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Barley RIC171 interacts with RACB *in planta* and supports entry of the powdery mildew fungus

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Summary

RHO-like GTPases of plants (ROPs, also called RACs) are involved in plant development and interaction with the environment. The barley ROP protein RACB is involved in susceptibility to the fungal pathogen *Blumeria graminis* f.sp. *hordei* (*Bgh*). By screening barley sequence databases for potential protein interactors of plant RHO-like proteins, we identified a ROP interactive CRIB (CDC42/RAC interactive binding) motif containing protein of 171 amino acids (RIC171). The protein interacted with constitutively activated RACB in a targeted yeast two hybrid assay. By use of split yellow fluorescing protein fusions we demonstrated that RIC171 interacts with constitutively activated (CA) RACB-G15V but not with dominant negative RACB-T20N *in planta*. Transient over-expression of RIC171, similar to over-expression of CA RACB-G15V, rendered epidermal cells more susceptible to penetration by *Bgh*. In contrast, expression of a 46 amino acid RIC171-CRIB peptide, which was sufficient to interact with CA RACB-G15V, had a dominant negative effect and reduced susceptibility to *Bgh*. A red fluorescing DsRED-RIC171 fusion protein co-localized with green fluorescing GFP-RACB-G15V at the cell periphery. Co-expression with CA RACB-G15V but not with RACB-T20N increased peripheral localization of DsRED-RIC171. Additionally, DsRED-RIC171 accumulated at sites of fungal attack, suggesting enhanced ROP-activity at sites of attempted fungal penetration.

Introduction

Early interaction of plants with the biotic and abiotic environment requires perception and transduction of extracellular signals. The processing of extracellular signals frequently involves GTP-binding proteins. The RHO-related subclass of plant small monomeric GTPases is called RAC or ROP (ROP: Rho of plants) (Winge *et al*., 2000; Yang, 2002). ROPs act as molecular switches in cell polarity, hormone signaling and plant defense. Activation of downstream effectors by ROP proteins requires binding of GTP, and in turn GTP-mediated ROP activity is abolished by intrinsic or stimulated GTPase activity. Hence, ROP proteins can be constitutively activated (CA) or rendered dominantly negative (DN) by mutation of the intrinsic GTPase function. In contrast, wild-type ROP activity is strictly regulated, in plants most likely by upstream receptor-like kinases, guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) (Borg *et al*., 1999; Bischoff *et al.*, 2000; Wu *et al*., 2001; Berken *et al*., 2005; Gu *et al*., 2006; Zhang and McCormick, 2007). Winge *et al.*, (2000) subdivided the 11 *Arabidopsis* ROP proteins into two major subgroups that can be distinguished by length due to an additional exon in group II. Cereals appear to express 6 to 9 *ROP* genes (Christensen *et al., 2*003; Schultheiss *et al., 2*003). Six barley full length ROP cDNAs have been isolated, and no further ROP family members could be identified in more than 400.000 expressed sequence tags (Schultheiss *et al., 2*003).

Plant susceptibility to biotrophic fungi such as *Blumeria graminis* f.sp. *hordei* (*Bgh*) is little understood (O'Connell and Panstruga, 2006). Successful pathogens target host proteins to bypass or to suppress basic defense mechanisms. Seemingly, such target proteins are essential to the host, because their resistant mutants often show pleiotropic growth or cell death phenotypes (Vogel and Somerville, 2000). Barley susceptibility to *Bgh* is dependent on the host receptor-like MLO protein. MLO is

believed to be corrupted by the fungus for host defense suppression (Schulze-Lefert and Panstruga, 2003). Mutants that do not express functional MLO are completely resistant to penetration by *Bgh*. Both, *mlo*-mediated resistance and basal resistance are limited in *ror1* and *ror2* (*required for mlo-specified resistance*) mutants (Freialdenhoven *et al*., 1996, Collins *et al., 2*003). ROR1 has not been identified yet but ROR2 is a plasma membrane syntaxin, which locally accumulates at the site of attempted fungal penetration, where it appears to be involved in exocytosis of defense-related compounds (Collins *et al*., 2003; Assaad *et al*., 2004; Bhat *et al*., 2005).

Transient transformation-mediated over-expression and RNA interference are used to study gene function in the interaction of cereals with powdery mildew fungi (Nielsen *et al*., 1999; Schweizer *et al*., 1999; 2000; Panstruga, 2004). Transient RNA interference-mediated knock-down of the barley *ROP* gene *RACB* renders epidermal cells more resistant to penetration by *Bgh.* In contrast, transient over-expression of the CA *RACB*-mutant *RACB-G15V* but not of wild-type *RACB* supports fungal penetration (Schultheiss *et al*., 2002; 2003). Hence, abundance and activity of RACB appear to modulate single cell susceptibility to *Bgh*. Analyses of genetically transformed susceptible barley plants, expressing CA *RACB-G15V,* support the role of RACB in susceptibility to *Bgh* and suggest an involvement of RACB in plant development and in response to abiotic stress (Schultheiss *et al., 2*005).

The mechanism of RACB activity is not known. However, RACB effects on susceptibility are dependent on functional *MLO* and *ROR1* (Schultheiss *et al*., 2002; 2003). RACB is a subgroup I ROP such as maize ROP2, ROP4 and ROP9, rice RACB and RACD, and *Arabidopsis* ROPs 1 to 6 (Christensen *et al*., 2003; Schultheiss *et al*., 2003). RACB is a plasma membrane-associated protein. This localization is mediated by a CAAX-box prenylation motif at the C-terminus, which is

also required for RACB function in susceptibility to *Bgh* (Schultheiss *et al*., 2003). Mechanistically, the function of RACB appears to be linked to the actin cytoskeleton because single cell RACB over-activation hampers actin-filament reorganization in *Bgh*-attacked cells, whereas RACB knock-down promotes polarization. The influence of RACB on actin is also seen independently from susceptibility because CA RACB-G15V modulates polarity of actin in fully resistant *mlo*-mutants, where CA RACB-G15V does not induce susceptibility (Opalski *et al*., 2005). Perhaps in contrast to barley RACB, rice RAC1 is functioning in the oxidative burst and defensive cell death in response to the hemibiotrophic fungus *Magnaporthe grisea* (Ono *et al*., 2001). Thus, small ROP G-proteins appear to be active in resistance and susceptibility to plant pathogenic fungi.

To uncover the mode of action of ROP proteins, there is an increasing interest in identifying ROP-interacting proteins. In the monocot rice, cinnamoyl-CoA reductase 1, plasma membrane NADPH oxidases, heat shock proteins and RAR1 (REQUIRED FOR MLA12 MEDIATED RESISTANCE) are described as RAC1-interactors (Kawasaki *et al*., 2006, Thao *et al.*, 2007, Wong *et al.,* 2007). The perhaps best studied *Arabidopsis* ROP-interacting proteins are novel plant GEFs (Berken *et al*., 2005; Gu *et al*., 2006) and RICs (ROP-interacting, CDC42/RHO interactive binding (CRIB) domain containing proteins; Wu *et al., 2*001). Because GEFs are ROP activating proteins, they predominantly interact with ROP-GDP, whereas RICs function downstream of GTP-bound ROPs. *Arabidopsis* RICs have been demonstrated to function in tip growth of pollen tubes and development of epidermal pavement cells (Wu *et al., 2*001; Fu *et al., 2*005; Gu *et al., 2*005). Thereby, RICs Ca^{2+}-dependently regulate antagonistic pathways, which modulate actin and tubulin organization. *In planta* ROP-RIC interaction has been demonstrated by fluorescence resonance energy transfer (FRET) of fluorescence-tagged ROPs and RICs (Fu *et al., 2*005; Gu *et al., 2*005).

Here, we used a candidate gene approach and a targeted yeast two hybrid screening to identify the barley CRIB protein RIC171, which similarly to RACB supports susceptibility to *Bgh* and appears to indicate ROP-activity at sites of fungal invasion.

