

1 **Barley RIC157 is involved in RACB-mediated susceptibility to** 2 **powdery mildew**

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8

9 **Abstract**

10 Successful pathogens often benefit from certain cellular host processes. For the
11 biotrophic ascomycete fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*) it has been shown
12 that barley RACB, a small monomeric G-protein (ROP, RHO of plants), is required for
13 full susceptibility to fungal penetration. The susceptibility function of RACB probably
14 lies in its role in cell polarisation, which may be co-opted by the pathogen for invasive
15 ingrowth of its haustorium. However, the actual mechanism of how RACB supports the
16 fungal penetration success is little understood. RIC proteins are considered scaffold
17 proteins which can interact directly with ROPs via a conserved CRIB motif. Here we
18 describe a yet uncharacterised RIC protein, RIC157, which can interact directly with
19 RACB. We could show that RIC157 undergoes a recruitment from the cytoplasm to the
20 cell periphery in the presence of activated RACB. During fungal infection, RIC157 and
21 activated RACB colocalise at the penetration site, particularly at the haustorial neck. In
22 a RACB-dependent manner, transiently overexpressed RIC157 renders barley
23 epidermal cells more susceptible to fungal penetration. We conclude that RIC157
24 promotes fungal penetration into barley epidermal cells via its function as downstream
25 executor in RACB-signaling.

26

27 **Introduction**

28 Plants have developed a multilayer immune system to cope with the constant threat of
29 microbial invasion, consisting of pre-formed barriers and induced defences based on
30 the receptor-mediated recognition of microbe-derived and endogenous elicitors (Boller
31 and Felix 2009; Stukenbrock and McDonald 2009). Except for necrotrophs, invading
32 microbes rely to different extents on a living host to establish an infection. With the help

33 of secreted effector proteins, pathogens undermine plant immune reactions and
34 influence the host metabolism to render the cellular environment more favourable
35 (Białas *et al.* 2018, Han and Kahmann 2019). The co-evolution between microbial
36 effectors and their specific host target molecules can lead to an increase in host
37 specialisation, with mutualistic relationships demonstrating extreme examples.
38 Regarding pathogens, especially in genomes of cereal powdery mildew fungi it has
39 been observed that the amount of genes encoding for metabolic enzymes is massively
40 reduced, concomitantly with the proliferation of the putative effector gene pool and
41 transposable elements (Spanu *et al.* 2010, Wicker *et al.* 2013, Frantzeskakis *et al.*
42 2018). Plant targets of these effectors are not necessarily involved in resistance
43 mechanisms, but also in cellular processes that, when controlled by the pathogen, can
44 become disadvantageous for the host and support the susceptibility towards the
45 invading pathogen. With the current possibilities to use a plethora of different breeding
46 technologies, durable crop resistance based on the loss of these susceptibility gene
47 product functions is within reach (Engelhardt *et al.* 2018).

48 Powdery mildew fungi are notorious pathogens known to infect a wide variety of
49 monocot and dicot plants causing massive yield losses in crops. The ascomycete
50 fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*) is the specific causal agent of the
51 agronomically important powdery mildew disease on barley (*Hordeum vulgare*) in
52 Europe (Jørgensen and Wolfe 1994). As an obligate biotrophic parasite, *Bgh* requires
53 living epidermal cells to complete its life cycle (Hückelhoven and Panstruga 2011).
54 Airborne conidia germinate on the leaf surface and form an appressorium to penetrate
55 the cuticle and the cell wall with the help of an immense turgor pressure and the release
56 of cell wall-degrading enzymes (McKeen and Rimmer 1973; Schulze-Lefert and Vogel
57 2000; Hückelhoven and Panstruga 2011). A successful fungal infection is
58 characterised by the formation of a haustorium inside the host cell which is essential
59 for nutrient uptake and effector protein delivery (Hahn and Mendgen 2001, Voegelé *et al.*
60 2001, Panstruga and Dodds 2009). The haustorium is separated from the host
61 cytosol by the extrahaustorial matrix and surrounded by the extrahaustorial membrane
62 (EHM), which is continuous with the plant plasma membrane, but differs functionally and
63 biochemically from it (Koh *et al.* 2005). It is feasible to imagine a pathogen-triggered
64 active contribution of the plant to accommodate the fungal haustorium.

65 ROPs (RHO (RAS homologue) of plants, or RACs, for rat sarcoma (RAS)-related C3
66 botulinum toxin substrate) form a unique subfamily of small monomeric RHO GTPases

67 in plants, since they do not fall into the phylogenetic RHO subclades of RAC, CDC42
68 and RHO GTPases found in yeast or animals (Brembu *et al.* 2006). G-proteins are
69 paradigms of molecular switches due to their ability to bind and hydrolyze GTP. The
70 GTP-bound form represents the activated state, and a plasma membrane association
71 of ROP-GTP via posttranslational lipid modifications is required for downstream
72 signalling (Yalovsky 2015). Upon GTP hydrolysis, GDP-bound or nucleotide-free
73 ROPs are inactive. The cycling between activated and inactive state needs to be
74 spatiotemporally controlled by regulatory partners. Guanine nucleotide exchange
75 factors (GEFs) positively regulate ROP activity by facilitating the GDP/GTP exchange.
76 In plants, three different sorts of ROP GEFs can be distinguished based on their
77 particular GEF domain: PRONE (plant-specific Rop nucleotide exchanger), DHR2
78 (DOCK homology region 2, found in SPIKE1) and a less well characterized DH-PH
79 domain (B-cell lymphoma homology-pleckstrin homology) described in a plant
80 homolog of human SWAP70 (Berken *et al.* 2005, Meller *et al.* 2005; Gu *et al.* 2006,
81 Basu *et al.* 2008, Yamaguchi and Kawasaki 2012, Yamaguchi *et al.* 2012, He *et al.*
82 2018). The interaction of ROPs with a GTPase Activating Protein (GAP) enhances the
83 intrinsic GTP hydrolysis activity, followed by ROP inactivation (Berken and Wittinghofer
84 2008). Beside their putative involvement in ROP recycling, Guanine nucleotide
85 Dissociation Inhibitors (GDIs) bind and sequester inactive ROPs in the cytoplasm and
86 are therefore considered negative regulators of ROP activity (Klahre *et al.* 2006,
87 Boulter and Garcia-Mata 2010). ROPs are involved in the regulation of a multitude of
88 cellular processes. For instance, the cytoskeleton organisation and consequentially
89 cell shape and function is subject to RHO-like GTPase control (Chen and Friml 2014).
90 In *Arabidopsis thaliana* xylem vessels, AtROP11 signaling promotes cell wall
91 apposition and shapes cell wall pit boundaries (Sugiyama *et al.* 2019). Different ROPs
92 are involved in polar cell growth and even function antagonistically during the
93 generation of *Arabidopsis thaliana* pavement cells (Craddock *et al.* 2012). Beside cell
94 polarisation and cytoskeleton organisation, ROPs have been also implicated in
95 membrane trafficking and auxin signaling (Yalovsky *et al.* 2008, Wu *et al.* 2011).
96 OsRac1 from rice (*Oryza sativa*), a great example for demonstrating the versatility of
97 ROPs, enhances cell division by regulating OsMAPK6, thereby promoting rice grain
98 yield (Zhang *et al.* 2019). It has also been shown to regulate immune-related processes
99 like ROS production, defense gene expression and cell death. OsRac1 becomes
100 activated by OsRacGEF1 upon receptor-mediated perception of fungal-derived chitin

101 by OsCEBiP and OsCERK1 (Akamatsu *et al.* 2013). Chitin-perception might also lead
102 to the activation of OsRAC1 by OsSWAP70 (Yamaguchi *et al.* 2012). Downstream
103 signaling by OsRAC1 is also triggered after recognition of pathogen effector proteins:
104 Plasma membrane-localised Pit, a nucleotide binding-leucine rich repeat resistance
105 (NLR) protein for the rice blast fungus *Magnaporthe oryzae*, associates with DOCK
106 family GEF OsSPK1, thereby likely activating OsRac1 (Kawano *et al.* 2010, Kawano
107 *et al.* 2014, Wang *et al.* 2018). A recent report regarding an involvement in defence
108 reactions against rice blast mediated by the NLR protein PID3 (Zhou *et al.* 2019) opens
109 up the possibility of OsRac1 being a downstream hub of other rice NLR proteins.
110 In the barley-powdery mildew interaction, several barley proteins involved in ROP
111 signaling or ROP activity regulation have been shown to influence fungal penetration
112 success. The barley ROP RACB has been shown to act as susceptibility factor
113 (Schultheiss *et al.* 2002, Schultheiss *et al.* 2003, Hoefle *et al.* 2011). In the absence of
114 the pathogen, RACB appears to be involved in cell polarization processes, as stable
115 RACB silencing affects stomatal subsidiary cell and root hair development (Scheler *et*
116 *al.* 2016). The expression of a constitutively activated GTP-bound RACB increased
117 fungal penetration efficiency into barley epidermal cells, whereas silencing RACB by
118 RNA interference (RNAi) renders epidermal cells less susceptible against fungal
119 invasion. Two RACB-interacting proteins have been described as negative regulators
120 of RACB function in susceptibility. First, the Microtubule-Associated ROP-GAP1
121 (MAGAP1) is recruited to the cell periphery by activated RACB, where it limits
122 susceptibility to powdery mildew likely by enhancing the GTP-hydrolyzing activity of
123 RACB (Hoefle *et al.* 2011). Second, activated RACB recruits the cytoplasmic ROP
124 binding kinase1 (RBK1) to the cell periphery and enhances its kinase activity *in vitro*
125 (Huesmann *et al.* 2012). Transient silencing of RBK1 or RBK1-interacting protein SKP1
126 (type II S-phase kinase1-associated protein) suggested that RBK1 acts in negative
127 regulation of RACB protein stability and hence in disease resistance (Reiner *et al.*
128 2016). Additionally, RBK1 also appears to be involved in cytoskeleton regulation by
129 positively controlling microtubule stability.

130 In order to regulate cellular processes, ROPs need to activate or deactivate
131 downstream executors (also called ROP effectors). The interaction with some of these
132 executors is often indirect and achieved via scaffold proteins bridging the activated
133 ROPs to their signal destination targets. Two different classes of scaffold proteins have
134 been described so far, ICR/RIPs and RICs. ICR/RIPs (Interactor of Constitutive Active

135 ROP/ROP Interactive Partners) have been shown as ROP interactors to be required
136 for cell polarity, vesicle trafficking and polar auxin transport (Lavy *et al.* 2007, Hazak *et*
137 *al.* 2014). In barley, RIPb interacts directly with RACB and enhances disease
138 susceptibility towards powdery mildew (McCollum *et al.* 2019 Preprint). RIC (ROP-
139 Interactive and CRIB-domain containing) proteins, another class of scaffold proteins in
140 ROP signaling, share a highly conserved CRIB motif (Cdc42/Rac Interactive Binding
141 motif, Burbelo *et al.* 1995), which is essential for the direct interaction with ROPs. The
142 CRIB domain is also present in a subset of ROP GAPs such as barley MAGAP1
143 (Schaefer *et al.* 2011). In barley, the knowledge about RIC protein function is quite
144 limited. RIC171, however, has been shown to not only interact directly with RACB, but
145 also to increase fungal penetration efficiency in barley epidermal cells upon
146 overexpression. Activated RACB recruits RIC171 to the cell periphery and, in the
147 presence of *Bgh*, RIC171 accumulated at the haustorial neck close to the penetration
148 site (Schultheiss *et al.* 2008). In *Arabidopsis thaliana* (At), 11 different RIC proteins
149 have been identified, that do not share any clear sequence homology outside their
150 CRIB domain (Wu *et al.* 2001). By directly interacting with AtROPs, AtRIC proteins are
151 involved in numerous cellular processes. During salt stress, AtROP2 regulates
152 microtubule organisation in an AtRIC1-dependent manner (Li *et al.* 2017). AtRIC1 also
153 interacts with AtROP6 in pavement cells to enhance the ordering of cortical
154 microtubules upon hormonal signals (Fu *et al.* 2009) and is involved in cell elongation
155 during pavement cell morphogenesis (Higaki *et al.* 2017). AtRICs counteract each
156 other to a certain extent as well, as seen with AtROP1-interacting AtRIC3 and AtRIC4
157 during pollen tube growth. AtRIC3 regulates calcium influx and triggers actin
158 depolymerisation, whereas AtRIC4 enhances actin polymerisation (Gu *et al.* 2005).
159 Light-induced stomatal opening is regulated via the AtROP2-AtRIC7 pathway. AtROP2
160 and AtRIC7 are likely to impinge on vesicular trafficking by inhibiting AtExo70B1 which
161 results in a diminished stomatal opening (Hong *et al.* 2016). These examples
162 emphasize the importance of ROP proteins as signaling hubs for various
163 developmental processes as well as the role RIC proteins play in finetuning the specific
164 cellular responses. However, the function of RICs during the interaction with pathogens
165 still remains to be elucidated.

