Distinct ROPGEFs successively drive polarization and outgrowth of root hairs

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SUMMARY

Root hairs are tubular protrusions of the root epidermis that significantly enlarge the exploitable soil volume in the rhizosphere. Trichoblasts, the cell type responsible for root hair formation, switch from cell elongation to tip growth through polarization of the growth machinery to a pre-defined root hair initiation domain (RHID) at the plasma membrane. The emergence of this polar domain resembles the establishment of cell polarity in other eukaryotic systems [1-3]. Rho-type GTPases of plants (ROPs) are among the first molecular determinants of the RHID [4,5] and later play a central role in polar growth [6]. Numerous studies have elucidated mechanisms that position the RHID in the cell [7-9] or regulate ROP activity [10-18]. The molecular players that target ROPs to the RHID and initiate outgrowth, however, have not been identified. We dissected the timing of the growth machinery assembly in polarizing hair cells and found that positioning of molecular players and outgrowth are temporally separate processes that are each controlled by specific ROP guanine nucleotide exchange factor (GEFs). A functional analysis of trichoblast-specific GEFs revealed GEF3 to be required for normal ROP polarization and thus efficient root hair emergence, while GEF4 predominantly regulates subsequent tip growth. Ectopic expression of GEF3 induced the formation of spatially confined, ROP-recruiting domains in other cell types, demonstrating the role of GEF3 to serve as a membrane landmark during cell polarization. Our findings suggest that morphogenetic programs in plants employ distinct regulatory modules for the alignment and activation of the cellular growth machinery.

RESULTS AND DISCUSSION

Temporal analysis of hair cell polarization reveals phased deployment of the tip-growth machinery

To dissect the process of breaking cellular symmetry and initiating polar growth in plants, we analyzed the gradual assembly of the tip growth machinery in trichoblasts using specific markers for cytoskeletal rearrangement, plasma membrane specialization, cell wall modification, vesicle trafficking, and Rho-GTPase signaling. Using stable transgenic *Arabidopsis* lines expressing fluorescently labeled versions of these protein markers, we determined the timing of their polarization at the RHID during trichoblast differentiation. As the *Arabidopsis* root represents a time-axis of development from stem cells to mature cells, we numbered the developmental stages (-7 to +3) within a cell file, with the first cell to exhibit a detectable bulge as stage +1 (**Figure 1A**). We used the integral plasma membrane marker GFP-LTI6B [19] as a reference and calculated the polarity index of fluorescently labeled marker proteins to quantify protein accumulation at the RHID (**Figure 1B**). Our survey unveiled a two-phase assembly of the tip growth machinery: an initiation phase, during which the RHID is positioned and predefined, followed by the tip growth phase. Consistent with previous reports [4,5], mCitrine-labeled (mCit) GTPase ROP2 associated with the RHID during the early initiation phase (stage -4), well before any detectable cell bulging (**Figure 1B, C, Supplemental Figure S1**). The timing of mCit-ROP2 polarization appeared to be largely independent from promoter activity, as analysis of an estradiol-inducible reporter construct for mCit-ROP2 resulted in a similar timing of RHID association, albeit with higher cytosolic signal (**Figure 1C, Supplemental Figure S1**). Closely related GTPases ROP4 and ROP6 exhibited a similar timing as ROP2, when inducibly expressed (**Figure 1C, Supplemental Figure S1**), which indicates that ROP GTPases are prepositioned well before growth is activated. The only tested ROP-effectors that were found to polarize after ROPs but still during the initiation phase, were the PHOSPHATIDYLINOSITOL-4-PHOSPHATE 5-KINASE 3 (PIP5K3) [20], and the NADPH oxidase ROOT HAIR DEFECTIVE 2 (RHD2) [21,22]. PIP5K3 and RHD2 have both been shown to be involved in ROP-dependent root hair growth regulation by modulating vesicle traffic to the plasma membrane and cell wall rigidity, respectively.

The second phase of root hair emergence was characterized by the polarization of known ROP effectors such as (ARPC2), a subunit of the actin polymerizing ARP2/3 complex [23], and the markers for vesicle transport and secretion SYP123 and SEC3A [24-26] (**Figure 1C, Supplemental Figure S1**). The onset of bulge formation was further characterized by local elevation of cytosolic Ca^{2+} as reported by the sensor RGECO-1 [27,28], as well as the formation of $PI(4,5)P_2$ at the emerging apex as indicated by the reporter P15Y [29]. The receptor-like kinase FERONIA, which activates ROPs during root hair tip growth [30], accumulated after bulging in stage +2 (**Figure 1C, Supplemental Figure S1**). The temporal separation of ROP positioning and growth activation led us to the conclusion that ROP regulation must also be temporally controlled.

Therefore, we hypothesized that upstream regulators of ROP GTPases exist, that are in charge of phase-specific ROP recruitment and control their activation.

ROPGEFs exhibit specific spatiotemporal patterns of expression and subcellular localization

ROPs have been extensively studied during cell elongation [31], stomata regulation [32], pavement cell development [33,34], and in regulation of the two tip-growing plant cell types, pollen tubes [35] and root hairs [4,5]. As molecular “switches”, the activity-state of GTPases depends on regulators that “toggle” the switch between active or inactive [36,37]. GTPase-activating proteins (GAPs) facilitate the catalytic cleavage of a bound GTP to GDP, rendering the GTPase inactive. Guanyl-nucleotide exchange factors (GEFs) replace the bound GDP for a GTP, thereby switching the GTPase into an active state and enabling the interaction with downstream effectors [37,38].

To identify candidate ROP regulators that play potential roles in root hair initiation and growth regulation we mined cell-type-specific gene expression data [39-41] and compared expression profiles of ROPGEFs of the PRONE family [42] and SPK1, the only DOCK family GEF [43], in root trichoblasts and atrichoblasts. Among these ROPGEFs (hereafter abbreviated as GEFs), the expression of GEF3 and GEF4 showed the strongest root hair-specificity, followed by GEF12, GEF11, GEF14, and GEF10 in descending order (**Supplemental Figure S2**). Other GEFs, including SPK1, did not exhibit stronger expression in hair cells and were therefore excluded from further analysis. To determine in which stage of hair cell maturation the candidate GEFs play their roles, we created translational fusions to mCitrine under the control of the respective native promoters and analyzed their expression pattern in growing roots. We observed a diverse distribution of expression patterns among the remaining GEFs, with a surprisingly unique pattern for each gene (**Figure 2A**). We further analyzed the timing of polarization at the RHID for each mCit-labeled GEF and compared it to the timing of mCit-ROP2. We found that mCit-GEF3 and mCit-GEF14 reached significant polarization consistently one cell stage earlier than mCit-ROP2 (**Figure 2B**). A major difference between both expression patterns was that GEF14 expression lasted from the meristematic zone (MZ) to the elongation zone (EZ), whereas GEF3 expression was first observed in the root transition zone (TZ, in-between MZ and EZ) and lasted beyond hair emergence (**Figure 2A**). The beginning of gene expression of GEF4 and GEF12 was detected first in EZ and the polarization of both proteins at the RHID coincided with the onset of bulge formation (**Figure 2A, B**). This specific behavior of highly related genes indicates that they take over specialized functions during defined time windows in differentiating cells.