Results

Identification and isolation of a RACB-interacting RIC protein

To decipher RACB signaling in barley, we started an *in silico* screening for barley ESTs coding for putative barley proteins that contain a CRIB domain. One candidate was isolated by RT-PCR based on its sequence (EST accession BF628722.2, cDNA accession AK249700) encoding a RIC-like protein (Fig. 1A). Because RIC proteins are diverse, and no common nomenclature of RICs exists, we designated the barley CRIB protein gene covering an open reading frame of 171 amino acids *HvRIC171*. RACB-binding and binding-specificity of HvRIC171 was assessed in targeted yeast two hybrid assays. We first used RACB and CA RACB-G15V as bait proteins. This revealed that RIC171 bound to wild type RACB and RACB-G15V and activated interaction-specific reporter genes including the galactosidase reporter in yeast. Hence, corresponding yeast clones grew on interaction-selective medium and gave blue signals on X-gal containing medium (Fig. 1B). Autoactivity of the RIC protein was excluded by prey-transformation together with the bait vector lacking any insert (Fig. 1B). It appeared that RIC171 bound effectively to both CA RACB-G15V and the barley CA type II ROP RAC3-G17V in yeast. However, we obtained only weak yeast colony growth when we used CA type II ROP6-G15V (Fig. 1B) and no colonies with CA type I RACD-G15V or CA type II RAC1-G23V as bait proteins respectively (not shown). Together, RIC171 interacts with three of five epidermis-expressed barley CA ROPs in yeast. Sequence analysis of *HvRIC171* (GenBank accession number AM931297) by homology based searches did not detect protein domains or motifs with known function except the CRIB motif itself, which is highly

conserved (Fig. 1A). RIC171 also possesses a cationic stretch at amino acid positions 122-135 that is conserved in *Arabidopsis* RIC6, RIC7 and RIC8 (Wu *et al*., 2001). BLASTP searches (Altschuhl *et al*., 1997) of full length RIC171 in GenBank did not detect highly similar proteins in other organisms. The most similar hit was a predicted RIC-protein from rice (GenBank accession XP_464195). *Arabidopsis thaliana* AtRIC6 (GenBank accession NP_179633) and AtRIC10 (not shown) also show some similarity to HvRIC171 (Fig. 1A). Our BLAST searches identified only one other barley EST encoding a CRIB domain protein. However, thus far we could not isolate a corresponding full length open reading frame.

RIC171 interacts with RACB-G15V in planta

To test barley RIC171 for *in planta* interaction with RACB and RACB-G15V, we established the split yellow fluorescent protein (YFP)-mediated bimolecular fluorescence complementation (BiFC) assay as described by Walter *et al*., (2004). We fused full length RACB, CA RACB-G15V or DN RACB-T20N and RIC171 to truncated versions of YFP (C-terminal and N-terminal fragments; YFP_C and YFP_N) and transiently co-expressed them in barley epidermal cells. This should allow for fluorescence complementation of YFP only when RIC171 and RACB would physically interact in barley. We used standardized microscope settings and scanned 20-40 cells per split YFP pair in each of four independent experiments (total n > 100 per pair) with a confocal laser scanning microscope. We detected strong YFP fluorescence complementation in 80-90 % of transformed cells when we co-expressed either YFP_C-RACB with RIC171-YFP_N or YFP_C-RACB-G15V with RIC171-YFP_N. We detected background BiFC signals when we co-expressed YFP_C-RACB-G15V with non-fused YFP_N. This signal was faint when compared to that of pairs of RACB- with RIC171-fusions (Fig. 2, columns one to three). However, we did not detect YFP fluorescence complementation when we co-expressed YFP_C-RACB-T20N with RIC171-YFP_N or non-fused YFP_C with RIC171-YFP_N (Fig. 2).

Fluorescence complementation appeared mainly at the cell periphery most likely representing the plasma membrane (see below). This supported specific interaction of the proteins, where active RACB could be expected from previous findings (Fig. 2 and Schultheiss *et al*., 2003). In some cells, BiFC signals from the RACB-G15V-RIC171 complex were also detected around the nucleus (see Figure 4C). Because DN RACB-T20N did not interact with RIC171, data suggest that RIC171 does not interact with RACB-GDP but may operate downstream of active RACB-GTP.

RIC171 induces susceptibility to Bgh

To test if RIC171 has a role in interaction with *Bgh*, we silenced and over-expressed RIC171 in single epidermal cells of barley for subsequent challenge by inoculation with *Bgh* and evaluation of fungal penetration success on transformed cells. Double-stranded RNA (dsRNA) of *RACB* induces resistance by transiently-induced gene silencing of *RACB* (Schultheiss *et al*., 2002; 2003). DsRNA-mediated transiently-induced gene silencing of RIC171 did not affect susceptibility to *Bgh* in single epidermal cells (not shown). However, in five independent experiments, over-expression of RIC171 rendered epidermal cells by average 50 % more susceptible to penetration by *Bgh* (Fig. 3). This value was similar to that observed for over-expression of CA RACB-G15V. When we co-expressed CA RACB-G15V and RIC171, penetration success of the fungus did not exceed the value observed after single expression of each gene. Over-expression of RIC171 did not breach resistance of a fully resistant *mlo5*-mutant genotype, and neither did CA RACB-G15V (data not shown and Schutheiss et al. 2003). Together RIC171 and active RACB have similar effects in supporting susceptibility to penetration by *Bgh*.

RIC171 co-localizes with RACB at the cell periphery

Because RIC171 and RACB interacted at the cell periphery (Fig. 2), we fused red fluorescing DsRED to the amino terminus of RIC171. DsRED-RIC171 was mainly detected at the cell periphery clearly surrounding the cytoplasm, which we labeled by

GFP (Fig. 4A). A minor portion of red fluorescence was observed in the cytoplasm and around the nucleus (see Fig. 6). Peripheral localization of RIC171 was reminiscent of the localization of barley ROP proteins (Schultheiss *et al*., 2003). When we expressed green fluorescing GFP-RACB-G15V together with DsRED-RIC171, fluorescence of both proteins overlapped at the cell periphery but not in the nucleoplasm where GFP-RACB-G15V was also detected. To confirm peripheral localization of the RACB-G15V-RIC171-complex, we induced plasmolysis of transformed epidermal cells by 20 % glycerol and observed Hechtian strands connecting the protoplast with the cell wall (Fig. 4C). Additionally, measurement of pixel intensities in YFP-BiFC or DsRED channels, respectively, revealed strong peripheral signals from the RACB-G15V-RIC171-complex contrasted by red signals in the nucleus and cytoplasm (Fig. 4D). Clear subcellular localization of RIC171 was astonishing because the protein lacks any known motifs for membrane targeting. Together this suggested specific interaction of RACB and RIC171 at the plasma membrane and a potential dependency of the localization of RIC171 on the interaction with RACB.

RACB and fungal attack locally recruit RIC171

To test whether activity of RACB might influence localization of RIC171, we co-expressed soluble GFP and DsRED-RIC171 with either CA RACB-G15V or DN RACB-T20N and measured intensity of red fluorescence at the cell periphery adjacent to anticlinal cell walls. We carried out red channel background subtraction and data normalization by fluorescence intensities of GFP in the nucleus, which was co-expressed from the same promoter. Data indicated that RIC171 localization at the cell periphery strongly increased when RACB was constitutively activated and somewhat decreased when RACB was DN (Fig. 5). Hence, activated RACB appeared to recruit RIC171 to the cell periphery. Since RIC171 localization seemed dependent on RACB-activity, we wondered whether RIC171 is differentially localized when *Bgh* attacks epidermal cells. Similarly to what was

observed in non-inoculated leaves, DsRED-RIC171 was localized at the periphery of cells attacked by *Bgh*. However, fluorescence was most intense at cell wall appositions that are built in response to fungal attack (Fig. 6A-F). *In situ* spectral analysis at cell wall appositions confirmed that red fluorescence originated from DsRED-RIC171 (data not shown). DsRED-RIC171 also often attached to the surface of penetrated cell wall appositions (Fig. 6A). Because the activity of RACB was crucial for localization of RIC171, spatial accumulation of RIC171 may indicate activity of RACB or of related ROPs at sites of fungal attempts to penetrate. We also co-expressed DsRED-RIC171 and DN RACB-T20N in attacked cells. Despite a generally lower fluorescence of DsRED-RIC171 at the cell periphery, when DN RACB-T20N was co-expressed, fluorescence at sites of fungal attack was still visible (Fig. 6G-I). Accordingly, DN RACB-T20N had no significant effect on susceptibility (Schultheiss *et al.*, 2003).

