

1 **The RopGEF KARAPPO is Essential for the Initiation of**
2 **Vegetative Reproduction in *Marchantia***

3

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24

25 **SUMMARY**

26 Many plants can reproduce vegetatively, producing clonal progeny from vegetative cells;
27 however, little is known about the molecular mechanisms underlying this process.
28 Liverwort (*Marchantia polymorpha*), a basal land plant, propagates asexually via gemmae,
29 which are clonal plantlets formed in gemma cups on the dorsal side of the vegetative
30 thallus [1]. The initial stage of gemma development involves elongation and asymmetric
31 divisions of a specific type of epidermal cell, called a gemma initial, which forms on the
32 floor of the gemma cup [2, 3]. To investigate the regulatory mechanism underlying gemma
33 development, we focused on two allelic mutants in which no gemma initial formed; these
34 mutants were named *karappo*, meaning “empty”. We used whole-genome sequencing of
35 both mutants, and molecular genetic analyses to identify the causal gene, *KARAPPO* (*KAR*),
36 which encodes a Rop guanine nucleotide exchange factor (RopGEF) carrying a PRONE
37 catalytic domain. *In vitro* GEF assays showed that the full-length KAR protein and the
38 PRONE domain have significant GEF activity toward MpRop, the only Rop GTPase in *M.*
39 *polymorpha*. Moreover, genetic complementation experiments showed a significant role for
40 the N- and C-terminal variable regions in gemma development. Our investigation
41 demonstrated an essential role for KAR/RopGEF in the initiation of plantlet development
42 from a differentiated cell, which may involve cell polarity formation and subsequent
43 asymmetric cell division via activation of Rop signaling, implying a similar developmental
44 mechanism in vegetative reproduction of various land plants.

45

46 **KEYWORDS**

47 asexual reproduction, small GTPase, cell polarity, evolution

48

49 **RESULTS AND DISCUSSION**

50 **Gemma development in *Marchantia polymorpha***

51 Vegetative reproduction is a form of asexual reproduction in which clonal individuals
52 develop directly from vegetative tissues, such as leaves, stems, and roots. Vegetative
53 reproduction is a developmental process based on totipotency, which is the potential for a
54 cell, even a differentiated cell, to regenerate organs or whole plantlets [4-6]. Many plants in
55 diverse lineages exhibit vegetative reproduction, *e.g.* potato (*Solanum tuberosum*), which
56 produces tubers in underground stems, *Kalanchoe diagremontiana*, which forms plantlets
57 at the leaf margins, the Dhalia family, which develop root tubers, and the hen and chicken
58 fern (*Asplenium bulbiferum*), which grows small bulbils on the top of fronds [7]. However,
59 very little is known about the underlying molecular mechanisms of vegetative
60 reproduction.

61 One of the most basal lineages in extant land plants, the liverwort *Marchantia*
62 *polymorpha*, has the ability to propagate asexually by forming clonal plantlets, called
63 gemmae, in a cupule or “gemma cup”, a cup-like receptacle formed on the dorsal side of
64 the thallus, which is the gametophyte plant body (Figure S1A). The development of the
65 gemma and gemma cup in *M. polymorpha* has been described on the basis of histological
66 observations [2, 8]. In the basal floor of the gemma cup, epidermal cells undergo cell
67 elongation followed by two cycles of asymmetrical cell division to form an apical gemma
68 cell and a basal cell (Figure S1E). The gemma cell continues to divide and finally produces
69 the discoid gemma with two laterally developed apical notches. The basal cell does not
70 divide any further and differentiates into a stalk cell [1, 2]. Mucilage papillae also develop
71 from individual epidermal cells located in the floor of gemma cups [2, 3]. In our
72 histological observations, various stages of developing gemmae and single-celled mucilage

73 papillae (large club-shaped cells) were observed in the basal floor of the gemma cup
74 (Figure S1B–D). The elongated morphology of mucilage papillae was distinct, with a
75 number of single-membrane vesicles in their cytosol (Figure S1C). At the initial stage of
76 gemma development, the basal stalk cell was already vacuolated, and the apical gemma cell
77 underwent several rounds of periclinal cell divisions. In most cases, an anticlinal cell
78 division was observed in the basal floor cell attached to the early stage of the developing
79 gemma, while there was no cell division observed in the basal floor cell attached to the
80 mucilage cell (Figure S1B–E).