In addition to the specific expression pattern of GEF3, its localization stood out in comparison to all other GEFs, as mCit-GEF3 appeared highly polarized at the cell periphery of the RHID (**Figure 2A, C**). While other GEFs, like mCit-GEF4, exhibited a lower degree of polarization and association with the plasma membrane (**Figure 2A, D**). mCit-GEF3 appeared highly concentrated in a circular polar domain with a diameter of 9.6 µm (± 2.5 µm), being barely detectable outside this domain (polarity index at cell "+1": 14.4 ± 2.2) (**Figure 2C, Supplemental Figure S3**). We tested

131 whether the timing of GEF polarization was dependent on the timing of gene expression and
132 analyzed the polarity indices of mCit-GEF3 and mCit-GEF4 under the control of estradiol-inducible
133 promoters. In both cases, the earlier gene expression did not result in an earlier RHID polarization
134 of the protein (**Supplemental Figure S1**), but rather delayed the time point of significant
135 polarization, likely due to higher cytosolic levels that affected the polarity index calculation. Its
136 independence from gene expression indicates that the timing of polarization is an intrinsic feature
137 that may be controlled by further partners of unknown identity.
138 To determine the functional role of the investigated GEFs we analyzed T-DNA insertion lines for
139 phenotypes in root hair initiation and development. Root hair density was used as a measure for
140 the general ability to develop root hairs, distance of the first bulge from the root tip was used to
141 analyze delays in the timing of root hair initiation and root hair length was used as measure for
142 regulation of tip growth. Only in *gef3* mutant alleles, root hair density was reduced and root hair
143 initiation was delayed, (**Figure 2E - G, Supplemental Figure S4**), while hair length seemed only
144 slightly affected (**Figure 2H**). In *gef3-1*, which lacks almost all full length transcript, the observed
145 phenotypes could be rescued with a GEF3::mCit-GEF3 construct (**Supplemental Figure S4**),
146 further verifying that loss of GEF3 function causes the phenotypes and confirming the functionality
147 of the fusion construct. Furthermore, other mutant alleles of *gef3* showed a similar delay in root
148 hair initiation (**Supplemental Figure S4**). All other *gef* mutants, including *gef4-2*, exhibited no
149 negative effects on bulge distance or root hair density (**Figure 2E - G**), but *gef4-2* lines did show a
150 reduced hair length (**Figure 2H**). This indicates distinct roles for GEF3 and GEF4, where GEF3 is
151 responsible for root hair initiation and bulging, while GEF4 is regulating root hair tip growth.
152
153 **GEF3 is required for efficient root hair emergence and polar recruitment of ROP2**
154 We have shown that GEF3 accumulated very specifically at the RHID, preceded ROP2
155 accumulation, and was required for proper root hair development. Therefore, GEF3 was selected
156 as a promising potential regulator of ROP dependent root hair initiation.
157 To determine the temporal relationship of GEF3 and ROP2 recruitment to the RHID in detail,
158 we observed the localization of both proteins at higher resolution over time. We employed a new
159 microfluidic device, termed RootChip-8S, for long-term imaging of growing *Arabidopsis* roots. The
160 RootChip-8S, is an adapted version of previously published RootChip designs [44-46] and consists
161 of eight separate observation chambers that can be individually perfused to provide stable growth
162 conditions and restrict root movement in Z to prevent focal drift (**Supplemental Figure S5**). The
163 first detectable signal of GEF3::mCit-GEF3 was observed in the transition zone of the root, shortly
164 after the last cell division (**Figure 2 A and 3 A**). Time-lapse imaging showed early targeting of mCit-
165 GEF3 to the rootward cell pole (**Figure 3 A**). As the protein gradually enriched at the RHID over
166 approximately 30 min, its localization at the rootward cell pole vanished (**Figure 3 A, B**). The GEF3
167 domain eventually stabilized at a consistent diameter of 9.6 µm (± 2.5 µm; *n*=17; FWHM), with its
168 center 9.0 µm (± 2.2 µm) from the rootward cell pole (**Figure 3 B, Supplemental Figure S3**). To
169 validate the earlier RHID accumulation of GEF3 compared to ROP2 through simultaneous

170 visualization in the same cells, we imaged their localization in growing roots co-expressing
171 fluorescently labeled versions of both proteins and found that mCit-GEF3 accumulated at the RHID
172 one stage earlier than mTurquoise2-ROP2 (mTq2-ROP2) (**Figure 3 C**).
173 To test if GEF3 directly influences ROP2 recruitment to the RHID, we expressed mCit-ROP2 in
174 the *gef3-1* T-DNA insertion mutant. Strikingly, we did not observe any significant polarization of
175 mCit-ROP2 in the *gef3-1* mutant (**Figure 3 D, E**). Some cells in *gef3-1* were still able to produce
176 bulges, but these bulges lacked polar ROP2 accumulation (**Figure 3 D, E**). Interestingly, the
177 general membrane association of mCit-ROP2 was unaffected, as the localization appeared similar
178 to that in other cell types. This shows that GEF3 function is required for ROP2 recruitment
179 specifically to the RHID.
180 If the lack of ROP activity is responsible for the phenotypes observed in *gef3-1* mutants, loss of
181 function mutants of ROPs should have similar phenotypes. We combined 2 previously described
182 T-DNA lines for ROP2 and ROP4 [33,47], and analyzed the single mutants and the double mutant
183 line for phenotypes in root hair initiation. For both traits, root hair density and distance of the first
184 bulge to the root tip, the double mutant *rop2-1/rop4-1* showed a clear phenotype, while the single
185 mutant lines showed no significant effects (**Figure 3 F - H**). Thus, lack of GEF3 or ROP2/ROP4
186 results in similar phenotypes, indicating a functional link between these proteins. In contrast to the
187 loss of ROP2 polarity in *gef3-1* mutants, mCit-GEF3 polarity is still observed in *rop2-1/rop4-1*
188 mutants (**Figure 3 I, K**). mCit-GEF3 polarity was unaffected in the early elongation zone (**Figure 3**
189 **I, K; Stage I**), but was slightly reduced in later stages (**Figure 3 I, K; Stage II & III**) compared to
190 mCit-GEF3 polarity in Col-0 background (**Supplemental Figure S1**). This indicates that initial
191 GEF3 polarization is largely ROP independent, while in later stages of root hair development GEF3
192 polarity is positively affected by ROPs. The question remained how GEF3 fulfills its function as a
193 factor directing polarization of ROP2. To test if GEF3 was capable of recruiting ROP2 through
194 physical interaction, we used a ratiometric bimolecular fluorescence complementation (rBiFC)
195 assay with GEF3-cYFP with either an nYFP-carrying ROP2 (wild-type), a constitutively active CA-
196 ROP2 (G14V), a dominant-negative DN-ROP2 (D120A), or a truncated *rop2*ΔN (Δ1-79) that lacks
197 its interaction domain (**Supplemental Figure S6**) [5,48], co-expressed transiently in *Nicotiana*
198 *benthamiana* leaf epidermis cells [49]. All GEF3-cYFP/nYFP-ROP2 combinations, except for
199 *rop2*ΔN, resulted in detectable YFP fluorescence (**Figure 3 L, M, Supplemental Figure S6**). This
200 finding was corroborated by a split-ubiquitin assay in yeast [50], testing the same putative interactor
201 combinations. Except for *rop2*ΔN, all forms of ROP2 yielded yeast growth, indicating physical
202 interaction with GEF3 (**Supplemental Figure S6**). This shows that GEF3 physically interacts with
203 the active and inactive form of ROP2 via its interaction domain. Consistently, mCit-*rop2*ΔN
204 expressed in *Arabidopsis* trichoblasts did not assume polar localization at the RHID (stage +1,
205 **Figure 3 N, O**), demonstrating that the interaction domain of ROP2 is required for its RHID
206 recruitment.

