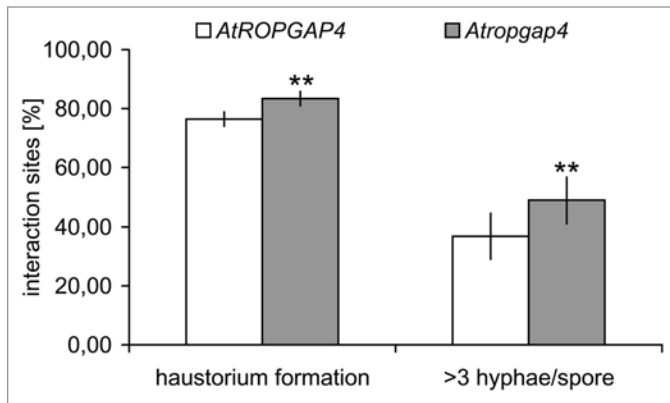


Figure 2. Fungal development on *Atropgap4* and on wild type at 48 h after inoculation. Each 10 Arabidopsis rosette leaves of *Atropgap4* (SALK_038694) and wild type *AtROPGAP4* (Col-0) have been inoculated with conidia of *E. cruciferarum* and fixed for microscopy 48 h later according to Hoeffle et al.¹³ Analysis of 150–200 individual interaction sites on each leaf revealed differences in the fungal success to form a haustorium in the first attacked epidermal pavement cell and to develop more than 3 epiphytic hyphae per spore at this time of the interaction. ** indicates significant differences between wild type and *Atropgap4* at $p < 0.01$ (unpaired Student's t-test). Repetition of the experiment led to very similar results.

mildew fungus rather than being directly required for basal resistance. Together with the data from barley, where HvRACB and HvMAGAP1 influence leaf cell polarity, we speculate that AtROPGAP1 and AtROPGAP4 limit the activity of AtROPs, which are involved in cell polarity of epidermal pavement cells such as AtROP2, AtROP4 and AtROP6.^{22,23} AtROPGAP1 and AtROPGAP4 can bind to all of these AtROPs in yeast, in vitro or in planta (Fig. 1).^{13,15,19} However, it remains to be analyzed whether AtROP-mediated organization of microtubules, which is increasingly well understood in Arabidopsis,^{22–25} is involved in susceptibility to powdery mildew. Alternatively, the recently established FERONIA receptor like kinase-ROPGEF-ROP pathway, involved in polar growth of root hairs in Arabidopsis,²⁶ might also function in leaves, because FERONIA is also required for susceptibility to powdery mildew.²⁷ In such a scenario ROPGAPs would limit FERONIA mediated processes involved in compatibility with the invading fungus.

Based on these results, we propose that ROPGAPs act as antagonistic players of ROP downstream signaling that limit susceptibility to adapted powdery mildew in Arabidopsis similar to the situation in barley (Fig. 3). However, it is not yet understood which ROP downstream signaling events and cellular responses are involved in Arabidopsis susceptibility to powdery mildew. Regulation of NADPH oxidases via the ROP-ROPGAP rheostat as suggested by Baxter-Burrell et al.¹⁹ might be important for limiting susceptibility to powdery mildew because NADPH oxidase RBOHF2 of barley is involved in both susceptibility and

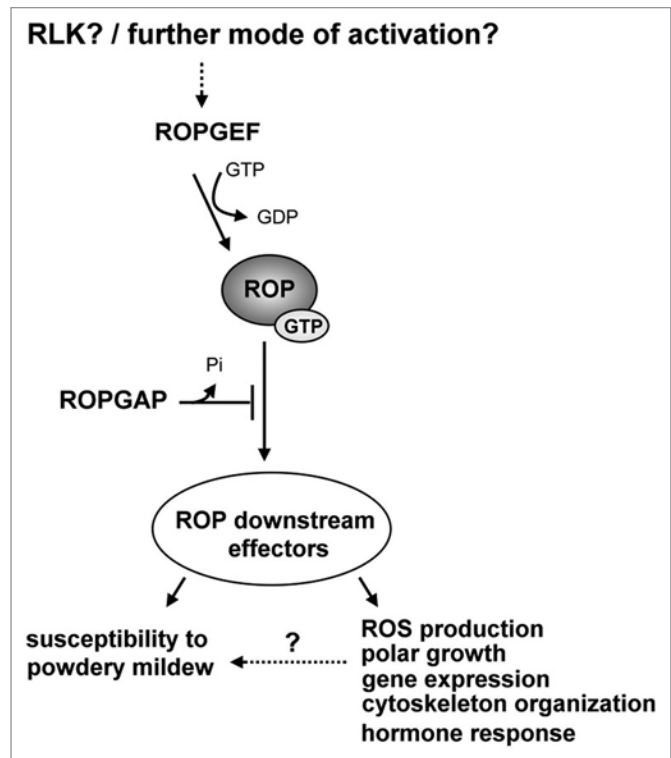


Figure 3. Hypothetical model for the role of Arabidopsis ROPGAPs in ROP mediated signaling. During pathogen attack, ROPs are activated via ROPGEFs, which catalyze the exchange of ROP bound GDP for GTP. RLKs were identified as possible upstream activators of ROPGEFs, but other modes of activation should not be excluded. Active ROPs might support via downstream effectors susceptibility to powdery mildews, while ROPGAP mediated inactivation of ROPs act antagonistic to these processes. There is also a possible influence of known ROP-regulated processes such as ROS production, gene expression, cytoskeleton organization or hormone responses on susceptibility to powdery mildew (shown as dotted line).

resistance to fungal penetration depending on the plant developmental status in which gene function is analyzed.^{28,29} This could even involve regulation of ADH because ADH of barley was recently shown to be involved in susceptibility to powdery mildew.^{30,31}

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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