166 Here we show results on barley RIC157, another CRIB domain-containing protein that
167 interacts CRIB-dependently with RACB in yeast and *in planta*. Overexpression of
168 RIC157 increases the powdery mildew penetration efficiency in barley leaf epidermal

169 cells in a RACB-dependent manner. Cytosolic RIC157 is recruited to the cell periphery
170 specifically by activated RACB and both proteins co-localise at the haustorial neck
171 during the compatible interaction with *Bgh*. Our findings indicate a possible role of the
172 RACB-RIC157 signaling pathway in promoting fungal penetration, thereby increasing
173 susceptibility towards *Bgh*.

174

175 **Results**

176 **Identification of RIC proteins in barley**

177 RIC proteins are characterised by their low molecular weight and a lack of sequence
178 homology to any proteins in the database outside their CRIB domain (Wu *et al.* 2001).
179 This conserved motif has been shown to interact directly with activated small GTPases
180 (Burbelo *et al.* 1995, Aspenström 1999). In order to identify additional RIC proteins in
181 barley, we performed a BLAST search using the CRIB motif of previously described
182 RACB interacting protein RIC171 (Schultheiss *et al.* 2008) against the 2019 annotation
183 of all barley coding sequences (Barley all CDS Morex v2.0 2019, [https://webblast.ipk-](https://webblast.ipk-gatersleben.de/barley_ibsc/)
184 [gatersleben.de/barley_ibsc/](https://webblast.ipk-gatersleben.de/barley_ibsc/)). Beside RIC171, we identified another seven proteins
185 sharing the properties of RIC proteins, and named them according to their predicted
186 amino acid sequence length RIC153 (HORVU.MOREX.r2.3HG0258770), RIC157
187 (HORVU.MOREX.r2.6HG0469110), RIC163 (HORVU.MOREX.r2.5HG0443720),
188 RIC168 (HORVU.MOREX.r2.2HG0170820), RIC170
189 (HORVU.MOREX.r2.6HG0521090), RIC171 (HORVU.MOREX.r2.2HG0164690),
190 RIC194 (HORVU.MOREX.r2.3HG0258620), RIC236
191 (HORVU.MOREX.r2.2HG0122110). An amino acid sequence alignment of all eight
192 barley RIC proteins illustrated no general domain homologies beyond the highly
193 conserved CRIB motif (Fig. 1). Interestingly, the CRIB motif was more C-terminally
194 located in RICs 153, 163 and 194, similar to RIC2 and RIC4 of *Arabidopsis thaliana*
195 (Wu *et al.* 2001), while the other RICs (157, 168, 170, 171 and 236) contained the
196 CRIB motif closer to their N-terminal end. However, we didn't identify additional
197 conserved domains shared by all members of the barley RIC protein family except for
198 the CRIB domain.

199 RACB-mediated susceptibility towards powdery mildew is determined exclusively in
200 barley leaf epidermal cells. Hence, in order to unravel downstream signaling
201 components of RACB, we focused on barley leaf-expressed RIC proteins. Using online
202 available gene expression databases (<https://webblast.ipk->

203 gatersleben.de/barley_ibsc/), we identified five leaf-expressed *RIC* genes (*RIC153*,
204 *RIC157*, *RIC163*, *RIC194* and previously published *RIC171* (Schultheiss *et al.* 2008).
205 Despite there is little sequence conservation between RIC proteins outside their CRIB
206 domain, we compared primary sequences of barley, *Arabidopsis thaliana* and rice
207 (*Oryza sativa*) RICs using an available online amino acid motif discovery software
208 (<http://meme-suite.org/tools/meme>). Interestingly, this discovered three more amino
209 acid motifs shared by individual barley (*RIC157*, *RIC168*, *RIC171*), rice (Os02g06660.1,
210 Os04g53580.1) and Arabidopsis RICs (*RIC10*, *RIC11*) (Suppl. Fig. S1).

211

212 **RIC157 increases susceptibility of barley towards *Bgh* in a RACB-dependent** 213 **manner**

214 To investigate a potential biological function of *RIC157* during the barley-powdery
215 mildew interaction, we analysed the penetration success of *Bgh* on barley epidermal
216 cells during various conditions (Fig. 2). Single cell transient overexpression of *RIC157*
217 had a strong effect on the penetration success of *Bgh* into barley epidermal cells (Fig.
218 2A). The susceptibility increased by about 50% compared to control treatments, an
219 outcome that is reminiscent of susceptibility levels observed after transient RACB or
220 *RIC171* overexpression (Schultheiss *et al.* 2003, 2008). Interestingly, we didn't
221 observe the opposite effect, meaning a decreased fungal penetration after RNA
222 interference (RNAi)-mediated silencing of *RIC157* compared to control levels (Fig. 2B).
223 This suggests additional factors being involved in RACB-mediated susceptibility
224 towards *Bgh*. To check if this elevated susceptibility of barley epidermal cells after
225 overexpression of *RIC157* is dependent on endogenously present RACB, we analysed
226 the fungal penetration efficiency by simultaneously overexpressing *RIC157* and
227 transiently silencing endogenous RACB expression via RNAi (Fig. 2c). Interestingly,
228 we did not observe an elevated fungal success rate, indicating that *RIC157* increases
229 barley epidermal cell susceptibility in a RACB-dependent manner. We confirmed the
230 efficiency of both RNAi silencing constructs via co-expression of fluorescence tag-
231 labelled targets and ratiometric fluorescence measurements (Suppl. Fig. S2).

232

233 **RIC157 interacts directly with RACB in yeast and *in planta***

234 RACB can directly interact with CRIB motif-containing proteins *RIC171* and *MAGAP1*
235 (Schultheiss *et al.* 2008, Hoefle *et al.* 2011). Therefore, it appeared likely that *RIC157*
236 can directly interact with RACB via its CRIB domain. We conducted different

237 approaches to test for direct protein-protein interaction between RIC157 and RACB. In
238 a targeted yeast-2-hybrid experiment, we showed that RIC157 directly interacts with
239 RACB and we also confirmed the CRIB motif being essential for this interaction (Fig.
240 3A, Suppl. Fig. S3). In yeast, RIC157 directly interacts with RACB and with the
241 constitutively activated RACB mutant CARACB(G15V), but not with the dominant-
242 negative, GDP-bound RACB mutant DNRACB(T20N). Interestingly, we also observed
243 a certain level of interaction between RIC157 and lower nucleotide affinity RACB
244 mutant DNRACB(D121N). This particular mutation has been shown previously in RAS
245 mutant D119N to behave either in a constitutively activated or a dominant-negative
246 way, depending on the experimental setup (Cool *et al.* 1999). To substantiate these
247 results, we aimed to prove the fusion protein stability by immunoblotting (Suppl. Fig.
248 S4). While all RACB variants were stably expressed and detectable, we were unable
249 to confirm the stability of RIC157 variants in yeast in most of several independent
250 experiments. Together with the fact that the yeast growth on selective medium was
251 quite slow but dependent on the RIC157 construct, this suggests a high RIC157
252 turnover in yeast.

253 In order to investigate the *in planta* interaction between RACB and RIC157, we
254 performed Bimolecular Fluorescence Complementation (BiFC, synonym split-Yellow
255 Fluorescent Protein (YFP)) experiments (Walter *et al.* 2004). We therefore fused N-
256 terminally N- and C-terminal YFP truncations to RIC157 and RACB variants and
257 transiently co-expressed complementary split-YFP fusion proteins in barley epidermal
258 cells via particle bombardment. YFP fluorescence reconstitution was ratiometrically
259 quantified against a co-expressed cytosolic mCherry fluorescence marker. As shown
260 in Fig. 3C (and Suppl. Fig. S5A), YFP fluorescence was reconstituted to a significantly
261 higher extent when split-YFP fusions of RIC157 were co-expressed with split-YFP
262 fusions of RACB and CARACB(G15V), compared to co-expressions with
263 DNRACB(T20N) and DNRACB(D121N). This suggests that *in planta*, RIC157 might
264 preferentially interact with activated RACB. We confirmed the stability of split-YFP
265 fusion proteins via immunoblot analysis of total protein samples extracted from
266 transformed barley mesophyll protoplasts (Suppl. Fig. S5B).

267 Since split-YFP experiments do not unequivocally reveal direct protein-protein
268 interaction, we further analysed the interaction between RACB and RIC157 by FLIM-
269 FRET and in particular the Green Fluorescent Protein (GFP) lifetime reduction (Fig.
270 3B). We fused mCherry N-terminally to RIC157 and GFP N-terminally to the activated

271 CARACB(G15V) and dominant-negative DNRACB(D121N) form and transiently co-
272 expressed mCherry and GFP fusion combinations in barley epidermal cells via particle
273 bombardment. Co-expression of GFP-DNRACB(D121N) and mCherry-RIC157
274 resulted in an average GFP lifetime of about 2.6ns, which is very similar to the value
275 we measure for GFP donor only expression. In contrast to that, the co-expression of
276 mCherry-RIC157 with GFP-CARACB(G15V) led to a small but highly significant
277 reduction in GFP lifetime to approximately 2.5ns. This GFP lifetime reduction clearly
278 demonstrates a direct protein-protein interaction between RIC157 and activated RACB
279 *in planta*.

280

281 **Recruitment of RIC157 to the cell periphery is RACB dependent**

282 Split-YFP fluorescence and GFP fluorescence during FLIM measurements localised
283 predominantly to the cell periphery. To investigate the subcellular localisation of
284 RIC157, we fused GFP N-terminally to RIC157 and transiently overexpressed this
285 construct in barley epidermal cells via biolistic transformation. As shown in Figure 4A
286 (upper row), RIC157 localises to the cytosol, but a lack of GFP fluorescence in the
287 nucleoplasm indicated that GFP-RIC157 is excluded from the nucleus. However, we
288 repeatedly observed a strong fluorescent signal around the nucleus suggesting a
289 potential affinity of RIC157 to the nuclear envelope/endoplasmic reticulum membrane
290 or proteins associated to it.

291 Since we found a direct protein-protein interaction between RIC157 and RACB, we
292 checked the potential impact of RACB on the subcellular localisation of RIC157 by
293 transiently co-expressing GFP-RIC157 with various untagged RACB forms (Suppl. Fig.
294 S6). The cytoplasmic localisation of RIC157 is not significantly affected in the presence
295 of RACB or the dominant negative DNRACB(D121N) mutant. However, we observed
296 a decrease in cytoplasmic and nuclear envelope-localised GFP fluorescence and an
297 accumulation of GFP fluorescence at the cell periphery when GFP-RIC157 was co-
298 expressed with the constitutively activated CARACB(G15V) form.

299 Because untagged proteins cannot be properly monitored, we extended our analysis
300 in barley epidermal cells using mCherry-tagged variants of RACB (Fig. 4A). Similarly,
301 we detected GFP-RIC157 in the cytoplasm where it co-localised with mCherry fusions
302 of RACB and DNRACB(D121N). In contrast to that, as with the untagged activated
303 RACB, we detected a similarly strong re-localisation of GFP-RIC157 to the cell
304 periphery in the presence of mCherry-tagged CARACB(G15V) that likewise

305 accumulated at this site. This suggests that activated RACB, like other ROPs
306 associating with the plasma membrane probably via its C-terminal prenylation and
307 possible palmitoylation (Schultheiss *et al.* 2003, Yalovsky 2015), recruits RIC157 to
308 the cell periphery.

309 In order to check if this RIC157 recruitment to the cell periphery is due to the co-
310 expression with activated RACB, we simultaneously transformed barley epidermal
311 cells with a RNAi construct to silence RACB (Fig. 4B). Without RACB silencing, co-
312 expression of GFP-RIC157 and mCherry-CARACB(G15V) again lead to accumulation
313 of both fusions proteins at the cell periphery. RNAi-mediated silencing of RACB on the
314 other hand did not only diminish the mCherry fluorescence to almost non-detectable
315 levels demonstrating a successful RACB silencing, it also decreased GFP
316 fluorescence at the cell periphery and increases GFP fluorescence in the cytoplasm.
317 This result clearly supports that the observed recruitment of RIC157 to the cell
318 periphery is mediated by activated RACB.

319

320 **RIC157 and RACB co-localise and accumulate at penetration site**

321 Co-localisation experiments in unchallenged barley epidermal cells demonstrated a
322 recruitment of RIC157 to the cell periphery in the presence of activated RACB. In order
323 to investigate, if both proteins co-localise at a more specific subcellular site during the
324 interaction with the powdery mildew fungus, we analysed the localisation of transiently
325 co-expressed RIC157 and CARACB(G15V) in epidermal barley cells 18-24 hours after
326 inoculation with *Bgh*. As shown in Fig. 5, fluorescent protein fusions of RIC157 and
327 activated RACB accumulate close to the fungal penetration site, forming a cone
328 outlining the neck of a developing haustorial initial. The RIC157/CARACB(G15V) co-
329 localisation was much more defined than the fluorescence signal of simultaneously
330 expressed cytoplasmic mCherry (Fig. 5B), indicating a specific membrane-associated
331 co-localisation in epidermal cells that are successfully penetrated by the fungus.