207 These results show that GEF3 is necessary for ROP polarization by recruiting ROP2 to the
208 RHID via direct physical interaction. Furthermore, ROP activity is not essential for GEF3
209 polarization, but enhances GEF3 polarity.
210
211 **GEF3 defines the RHID and guides ROP polarization**
212 To investigate whether GEF3 is not only necessary but also sufficient for ROP2 polarization, we
213 studied transgenic lines, inducibly overexpressing mCit-GEF3 (mCit-GEF3ox) in Col-0 or in
214 ROP2::mTq2-ROP2 background. Upon overexpression of mCit-GEF3 we observed that additional
215 patch-like GEF3 domains formed in trichoblasts and, surprisingly, also in atrichoblasts (**Figure 4**
216 **A**). Interestingly, in overexpression lines, we frequently observed mCit-GEF3 accumulation at the
217 shootward sides of cells in the meristem or the early elongation zone (**Figure 4 A**). The final position
218 of the primary RHID was not altered compared to Col-0 (**Supplemental Figure S7**). All observed
219 normal and ectopic mCit-GEF3 accumulations resulted in the simultaneous recruitment of mTq2-
220 ROP2 (**Figure 4 A**).
221 We took advantage of the ubiquitous overexpression of mCit-GEF3 and determined its
222 localization in cells outside the root. In epidermal cells of the hypocotyl, we observed multiple
223 occurrences of ectopic GEF3 accumulation into domains that strikingly resembled those of RHIDs
224 in trichoblasts (**Figure 4 B**). Consistently, these ectopic mCit-GEF3 domains also lead to
225 recruitment of mTq2-ROP, which never occurred in hypocotyl cells under control conditions (**Figure**
226 **4 B**). Except for trichoblasts, mCit-GEF3ox did not lead to bulge formation in other cells, indicating
227 that ROP polarization alone was not sufficient to trigger outgrowth and further trichoblast-specific
228 factors are required.
229 Since the analysis of gene expression, protein localization and loss-of-function phenotypes all
230 suggested a differential timing of GEF3 and GEF4 function, we hypothesized that gain-of-function
231 phenotypes should also reflect their specific roles in root hair initiation and growth. We therefore
232 compared the phenotypic effects of mCit-GEF3ox to mCit-GEF4ox, which is known to induce root
233 hair branching in trichoblasts [51]. In mCit-GEF4ox lines, bulges with branched tips, which failed to
234 form long tube-like hairs, occurred in 30.5% of the observed cells (**Figure 4 C, D**). mCit-GEF3ox
235 roots also displayed root hairs with branched tips in 20.6% of all trichoblasts, resembling mCit-
236 GEF4 overexpression (**Figure 4 C, D**). However, mCit-GEF3ox further led to the formation of
237 additional bulges, with 5.5% of trichoblasts showing multiple unbranched bulges and 21.4% of the
238 cells showing multiple branched bulges within one cell (**Figure 4 C, D**). This phenotype bears
239 striking similarities to *scn1* mutants that lack a ROP-inhibiting RhoGDI [11], which further points
240 towards a gain of ROP activity in mCit-GEF3ox roots at an early stage during RHID positioning.
241 We observed mCit-GEF3 accumulations in tips of branched root hairs and mTq2-ROP2 was
242 generally present in the same locations (**Figure 4 E**), thus confirming that mCit-GEF3ox was also
243 responsible for the formation of additional polar domains in branching hairs. These differential
244 effects of GEF3 and GEF4 overexpression resemble the differential timing of their polar localization
245 and further support the hypothesis that both proteins serve specific functions during different

246 phases of root hair development, with GEF3 being decisive for the early formation of the RHID and
247 ROP2 recruitment, and GEF4 being involved in downstream activation of ROP2 to trigger growth.
248
249 **GEFs serve as positional cues during cell polarization**
250 To our knowledge, GEF3 is not only the first ROPGEF member reported to precede ROP
251 polarization during tip growth initiation, but it is also the first example for a plant protein that, when
252 overexpressed, is capable of inducing the ectopic formation of polar, ROP-recruiting domains. Our
253 finding, that GEF3 acts upstream of ROP2, challenges the idea that ROPs are self-organizing key
254 initiators of cell polarization [52]. Our results suggest a GEF3-mediated recruitment and temporally
255 controlled ROP activation by differentially timed GEFs at this predefined location. Therefore, the
256 previously modeled self-assembly mechanism of ROP2 at the RHID may not be the only driving
257 force for polarization.
258 Evidence for a GTPase-positioning function of GEFs can also be found in other organisms.
259 Recent work shows that during cellularization in Drosophila embryos, the GEF Dizzy and the
260 heteromeric GEF complex ELMO-Sponge are required for polarization of the GTPase Rap1
261 [38,53]. It remains, however, challenging to establish the molecular sequence of GEF and GTPase
262 functions in the context of a developing embryo. In budding yeast, Cdc42 is activated by its GEF
263 Cdc24 during both budding and shmoo formation [54-56]. To initiate budding in yeast, the GEF
264 Bud5 is recruited to the edge of the previous cell-division site by the landmark Axl2 [57]. Stabilized
265 by scaffold proteins, Bud5 and the GEF Cdc24 then locally activate the GTPases Rsr4 and Cdc42
266 [58-63].
267 Using a GEF-scaffold complex as landmark for the targeted deployment of the growth machinery
268 may therefore represent a common design principle among eukaryotic cells to prevent
269 spontaneous and random polarization and, instead, drive growth in a directed manner. Yet, the fact
270 that ROPGEFs share no conserved features with animal or fungal GEFs [42], indicates that the
271 problem of robust cell morphogenesis has been solved several times during evolution to account
272 for specific differences in cellular growth mechanisms, organ development and lifestyle.

MATERIALS AND METHODS

Plant material

Arabidopsis thaliana ecotype Col-0 was used as wildtype in this study. The mutant lines *rop2-1* (SALK_055328C), *gef3-1* (SALK_079879C), *gef3-2* (SALK_079885), *gef3-3* (SAIL_294_A09), *gef3-4* (SALK_021751) *gef3-5* (SALK_046978C) *gef4-2* (SALK_107520) were obtained from NASC (Nottingham Arabidopsis Stock Centre http://arabidopsis.info/). The *rop4-1* mutant [34] was kindly provided by Markus Grebe (Potsdam). Double mutants were made by crossing and selection for double homozygous plants by PCR (Primers used for genotyping, see Table S3). The GFP-SYP123 and GFP-SYP132 lines [24] were kindly provided by Masa H. Sato (Kyoto), SEC3A-GFP lines [25] were kindly provided by Tijs Ketelaar (Wageningen), the GFP-ARPC2A line [23] was kindly provided by Kateřina Schwarzerová (Prague), and the PI(4,5)P_2-reporter line (P15Y) [29] was kindly provided by Yvon Jaillais (Lyon). To generate transgenic Col-0 lines expressing GFP-tagged FER, Col-0 was transformed with pFER::FER-GFP containing pMDC111 [64], kindly provided by Ueli Grossniklaus (Zurich).
Information on all genes referenced in this work, including mutant alleles and sources is provided in Supplementary Information, **Table S1**.