The G-protein binding domain of RIC171 interferes with fungal penetration success

The observation that RIC171 is locally enriched where *Bgh* attacks provoked us to test whether a presumably nonfunctional fragment of RIC171 might inhibit fungal establishment. We first tested whether a CRIB peptide of 46 amino acids (CRIB46, amino acid positions 23-68) would be sufficient to interact with CA RACB-G15V. To this end, we cloned a corresponding PCR product into the split YFP_N vector. BiFC experiments demonstrated that CRIB46-YFP_N was sufficient to mediate interaction with CA YFP_C-RACB-G15V. However, CRIB46-YFP_N did not interact with DN YFP_C-RACB-T20N (Fig. 7A). Data indicate that the RIC171-CRIB motif alone suffices to interact with CA RACB-G15V *in planta*. Importantly, CRIB46 reduced penetration success of *Bgh* when over-expressed in single epidermal cells (Fig. 7B). CRIB46 reduced fungal penetration significantly by 23 % ($p<0.05$, Student´s *t* test). Although the effect was weaker than that of knock-down of RACB by dsRNA interference (Schultheiss *et al*., 2002), data support

that CRIB proteins such as RIC171 are involved in RAC/ROP-dependent susceptibility to *Bgh*.

Discussion

Here, we provide evidence for a barley ROP interactive protein of the RIC family that appears to function as a ROP downstream protein. Barley RIC171 interacts with CA RACB-G15V *in planta*, and, similar to CA RACB-G15V, supports susceptibility to penetration by *Bgh*. Because activated RACB-G15V recruits RIC171 to the cell periphery and RIC171 concentrates, where *Bgh* attempts to penetrate, we propose that the spatial accumulation of RIC171 indicates local ROP activity that supports fungal accommodation in epidermal cells of barley. RIC171 might represent a ROP-GTP interacting protein involved in pathogenesis of powdery mildew.

In targeted and non-targeted (not shown) yeast two hybrid assays, we identified several barley proteins that interact with barley RACB or barley RAC3 in yeast. Because RIC proteins are considered as ROP downstream effectors that link ROP activity to specific cellular responses (Wu *et al*., 2001), we first focused on RIC171 function. Apart from the CRIB domain for binding of ROP-GTP, sequences of RIC proteins are little conserved (Wu *et al*., 2001, Fig. 1). RICs represent a highly diverse protein family, which suggests that they may confer specificity to redundant ROP proteins (Wu *et al*., 2001). We found that RIC171 interacts in yeast with three out of five tested barley ROPs expressed in the epidermis. Interestingly, all RIC171-interacting CA ROPs of barley confer enhanced susceptibility when over-expressed in single epidermal cells (compare Fig. 1B and Schultheiss *et al.*, 2003). To confirm that RIC171 can interact *in planta* with barley RACB, we used split YFP-based bimolecular fluorescence complementation of YFP_C-RACB and RIC171-YFP_N. This revealed that RACB interacts with RIC171 at the cell periphery, where both proteins co-localize (Fig. 2 and 4). RACB and RIC171 did not interact in the

nucleus although at least RACB appeared to be present. This seems to indicate site-specific interaction of both proteins that could involve additional proteins *in planta*. Wild type RACB and CA RACB-G15V interacted *in planta* with RIC171, whereas DN RACB did not. This supports specificity of the interaction of RACB and RIC171 *in planta* because a single amino acid exchange in RACB completely abolished fluorescence complementation. CA RACB-G15V interaction with RIC171 is most likely mediated by the CRIB domain because the CRIB46 peptide containing the corresponding conserved amino acids was sufficient to mediate fluorescence complementation of split YFP fusions. These data support previous findings that RIC proteins interact with GTP-bound activated ROPs but not with GDP-bound ROPs and thus link RICs to ROP-downstream effects (Wu *et al*., 2001; Fu *et al*., 2005; Gu *et al*., 2005). This is further supported by the fact that RIC171 alone was sufficient to induce CA RACB-G15V-like effects in barley susceptibility to *Bgh* (Fig. 3). The strength of this effect was similar to that of CA RACB-G15V and could not be further enhanced by simultaneous expression of both proteins. This indicates that both proteins induce similar downstream effects because otherwise one would expect additive effects. Interestingly, CRIB46 had a dominant negative effect on fungal success, suggesting that CRIB-proteins like RIC171 are involved in fungal entry. CRIB46 induced a 23 % reduction in fungal success whereas knock-down of HvRACB can have effects of up to 60 % (Schultheiss *et al.*, 2002, and data not shown). Potentially, CRIB46 also blocked other CRIB motif containing proteins such as ROP-GAPs (Wu *et al*,. 2000) from interaction with ROP-GTP. Since ROP-GAPs are considered as negative regulators of ROP activity, this would explain a limited effect of CRIB46. Alternatively, binding of CRIB46 to ROPs did not fully block interaction with other ROP downstream proteins.

RIC171 might function in susceptibility to *Bgh* when recruited by fungal attack. To test a potential recruitment of RIC171 to the cell periphery, we analyzed localization of RIC171

depending on RACB activity and on fungal attack. The peripheral localization of RIC171 was striking because RIC171 lacks any known protein targeting sequence or predicted transmembrane domain. Hence, localization of RIC171 might depend on membrane-anchors of interacting partners such as RACB, which is predicted to be posttranslationally prenylated at a carboxy-terminal cysteine residue within a CAAX-box motif (Schultheiss *et al*., 2003). Specific localization of RIC171 and *in planta* interaction with CA RACB-G15V provoked the idea that the protein might be recruited into active ROP signaling complexes. RACB is supposed to operate at the cell periphery because a truncated mutant of CA RACB-G15V, lacking the CAAX-box, was dislocalized from the plasma membrane and compromised in supporting susceptibility to *Bgh* (Schultheiss *et al*., 2003). To test potential translocation into membrane-associated RACB complexes, we measured RIC171 concentrations at the cell periphery depending on presumptive nucleotide-loading of RACB. CA RACB-G15V activity obviously led to accumulation of RIC171 at the cell periphery (Fig. 5) and a bimolecular CA RACB-G15V-RIC171 complex was detected in Hechtian strands of plasmolyzed cells (Fig. 4C). Data suggest that local concentrations of RIC171 are dependent on the activity of RACB and thus can be taken as a measure for local ROP activity. This appears important because RIC171 concentrated at sites of fungal attack, suggesting ROP activity at sites of attempted fungal invasion. Our data support the view that attack from *Bgh* locally activates barley ROP proteins, which promotes susceptibility of epidermal cells to fungal invasion. Interestingly, ROPs were recently reported to localize at sites of interaction of cultured plant cells with *Phytophthora infestans* (Schütz *et al.*, 2006).

DN RACB-T20N had a minor effect on localization of DsRED-RIC171 (Figs. 5 and 6G) and did not affect susceptibility to *Bgh* (Schultheiss *et al.*, 2003). This may indicate insufficiency of DN RACB-T20N to outcompete endogenous ROPs of barley or

redundancy of RIC171 in fungal entry. Alternatively, residual RIC171 concentrations at sites of fungal attack were sufficient to retain susceptibility.