332

333

334 **Discussion**

335 The activity of the ROP protein RACB appears to be disadvantageous for barley in the
336 presence of *Bgh*. To date, however, our knowledge about the exact RACB-regulated
337 cellular process(es), the fungus takes advantage of, is still quite limited. Albeit we
338 observed a role of RACB or RACB-interacting proteins in polar cell development and

339 cytoskeleton organization (Opalski *et al.* 2005; Hoefle *et al.* 2011; Huesmann *et al.*
340 2012; Scheler *et al.* 2016; Nottensteiner *et al.* 2018), a direct mechanistic link between
341 RACB-mediated susceptibility and RACB-regulated cytoskeleton organisation or polar
342 membrane trafficking is still missing. Our studies, however, open up the prospect of a
343 RACB-regulated pathway via a ROP-specific scaffold protein that might be exploited
344 in barley epidermal cells by *Bgh* to support susceptibility towards powdery mildew.

345

346 **RIC proteins as scaffolds in RACB downstream signalling**

347 In a signalling cascade, scaffold proteins are as vital for mediating the molecular
348 response as upstream signalling hubs or downstream executors. These scaffolds
349 establish not just a hub-executor connection, but by doing so they represent also the
350 first branching point in the signalling cascade that eventually leads to specific effects
351 without possessing any kind of enzymatic activity themselves (Zeke *et al.* 2009, Good
352 *et al.* 2011). Regarding signalling pathways, ROPs function as signalling hubs and
353 have been shown to be involved in loads of cellular processes and for some ROPs the
354 regulatory role in different, sometimes even antagonistic signalling pathways has been
355 described (Gu *et al.* 2005; Nibau *et al.* 2006; Feiguelman *et al.* 2018). If ROPs do not
356 interact directly with downstream executors, RIC proteins (and also ICRs/RIPs, Mucha
357 *et al.* 2010, McCollum *et al.* 2019 Preprint) are considered bridging units creating the
358 scaffold for specific branches of ROP signalling (Schultheiss *et al.* 2008, Craddock *et al.*
359 *et al.* 2012, Zhou *et al.* 2015, Hong *et al.* 2016). The presence of a CRIB motif without
360 any further sequence homology to known proteins defines a RIC protein (Wu *et al.*
361 2001). The CRIB motif enables RIC proteins to directly interact with ROPs, as for
362 instance described for the *Arabidopsis thaliana* ROP2-RIC4 and ROP6-RIC1
363 interaction or for the barley RACB-RIC171 interaction (Fu *et al.* 2005, Schultheiss *et al.*
364 *et al.* 2008, Fu *et al.* 2009). Beside RIC171, we identified another seven proteins of
365 different sizes in barley that, by the above-mentioned definition, we consider RIC
366 proteins. Apparently there is a difference between monocots and dicots regarding the
367 number of RIC proteins (Wu *et al.* 2001), similar to RIP proteins (McCollum *et al.* 2019
368 Preprint), suggesting a higher probability of either functional redundancies,
369 diversification or antagonistic partners in dicots (Gu *et al.* 2005). Redundancies
370 between the barley leaf-expressed RIC proteins are, considering the low primary
371 sequence conservation, hard to predict and not known yet. The partial similarities
372 between leaf-expressed RIC157 and RIC171 (Suppl. Fig. 1) could however indicate

373 that these two RIC proteins are either essential co-ligands, redundant scaffold proteins
374 or even antagonizing members of the same signalling pathway.

375

376 **RIC157 increases susceptibility towards powdery mildew**

377 In this study, we concentrated on leaf-expressed RIC157 and show, that the transient
378 RIC157 overexpression leads to a strong increase in barley epidermal cell
379 susceptibility towards powdery mildew infection (Fig. 2A). Thus, the fungus explicitly
380 benefits from a highly abundant RIC157. The RNA interference-mediated silencing of
381 RIC157, however, did not lead to a higher resistance compared to control-treated cells
382 (Fig. 2B). This could have different reasons. Although we have shown a significant
383 reduction of RIC157 protein levels in the presence of the RIC157 RNAi silencing
384 construct (Suppl. Fig. S2), it must be noted that RNAi-based silencing is never 100%
385 efficient. Remaining endogenous RIC157 transcript levels might be sufficient to allow
386 for control level penetration efficiencies. Additionally, the protein turnover rate of
387 RIC157 is unknown, meaning even with a high RNAi silencing efficiency it is still
388 possible that RIC157 protein, expressed before the transformation with the RNAi
389 silencing construct, is present throughout the infection assay and sufficiently abundant
390 to mask an otherwise more resistant phenotype. Another very important point that
391 needs to be stressed is the function of RACB or ROPs in general as signalling hubs
392 (Nibau *et al.* 2006). The signalling via RIC157 is likely not the only RACB-regulated
393 path that leads to susceptibility. Shutting down the particular RACB-RIC157 route
394 probably still leaves other RACB signalling pathways functional, which are also
395 involved to a certain extent in RACB-mediated susceptibility. An increased resistance
396 towards fungal infection is only achieved once the signaling hub is removed or switched
397 off by silencing RACB or by transient overexpression of MAGAP1 (Hoeftle *et al.* 2011).
398 In accordance with this, RACB-dependency of the RIC157-promoted susceptibility
399 (Fig. 2C) suggests that even abundant RIC157 still requires the presence of RACB to
400 function as a susceptibility factor.

401

402 **RIC157 interacts with RACB and is recruited to the cell periphery**

403 We have demonstrated that RIC157 can interact directly with RACB in yeast and *in*
404 *planta* (Fig. 3). In barley epidermal cells, RIC157 showed interaction with activated
405 RACB, but not with either dominant negative form. The observed interaction in yeast
406 between RIC157 and the dominant-negative form DNRACB(D121N) could be

407 explained by the different experimental setups and/or biological backgrounds. G-
408 proteins with this particular mutation have been observed previously to behave either
409 dominant-negative or constitutively activated (Cool *et al.* 1999). DNRACB(D121N) has
410 an intrinsic lower nucleotide affinity, however in yeast this form might be GTP bound
411 and hence resemble the activated RACB.

412 The subcellular *in planta* RACB-RIC157 interaction site seen in BiFC and FLIM-FRET
413 experiments appeared to be at the cell periphery. This seems conclusive, because
414 RIC157 preferentially interacted with activated RACB. Activated ROPs are supposed
415 to be associated with negatively charged phospholipids in plasmamembrane
416 nanodomains, which is additionally promoted via posttranslational prenylation and S-
417 acylation (Yalovsky 2015, Platre *et al.* 2019). Fluorescence-tagged RIC157 alone did
418 not show any specific localisation in the absence of the fungus, although it seems to
419 be excluded from the nucleus (Fig. 4). In the presence of activated RACB, however,
420 RIC157 undergoes a relocalisation from the cytoplasm to the cell periphery, where the
421 interaction with the activated ROP takes place. It had been previously shown that
422 expression of activated GFP-RACB alone leads to its preferential localisation at the
423 cell periphery with a cytosolic background (Schultheiss *et al.* 2003). We assume that a
424 potential influence of endogenous levels of RIC157 or activated RACB on the
425 localisation of their overexpressed interaction partner is probably negligible in our
426 experimental setup. A similar recruitment to the cell periphery has been observed with
427 other proteins that directly interact with activated RACB (Schultheiss *et al.* 2008,
428 Huesmann *et al.* 2012, McCollum *et al.* 2019 Preprint), reinforcing the model that
429 RACB activation is preceding the recruitment of interaction partners and that
430 downstream RACB signaling is initiated at the plasma membrane. This is further
431 supported since a RACB mutant version lacking the C-terminal CSIL motif for
432 prenylation localizes to the cytoplasm and is inactive in promoting susceptibility
433 (Schultheiss *et al.* 2003).

434 With regard to RACB's and RIC157's capability to support fungal infection, the
435 recruitment of RIC157 to the cell periphery becomes even more interesting. Our data
436 suggest a recruitment of RIC157 to the attempted fungal penetration site (Fig. 5). The
437 cone-like structure surrounding the haustorial neck indicates a subcellular co-
438 localisation with activated RACB. However, the co-localisation of RACB and fungal
439 infection-supporting RACB-interactors is not exclusive to RIC157. RIC171 and RIPb,
440 two proteins also considered scaffolds in RACB signalling, co-localise at the haustorial

441 neck (Schultheiss *et al.* 2008, Hückelhoven and Panstruga 2011; McCollum *et al.* 2019
442 Preprint). Future experiments may show, whether subcellular co-concentration of
443 RACB and RACB-interacting proteins indicate a specific lipid composition of the
444 haustorial neck, which then recruits activated ROPs, or a membrane domain of high
445 ROP activity due to local GEF activity, or perhaps indicates an exclusion of ROPs from
446 further lateral diffusion into the EHM, which was suggested to be controlled at the
447 haustorial neck (Koh *et al.* 2005).

448

449 **RIC157 and susceptibility**

450 RIC157 transiently overexpressed in barley epidermal cells localises to the cytoplasm
451 and enhances fungal penetration efficiency in a RACB-dependent manner. Activated
452 RACB, however, associates with the plasma membrane where it likely recruits RIC157
453 for downstream signalling. Thus, transiently overexpressed and endogenous RIC157
454 might promote RACB-mediated susceptibility upon recruitment to the cell periphery by
455 endogenously present activated RACB. Only a small fraction of overexpressed RIC157
456 is possibly recruited to the cell periphery without concomitant co-overexpression of
457 activated RACB. This means that such a minute fluorescence localisation change
458 might prove difficult to be detected, but it needs to be emphasized that we do not
459 assume RIC157 promoting susceptibility to powdery mildew from a solely cytoplasmic
460 site. Indeed, in front of a cytoplasmic background, RIC157 is also visible at the cell
461 periphery without co-expression of CARACB(G15V), possibly reflecting partial
462 recruitment by endogenous ROPs (Fig. 4A).

463 The domain and sequence similarity between barley RIC157 and *Arabidopsis thaliana*
464 RIC10 and RIC11 (Suppl. Fig. 1) does not necessarily indicate similar functions of both
465 proteins. Beside AtRIC10 and AtRIC11, for which nothing is known to date about their
466 biological function, RIC157 also shares some amino acid motif similarity with AtRIC1,
467 AtRIC3 and AtRIC7 (Suppl. Fig. 7), for which an involvement in cytoskeleton
468 organization has previously been demonstrated. From these three *Arabidopsis* RIC
469 proteins, AtRIC1 appears to be an interesting candidate from which a RIC157 function
470 could be deduced. AtRIC1 has been shown to interact with ROP6 to activate the p60
471 subunit of Katanin (KTN1), a microtubule-severing enzyme (Lin *et al.* 2013), as
472 opposed to the interaction with AtROP2 that negatively regulates the action of AtRIC1
473 on microtubules (Fu *et al.* 2005). The microtubule-severing activity of KTN1 might even
474 be regulated by AtROP2, AtROP4 and AtROP6 (Ren *et al.* 2017) via AtRIC1. Whether

475 barley RIC157 also fulfils such a regulatory role in organising microtubules still remains
476 to be seen. Regarding the subcellular localisation there are, however, clear
477 differences: Barley RIC157 localises to the cytoplasm, AtRIC1 associates with
478 microtubules (Fu *et al.* 2005). However, when microtubule arrays in penetrated and
479 attacked but non-penetrated barley epidermal cells were compared, penetration
480 success was strongly associated with parallel non-polarized microtubule arrays in the
481 cell cortex and a diffuse or depleted microtubule structure at the haustorial neck (Hoefle
482 *et al.* 2011). AtRIC3 has been shown to be involved in the pollen tube growth process,
483 where its function leads to actin disassembly in a ROP1-dependent manner upon
484 calcium influx into the cytoplasm (Gu *et al.* 2005, Lee *et al.* 2008). Likewise, AtRIC7
485 was recently reported to influence vesicle trafficking in stomata resulting in the
486 suppression of an elevated stomatal opening after ROP2-dependent inhibition of the
487 exocyst complex *via* Exo70B1 (Hong *et al.* 2016). Both F-actin organization and
488 exocyst function are important in penetration resistance to *Bgh* (Opalski *et al.* 2005;
489 Miklis *et al.* 2007; Ostertag *et al.* 2012). Therefore, the discovery of RIC157 as a RACB-
490 dependent susceptibility factor may pave the way to a better understanding of ROP-
491 steered processes that are pivotal for fungal invasion into barley epidermal cells. The
492 challenge will be to find the downstream factors that RIC157 activates and to
493 understand how RIC157 interacts with RACB in the presence of several other RACB
494 interactors. We assume that several diverse RICs and ICR/RIP proteins could form a
495 cooperative network for orchestrating F-actin, microtubule and membrane organization
496 at the site of fungal entry.