81

82 **Isolation of *karappo-1* and *karappo-2* mutants**

83 In recent years, *M. polymorpha* has been exploited as a basal plant model system due to the
84 availability of whole-genome sequence information, high-efficiency transformation
85 methods, and genetic modification techniques [9-16].

86 To identify key regulator(s) involved in the initial stage of gemma development in
87 *M. polymorpha*, we focused on two mutants, named *karappo-1* (*kar-1*) and *karappo-2*
88 (*kar-2*), that show a common phenotype of impaired gemma formation. These two mutants
89 were isolated independently; *kar-1* was isolated during the screening of T-DNA-tagged
90 lines for morphological phenotypes of the gametophyte thallus [17], and *kar-2* was isolated
91 from transgenic lines generated by biolistic delivery of a plasmid [18]. In the wild type as
92 well as in *kar-1* and *kar-2* mutants, gemma cups formed at intervals on the dorsal side of
93 thalli along the midrib (Figure 1A, B, and C). Numerous mature gemmae were observed
94 from the top of the wild-type gemma cup; however, no gemmae were found in the *kar-1*
95 and *kar-2* mutants (Figure 1F, G, and H). Transverse sections of the gemma cup showed no
96 developing gemmae in the gemma-cup of the *kar-1* and *kar-2* mutants (Figure 1L, M, Q,

97 and R), while various stages of developing gemmae were observed in the wild type (Figure
98 1K and P). In contrast, mucilage papillae were formed from the basal epidermis of the
99 gemma cup in the *kar-1* and *kar-2* mutant as in the wild type (Figure 1U, V, and W). There
100 was no distinct impairment in the other aspects of vegetative development in the *kar-1* and
101 *kar-2* mutants compared to the wild type (*i.e.* growth rate of thalli, air chamber formation,
102 and rhizoid development). These observations suggest that the initial stage of gemma
103 development is defective in *kar-1* and *kar-2* mutants.

104

105 **Molecular characterization of *kar* mutants**

106 The segregation ratio of the mutant gemma phenotype in an F₁ population generated from a
107 cross between the *kar-1* mutant, which is a female, and the wild-type (WT) male accession
108 Takaragaike-1 (Tak-1) was 102:108 (*kar*:WT). This fit the expected 1:1 ratio as indicated
109 by the chi-squared test (p<0.01), suggesting the involvement of a single genetic locus in the
110 *kar* phenotype. However, the *kar* phenotype in F₁ progenies segregated independently from
111 the hygromycin-resistant marker in the transformed T-DNA fragment, indicating that the
112 *kar-1* mutation is independent of the T-DNA insertion. The *kar-2* mutant, which is a male
113 line, was infertile in several attempts at crossing with the wild-type female accession
114 Takaragaike-2 (Tak-2).

115 To identify the causal gene of the *kar-1* and *kar-2* mutant phenotype, we
116 sequenced the whole genomes of these mutants by next-generation DNA sequencing, and
117 mapped the obtained reads on the reference genome of *M. polymorpha* [9]. Compared to
118 the wild type, the *kar-1* mutant carried a 9-bp deletion and an 18-bp insertion at the
119 junction of the 5th exon and 5th intron of *Mapoly0171s0028* [9]. As a result, the cDNA
120 sequence of *Mapoly0171s0028* in the *kar1* mutant showed an 11-bp deletion and a 1-bp

121 insertion in the coding sequence, which caused a frame-shift and generated a truncated
122 protein (Figure S2A–C). Furthermore, we identified a deletion of an approximately 20-kb
123 genomic locus containing the entire coding sequence of *Mapoly0171s0028* in the *kar-2*
124 mutant (Figure S2D and E).