Spatially controlled ROP activity is involved in initiation and maintenance of tip growth in root hairs and pollen tubes and in pavement cell interdigitation (Molendijk *et al*., 2001; Wu *et al*., 2001; Carol *et al*., 2005; Fu *et al*., 2005; Gu *et al*., 2005; Jones *et al*., 2007). Fungal invasion and haustoria formation in intact epidermal cells requires a rapid growth of the membrane surface and creation of the extrahaustorial matrix, a new apoplastic compartment. This process has been compared to inverted tip growth (Schultheiss *et al*., 2003). In *Arabidopsis*, plasma membrane invaginations build locally, where *Golovinomyces cichoracearum* attempts to penetrate (Koh *et al*., 2005). *Arabidopsis* RICs are involved in pollen tube growth and polar growth during pavement cell development (Wu *et al*., 2001, Fu *et al*., 2005, Gu *et al*., 2005). Interestingly, over-expression of ROP1 in pollen tubes recruits GFP-AtRIC6 to the cell periphery of pollen tubes, whereas localization of GFP-AtRIC6 alone is more diffuse with some accumulation at the peripheral apex (Wu *et al*., 2001). Hence, AtRIC6, an *Arabidopsis* RIC related to barley RIC171, functions in polar growth processes and locates similarly when an interacting ROP-protein is co-expressed. It appears to be an attractive model that virulent *Bgh* directs the plant to follow a tip-growth mimicking program by highjacking host signaling via ROP and RIC proteins for accommodation of the fungal haustorium. Similarly, effectors of bacterial agents of human diseases activate RHO-like host proteins to promote membrane ruffling and bacterial uptake into non-phagocytic cells (Gruenheid and Finlay, 2003).

Experimental procedures

Plant materials, pathogen and inoculation

The barley (*Hordeum vulgare* L.) line Ingrid was obtained from Lisa Munk (Royal Veterinary and Agricultural University, Copenhagen, Denmark). Plants were grown in a growth chamber at 18°C with 60 % relative humidity and a photoperiod of 16 h (240 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 150 conidia mm^{-2}. *Bgh* was maintained on barley cv. Golden Promise under the same conditions.

Isolation of the barley RIC171 cDNA, cloning and sequencing.

We isolated cDNA fragments by using the one-step RT-PCR kit (Qiagen, Hilden, Germany) and a complex RNA pool from green leaves described previously (Schultheiss *et al*., 2002). Primers were designed using EST data base information for specific barley EST sequences. Primers 5'-ACTAGTCAATACATTTGCAGCCTTGTC-3' (5' primer, containing a SpeI restriction site) and 5'-CCCGGGCGATCGATGGCGTCCAACTAC-3' (3' primer, containing a SmaI restriction site) were used. cDNAs were isolated from gels and cloned into the pGEM-T cloning vector (Promega, Mannheim, Germany), resulting in pGEM-T-RIC171. cDNAs were sequenced from plasmids by use of the Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing Kit (Amersham, Freiburg, Germany) and analysed for similarities in the GenBank database using the BLAST algorithm (Altschul *et al*., 1997).

For transient transformation assays (see below) the complete open reading frame of the *RIC171* cDNA was subcloned into the plant expression vector pGY1 (Schweizer *et al*., 1999), which contains a 540 bp fragment of the cauliflower mosaic virus 35S (CaMV 35S) promoter. RIC171 was cut from pGEM-T-RIC171 using the SpeI restriction sites and ligated into the XbaI site of pGY1, resulting in pGY1-RIC171. The CRIB46 fragment of RIC171 was amplified from pGY1-RIC171 by PCR using primer 5'-

GTCGACATGGCGAAGGAGCAGGAGAT-3' (5' primer introducing an ATG codon for initiation of protein translation) and primer 5'-CTACGACGACCCCTCGATGA-3' (3' primer introducing a stop codon). The PCR fragment was ligated into the pGEM-T cloning vector (Promega, Mannheim, Germany), sequenced and then subcloned into the SalI restriction site of pGY1, resulting in pGY1-CRIB46.

Targeted yeast two hybrid assay

We performed a targeted yeast two hybrid assay to examine the interaction of different barley RAC/ROP proteins with RIC171. For the construction of pGADT7-RIC171, the RIC171 coding fragment was cut out of pGEM-T-RIC171 using the SpeI and SmaI restriction sites of the RIC171 primers (see above) and subcloned in frame with the activation domain into the XbaI and SmaI restriction sites of the pGADT7-Rec AD vector (Matchmaker™ Two-Hybrid Library Construction & Screening Kit, Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France).

Barley RAC/ROPs were amplified from plasmids (Schultheiss *et al*,. 2003) by PCR, ligated into the pGEM-T cloning vector (Promega, Mannheim, Germany), and, after sequence confirmation, subcloned in frame with the DNA-binding domain into the pGBKT7 DNA-BD vector (Matchmaker™ Two-Hybrid Library Construction & Screening Kit, Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France). To facilitate transport to the nucleus, it was necessary to remove the protein's prenylation and palmitoylation sites respectively, which mediate membrane association. In case of RACD this step was dispensable since the protein was found to enter the nucleus of barley epidermal cells (H. Schultheiss, unpublished results). The cDNAs were amplified from plasmids that either contained the wild type RAC/ROP or a constitutively activated mutant RAC/ROP (Schultheiss *et al*., 2003). Primer sequences for cDNA amplification and restriction sites for subcloning from pGEM-T into pGBKT7 DNA-BD can be taken from table 1.

Table 1. Primer sequences for cDNA amplification and restriction sites for subcloning from pGEM-T into pGBKT7 DNA-BD

Construct	5' and 3' primer sequences	Restriction sites used for subcloning
pGBKT7-RACBΔCSIL	5'-GGATCCTGATGAGCGCGTCCAGGTT-3'	BamHI
	5'-GTCGACTCAAGCCCCCCTCTGCGCCTTTTT-3'	SalI
pGBKT7-RACB-G15VΔCSIL	5'-GGATCCTGATGAGCGCGTCCAGGTT-3'	BamHI
	5'-GTCGACTCAAGCCCCCCTCTGCGCCTTTTT-3'	SalI
pGBKT7- RAC3-G17VΔloc	5'-GGATCCCCGCGCGGCGAGCCATG-3'	BamHI
	5'-CGTCGGGGATGATCAATGATGAAC-3'	SalI
pGBK T7-RAC1-G23VΔloc	5'-GGATCCGCTGGAGAGGAGAGGAGAGG-3'	BamHI
	5'-GTCGACCTAACTAGAGCGTGTCCGAG-3'	SalI
pGBKT7-ROP6-G15VΔloc	5'-AATTGGGAATTCCGTCGCATGCTCCG-3'	EcoRI
	5'-GGAGCACCCGGGGCTTCACCTGGT-3'	SmaI
pGBKT7-RACD-G15V	5'-GGATCCTGATGAGCGCATCTCGG-3'	BamHI
	5'-GTCGACGCGAGACACTGCAAAACAAA-3'	SalI

For the targeted yeast two hybrid assays, the bait (pGBKT7 DNA-BD) and prey (pGADT7-Rec AD) vectors were co-transformed into the yeast strain AH109 by using the lithium acetate method (Matchmaker™ Two-Hybrid Library Construction & Screening Kit, Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France). Transformed yeast cells were transferred onto 2 % agar plates with selective medium (synthetic dropout (SD) minimal medium containing 0.17 % nitrogen base without amino acids, amino acids and nucleotide bases) lacking leucine and tryptophan and containing 2 % glucose as carbon source. After 4 days of growth at 30°C, several colonies were picked from each plate and resuspended. For yeast drop assay, yeast suspensions were adjusted to a concentration of 200 cells μL^{-1} and then serial 10-fold diluted. 5 µl of each dilution were dropped onto 4 fold selective SD minimal agar plates, lacking leucine, tryptophan, histidine and adenine, and supplemented with 20 µg/ml X-α-Gal (5-Bromo-4-chloro-3-indolyl- α-D-galcactopyranoside). Yeast cells were subsequently incubated for 7 days at 30°C.

Transient transformation and evaluation of fungal development

A transient transformation protocol, originally developed for wheat to assess gene function in the interaction with powdery mildew, was used to deliver over-expression constructs of the ROP proteins into epidermal cells of barley leaf segments as described by Schweizer *et al*., (1999; 2000) and Schulheiss *et al*., (2002).