497

498 **Experimental procedures**

499 **Plant and fungal growth conditions**

500 Wildtype barley (*Hordeum vulgare*, cultivar „Golden Promise“) was cultivated in long
501 day conditions (16 hours day light, 8 hours darkness) at a temperature of 18°C with a
502 relative humidity of 65% and a light intensity of 150 μ mol s⁻¹ m⁻².

503 The biotrophic powdery mildew fungus *Blumeria graminis* f.sp. *hordei* A6 was used in
504 all experiments. It was cultivated and propagated on barley „Golden Promise“ under
505 the same condition described above.

506

507 **Cloning of constructs**

508 Via a Two-Step Gateway cloning approach, in a first PCR *RIC157*
509 (HORVU.MOREX.r2.6HG0469110) was amplified from a barley cDNA pool prepared
510 from leaf and epidermal peels using gene-specific primers RIC157_GW_for and
511 RIC157_GW_rev+STOP (Suppl. Table 1) creating an incomplete Gateway attachment
512 site overhang. A second PCR using primers attB1 and attB2 completed the attachment
513 sites. To create a Gateway entry clone of *RIC157*, the amplified product was
514 recombined into pDONR223 (Invitrogen) via BP-reaction using Gateway BP Clonase™
515 II according to manufacturer's instruction (Thermo Fisher Scientific). To clone
516 *RIC157ΔCRIB*, both fragments upstream and downstream of CRIB motif were
517 amplified separately using primers RIC157_GW_for and RIC157delCRIB_rev for
518 PCR1, RIC157delCRIB_for and RIC157_GW_rev+STOP for PCR2, creating
519 overlapping overhangs. In PCR3 both fragments together with primers
520 RIC157_GW_for and RIC157_GW_rev+STOP completed *RIC157ΔCRIB* with
521 incomplete Gateway attachment sites overhangs. Completion of attachment sites
522 and creating an entry clone in pDONR223 was done as described above. To clone
523 *RIC157-H37Y-H40Y*, a site-directed mutagenesis using primers
524 CRIB157H37&40Y_for and CRIB157H37&40Y_rev was performed according to
525 QuikChange® Site-Directed Mutagenesis Protocol (Stratagene). For cloning entry
526 constructs of *RACB* variants, primers RACB_GW_for and RACB_GW_rev were used
527 to amplify *RACB* from previously described constructs (Schultheiss *et al.* 2003) and
528 cloned into pDONR223 via BP as described above. To clone *DNRACB(D121N)*, a site-
529 directed mutagenesis using primers RACB_D121N_fw and RACB_D121N_rv was
530 performed according to QuikChange® Site-Directed Mutagenesis Protocol
531 (Stratagene).

532 For RNA interference (RNAi) silencing of *RIC157* in barley, we PCR-amplified two
533 *RIC157* fragments, a 97bp fragment with primers RIC157_RNAi_NotI_for and
534 RIC157_RNAi_EcoRI_rev containing NotI and EcoRI restriction sites, and a 324bp
535 fragment with primers RIC157_RNAi_EcoRI_for and RIC157_RNAi_XbaI_rev
536 containing EcoRI and XbaI restriction sites. After restriction digest of all sites, ligating
537 into pIPKTA38 via NotI and XbaI sites we created a *RIC157_RNAi* entry construct
538 lacking the CRIB motif nucleotide sequence to prevent off-target silencing of other
539 CRIB-domain containing RNAs. The *RIC157_RNAi* sequence was then cloned via LR
540 reaction using Gateway LR Clonase™ II according to manufacturer's instruction

541 (Thermo Fisher Scientific) into RNAi expression plasmid pIPKTA30N to create a
542 double-strand RNAi expression construct (Douchkov *et al.* 2005).

543 For Yeast-2-Hybrid expression clones, entry clones of *RIC157* and *RACB* variants
544 were introduced into prey plasmid pGADT7-GW and pGBKT7-GW via LR reaction
545 using Gateway LR Clonase™ II according to manufacturer's instruction (Thermo Fisher
546 Scientific). pGADT7-GW and pGBKT7-GW have been modified from pGADT7 and
547 pGBKT7 (Clontech) into a Gateway-compatible form using Gateway™ Vector
548 Conversion System (Thermo Fisher Scientific). To create *RACB* variants lacking C-
549 terminal prenylation sequence, a premature STOP-Codon was introduced by site-
550 directed mutagenesis as described above using primers delCSIL_for and delCSIL_rev.
551 In order to clone BiFC constructs, we PCR amplified a *RIC157* full-length fragment with
552 primers RIC157_BamHI_for and RIC157_KpnI_rev containing BamHI and KpnI
553 restriction sites. After restriction digest, we ligated this construct into pUC-
554 SPYNE(R)173 (Waadt *et al.* 2008).

555 For localisation and overexpression studies in barley, *RIC157* and *RACB* variants in
556 pDONR223 were used as entry constructs to clone them into various pGY1-based
557 CaMV35S promoter-driven expression vectors (Schweizer *et al.* 1999) via LR reaction
558 as described above. Empty pGY1 (encoding for no tag) was rendered Gateway-
559 compatible via Gateway™ Vector Conversion System (Thermo Fisher Scientific). To
560 create expression vectors for proteins C- or N-terminally tagged by GFP or mCherry,
561 Gateway Reading Frame Cassettes for C- and N-terminal fusions, respectively, were
562 integrated into a pGY1-plasmid backbone upon XbaI digestion and combined at 5' or
563 3' with sequences of monomeric GFP or mCherry. Cloning procedure was performed
564 using In-Fusion HD cloning kit (Takara Bio USA). Constructs for GFP and mCherry
565 upstream or downstream of the Gateway cassette were amplified using primers
566 GW_RfA_mCherry-F, GW_RfA_mGFP-F, GW_RfA_Xba-R, GW_Xba_RfB-F,
567 GW_RfB-R, mGFP-STP-F, mCherry-STP-F, XFP-noSTP_Xba-F, XFP-noSTP-R,
568 mGFP-noSTP-R, mCherry-STP_Xba-R and mGFP-STP_Xba-R.

569 The *RACB* RNAi construct, *RACB* BiFC constructs have been described previously
570 (Schultheiss *et al.* 2003, Schultheiss *et al.* 2008, Schnepf *et al.* 2018, McCollum *et al.*
571 2019 Preprint).

572

573 **Barley epidermal cell transformation and penetration efficiency assessment**

574 For transient overexpression in barley, primary leaf epidermal cells of 7d old plants
575 were transformed using biolistic bombardment with 1µm gold particles that were
576 coated with 2µg of each test plasmid and additionally with 1µg of a cytosolic
577 transformation marker. After mixing the gold particles with plasmid combinations,
578 CaCl₂ (0.5M final concentration) and 3.5µl of 2mg/ml Protamine (Sigma) were added
579 to each sample. The gold particle solution was incubated at room temperature for
580 30min, washed twice with 500µl Ethanol (first 70%, then 100%) and eventually
581 dissolved in 6µl 100% ethanol per biolistic transformation. After shooting, leaves were
582 incubated at 18°C.

583 For localisation and BiFC experiments, leaves were analysed 2 days after
584 transformation. For FRET-FLIM analysis of RACB-RIC157 interaction, barley primary
585 leaves of 7d old plants were transiently transformed. Therefore, 2ug of meGFP-RACB
586 and 1ug mCherry-RIC157 containing plasmids were coated on gold particles for
587 biolistic transformation of single barley epidermal cells.

588 For inoculation with *Bgh*, fungal spores were manually blown in a closed infection
589 device over transformed leaves either 6 hours after transformation (for microscopic
590 analyses 16 hours after inoculation) or 1 day after transformation (to check penetration
591 efficiency 48 hours after inoculation).

592 To analyse penetration efficiency, a transient assay system based on a cytosolic GUS
593 marker was used as described previously (Schweizer *et al.* 1999). The reporter gene
594 construct pUbiGUSPlus was a gift from Claudia Vickers (Addgene plasmid # 64402;
595 <http://n2t.net/addgene:64402>; RRID:Addgene_64402, Vickers et al. 2003). Additionally
596 to overexpression or RNAi silencing constructs, each barley leaf was co-transformed
597 with pUbiGUSPlus. 48 hours after *Bgh* inoculation, leaves were submerged in GUS
598 staining solution (0.1M Na₂HPO₄/NaH₂PO₄ pH 7.0, 0.01 EDTA, 0.005M Potassium
599 hexacyanoferrat (II), 0.005M Potassium hexacyanoferrat (III), 0.1% (v/v) Triton X-100,
600 20% (v/v) Methanol, 0.5mg/mL 1.5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid). For
601 the solution to enter the leaf interior, a vacuum was applied. The leaves were incubated
602 at 37°C over night in GUS staining solution and subsequently for at least 24 hours in
603 70% Ethanol. Fungal structures were stained with ink-acetate solution (10% ink, 25%
604 acetic acid). Transformed cells were identified after GUS staining with light microscopy.
605 An established haustorium was considered a successful penetration and for each
606 sample at least 50 interactions were analysed. Barley epidermal cells transformed with
607 the empty expression plasmid were used as negative control.

608

609 **Barley protoplast preparation and transformation**

610 To prepare protoplasts from barley mesophyll cells, the lower epidermis of primary
611 leaves from 7 day-old barley plants was peeled and the leaves were incubated 3 to 4
612 hours at room temperature in the darkness while floating with the open mesophyll
613 facing downwards on an enzymatic digestion solution: 0.48M mannitol, 0.3% (w/v)
614 Gamborg B5, 10mM MES pH 5.7, 10mM CaCl₂, 0.5% (w/v) Cellulase R10, 0.5% (w/v)
615 Driselase, 0.5% Macerozyme R10. After enzymatic treatment, an equal amount of W5
616 solution was added: 125mM CaCl₂, 154mM NaCl, 5mM KCl, 2mM MES pH 5.7. Upon
617 filtering through a 40µm nylon mesh, the protoplasts were pelleted 5min at 200g and
618 carefully resuspended in 10ml W5 solution. After another centrifugation step, the
619 protoplast concentration was adjusted to 2 x 10⁶ cells per mL in MMG solution: 0.4M
620 mannitol, 15mM CaCl₂, 2mM MES pH 5.7. For each transformation sample, 1mL
621 protoplast solution was mixed with 50µg of each plasmid and 1.1mL PEG solution
622 (40% (w/v) PEG4000, 0.1M mannitol, 0.2M CaCl₂) and incubated 20min at room
623 temperature in the darkness. Afterwards, 4.4mL of W5 solution was added to each
624 transformation and gently mixed. After another pelleting at 200g, the protoplasts were
625 resuspended in 1mL W1 solution (0.5M mannitol, 20mM KCL, 4mM MES pH 5.7) and
626 incubated in the darkness at room temperature for at least 16 hours.

627

628 **Yeast-2-Hybrid**

629 Yeast strain AH109 was transformed with bait (pGBKT7) and prey (pGADT7)
630 constructs by following the small scale yeast transformation protocol from
631 Yeastmaker™ Yeast Transformation System 2 (Clontech). Upon transformation, yeast
632 cells were plated on Complete Supplement Medium (CSM) plates lacking leucine and
633 tryptophan (LW) and incubated for 3 days at 30°C. A single colony was taken to
634 inoculate 5mL of LW-dropout liquid medium that was incubated with shaking over night
635 at 30°C. The next day, 2mL of culture was pelleted for immunoblot analyses. 7.5µL of
636 undiluted overnight culture (and additionally a 1:10, 1:100 and 1:1000 for control
637 purposes) were dropped on CSM plates lacking leucine and tryptophan, and also on
638 CSM plates lacking leucine, tryptophan and adenine. Plates were incubated for at least
639 3 days at 30°C. Growth on CSM-LW plates confirmed the successful transformation of
640 both bait and prey plasmids, while growth on CSM-LWAd plates indicated activation

641 of reporter genes. As control for a positive and direct protein-protein interaction we
642 routinely used murine p53 and the SV40 large T-antigen (Li and Fields 1993).

643

644 **Immunoblot analysis**

645 For total protein extraction from yeast, we followed the protocol described in Kushnirov
646 (2000) using 2mL of over night culture of yeast transformants grown in CSM-LW liquid
647 medium. The extraction of total protein from barley mesophyll protoplasts was
648 performed by pelleting transformed protoplasts 5min at 200g. The pellet was
649 resuspended thoroughly in 50 μ L of 2x SDS loading buffer by vortexing. A complete
650 protein denaturation was achieved by boiling protoplast samples 10min at 95°C. After
651 shortly spinning down the samples, the stability of fusion proteins in yeast and in planta
652 was assessed via Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-
653 PAGE) and immunoblotting on PVDF membranes. Antibodies used for detecting
654 protein bands on PVDF membranes came from SantaCruz Biotechnology
655 (<https://www.scbt.com/scbt/cart/cart.jsp>): anti-GFP(B-2), anti-cMyc(9E10), anti-HA(F-
656 7) and horseradish-peroxidase conjugated anti-mouse. Presence of antibodies on
657 membrane was visually detected by using SuperSignal West Femto
658 Chemiluminescence substrate (ThermoFisher Scientific). Equal protein loading and
659 blotting success was confirmed via Ponceau S-staining of the PVDF membrane.