RNA extraction and expression analysis

Seedlings were grown on mesh on a ¼ MS plate. Root tissue was harvested 10 dag, immediately frozen in liquid nitrogen and ground in a tissue lyser (Qiagen) with metal beads. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen). After DNAse digest (Thermo Fischer Scientific), 0.5 µg total RNA was used for cDNA synthesis with oligo(dT) primers (RevertAid First Strand cDNA Synthesis Kit, Thermo Fischer Schientific). To test for the presence of Gef3 mRNA, 2µl of this cDNA was used for PCR amplification (40 cycles). The primers used for the expression analysis are listed in Supplementary Information, **Table S2**.

Growth conditions and inducible expression

Plants were grown under long day conditions (16 h light) at 21°C. Seedlings used for experiments were grown on ¼ MS medium (pH 5.7) without sucrose, supplied with 0.8% plant agar (Duchefa). Transgenic plants transformed with an estradiol inducible construct were induced with ¼ MS medium containing 20 µM Estradiol (Stock 20 mM in Ethanol) and 0.01% Silwet L77. The time after induction differed between the individual expression constructs and ranged from 3 – 12 h to get sufficient signal for imaging. In general, for imaging of protein localization using inducible expression constructs, time after induction was as low as possible to avoid overexpression artefacts.

310 **Plant transformation and selection**

311 The agrobacterium strain GV3101::pMP90RK pre-transformed with the helper plasmid pSoup was
312 used for all transformations of *Arabidopsis thaliana*. Plants were transformed using floral dipping
313 and transgenic T1 plants were recovered after the appropriate selection. Selection of constructs
314 containing mTurquoise2 (mTrq2) was done on plates containing 50 µg/ml Kanamycin. T1 Selection
315 of constructs containing mCitrine (mCit) was done on soil spraying 3x within 2 weeks (7 – 21 day
316 old seedlings) with Basta®-Solution containing 200 µg/ml Glufosinate-ammonium and 0.05%
317 Tween20. Further generations were selected on plates containing Basta®-Solution to a
318 concentration of 7.5 µg/ml Glufosinate-ammonium. At least 10 independent transgenic lines per
319 expression construct were recovered and checked for fluorescence. The used lines were selected
320 upon good fluorescence and least phenotype and confirmed by PCR.
321

322 **Molecular cloning**

323 All cloning for expression vectors used in Arabidopsis was performed using the GreenGate system
324 [65] and base vector pGGZ003 was used for all expression constructs. A List of all expression
325 constructs including module composition and tagging site is provided in Supplementary
326 Information, **Table S3**. Where internal BsaI (Eco31I) sites were present, they were mutated fusing
327 two PCR fragments, with one mutating the internal BsaI site by an altered primer sequence at this
328 site. After cloning the individual modules into the entry vectors, the complete fragment was checked
329 by sequencing. To confirm the correct assembly of modules into pGGZ003, the presence and
330 orientation was checked by sequencing. As fluorophores, mCitrine (mCit) [66] and mTurqoise2
331 (mTrq2) [67] were fused to the gene of interest separated by a short GAGAGA-Linker. mCit was
332 cloned from the Ca^{2+} indicator CerTN-L15, containing the common folding mutations V163A and
333 S175G [68], and made monomeric by introducing the A206K mutation. mTrq2 was cloned from the
334 Addgene vector LifeAct-mTrq2 (ID #36201). A List of all primers used for molecular cloning is
335 provided in Supplementary Information, **Table S2**.
336 For estradiol inducible expression constructs [69], the XVE gene was fused to the promoter (635
337 bp) and terminator of *UBI10* (AT4G05320). This expression cassette was cloned in reverse
338 orientation into the entry vector pGGA000 together with the oLexA minimal 35S promoter element.
339 Both elements were separated by a 560 bp spacer. This resulted in a single module that could be
340 used as a promoter module for GreenGate cloning.
341 For expression of genes under control of their native promoter, the following sequences upstream
342 of the start codon were cloned into pGGA000: *GEF3* -1743 bp; *GEF4* -1500 bp; *GEF10* -1534 bp;
343 *GEF11* -1458 bp; *GEF12* -701 bp; *ROP2* -1503 bp; *PIP5K3* -1193 bp.
344 For the mbSUS and rBiFC analysis, constructs (listed in Supplementary Information, **Table S3**)
345 were generated using Gateway technology or the 2in1 cloning system (Grefen and Blatt 2012).
346 Coding sequences were PCR-amplified and inserted via 'BP' recombination into either pDONR207,
347 pDONR221-P1P4, or pDONR221-P3P2, respectively, and confirmed by sequencing. Resulting

348 ‘Entry constructs’ were cloned via ‘LR’ recombination into Destination vectors for either mbSUS or
349 rBiFC [70].
350

351 **Phenotyping of knockout and overexpression mutant lines**

352 Primary roots (7 dag) were used for all phenotyping experiments. For phenotyping of knockout
353 mutant lines the distance of the first bulge to the root tip was defined. The first visible swelling of
354 the cell outline was defined as first bulge, even when this morphological change was very little. The
355 root hair density was analyzed in the next 2 mm. The root hair length was analyzed measuring root
356 hairs growing perpendicular to the root in a region 3-6 mm away from the root tip. For phenotyping
357 the overexpression lines, roots were induced by spraying with medium containing 20 µM Estradiol.
358 The position of the root tip was marked just after induction and normal growing roots were imaged
359 and analyzed 24-26 hours after induction (h.a.i.). A region approximately 2-4 mm from the root tip
360 was used for the analysis of the overexpression phenotype.
361

362 **Propidium iodide staining**

363 Primary roots (7dag) were stained for 5 min in 10 ug/ml propidium iodide (diluted in 1/4MS, from
364 1mg/ml Stock), washed in medium and imaged directly. The staining of different root tissues is very
365 uneven. For better visualization of brighter and dimmer regions the gamma of the images was
366 adjusted (See image processing).
367

368 **Ratiometric Bimolecular Fluorescence analyses (rBiFC)**

369 *Agrobacterium*-mediated transient transformation of 4-6 week old *N. benthamiana* leaves with 2in1
370 rBiFC constructs as described previously [71]. Fluorescence intensities were recorded 36h post
371 infiltration for approximately 30 images per construct as described in [49]. Ratios between
372 complemented YFP fluorescence and RFP were calculated and plotted using BoxplotR.
373 Immunoblotting verified protein expression utilizing anti-MYC conjugated to peroxidase antibodies
374 and anti-HA peroxidase conjugated antibodies, respectively.
375

376 **Mating-based Split Ubiquitin Assay (‘Cyto-SUS’)**

377 Bait/Cub fusion and preys/Nub fusions were generated by cloning the coding region for *GEF3* and
378 different *ROP2* versions into the Gateway-compatible vectors pMetOYC-Dest and pNX35-Dest,
379 respectively. These were transformed into haploid yeast strains THY.AP4 (Cub fusion) and
380 THY.AP5 (Nub fusions) as described previously [72]. After mating, diploid yeasts were dropped in
381 10 times OD dilutions on vector- (CSM-Leu-, Trp-, Ura) and interaction-selective media (CSM-Leu-
382 , Trp-, Ura-, Ade-, His-) supplemented with 50 or 500µM methionine. The non-mutated N-terminal
383 ubiquitin moiety (NubI) was used as positive control, and NubI13G (empty vector) as negative

control [70]. Protein expression was verified in haploid yeast using antibodies against the VP16 domain for the Cub fusion or the HA epitope tag for the Nub fusions.