Barley leaf segments were bombarded with plasmid- and/or dsRNA-coated particles 24 hours before inoculation with *Bgh*, race A6. DsRNA was produced by *in vitro* transcription in sense and antisense orientation and subsequent hybridization as described previously (Schultheiss *et al*., 2002). Interaction outcome was judged 40 hours after inoculation by fluorescence and bright field microscopy. For each individual experiment, at least 100 interaction sites were evaluated. Transformed GFP expressing cells were identified under blue light excitation. Penetration efficiency of *Bgh* was judged by the frequency by which *Bgh* was able to establish haustoria in transformed cells. Surface structures of *Bgh* were detected by light microscopy or by fluorescence staining of the fungus with 0.3 % calcofluor (w/v in 50 mM TRIS, pH 9) for 30 seconds. Deviation of penetration efficiency [%] was used as a measure for susceptibility of cells that expressed GFP and a test-gene compared to those transformed with GFP and empty vector (pGY1). Penetration efficiency [%] was calculated as number of penetrated cells divided by total number of attacked cells multiplied by 100. The relative penetration efficiency was calculated as penetration efficiency compared to controls set as zero or 100 percent respectively.

Localization of fluorescent-tagged proteins and quantification of local fluorescence intensities by CLSM

For protein localization experiments, RIC171 was fused N-terminally with red fluorescing DsRED. pGY1-DsRED-RIC171 was constructed as follows: The DsRED

coding fragment was amplified from the p35S-DsRed plasmid (Dietrich and Maiss, 2002) using primer 5'-CCCGGGATGGTGCGCTCCTCCAAG-3' (5' primer, containing a SmaI restriction site) and primer 5'-CCCGGGACAACCGGTACCTCTAGAC-3' (3' primer, containing a SmaI restriction site), which eliminates the stop codon. DsRED was then inserted in frame into pGY1-RIC171 using the internal SmaI sites of pGY1-RIC171, i.e. at the N-terminal end of RIC171. Plasmids were checked by sequencing. Construction of GFP-fusions of RACB was described previously (Schultheiss *et al*., 2003). Transient transformation was performed as described above using 0.8 µg of each plasmid per shot.

The localization of the fusion proteins was detected by confocal laser scanning microscopy (CLSM; Leica TCS SP2, Leica Microsystems, Mannheim, Germany) 48 h after transformation. GFP-HvRACB was excited by a 488 nm laser line and detected at 505 - 530 nm. DsRED-RIC171 was sequentially excited by a 543 nm laser line and detected at 580 - 650 nm.

To measure RACB-dependency of local DsRED-RIC171 fluorescence intensities, we integrated fluorescence intensities in regions of interest at the cell periphery. Background autofluorescence was subtracted to ensure measurement of DsRED-RIC171. We normalized fluorescence of DsRED-RIC171 by GFP-fluorescence in the nucleus (GFP was expressed from the same CaMV35S promoter in pGY1-GFP). For each combination of constructs, we measured fluorescence intensities in regions of interests of 10 optical sections of at least 10 cells each and averaged data. Measurements were repeated in three independent experiments leading to similar results.

Bimolecular fluorescence complementation assay

We used split yellow fluorescent protein (YFP)-mediated bimolecular fluorescence complementation (BiFC) to verify *in planta* protein-protein interactions of RACB with

RIC171 or the CRIB46 peptide alone (Walter *et al*., 2004). For this, we fused RACB variants (RACB WT, CA RACB-G15V, DN RACB-T20N) with the C-terminal part of yellow fluorescing YFP and RIC171 or CRIB46 with the N-terminal part of YFP.

RACB, RACB-G15V and RACB-T20N coding fragments together with the CaMV35S terminator were cut out of the respective pGY1 expression constructs (Schultheiss *et al*., 2003) using BamHI and EcoRI respectively, and subcloned blunt/sticky into the pUC-SPYCE vector (Walter *et al*., 2004), which was cut open with BsrGI and EcoRI, in frame with the C-terminal fragment of YFP. The resulting fusion constructs were pUC-SPYCE-RACB, pUC-SPYCE-RACB-G15V and pUC-SPYCE-RACB-T20N.

The RIC171 fragment was amplified from pGY1-RIC171 using primer 5'-GTCGACATGGCGTCCAACTACAAG (5' primer, containing a SalI restriction site) and primer 5'-GGTACCAGATATCACACTATTGGTGTC-3' (3' primer, containing an Acc65I restriction site) and cloned into pGEM-T (Promega, Mannheim, Germany). After sequence confirmation, the RIC171-coding fragment was subcloned into the pUC-SPYNE vector (Walter *et al*., 2004) in front of and in frame with the YFP N-terminus using the SalI and Acc65I restriciton sites, resulting in pUC-SPYNE-RIC171. Similarly, the RIC171 CRIB46 fragment was amplified from pGY1-RIC171 by PCR using primer 5'-GTCGACATGGCGAAGGAGCAGGAGAT-3' (5' primer, containing a SalI restriction site) and primer 5'-GGTACCGGATGAGAAATCCGACGA-3' (3' primer, containing a Acc65I restriction site) and cloned into the pUC-SPYNE vector, resulting in pUC-SPYNE-CRIB46.

We used particle bombardment (see above) to co-transform fusion constructs in various combinations into barley epidermal cells together with DsRED as soluble protein marker. Each shot delivered 0.8 µg DsRED plasmid and 1 µg of each BiFC construct (pUC-SPYCE and pUC-SPYNE). Empty BiFC vectors were used as negative controls. 48 h after transformation, short transformed epidermal cells were checked for DsRED and

YFP fluorescence by CLSM (Leica TCS SP5, Leica Microsystems, Mannheim, Germany). DsRED was excited by a 561 nm laser line and detected at 585 - 635nm while YFP was sequentially excited at 514 nm and detected at 525-555 nm. For excitation and detection of BiFC, we used identical microscope settings for all samples. Argon laser was set to 20 % laser intensity, 514 nm laser line was set to 15 % of the maximum, and detector gain was 900V.