660

661 **Microscopy**

662 Localisation and BiFC experiments were analysed on a Leica TCS SP5 confocal laser
663 scanning microscope. The excitation laser wavelengths were 458nm for CFP, 488nm
664 for GFP, 514nm for YFP and 561nm for RFP and mCherry, respectively. The
665 fluorescence emission was collected from 462 to 484nm for CFP, from 500-550nm for
666 GFP, from 515 to 550nm for YFP and from 569 to 610nm for RFP and mCherry. Barley
667 epidermal cells were imaged via sequential scanning as z-stacks in 2 μ m increments.
668 Maximum projections of each z-stack were exported as Tiff files from the Leica LAS
669 AF software (version 3.3.0).

670 Localisation experiments of fluorescent protein fusions and BiFC analysis in barley
671 epidermal cells were conducted from 24 hours until 48 hours after biolistic
672 transformation. Regarding ratiometric BiFC quantification using Leica LAS AF
673 software (version 3.3.0), the fluorescence intensity was evaluated over the whole cell
674 area and the ratio between YFP and cytosolic mCherry fluorescence signal was

675 calculated. For each BiFC combination, the fluorescence of at least 20 cells was
676 measured.

677 For FRET-FLIM analysis of RACB-RIC157 interaction, the expression of the
678 fluorophore-fusion proteins was analysed 2 days after transformation using an
679 Olympus FluoView™3000 inverse laser scanning confocal microscope with an
680 UPLSAPO 60XW 60x/NA 1.2/WD 0.28 water immersion objective (Olympus,
681 Hamburg, Germany). Fluorescence of GFP was collected between 500-540nm and
682 mCherry emission was imaged between 580-620nm upon excitation with 488 and
683 561nm argon laser lines, respectively. For FRET-FLIM measurements the PicoQuant
684 advanced FCS/FLIM-FRET/rapidFLIM upgrade kit (PicoQuant, Berlin, Germany) was
685 used, comprising a 485nm pulsed laser line for GFP excitation (pulse rate 40 mHz,
686 laser driver: PDL 828 SEPIA II, laser: LDH-D-C-485), a Hybrid Photomultiplier Detector
687 Assembly 40 to detect GFP fluorescence and a TimeHarp 260 PICO Time-Correlated
688 Single Photon Counting module (resolution 25 ps) to measure photon life times. GFP
689 fluorescence was imaged at the aequatorial plane of epidermis cells to capture GFP
690 fluorescence at the cell periphery and possibly plasma membrane. For each interaction
691 at least 16 cells were analysed and 300 to 600 photons per pixel were recorded. Decay
692 data within a region of interest were fitted using an n-exponential reconvolution fit with
693 model parameters n=3 and calculated instrument response function of the PicoQuant
694 SymPhoTime 64 software.

695

696 **Supplemental Data**

697 **Suppl. Fig. S1: Barley RIC157 shows limited sequence similarity to RIC1 from**
698 ***Arabidopsis thaliana***

699 **Suppl. Fig. S2: RNA interference silencing efficiency**

700 **Suppl. Fig. S3: CRIB deletion and CRIB mutation in RIC157 prevents interaction**
701 **with RACB in yeast**

702 **Suppl. Fig. S4: Protein stability in yeast**

703 **Suppl. Figure S5: Raciometric BiFC analyses and BiFC fusion protein stability**

704 **Suppl. Fig. S6: RIC157 is recruited to the cell periphery by activated RACB**

705 **Suppl. Fig. S7: Barley RIC157 shows limited sequence similarity to RIC proteins**
706 **from *Arabidopsis thaliana*.**

707 **Suppl. Table 1: Primers used in this study**

708

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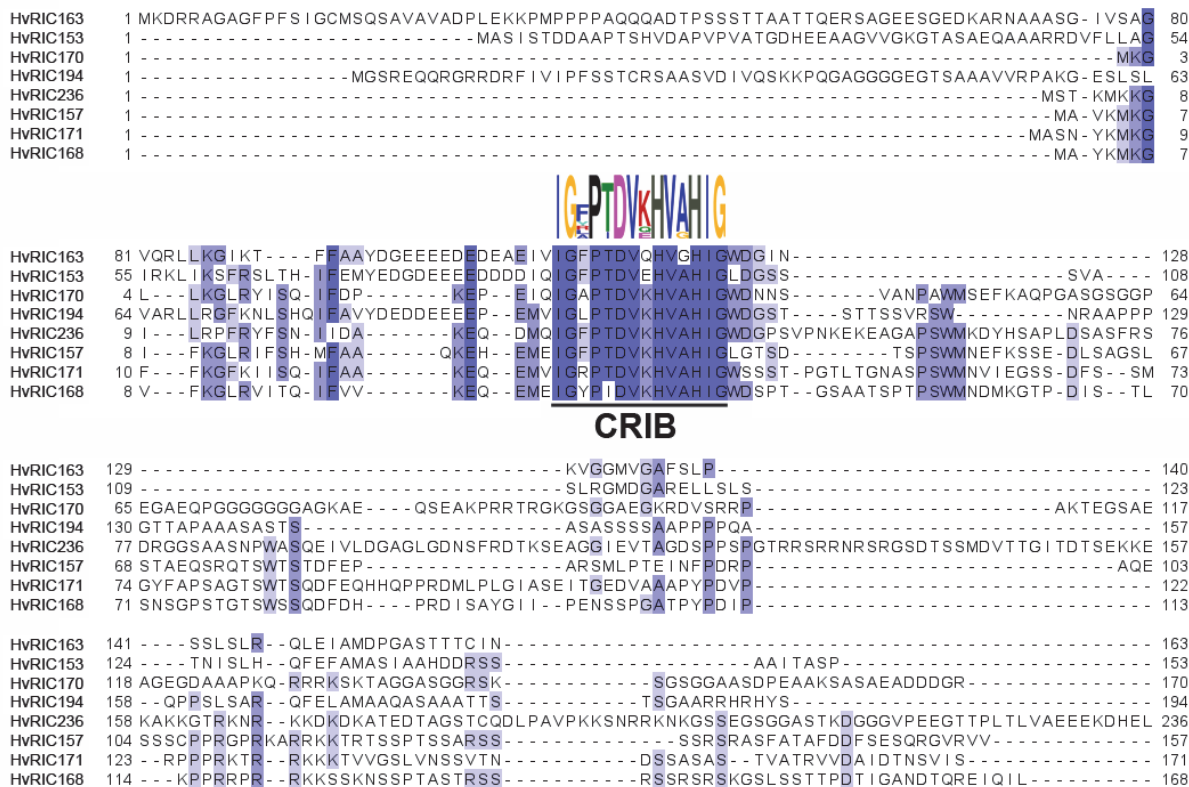
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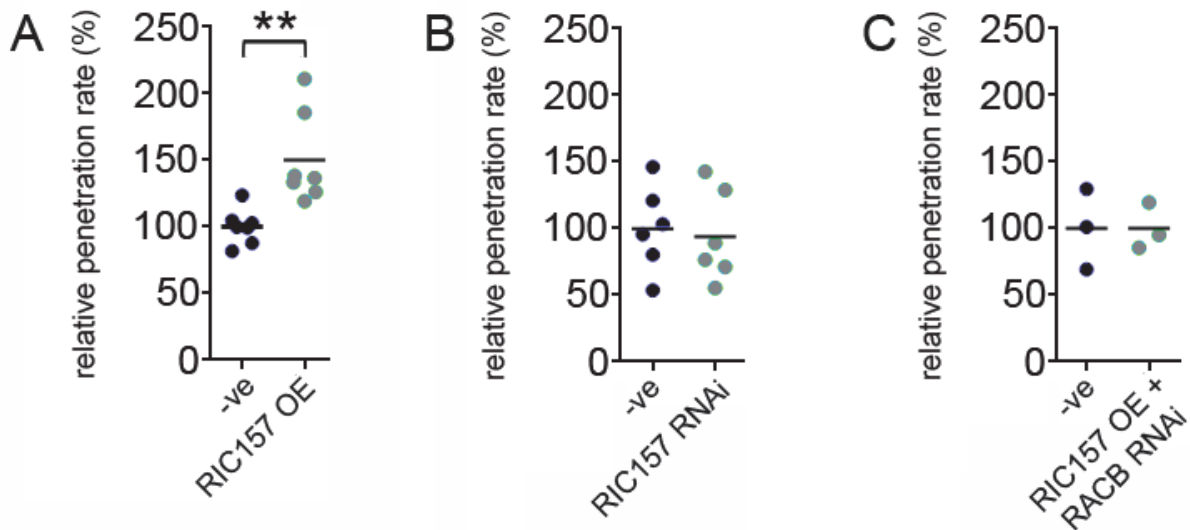
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1030 **Figures**



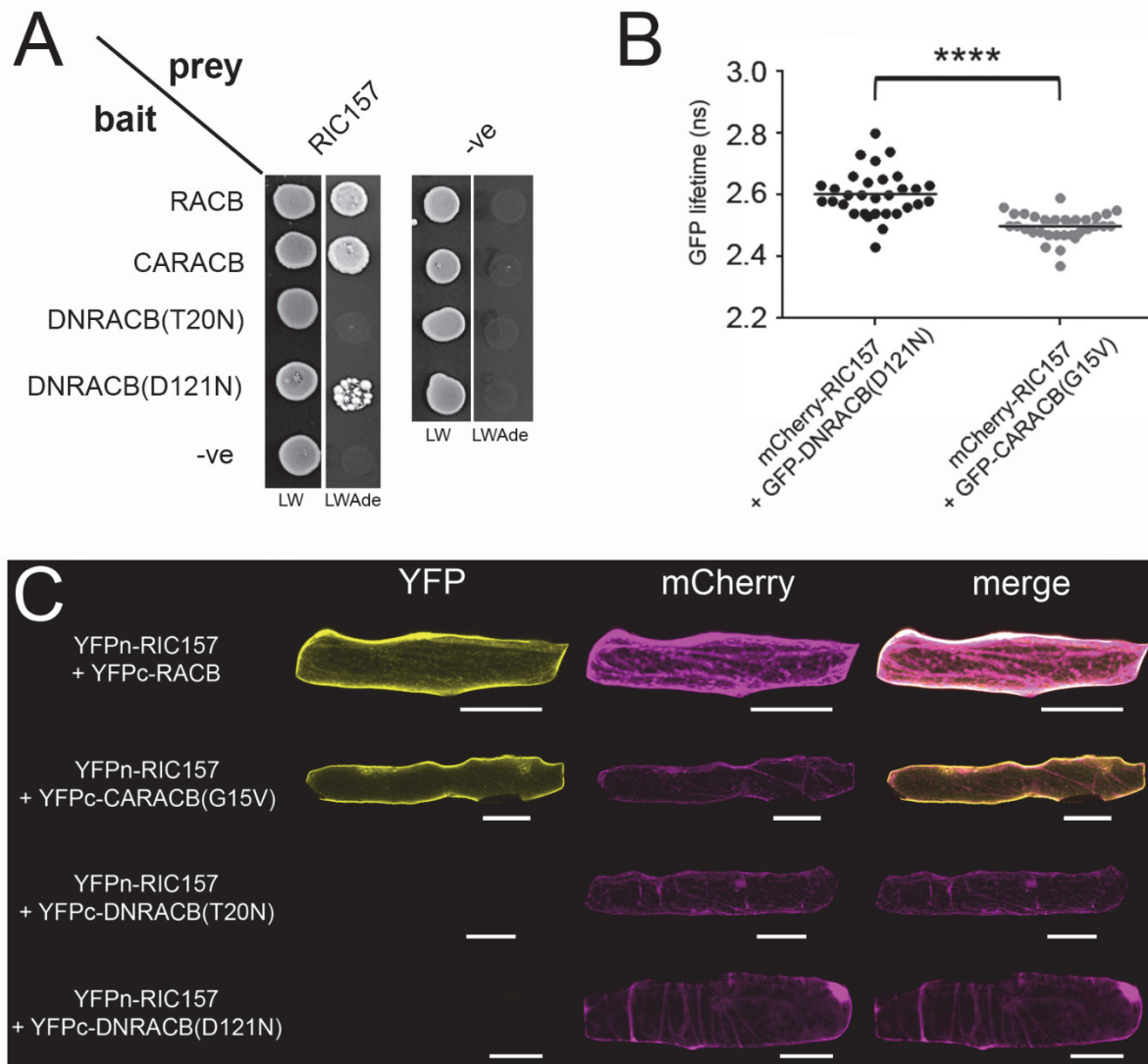


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1041 **Figure 2: RIC157 increases susceptibility in a RACB-dependent manner.**

1042 Epidermal cells of 7 day old primary barley leaves were transiently transformed by
1043 particle bombardment with either (A) an overexpression construct of RIC157, (B) a
1044 RNA interference construct of RIC157 or (C) simultaneously with an overexpression
1045 construct of RIC157 and a RNA interference construct of RACB. Empty overexpression
1046 or RNA interference plasmids were used as controls (-ve). The penetration efficiency
1047 of *Bgh* into transformed barley epidermal cells was analysed 48 after inoculation with
1048 fungal spores. Values are shown as mean of at least 3 independent biological
1049 replicates, relative to the mean of the control set as 100%. ** indicates significance P
1050 < 0.01 , Student's t-test.