Microfluidic device (RootChip-8S) fabrication and on-chip plant cultivation

Microfluidic device fabrication and on-chip plant cultivation were performed as described in [46]. Briefly, Arabidopsis seedlings were grown in pipette tips filled with solidified medium and interfaced with poly(dimethylsiloxane) (PDMS) RootChip-8S devices 4-5 days after germination under sterile conditions. Media perfusion through the microfluidic channels was controlled precisely using a peristaltic pump (DNE GmbH; volumetric flow rate in each channel, 5 µl/min).

Bright-field and fluorescence imaging

Live-cell fluorescence imaging was performed on a custom-built spinning-disk confocal microscope, consisting of a Nikon Ti-E stand, equipped with 20x multi-immersion (N.A. 0.75) and 60x water immersion (N.A. 1.2) objectives (Nikon), a motorized stage (Applied Scientific Instrumentation, USA), a spinning disk with customized hole pattern (CREST Optics, Italy), a motorized filter wheel (Cairn Research, UK), a laser launch (Omicron, Germany), and an EMCCD camera (Photometrics, USA). Image acquisition was operated through Nikon NIS Elements software or Micro-Manager [73]. mCitrine-labeled proteins were excited using a laser emitting at the wavelength of 515 nm; mTrq2-labeled proteins were excited at 440 nm. Bandpass filter specifications were 542nm/20 for mCit emission and 480nm/40 for mTrq2. Chlorophyll autofluorescence was recorded using 561 nm excitation and emission filter specifications of 630nm/92. Root hair phenotyping was performed by transmitted light microscopy on a Nikon SMZ18 stereo microscope, equipped with SHR Plan Apo 0.5x (N.A. 0.075) and 2x (N.A. 0.3) objectives (Nikon) and an Orca Flash 4.0 sCMOS camera (Hamamatsu, Japan). Imaging of the propidium iodide stained root tips expressing different *GEF*::mCit-*GEF* fusions was performed on a Leica SP5 point scanning confocal microscope with a 20x water immersion objective (N.A 0.7), using 1x Zoom, Pinhole 1AU, 1024x1024 pixel scanning field, 400 Hz scanning speed, 2x line average and 2µm Z-Steps as settings. mCit was excited with the 514 nm line of an Argon-Laser and emission light was collected between 520-550 nm. Propidium iodide was exited with a 561 nm laser and emission light was collected between 570-650 nm. Both channels were imaged in sequential scans to avoid crosstalk using Hybrid-Detectors (Gain 200).

Image Processing, data analysis and software

All image processing, image analysis and measurements was done using FIJI (ImageJ) [74], further data analysis was done in Microsoft Excel, if not mentioned otherwise. As statistical test, t-tests (two-tailed, unequal variances) were performed in Excel. A gaussian filter (radius 0.8) and a rolling ball (radius 100) background subtraction was applied to all shown images. The “Royal” lookup table

421 was used for images shown in intensity pseudo color. Kymographs were generated using the multi-
422 kymograph plugin in FIJI. For images of propidium iodide (PI) staining for visualization of the root
423 outlines, the gamma settings were adjusted to 0.75 to reduce the contrast between weakly and
424 strongly stained regions of the root. The adjustment was only done in the PI channel. The signal of
425 the mCitrine-fusions was not altered.
426 Polarity indices were analyzed on the original unprocessed images by measuring in 3 by 15 pixel
427 regions at the RHID, outside the RHID and outside the root, as a background value. This
428 background value was subtracted from both regions and the value outside the RHID was divided
429 by the RHID value. This polarity index was calculated for each cell within a root hair file, all files
430 were aligned to the first cell with a visible bulge and the average polarity index for each
431 developmental step was calculated. A signal was defined as polar, if its value differed (p-value
432 <0.05, according to two-tailed t-test with unequal variances) to the polarity index of the membrane
433 marker GFP-Lti6b.
434 Box plots were created using BoxPlotR (http://shiny.chemgrid.org/boxplotr/), an R-based web-tool
435 provided by the Tyers (Montreal) and Rappsilber (Edinburgh) labs. For all plots number of
436 measurements (n) is given. Center lines show the medians; box limits indicate the 25th and 75th
437 percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from
438 the 25th and 75th percentiles, outliers are represented by dots; crosses represent sample means;
439 bars indicate 95% confidence intervals of the means. For each box plot shown, the value of
440 significance (*p*) according to two tailed Student's t-test are indicated.

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AUTHOR CONTRIBUTIONS

The study was conceived by P.D. and G.G.; P.D., A.R., V.A.F.S., D.G.M., L.Y.A., and N.F.K. performed experiments; P.D., A.R., V.A.F.S., D.G.M., L.Y.A., C.G. and G.G. analyzed data. C.E.S. and G.G. designed the microfluidic device. J.F.E. designed the spinning disk microscope and gave advice about the imaging procedure. P.D., A.R. and G.G. prepared figures and wrote the manuscript with input from all coauthors. All authors read and approved the final version of the manuscript.

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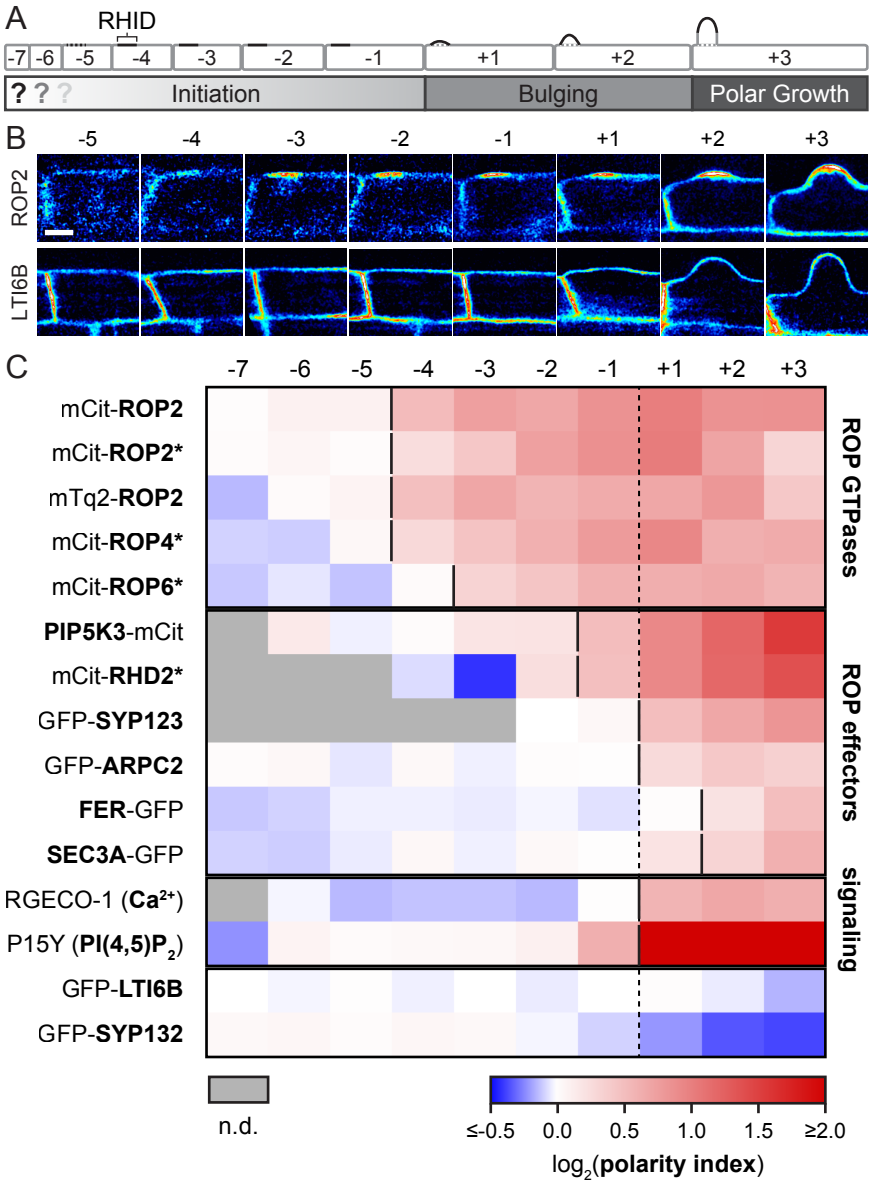