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Legends to the figures

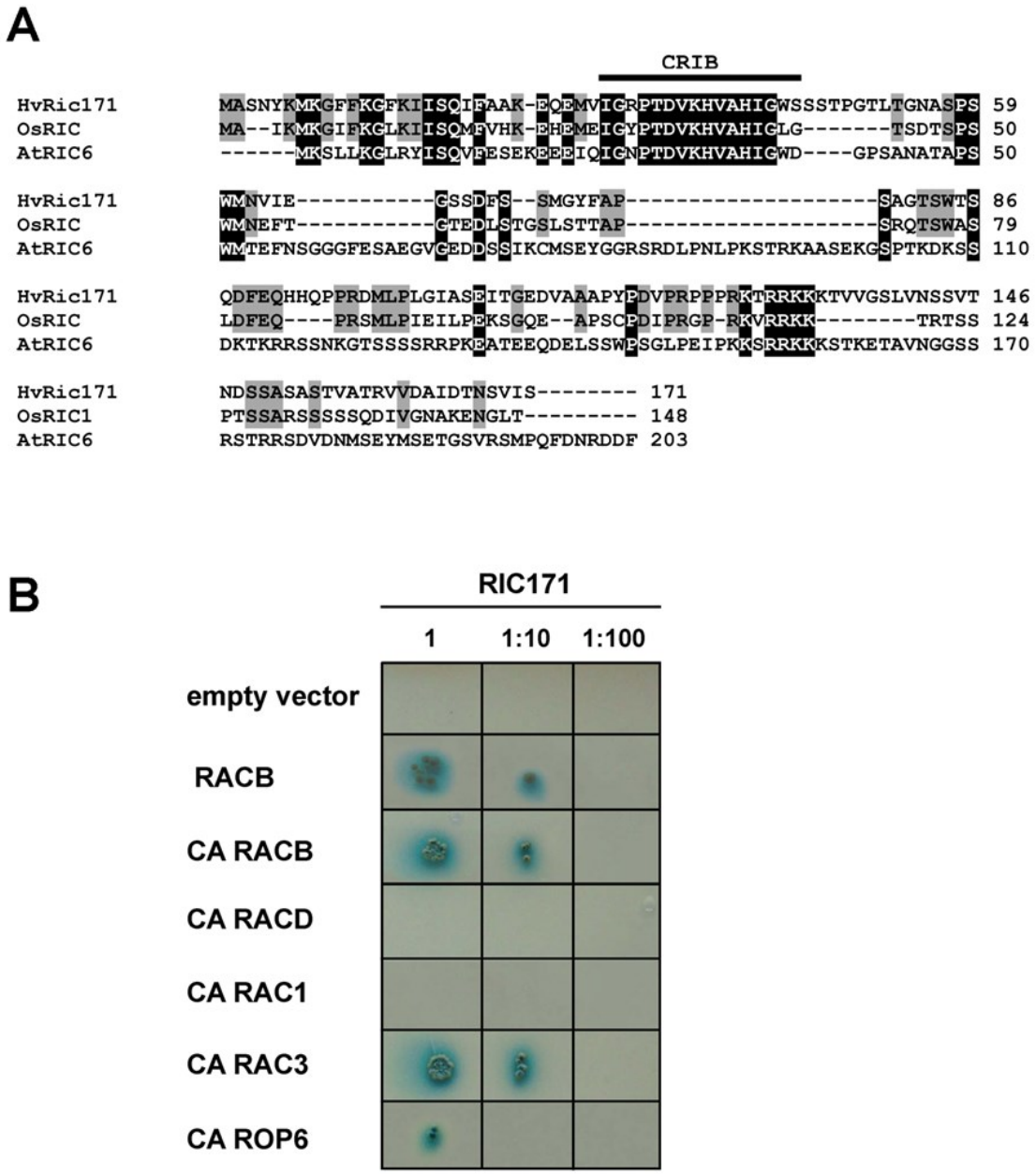


Fig. 1. RIC171, a CRIB protein, interacts with barley ROPs in yeast. **A,** Alignment of the deduced amino acid sequences of HvRIC171 (accession AM931297) and homologs from rice (OsRIC, accession XP_464195) and *Arabidopsis* (AtRIC6, accession NP_179633). The predicted CRIB domain (pfam00786) is highlighted by a bar. Black shaded amino acids are conserved in all homologs. Grey shaded amino

acids are conserved in barley and rice. **B.** Targeted yeast two hybrid assay with different barley ROPs as baits and RIC171 as prey. Picture shows the result of a drop assay on four-fold selective media lacking leucine, tryptophan, histidine and adenine and containing X-α-Gal. We dropped equal amount of yeast cells that had been pre-cultured in two-fold selective media lacking leucine and tryptophan.

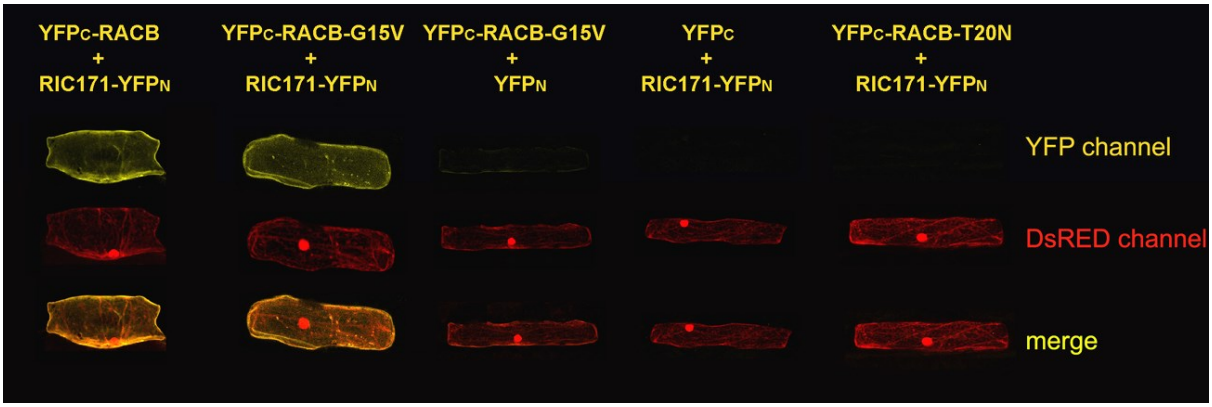


Fig. 2. Bimolecular fluorescence complementation by split YFP proteins fused to RACB and RIC171 from barley. *In planta* interaction of wild type RACB and activated RACB-G15V with RIC171 allows for strong YFP fluorescence complementation at the cell periphery (column one and two). Fluorescence is faint when one of the split YFP proteins is expressed alone instead of being fused to RIC171 or RACB (columns three and four). Fusion of YFP_N to the dominant negative mutant RACB-T20N does not interact with RIC171, and no fluorescence is observed (column five). Red fluorescing DsRED protein is localized in the cytoplasm and nucleoplasm and contrasts YFP fluorescence at the cell periphery. All pictures represent maximum projections of 20-30 optical sections through the entire cell at 2 µm increments. All signals have been recorded with the same laser beam intensity and detector voltage.

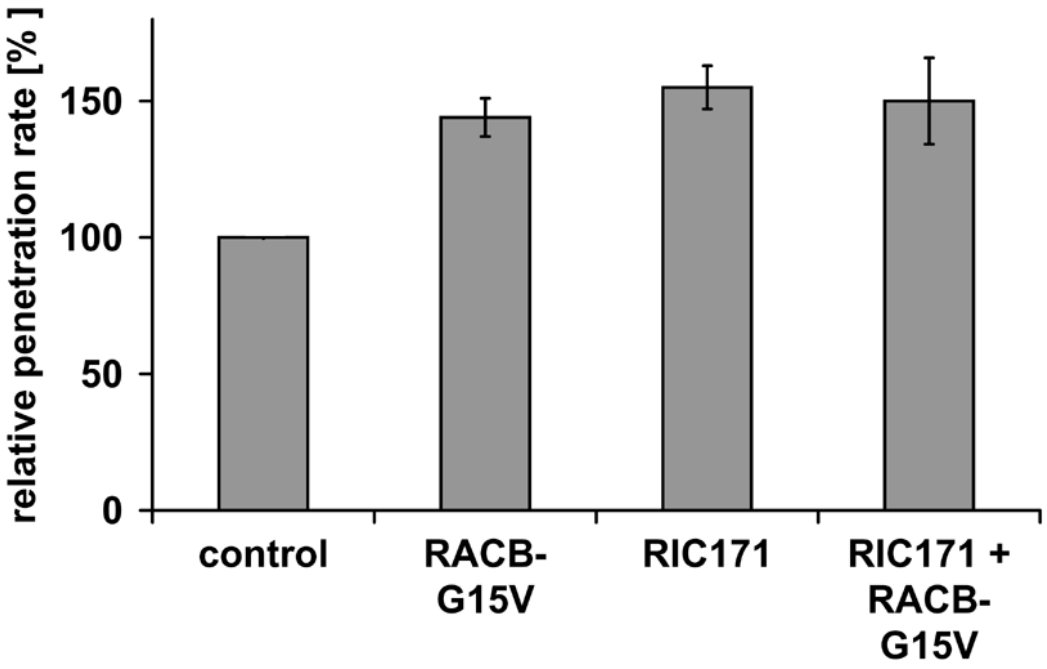
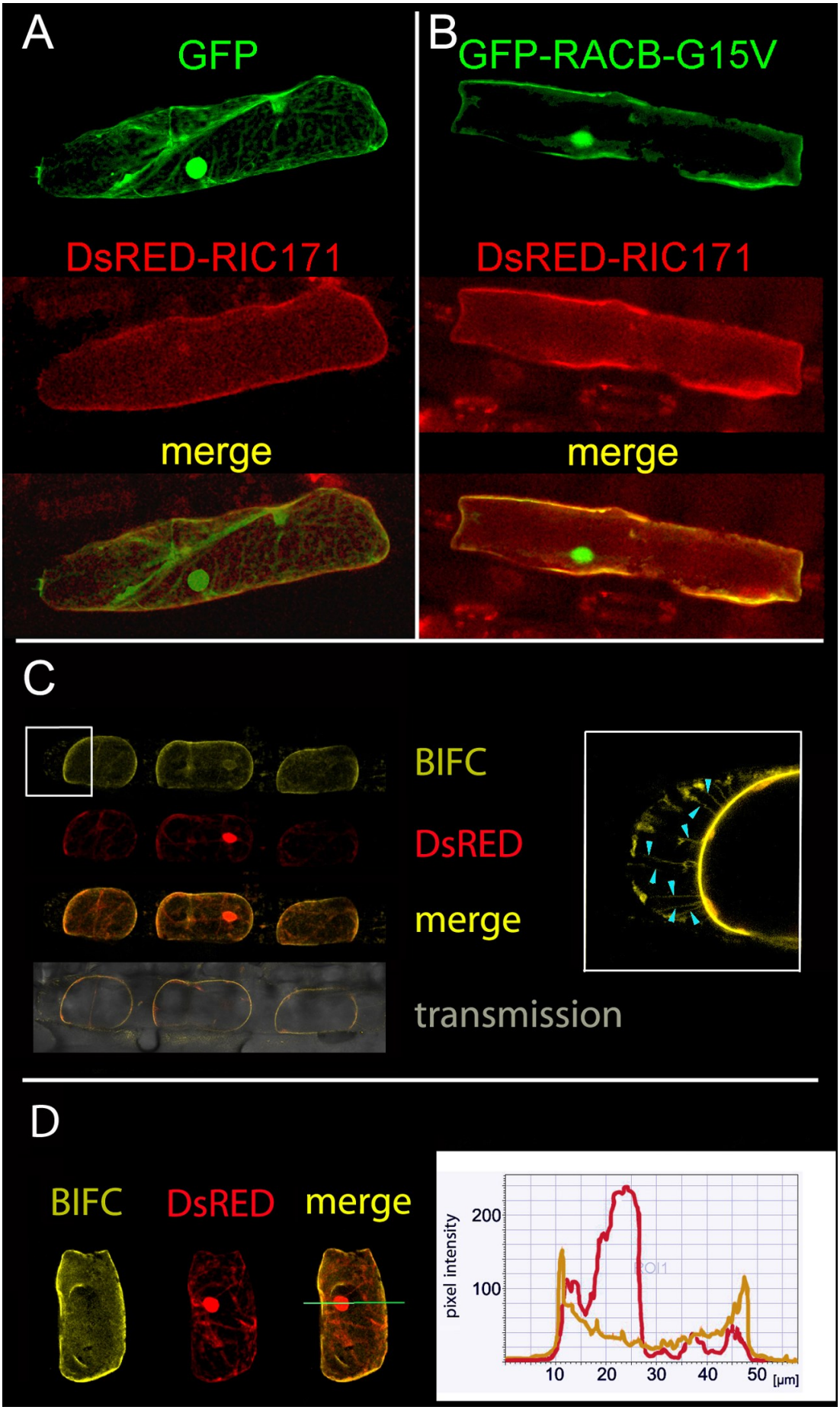