1051



1052

1053 **Figure 3: RIC157 interacts directly with RACB in yeast and in planta.** A) Yeast-2-

1054 Hybrid indicates direct interaction between RIC157 and RACB in yeast. Yeast strain

1055 AH109 was transformed with indicated bait and prey fusion constructs. Overnight

1056 cultures of yeast transformants were dropped onto Complete Supplement Medium

1057 plates either lacking leucine and tryptophan (LW) or lacking leucine, tryptophan and

1058 adenin (LWAd) and incubated at 30°C. Growth on LWAd medium indicates

1059 interaction between bait and prey fusion proteins. -ve denotes empty prey and bait

1060 plasmid. Photos were taken 2 days (LW) and 7 days (LWAd), respectively, after

1061 dropping. B) Quantification of FLIM analysis confirms direct protein-protein interaction

1062 between RIC157 and activated RACB *in planta*. GFP lifetime in barley epidermal cells

1063 transiently co-expressing indicated constructs was investigated at the aequatorial

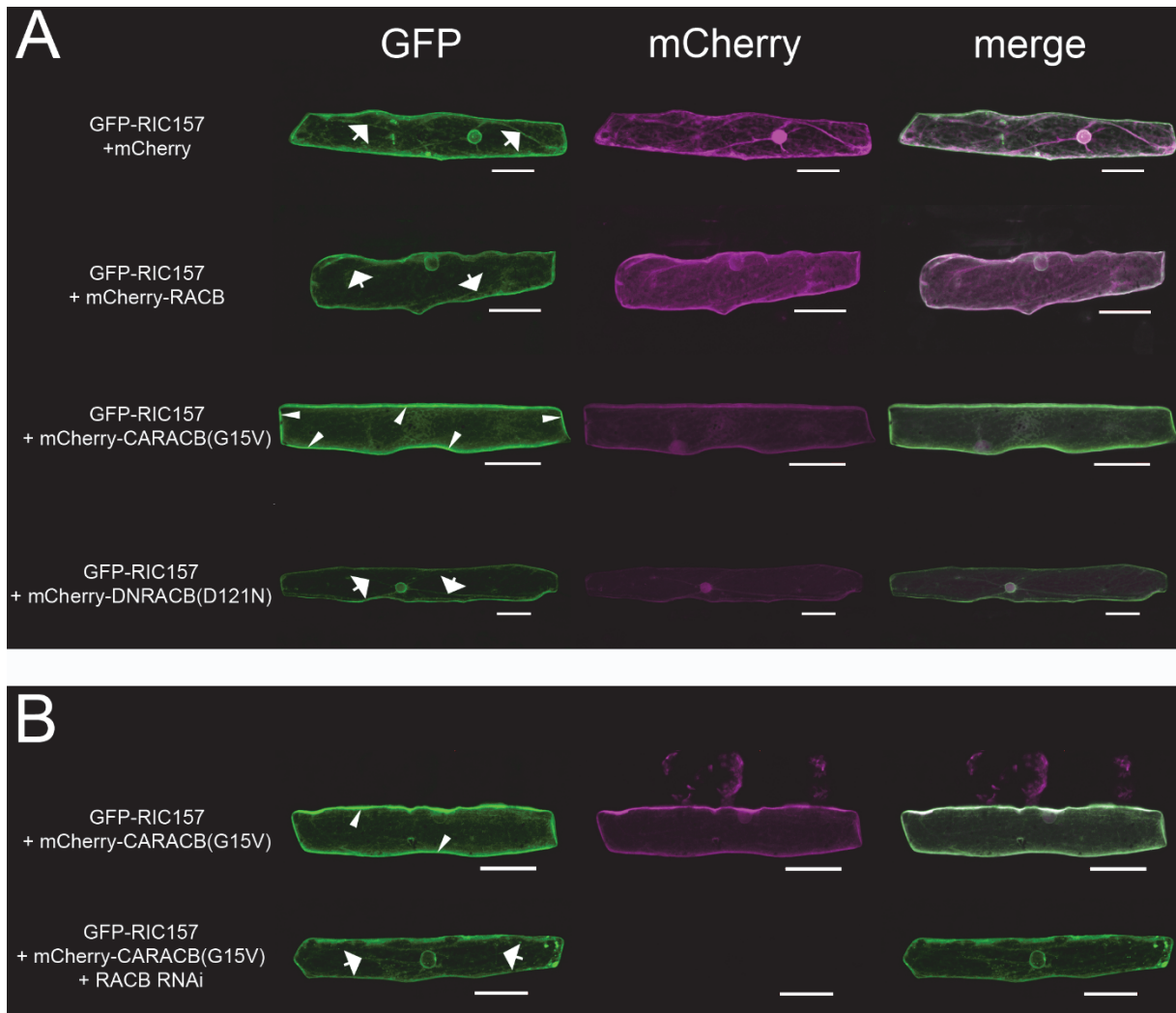
1064 plane 2d after transformation via particle bombardment. Graph shows result of 3

1065 independent biological replicates. **** indicates significance $P < 10^{-4}$, Student's t-test.

1066 C) BiFC shows close proximity between RIC157 and RACB *in planta*. Barley epidermal

1067 cells transiently co-expressing split-YFP fusion protein combinations and mCherry as
1068 cytosolic transformation marker after particle bombardment transformation. 2d after
1069 transformation, YFP fluorescence reconstitution was analysed via Confocal laser
1070 scanning microscopy. Shown are maximum projections of at least 15 optical sections
1071 taken at 2 μ m increments. Bars = 50 μ m.
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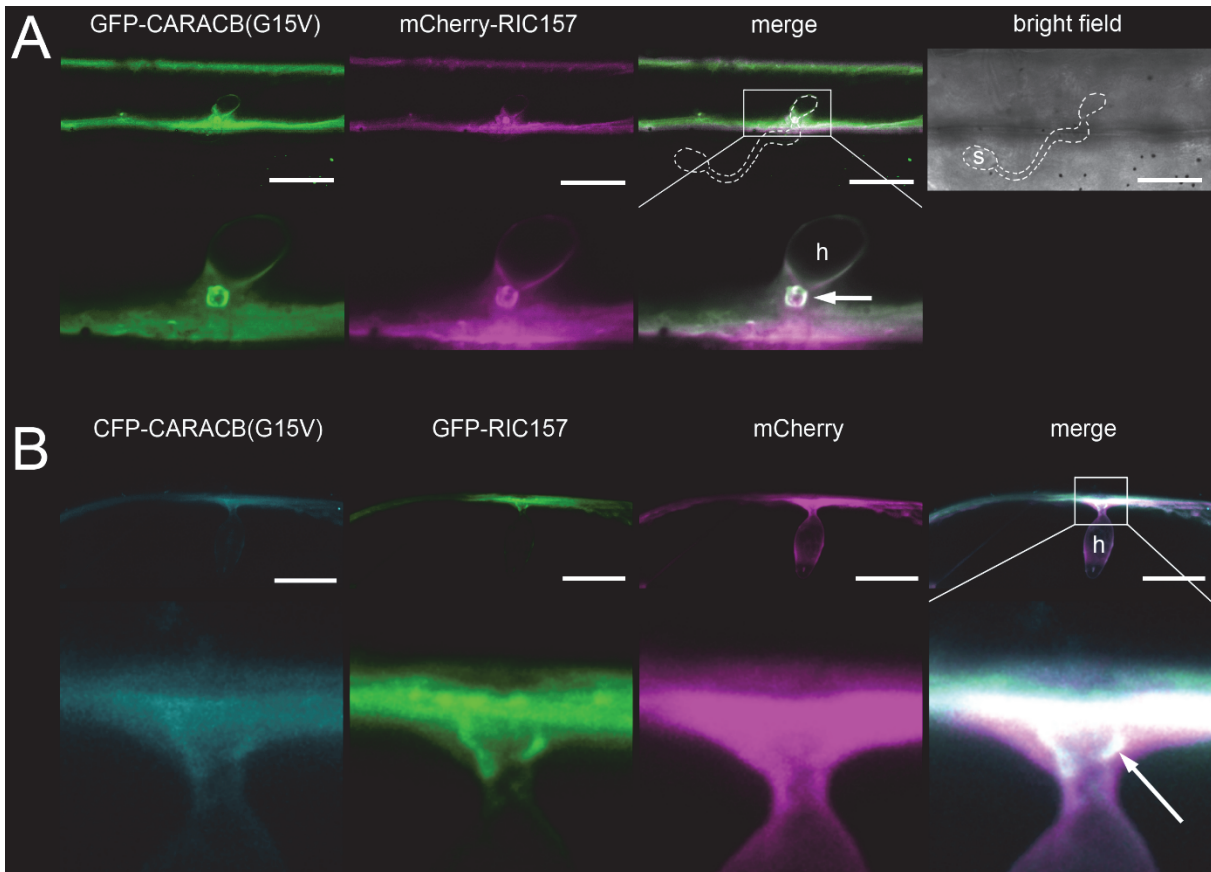
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1075 **Figure 4: RIC157 localisation is affected by RACB-activation status.** Confocal
1076 scanning microscopy of barley epidermal cells 1d after transformation via particle
1077 bombardment. A) GFP-RIC157 localises to the cytoplasm, but not the nucleus (upper
1078 row) and is recruited to the cell periphery exclusively by mCherry-CARACB(G15V), but
1079 not by mCherry-RACB or mCherry-DNRACB(D121N). B) Simultaneous RNA
1080 interference-mediated silencing of RACB attenuates RIC157 recruitment to the cell
1081 periphery. Arrows indicate cytoplasmic strands, arrow heads point towards GFP
1082 fluorescence accumulation at cell periphery. Microscopy pictures show maximum
1083 projections of at least 15 optical sections taken at 2µm increments. Bar = 50µm.

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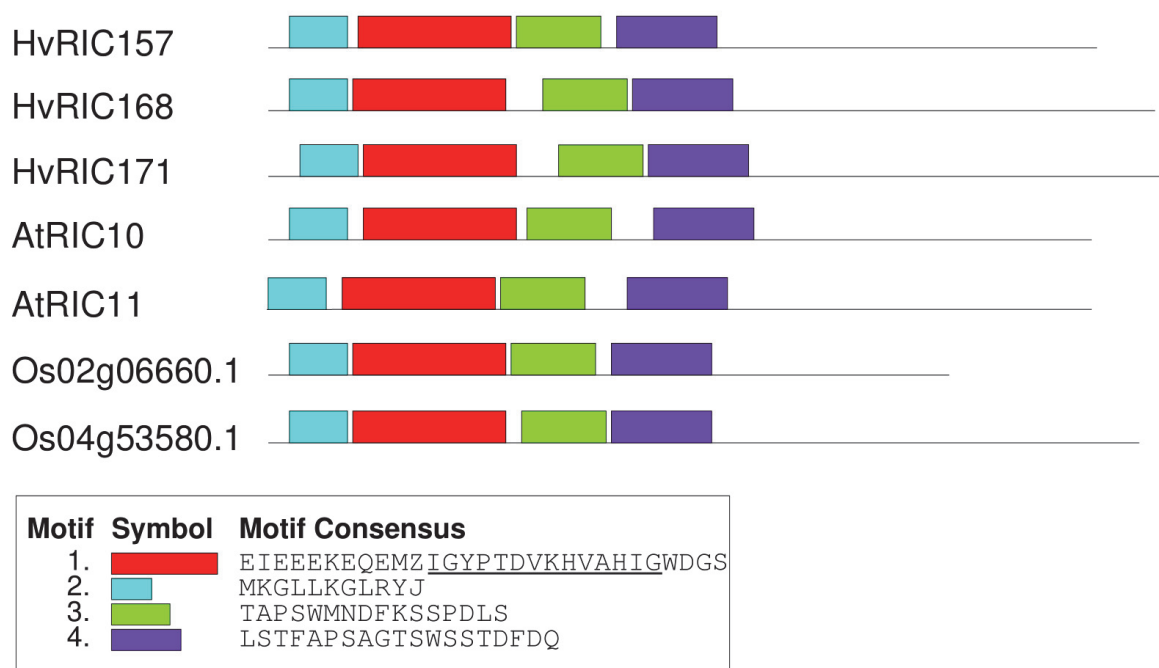
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Figure 5: RIC157 is recruited to penetration site where it colocalises with activated RACB. Confocal laser scanning microscopy of epidermal cells 1d after transformation via particle bombardment and 18-24h after inoculation with *Bgh*. Cells in A) and B) show successful fungal penetration due to haustorium formation (h). A) Transient co-expression of GFP-CARACB(G15V) and mCherry-RIC157. Area in white square is enlarged in lower panel. S = spore; Bar = 30μm. B) Transient co-expression of CFP-CARACB(G15V) and GFP-RIC157. Cytosolic mCherry was expressed to distinguish RIC157 and CARACB(G15V) localisation from cytoplasm at penetration site. Bar = 20μm. Arrows indicate approximate position of the haustorial neck. Contrast of images was equally slightly enhanced. Arrows indicate haustorial neck close to fungal penetration site.