Figure 1. Timeline analysis of protein accumulation at the RHID.

(**A**) Model of root hair development in an epidermal cell file. During the initiation phase the root hair initiation domain (RHID) emerges in elongating trichoblasts and the tip growth machinery is assembled using an unknown mechanism. Stages -1/+1 mark the bulge out at the RHID followed by the transition into polar growth. (**B**) Comparison of mCit-ROP2 and GFP-LTI6B protein localization at the RHID in different stages of root hair development. Scale bar, 10 µm. (**C**) Protein accumulation at the RHID throughout the early development of root hairs in 13 different marker lines. Genes are sorted by the timing of accumulation within their group of protein function. Color coding represents the polarity index (signal intensity in/out of RHID; $\log_2$ scale) for each developmental step. (n.d.: no data). For each stage average polarity index values from multiple cells are shown (see **Supplemental figure 1** for details). Stages -7 to -1 represent cells in the initiation phase before bulging, +1 to +3 cells after bulging. The homogeneously distributed membrane protein Lti6b is used as reference for protein accumulation. Listed genes were fused to either eGFP, mTurquoise2 (mTq2) or mCitrine (mCit), under control of their own promoters, except for UBQ10::P15Y (PI(4,5)P_2 reporter), 35S::LTI6B and genes under control of an estradiol inducible promoter (indicated by an asterisk).

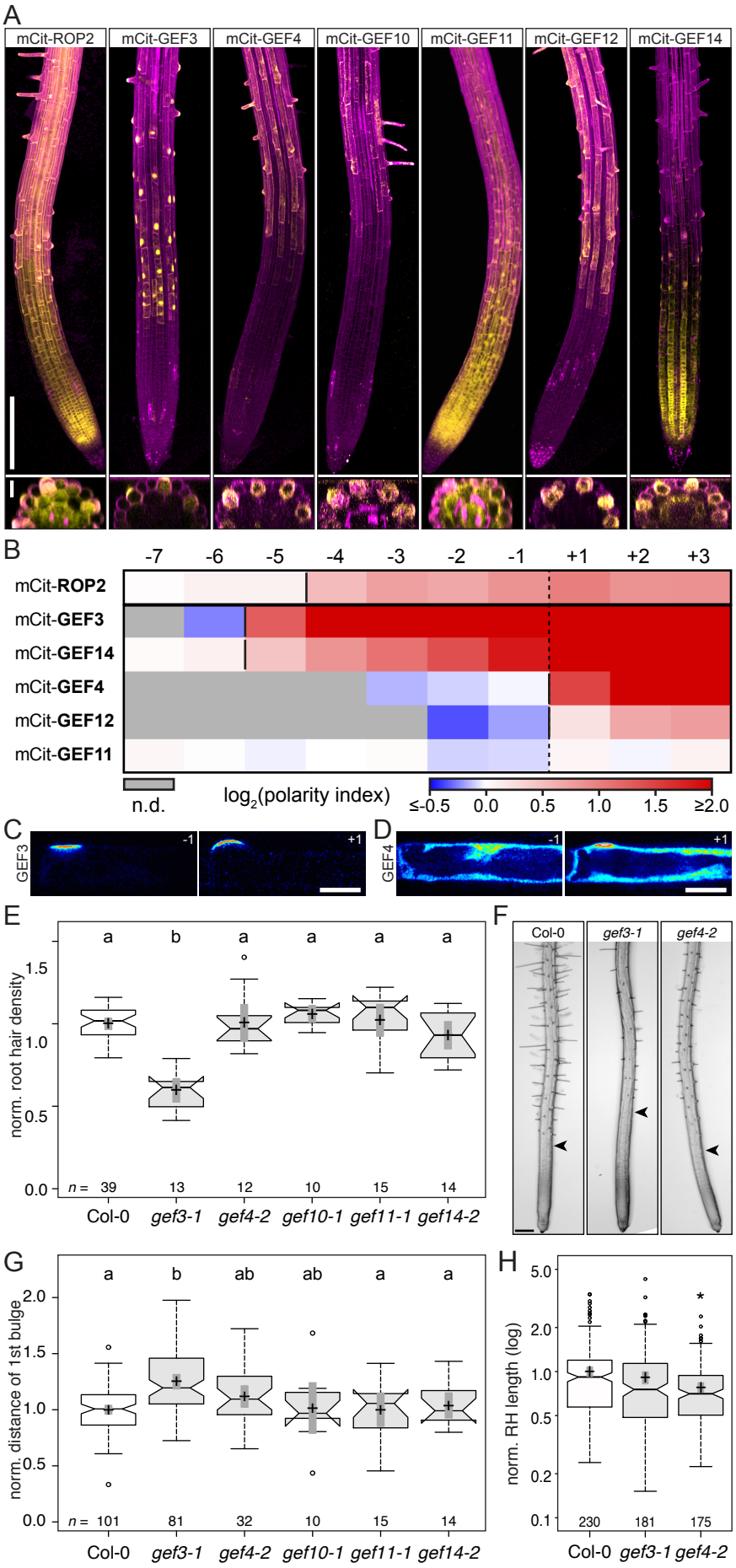
A
mCit-ROP2
mCit-GEF3
mCit-GEF4
mCit-GEF10
mCit-GEF11
mCit-GEF12
mCit-GEF14
B
-7 -6 -5 -4 -3 -2 -1 +1 +2 +3
mCit-ROP2
mCit-GEF3
mCit-GEF14
mCit-GEF4
mCit-GEF12
mCit-GEF11
n.d.
log2(polarity index)
≤-0.5 0.0 0.5 1.0 1.5 ≥2.0
C
GEF3
-1
+1
D
GEF4
-1
+1
E
norm. root hair density
a b a a a a
n = 39 13 12 10 15 14
Col-0 gef3-1 gef4-2 gef10-1 gef11-1 gef14-2
F
Col-0
gef3-1
gef4-2
G
norm. distance of 1st bulge
a b ab ab a a
n = 101 81 32 10 15 14
Col-0 gef3-1 gef4-2 gef10-1 gef11-1 gef14-2
H
norm. RH length (log)
230 181 175
Col-0 gef3-1 gef4-2

Figure 2. Functional analysis of ROPGEFs in the root epidermis identifies GEF3 to be required for efficient root hair emergence.