125 We then performed genetic complementation tests in the *kar-1* and *kar-2* mutants
126 by introducing the *Mapoly0171s0028* cDNA fragment under the control of its own
127 promoter (*proKAR:KAR*). The resultant transgenic lines in the *kar-1* and *kar-2* mutant
128 backgrounds had restored gemma formation (Figure 1I, J, N, O, S, and T). For further
129 confirmation, we disrupted *Mapoly0171s0028* in the wild type using homologous
130 recombination-mediated gene targeting [11] and isolated two independent knockouts of
131 *Mapoly0171s0028* (Figure 2A and B). The two knockout lines, *kar^{KO}* #1 and #2, showed a
132 complete loss of gemma formation similar to the *kar* mutants. The impaired gemma
133 formation was recovered by the introduction of citrine-fused wild-type cDNA of
134 *Mapoly0171s0028* (Figure 2C–E). These results indicated that the *kar* phenotype was
135 caused by a loss of function of *Mapoly0171s0028*. This gene was designated as *KARAPPO*
136 (*KAR*) after a Japanese word meaning “empty”, representing the characteristic phenotype
137 of the mutants with empty gemma cups.

138

139 ***KAR* encodes a potential activator of Rop GTPase signaling**

140 The deduced amino acid sequence of KAR encodes a highly conserved plant-specific Rop
141 nucleotide exchanger (PRONE) catalytic domain, which is characteristic of the guanine
142 nucleotide exchange factor (GEF) of the Rop GTPase [19], while the N- and C-terminal
143 regions outside of the PRONE domain were highly variable (Figure S3). In angiosperms,
144 Rop signaling mediated by RopGEF is involved in various developmental processes and

145 environmental responses [20-25]. *KAR* is the sole PRONE-type RopGEF gene in the *M.*
146 *polymorpha* genome. In addition, the *M. polymorpha* genome also contains only a single
147 copy of *Rop*, *Mapoly0051s0092*, designated as MpRop, which showed a high overall
148 similarity to *Rop* in various plant lineages (Figure S4A and C).

149 To determine whether *KAR* encodes a functional GEF that acts on MpRop, we
150 examined the interaction of KAR and MpRop by a yeast two-hybrid assay. Yeast cells
151 co-transformed with a combination of either AD::KAR and BD::MpRop or AD::MpRop
152 and BD::KAR grew on selective -W/L/H and -W/L/H/A medium (Figure 3A), indicating
153 that KAR and MpRop physically interact. The interaction between KAR and MpRop was
154 further confirmed by an *in vitro* pull-down assay. The predicted protein coding sequence of
155 KAR was fused to the C-terminus of the 6x Histidine-tag. Purified 6xHis-KAR fusion
156 proteins were pulled down with guanosine triphosphate (GTP)-bound, guanosine
157 diphosphate (GDP)-bound, or nucleotide-free forms of the glutathione S-transferase
158 (GST)-MpRop fusion protein and were detected using anti-His antibody. KAR fusion
159 proteins exhibited similar interactions with different forms of MpRop *in vitro* (Figure 3B).

160 Next, we examined the GEF activity of KAR toward MpRop (Figure 3C). In
161 *Arabidopsis thaliana*, there are 14 RopGEFs with a high degree of sequence similarity to
162 the residues that are involved in catalyzing GDP/GTP exchange [26]. In Arabidopsis, the
163 PRONE domain is sufficient for catalysis of nucleotide exchange on ROP [19] [26], while
164 the variable C-terminal domain of some RopGEFs autoinhibits GEF activity [26]. To test
165 the GEF activity and the potential regulatory role in the variable regions of KAR, we
166 purified the full-length KAR protein and a truncated version of KAR containing just the
167 PRONE domain (KAR-PRONE), and characterized their GEF activity toward MpRop
168 using radio-labelled [³⁵S]-GTP γ S. We detected significant GEF activities toward MpRop

169 with full-length KAR and KAR-PRONE, and the GEF activity of full-length KAR was
170 comparable to that of KAR-PRONE (Figure 3C).