Fig. 3. Over-expression of RIC171 supports fungal penetration into barley epidermal cells. Similar to active RACB, the potential RACB effector RIC171 enhances susceptibility to penetration by *Blumeria graminis* f.sp. *hordei*. Y-axis represents the relative penetration rate, when compared to the average control, which was set as 100 %. Error bars represent standard error of five independent biological repetitions.


A
GFP
DsRED-RIC171
merge
B
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C
BIFC
DsRED
merge
transmission
D
BIFC
DsRED
merge
pixel intensity
200
100
10
20
30
40
50
[µm]

Fig. 4. Subcellular localization of DsRED-RIC171. **A.** Maximum projection of 20 optical sections through an epidermal cell of barley co-expressing soluble GFP and DsRED-RIC171. **B.** Maximum projection of 20 confocal optical sections through an epidermal cell of barley co-expressing GFP-RACB-G15V and DsRED-RIC171. Note that DsRED-RIC171 is co-localized with GFP-RACB-G15V at the cell periphery but not in the nucleus. **C.** Protoplast fragmentation upon plasmolysis of an epidermal cell expressing DsRED and the BiFC pair YFPc-RACB-G15V and RIC171-YFPn. Note Hechtian strands (arrowheads) in the inset magnified on the right panel. Pictures represent maximum projections of 30 optical sections through the entire cell at 2 µm increments. **D.** Quantification of BiFC signal intensity of YFP_C-RACB-G15V and RIC171-YFP_N at the cell periphery. Pixel intensity was measured along the green line in a maximum projection of 25 optical sections at 2 µm increments. Right panel: Yellow BiFC signal is high in the cell periphery but weak in the nucleus and the cytoplasm. In contrast, red DsRED signal is weaker at the periphery and higher in the nucleus and in the cytoplasm.

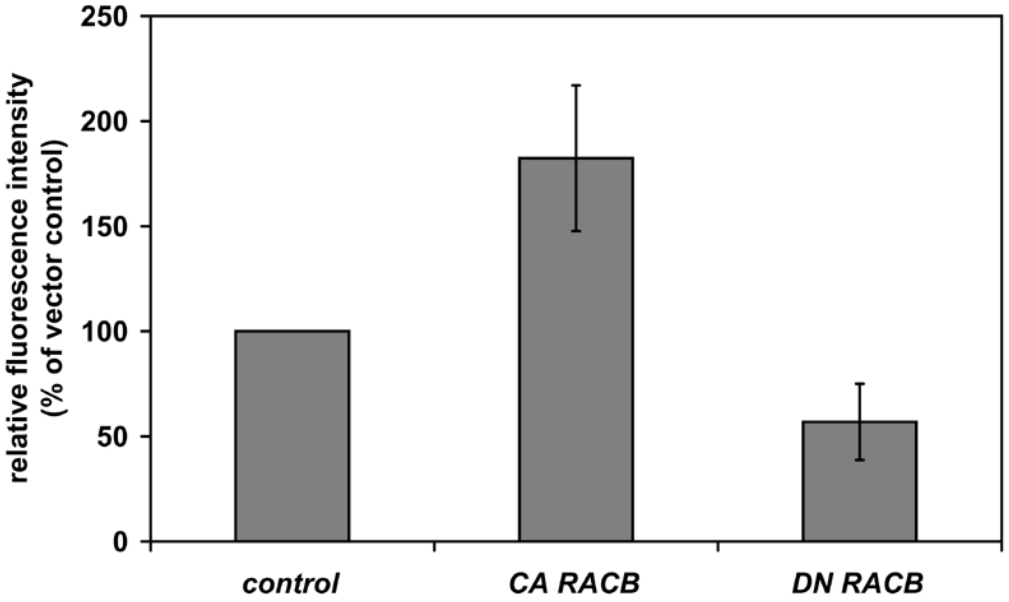

Fig. 5. Influence of RACB on subcellular localization of DsRED-RIC171. Relative fluorescence intensity of DsRED-RIC171 at the cell periphery was measured when an empty vector (control), CA RACB-G15V or DN RACB-T20N was co-expressed and normalized by fluorescence intensity of soluble GFP in the nucleoplasm. Error bars represent standard error of three independent biological repetitions.