(**A**) Roots expressing mCit fusion proteins (yellow) under their respective promoters. To visualize root and cell outlines, roots were stained with propidium iodide (magenta). Top panel: Maximum intensity projection of the root tip. Scale bar, 200 µm. lower Panel: Optical cross section through the root in the elongation zone to visualize the tissues expressing the respective constructs. Scale bar, 20 µm. (**B**) Timeline analysis of protein accumulation at the RHID for selected GEFs in comparison to ROP2. GEFs are sorted by timing of accumulation at the RHID. Polarity index measurement and calculation as in Figure 1. (**C, D**) Subcellular localization (intensity-coded representation) of mCit-GEF3 and mCit-GEF4 in cells just before (-1) and after bulging (+1). Scale bars, 20µm. (**E, G**) Quantification of root hair density (**E**) and distance (**G**) of first bulge to the root tip for selected GEFs, normalized to Col-0. Letters show results of an ANOVA-Test (significance value 0.01), with same letters indicating no significant differences. (**F**) Representative root tips of WT (Col-0), gef3-1 and gef4-2 mutant lines. Arrowheads indicate the first bulge. (H) Quantification of hair length 3-6 mm away from root tip in gef3-1 and gef4-2 mutant lines compared to Col-0. Normalized values are shown in log2 scale to account for the high variability in individual hairs. Asterisk indicates $p \leq 0.05$ according to two-tailed students t-test. Values in (**E, G, H**) were normalized to corresponding Col-0 values for each independent experiment.

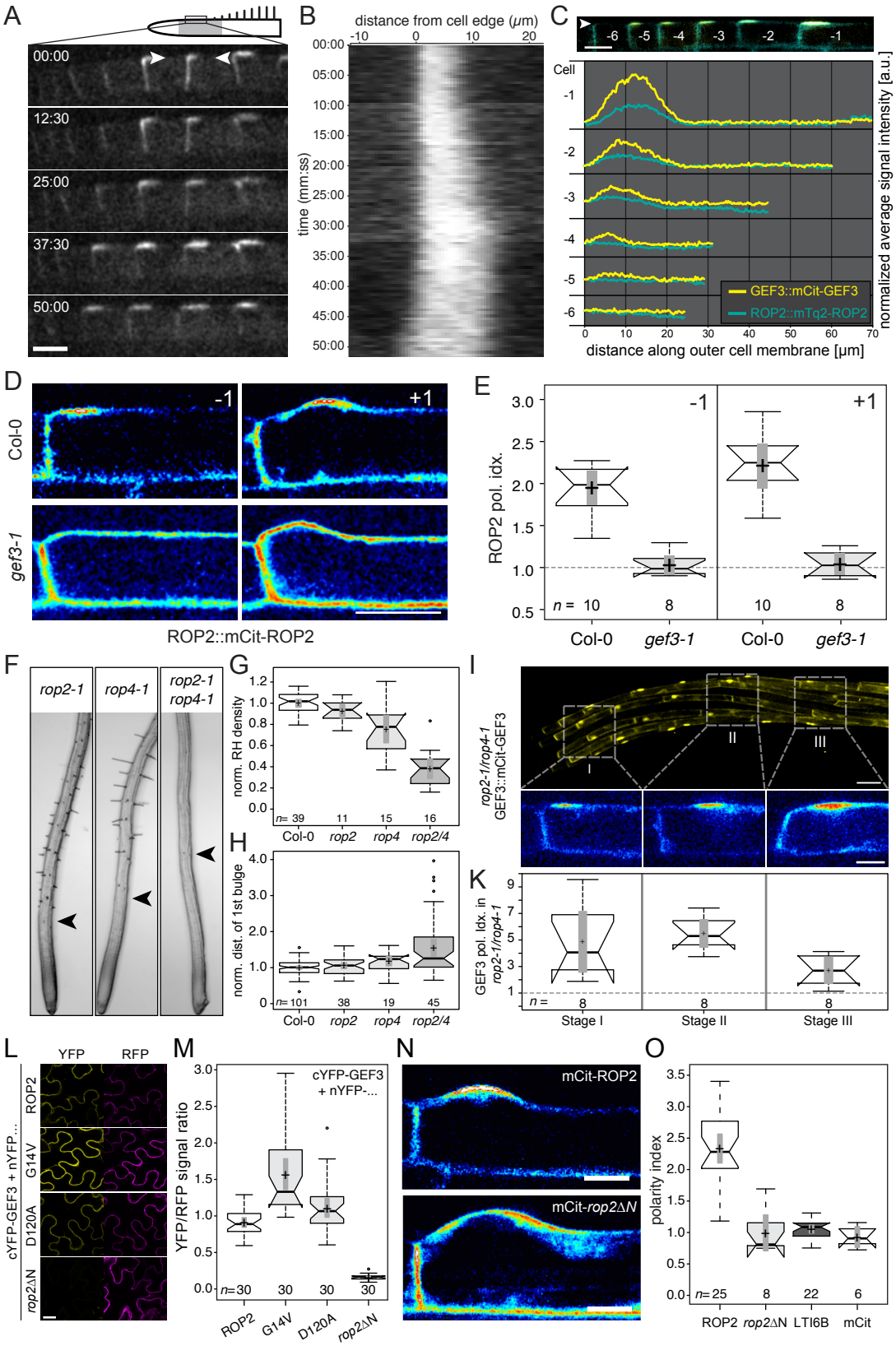
A
00:00
12:30
25:00
37:30
50:00
B
distance from cell edge (μm)
time (mm:ss)
C
Cell
normalized average signal intensity [a.u.]
GEF3::mCit-GEF3
ROP2::mTq2-ROP2
distance along outer cell membrane [μm]
D
Col-0
gef3-1
ROP2::mCit-ROP2
E
ROP2 pol. idx.
Col-0
gef3-1
F
rop2-1
rop4-1
rop2-1 rop4-1
G
norm. RH density
Col-0
rop2
rop4
rop2/4
H
norm. dist. of 1st bulge
I
rop2-1/rop4-1 GEF3::mCit-GEF3
Stage I
Stage II
Stage III
K
GEF3 pol. Idx. in rop2-1/rop4-1
L
YFP
RFP
cYFP-GEF3 + nYFP-...
ROP2
G14V
D120A
rop2ΔN
M
YFP/RFP signal ratio
cYFP-GEF3 + nYFP-...
N
mCit-ROP2
mCit-rop2ΔN
O
polarity index
ROP2
rop2ΔN
LTI6B
mCit

Figure 3. GEF3 is necessary for polar ROP2 recruitment during root hair initiation.