171 We tested the functionality of the *KAR-PRONE* coding sequence without the N-
172 and C-terminal region in gemma formation. Two lines with comparable expression levels
173 of the full-length *KAR* or the *KAR-PRONE* coding sequence were selected for further
174 analysis (Figure 3D). Introduction of citrine-fused full-length *KAR* cDNA restored to some
175 extent the formation of gemma with normal morphology in the *kar*^{KO} #2 background. By
176 contrast, the citrine-fused KAR-PRONE did not lead to the full recovery of gemma
177 formation; the most of the gemmae were abnormal in size and morphology with an
178 irregular periphery (Figure 3E). These results demonstrated that the N-terminal and
179 C-terminal variable regions of KAR play a significant role in proper gemma formation *in*
180 *vivo*, although they have no obvious effect on GEF activity *in vitro* (Figure 3C).

181

182 **Ubiquitous expression of *KAR* and *MpRop* in vegetative tissues**

183 To evaluate the expression pattern of *KAR* and *MpRop*, we generated transgenic *M.*
184 *polymorpha* lines expressing the β-glucuronidase (*GUS*) reporter gene under the control of
185 the *KAR* promoter (*proKAR:GUS*) and the *MpRop* promoter (*proMpRop:GUS*). In
186 *proKAR:GUS* and *proMpRop:GUS* lines, GUS staining was observed in the broader region
187 of the entire thallus, including the basal floor of the gemma cups containing developing
188 gemmae (Figure 4A and B). We further evaluated the expression pattern of *KAR* and
189 *MpRop* using reverse-transcription quantitative PCR (RT-qPCR). Transcripts of *KAR* and
190 *MpRop* were detected in all stages and organs in the vegetative thallus (Figure 4C). These
191 results suggest that *KAR* and *MpRop* are ubiquitously and simultaneously expressed in the
192 initial stage of gemma development.

193 Knockout plants of *MpRop* were generated by homologous
194 recombination-mediated gene targeting; however, two independent *MpRop*^{KO} lines showed
195 severe impairment of thallus growth and wilted before the formation of the gemma cup
196 (Figure S4B). This result suggests a much broader function for the sole Rop in *M.*
197 *polymorpha*, which is evidently essential for the growth and development of the
198 gametophyte thallus. Another type of GEF, SPIKE1 (SPK1), has been reported to regulate
199 cytoskeletal rearrangement and cell-shape change in response to growth signals in
200 angiosperms [27, 28]. SPK1 has a conserved DOCK homology region 2 (DHR2) domain,
201 which is distantly related to CZH [CDM (Ced-5, Dock180, Myoblastcity)-Zizimin
202 homology] RhoGEFs in animals and fungi [29]. *M. polymorpha* contains a single *SPK1*
203 homologue, MpSPK1 [9] (Figure S4C), which may have a critical function in controlling
204 Rop signaling in thallus growth.

205 The contrast between the specific developmental impairment in *kar* mutant lines
206 (Figure 1 and 2), and the ubiquitous promoter activity of *KAR* and *MpRop* (Figure 4),
207 suggests an upstream regulatory mechanism for KAR activity, which enables cell-type
208 specific activation of Rop in the gemma initial. Recent studies have shown that several
209 plasma membrane-localized receptor-like protein kinases (RLKs) function as upstream
210 regulators of Rop signaling through interaction with the C-terminal variable region of
211 RopGEF (*i.e.* the pollen receptor kinases in tomato (*Solanum lycopersicum*) and FERONIA
212 in *Arabidopsis thaliana*) [20, 21, 30]. Similar to their role in polarized cell growth mediated
213 by Rop signaling in angiosperms, RLK(s) might be involved in the specific function of
214 KAR in the initial stage of gemma development in *M. polymorpha*. Further functional
215 studies on the N-terminal and C-terminal variable regions of KAR will be needed to
216 understand the regulatory mechanism of Rop signaling in *M. polymorpha*.