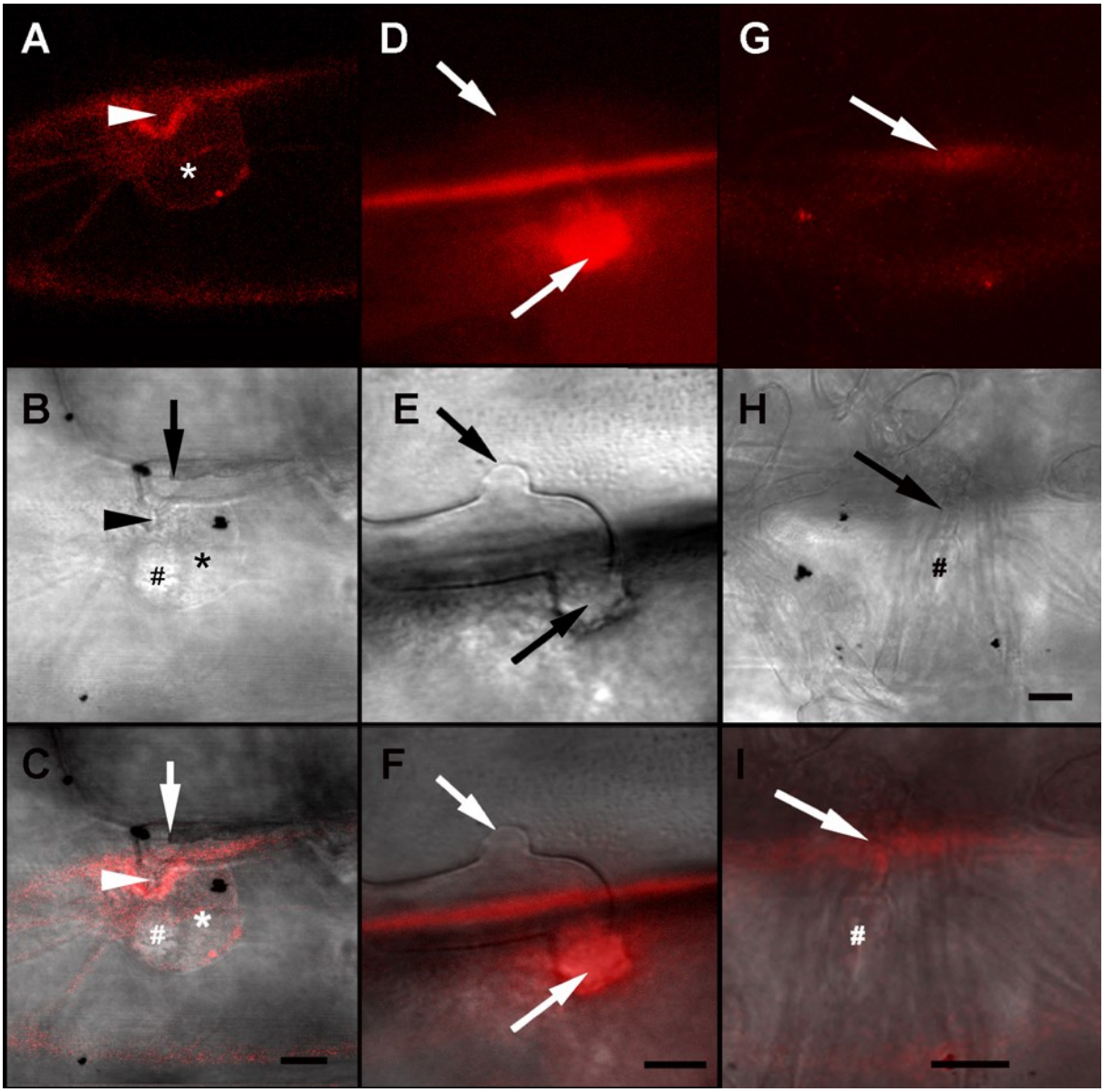

Fig. 6. Fungal attack recruits RIC171 to sites of interaction. **A-C.** Site of successful fungal penetration with local accumulation of DsRED-RIC171 at 18 h after inoculation. The fungus penetrated the cell wall apposition from its appressorium (arrow in B) by a penetration peg (arrowhead in B) and formed a haustorium (out of focus #). The asterisk marks the nucleus, which migrated to the site of interaction. **D-F.** Site of non-successful fungal penetration attempt at 24 h after inoculation. Note that DsRED-RIC171-specific fluorescence is visible in the periphery of the transformed cell (lower arrow) but not in the neighboring non-transformed cell, which is also attacked from the fungal appressorium (upper arrow). Size bars = 10 µm. **G-I.**

Site of successful fungal penetration with moderate local accumulation of DsRED-RIC171 during co-expression of DN RACB-T20N. The fungus penetrated (arrow) the cell wall apposition from its appressorium and formed a haustorium (#). Size bars = 10 µm

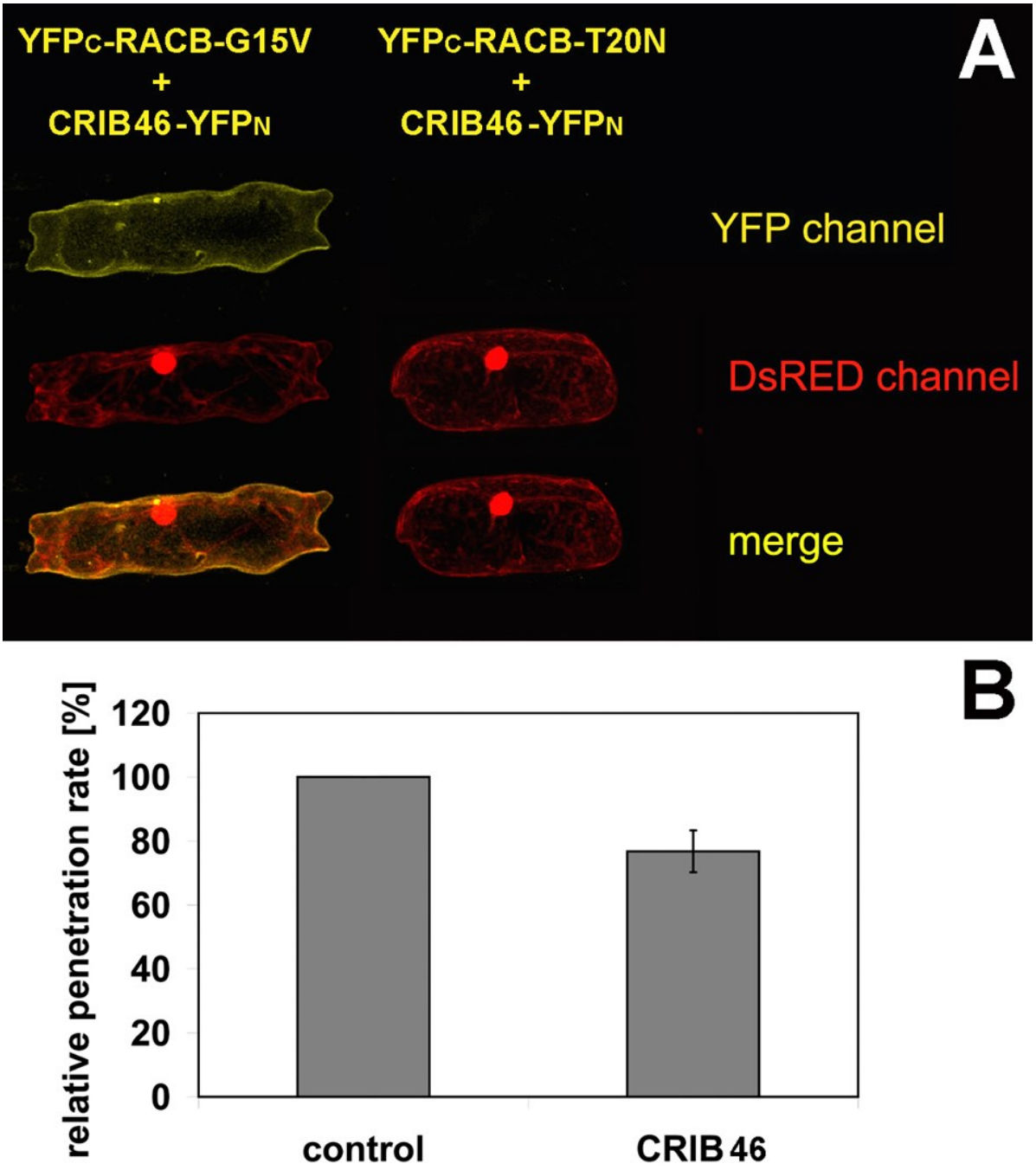

Fig. 7. A. Bimolecular fluorescence complementation by split YFP proteins fused to RACB and to the putative ROP-binding peptide CRIB46 from barley RIC171. *In planta* interaction of activated RACB-G15V with CRIB46 allows for strong YFP fluorescence complementation at the cell periphery (column one). Fusion of YFP_C to the DN mutant RACB-T20N does not interact with CRIB-YFP_N, and no fluorescence is observed (column

two). The red fluorescing DsRED protein is localized in the cytoplasm and nucleoplasm and contrasts YFP fluorescence at the periphery. Pictures represent 30 optical sections through the entire cell at 2 µm increments. **B.** Over-expression of the CRIB46 peptide reduces fungal penetration success by 23 %. Error bars represent standard error of five independent repetitions.