(**A**) Time-lapse imaging of GEF3::mCit-GEF3 in the RootChip-8S. A root hair cell file in the early elongation zone is shown. Multiple stages of protein accumulation can be seen in each still image. The time series was registered to compensate cell movement due to root growth. Numbers indicate time (mm:ss). Scale bar, 20µm. (**B**) The kymograph was generated along the outer cell flank (left-right orientation) between arrow heads in (**A**). The vertical axis depicts time; the horizontal axis depicts distance. Note the gradual expansion of the emerging mCit-GEF3 domain over time until approximately 30 min and a stable domain size after that. (**C**) Colocalization and intensity profiles of GEF3 and ROP2 along RH cell files. Top: exemplary root hair cell file expressing ROP2::mTq2-ROP2 (cyan) and GEF3::mCit-GEF3 (yellow). Cells are labeled from youngest cells (-6) to oldest cell (-1) before bulging. Bottom: average intensity profiles of mTrq2-ROP2 (grey) and mCit-GEF3 (black) along the outer cell periphery (line width 3 px) of 6 root hair cell files (arrowhead indicates direction of line measurement). (**D**) Subcellular localization and (**E**) quantification of mCit-ROP2 polarity at the RHID in Col-0 and the *gef3-1* mutant in root hair cells just before (-1) and after bulging (+1). Scale bar, 20µm. (**F**) Representative root tips of *rop2-1, rop4-1* and *rop2-1xrop4-1* mutant lines. Arrowheads indicate the first bulge. (**G, H**) Quantification of root hair density and distance of first bulge to the root tip, normalized to Col-0 (as reference, the data for Col-0 in Figure 3 are reused). Values are normalized to corresponding Col-0 values for each independent experiment. (**I**) GEF3::mCit-GEF3 expression and localization in *rop2-1xrop4-1* mutant background. Upper panel: overview of the elongation zone. Lower panel: subcellular localization of mCit-GEF3 in cells of the early (I), middle (II), and late (III) elongation zone. (**K**) Quantification of the polarity index of GEF3::mCit-GEF3 in *rop2-1xrop4-1* mutant background. Stages are chosen as indicated in (**I**). (**L**) Representative images of an rBiFC assay in tobacco epidermal cells for each combination tested. All images were recorded using identical conditions and settings, correction for brightness and contrast during image processing was kept equal except for the exemplary YFP image of *rop2*ΔN, which was set brighter to visualize the very low background signal. (**M**) Quantification of ratiometric BiFC experiments. YFP signals were normalized to the corresponding RFP signal to allow comparison of interaction strength. (N) Confocal image of root hair cells expressing estradiol inducible mCit-ROP2 or mCit-*rop2*ΔN. Scale bar, 10µm. (**O**) Quantification of polarity index of the constructs shown in (**N**) and compared to the non-polar membrane marker GFP-LTI6B and free mCitrine.

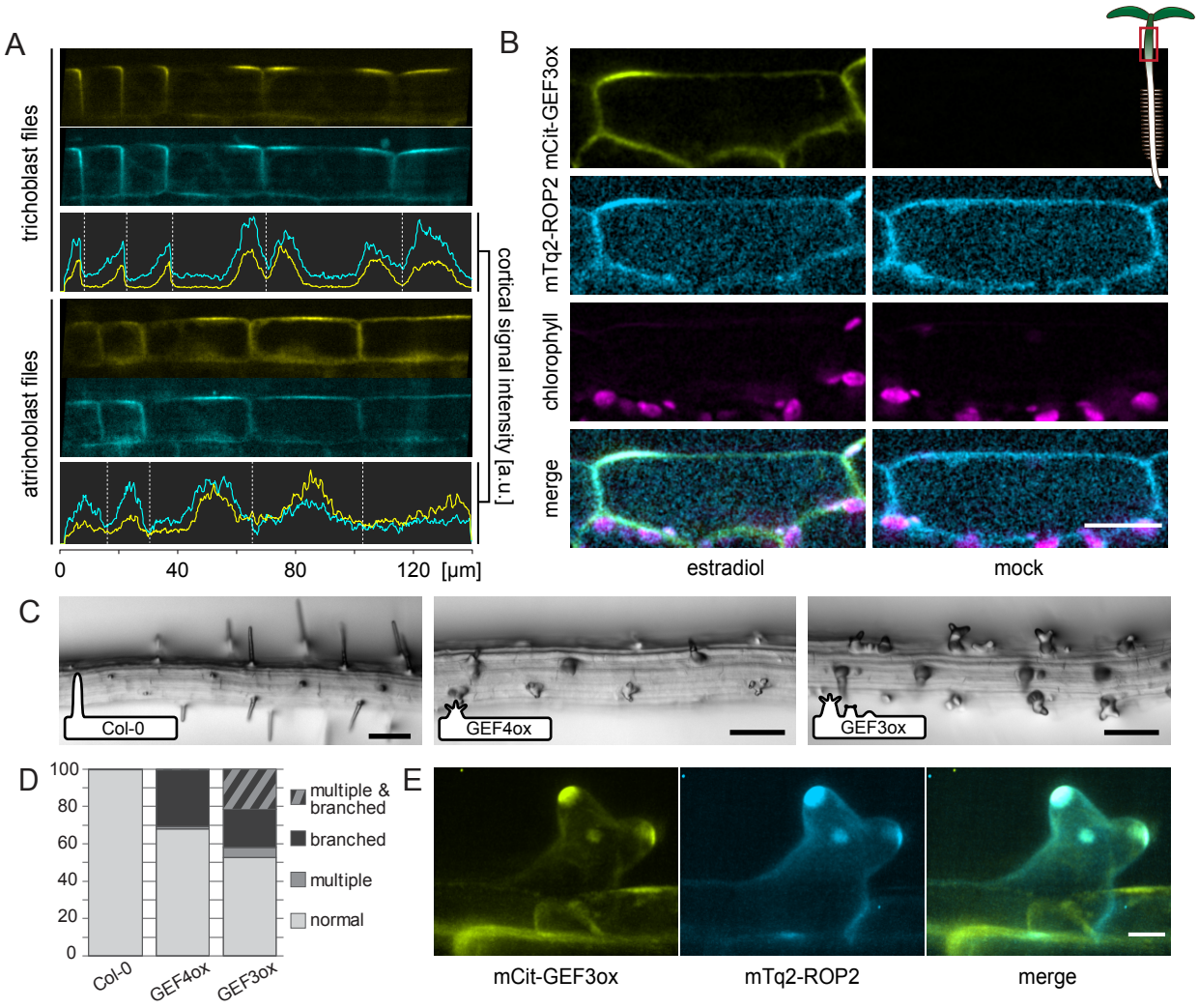

Figure 4. GEF3 initiates the formation of polar growth domains.

(**A**) Localization of ROP2::mTq2-ROP2 (cyan) in roots overexpressing mCit-GEF3 (yellow). Top: representative cell files of trichoblasts and atrichoblasts, both show ectopic accumulations of both markers. Bottom: intensity profiles along cell periphery of cells shown on the left. Dashed lines indicate cell borders. (**B**) Subcellular localization of ROP2::mTq2-ROP2 (cyan) in hypocotyl cells ectopically expressing mCit-GEF3 (yellow). Chlorophyll autofluorescence is shown in magenta. Scale bar, 20 µm. (**C**) Representative images of Col-0 (upper) and roots overexpressing mCit-GEF4 (middle) and mCit-GEF3 (lower) (24h after estradiol induction). Inserts show an idealized sketch of the predominant root hair shape. Scale bars, 100 µm. (**D**) Frequency of phenotypes (induction of multiple hairs, bulges with multiple tips, and combined phenotypes) induced by overexpression of lines shown in (**C**) in % of total observed hairs (n(Col-0)=337; n(mCit-GEF3ox)=238; n(mCit-GEF4ox)=233). (**E**) Colocalization of mCit-GEF3 (yellow) and ROP2::mTq2-ROP2 (cyan) in branched root hairs, 24 hours after estradiol induction of mCit-GEF3 overexpression. Scale bar, 20 µm.