1099

1100 **Suppl. Fig. S1: Barley RIC157 shows limited sequence similarity to barley RIC168**

1101 **and RIC171, but also to RIC proteins from *Arabidopsis thaliana* and *Oryza sativa*.**

1102 Primary sequences of RIC proteins (schematically shown in top part of the figure) were

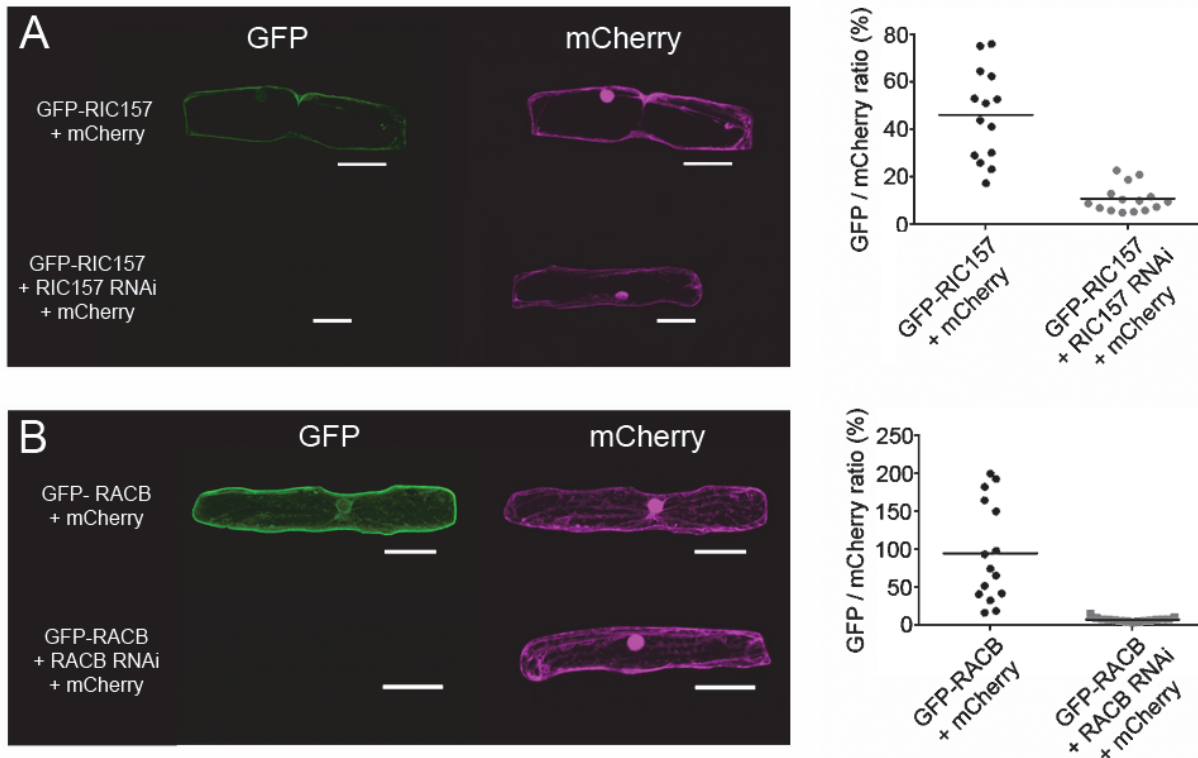
1103 analysed for domain homologies using MEME online software (<http://meme->

1104 [suite.org/tools/meme](http://meme-suite.org/tools/meme)). Consensus sequence of the four most similar motifs are shown

1105 below. Underlined sequence denotes CRIB domain. Hv = *Hordeum vulgare*, At =

1106 *Arabidopsis thaliana*, Os = *Oryza sativa*.

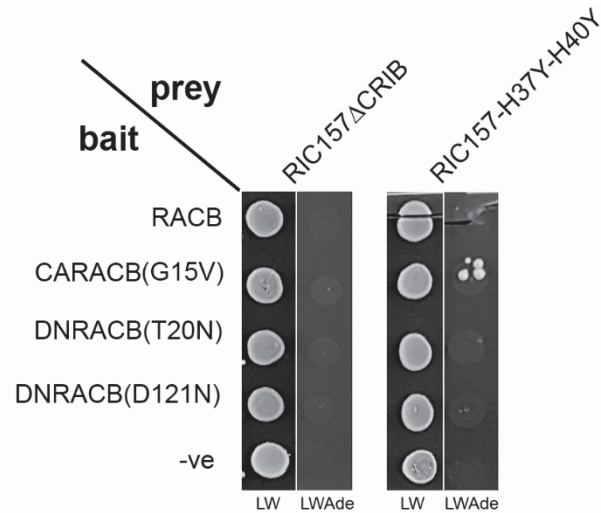
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1109 **Suppl. Fig. S2: RNA interference-mediated silencing efficiency.** Epidermal cells of
1110 7d old barley primary leaves were transiently transformed via particle bombardment
1111 with overexpression constructs of GFP fusions of RIC157 (A) and RACB (B) alone or
1112 together with RNAi silencing constructs. A construct to express cytosolic mCherry was
1113 simultaneously co-delivered for transformation efficiency and fluorescence
1114 quantification purposes. Microscopy images are maximum projections of at least 15
1115 optical sections taken at 2 μ m increments. Bars = 50 μ m. Each graph shows the mean
1116 of GFP fluorescence as percentage of mCherry fluorescence per transformed cell
1117 (whole cell area was taken as region of interest for measuring fluorescence intensity).
1118 Dots represent single measured cells.

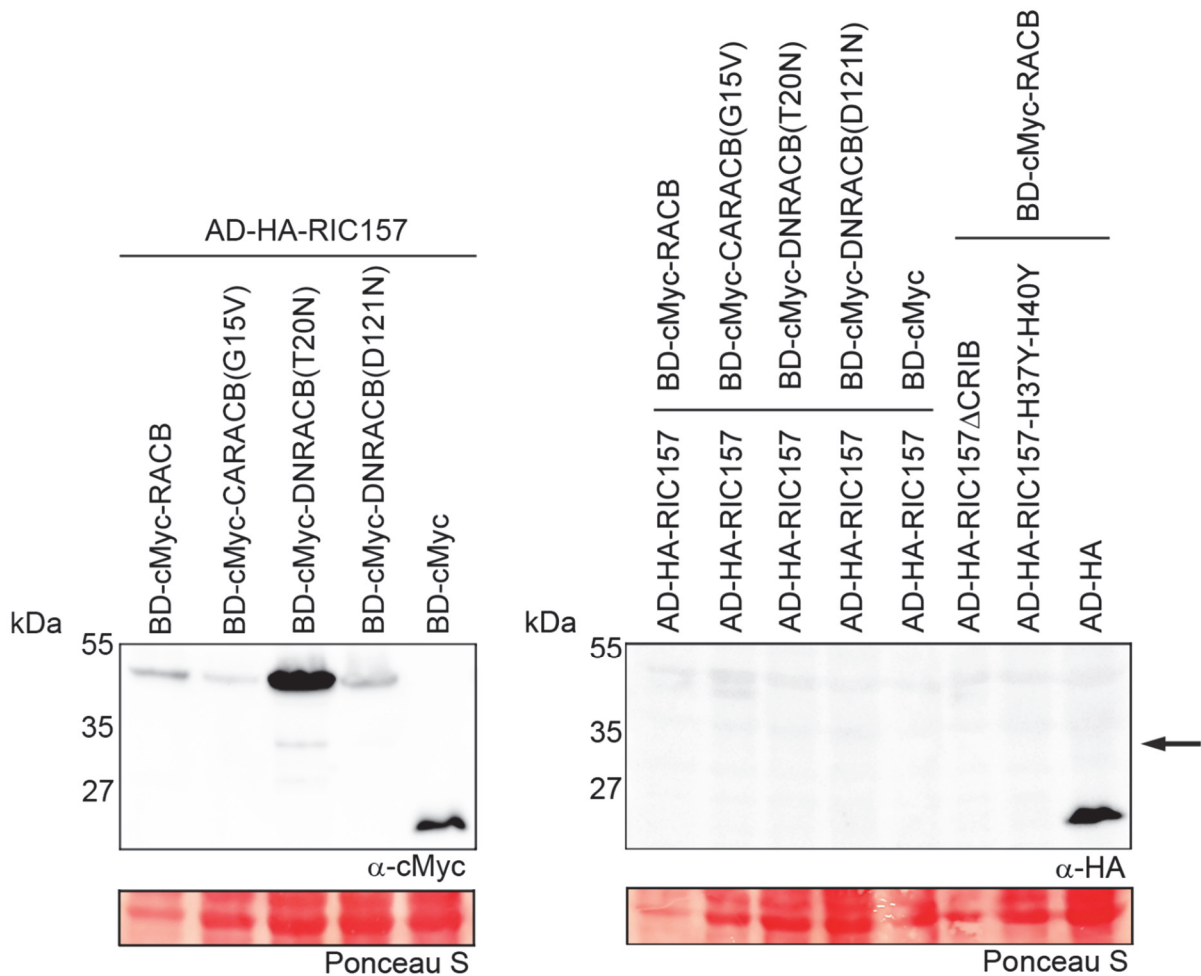
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1121 **Suppl. Fig. S3: CRIB deletion and CRIB mutation in RIC157 prevents interaction**
1122 **with RACB in yeast.** Yeast strain AH109 was transformed with indicated bait and prey
1123 fusion constructs. Overnight cultures of yeast transformants were dropped onto
1124 Complete Supplement Medium plates either lacking leucine and tryptophan (LW) or
1125 lacking leucine, tryptophan and adenin (LWAdE) and incubated at 30°C. Growth on
1126 LWAdE medium indicates interaction between bait and prey fusion proteins. -ve
1127 denotes empty prey and bait plasmid. Photos were taken 2 days (LW) and 7 days
1128 (LWAdE), respectively, after dropping.

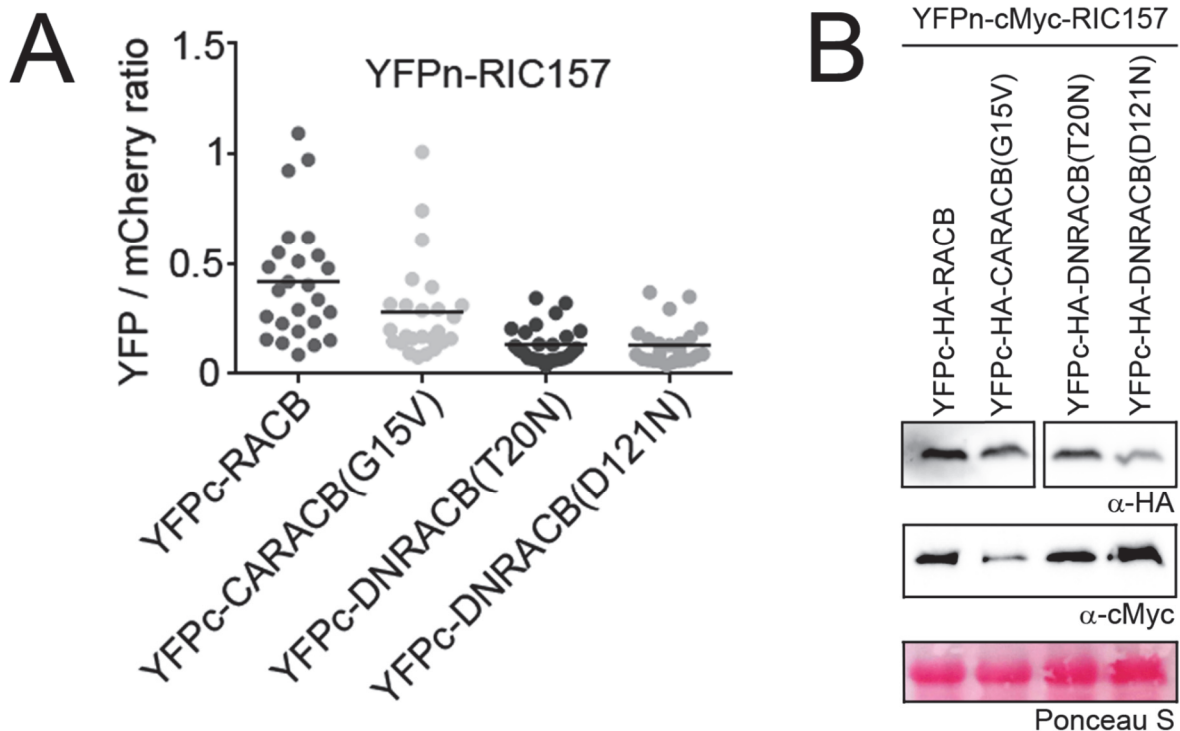
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1131 **Suppl. Fig. S4: Protein stability in yeast.** Immunoblots probed with α -cMyc to detect
 1132 RACB variants fused to GAL4 binding domain (BD; bait) encoded on pGBKT7 and α -
 1133 HA to detect RIC157 variants fused to GAL4 activation domain (AD; prey) encoded on
 1134 pGADT7 after transformation of yeast strain AH109. Total yeast protein was extracted
 1135 as described in Experimental Procedures. Molecular weight is indicated in kiloDalton
 1136 (kDa). Ponceau S staining shows protein loading. Stable expression of RIC157
 1137 variants fused to GAL4 activation domain could not be detected in immunoblots (arrow
 1138 indicates expected bandsizes).

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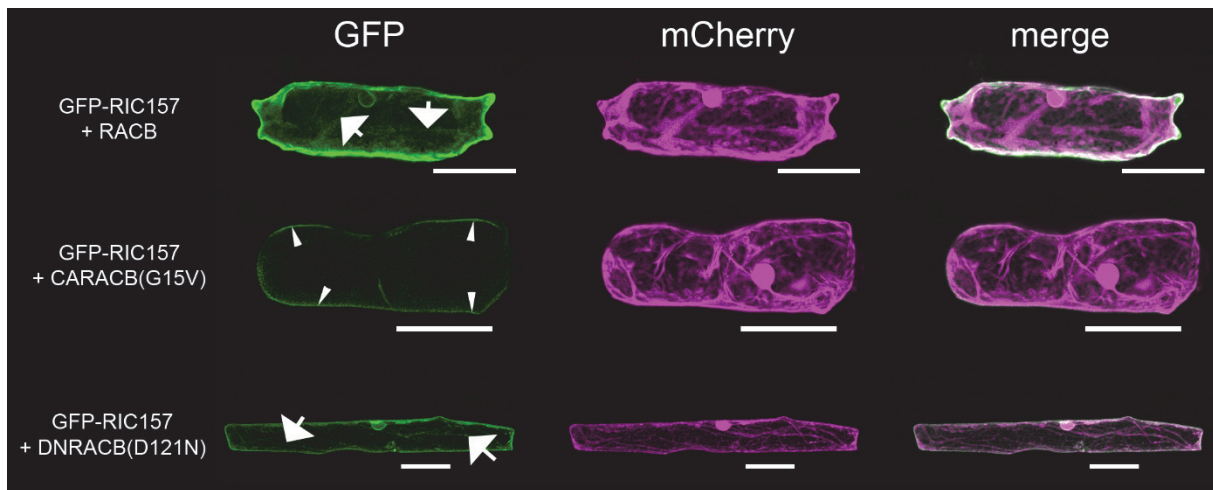


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1141 **Suppl. Figure S5: Ratiometric BiFC analyses and BiFC fusion protein stability.**

1142 A) Barley epidermal cells were transiently transformed with indicated constructs
1143 encoding for BiFC fusion proteins. Cytosolic mCherry expression was used in each
1144 sample as transformation marker and to quantify reconstituted YFP fluorescence
1145 ratiometrically. The whole cell area was taken as region of interest. Graph shows the
1146 mean of the YFP/mCherry fluorescence ratio, taken from at least 20 cells (shown as
1147 dots. B) Barley mesophyll protoplasts were prepared and transformed with BiFC
1148 constructs as described in Experimental procedures. To confirm stable *in planta*
1149 expression, immunoblots were probed with α -HA to detect YFPc-RACB fusion proteins
1150 and α -cMyc to detect YFPn-RIC157 fusion proteins. Ponceau S staining shows equal
1151 protein loading.

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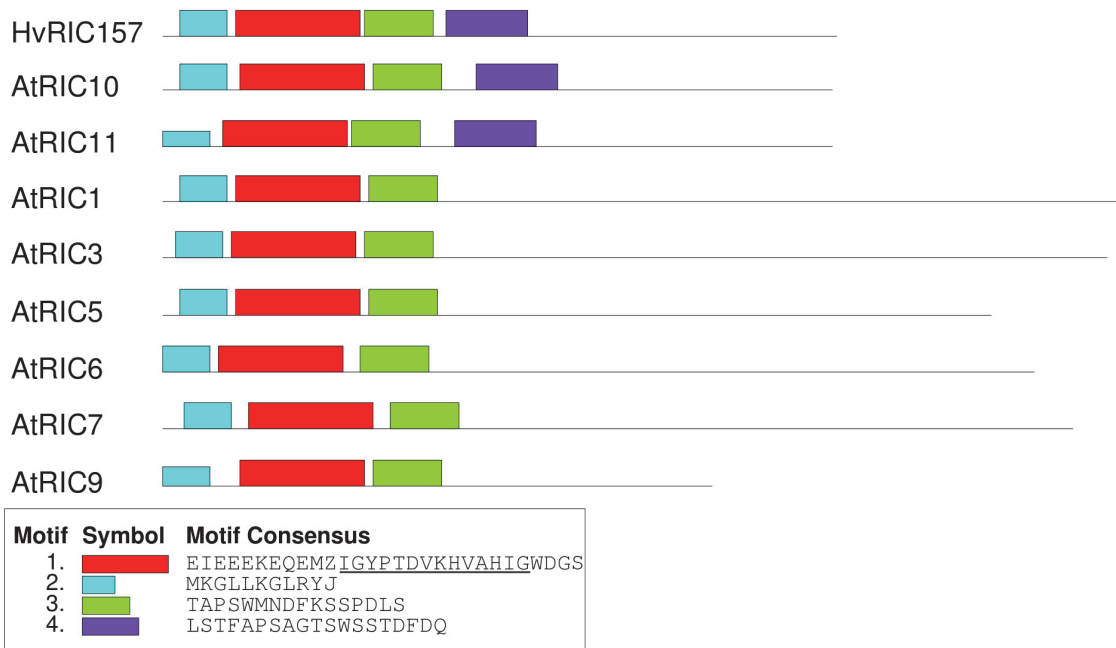


1153

1154 **Suppl. Fig. S6: RIC157 is recruited to the cell periphery by activated RACB.**

1155 Confocal laser scanning microscopy of barley epidermal cells 1d after transformation
 1156 via particle bombardment. GFP-RIC157 localises to the cytoplasm, but not the nucleus
 1157 and is recruited to the cell periphery exclusively by non-tagged CARACB(G15V), but
 1158 not DNRACB(D121N). A construct for cytosolic mCherry expression was
 1159 simultaneously used to check transformation efficiency. Arrows indicate cytoplasmic
 1160 strands, arrow heads point towards GFP fluorescence accumulation at cell periphery.
 1161 Microscopy pictures show maximum projections of at least 15 optical sections taken at
 1162 2µm increments. Bar = 50µm.

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1165 **Suppl. Fig. S7: Barley RIC157 shows limited sequence similarity to RIC proteins**
 1166 **from *Arabidopsis thaliana*.** Primary sequences of RIC proteins (schematically shown

1167 in top part of the figure) were analysed for domain homologies using MEME online
 1168 software (<http://meme-suite.org/tools/meme>). Consensus sequence of the four most
 1169 similar motifs are shown below. Underlined sequence denotes CRIB domain. Hv =
 1170 *Hordeum vulgare*, At = *Arabidopsis thaliana*.

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1172

Primer name	Gene (construct)	Sequence
RIC157_GW_for	<i>RIC157</i>	5'-AAAAAGCAGGCTCACAAATGGCGGTAAGATGAAGGG-3'
RIC157_GW_rev+STOP	<i>RIC157</i>	5'-AGAAAGCTGGGTACCAGCCTCCGGATCAGACGACTCGAACCCCTCTTTGC-3'
RIC157delCRIB_for	<i>RIC157 Δ CRIB</i>	5'-GCTCAAAAGGAGCATGAGATGGAATGGGCACCAGTGACACATC-3'
RIC157delCRIB_rev	<i>RIC157 Δ CRIB</i>	5'-CACTGGTGCCCAATCCATCTCATGCTCCTTTGAGC-3'
CRIB157H37&40Y_for	<i>RIC157-H37-40Y</i>	5'-CCTACAGATGTAAGATATGTGGCTTACATAGGTTTGGGCACCAGTGACACATCTCC-3'
CRIB157H37&40Y_rev	<i>RIC157-H37-40Y</i>	5'-GGAGATGTGTCACTGGTGCCCAACCTATGTAAGCCACATACCTTACATCTGTAGG-3'
attB1	<i>attB1</i>	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCACAA-3'
attB2	<i>attB2</i>	5'-GGGGACCACCTTTGTACAAAGAAAGCTGGGTACCAG-3'
RIC157_RNAi_NotI_for	<i>RIC157 RNAi</i>	5'-AATTGCGGCCGCAAGATGAAGGGAATCTTCAAAGGGC-3'
RIC157_RNAi_XbaI_rev	<i>RIC157 RNAi</i>	5'-AATTTCTAGAACGCCGTCGCGAAGGAGGCCCTCGACC-3'
RIC157_RNAi_EcoRI_for	<i>RIC157 RNAi</i>	5'-AATTGAATCTTGGGCACCAGTGACACATCTCC-3'
RIC157_RNAi_EcoRI_rev	<i>RIC157 RNAi</i>	5'-AATTGAATCTTCCATCTCATGCTCCTTTTGGAGC-3'
RIC157_BamHI_for	<i>RIC157</i>	5'-AATTGGATCCATGGCGGTAAGATGAAGGGAATC-3'
RIC157_KpnI_rev	<i>RIC157</i>	5'-AATTGGTACCCTAGACGACTCGAACCCCTCTTGC-3'
RIC157_for	<i>RIC157</i>	5'-ATGGCGGTAAGATGAAGG-3'
RIC157_rev	<i>RIC157</i>	5'-GACGACTCGAACCCCTCTTGC-3'
RACB_D121N_fw	<i>DNRACB(D121N)</i>	5'-CTCGTGGGAACAAAGCTTAATCTTCGAGATGACAAG-3'
RACB_D121N_rv	<i>DNRACB(D121N)</i>	5'-CTTGTCATCTCGAAGATTAAGCTTTGTTCCACGAG-3'
RACB_GW_for	<i>RACB</i>	5'-AAAAAGCAGGCTCACAAATGAGCGCTCCAGTTTCATAAAGTGC-3'
RACB_GW_rev	<i>RACB</i>	5'-AGAAAGCTGGGTACCAGCCTCCGGACAAGATGGAGCAAGCCCCC-3'
delCSIL_for	<i>RACB Δ CSIL</i>	5'-GAAGAAAAAGGCGCAGAGGGGGGCTTGATCCATCTTGATGTCGGAGCGGTG-3'
delCSIL_rev	<i>RACB Δ CSIL</i>	5'-CACCGCCTCCGGACTACAAGATGGATCAAGCCCCCTCTGCGCCTTTTCTTC-3'
GW_RfA_mCherry-F	<i>mCherry</i>	5'-GCTGTACAAGATCACAAAGTTTGTACAAAAAAGCTG-3'
GW_RfA_meGFP-F	<i>meGFP</i>	5'-GCTGTACAATAACAAAGTTTGTACAAAAAAGCTG-3'
GW_RfA_Xba-R	<i>RfA</i>	5'-TGCTGCAGGTCGACTCTAGAATCACCACCTTTGTACAAGAAAGCTG-3'
GW_Xba_RfB-F	<i>RfB</i>	5'-GGTACCCTGGGATCCTCTAGAATCAACAAGTTTGTACAAAAAAGCT-3'
GW_RfB-R	<i>RfB</i>	5'-TGCTCACCATATCAACCACCTTTGTACAAGAAAGCT-3'
meGFP-STP-F	<i>meGFP</i>	5'-AGTGGTTGATATGGTGAAGCAAGGGCGAGG-3'
mCherry-STP-F	<i>mCherry</i>	5'-AGTGGTTGATATGGTGAAGCAAGGGCGAGG-3'
XFP-noSTP_Xba-F	<i>XFP</i>	5'-GGTACCCTGGGATCCTCTAGAATGGTGAAGCAAGGGCGAGG-3'
XFP-noSTP-R	<i>XFP</i>	5'-AACTTGTGATCTTGTACAGCTCGTCCATGCC-3'
meGFP-noSTP-R	<i>meGFP</i>	5'-AACTTGTGATTTTGTACAGCTCGTCCATGCC-3'
mCherry-STP_Xba-R	<i>mCherry</i>	5'-TGCTGCAGGTCGACTCTAGATTACTTGTACAGCTCGTCCATGCC-3'
meGFP-STP_Xba-R	<i>meGFP</i>	5'-TGCTGCAGGTCGACTCTAGATTATTTGTACAGCTCGTCCATGCC-3'

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1174 **Suppl. Table 1: Primers used in this study**