217

218 **Role of KAR in the initial stage of gemma development**

219 In this study, we demonstrated that *KAR*, which encodes a RopGEF, is an essential factor
220 required for the initial stage of gemma development in *M. polymorpha*. Histological studies
221 have suggested the occurrence of cell protrusion and subsequent asymmetric cell divisions
222 in the initial stage of gemma development in the epidermal floor of the gemma cup (Figure
223 S1; [2]). A recent study in *M. polymorpha* demonstrated that a ROOT-HAIR DEFECTIVE
224 SIX-LIKE (RSL) class I basic helix-loop-helix (bHLH) transcription factor, MpRSL1,
225 controls the morphogenesis of structures derived from individual epidermal cells (*i.e.*
226 rhizoids, slime papillae, mucilage papillae, and gemmae) [3]. Similar to the
227 loss-of-function mutants of MpRSL1, in the *kar* mutants, we did not observe the one- or
228 two-cell stage of gemma development. On the other hand, the other epidermis-derived
229 structures, mucilage papillae and rhizoids, were generated normally in the *kar* mutants, but
230 are absent in Mprsl1 mutants [3]. Mucilage papillae and rhizoids do not undergo any
231 further cell division after polarized cell growth, whereas the development of gemmae
232 involves subsequent asymmetrical cell division and further cell divisions. These
233 observations suggest that KAR promotes the asymmetrical cell division(s) of the gemma
234 precursor cell after the specification of an epidermal cell controlled by MpRSL1.

235 KAR contains a highly conserved PRONE domain (Figure S3), which has been
236 implicated in the activity of the GEF of Rop GTPase [19, 26]. In this study, we
237 demonstrated the GEF activity of KAR on MpRop *in vitro* (Figure 3). The *Arabidopsis*
238 *thaliana* genome contains 14 RopGEFs, and Rop signaling mediated by RopGEF is
239 involved in the control of polar cell growth of pollen tubes and root hairs [20-23]. This
240 polar growth involves the coordination of cytoskeleton organization and vesicular

241 trafficking [31, 32]. In yeast and animals, the closest homologues of Rop, the Cdc42 Rho
242 GTPases, regulate polarity and play a key role in the control of asymmetrical cell division
243 [33, 34]. Recently in monocots, Rop was shown to be involved in the asymmetrical
244 division of the stomata mother cell by controlling cytoskeletal scaffolds and nuclear
245 positioning [35, 36]. KAR-mediated Rop signaling could function in the formation of cell
246 polarity and/or subsequent asymmetrical cell divisions during the initiation of gemma
247 development.

248 Vegetative reproduction can be considered as a type of naturally occurring somatic
249 embryogenesis, in which a meristem is regenerated from differentiated cells. In the
250 development of somatic embryos from single cells isolated from tissue cultures of carrot
251 (*Daucus carota* subsp. *sativus*), the first cell division occurs asymmetrically, and one of the
252 daughter cells gives rise to a three-dimensional cell mass from which one or more embryos
253 develop [4-6]. Asymmetrical cell division to produce daughter cells of a different cell fate
254 must be a common key process in the initial stage of organ/plantlet regeneration from
255 differentiated cells. RopGEF-mediated Rop signaling seems to have been acquired in the
256 common ancestor of land plants after the emergence of charophycean algae. PRONE-type
257 RopGEFs have highly diverged in the course of land plant evolution, while the basal land
258 plant *M. polymorpha* has a limited gene repertoire for Rop signaling (Figure S3 and S4).
259 The Rop-driven asymmetric cell division of differentiated cells to regenerate clonal
260 progenies could be a key innovation for sessile land plants to dominate the terrestrial
261 ecosystem, and this mechanism may have been co-opted to regulate numerous
262 physiological and developmental processes, probably also including vegetative
263 reproduction or organ regeneration, in the course of land plant evolution.

264

265 **CONTACT FOR REAGENT AND RESOURCE SHARING**

266 Further information and requests for resources and reagents should be directed to and will
267 be fulfilled by the Lead Contact, Kimitsune Ishizaki (kimi@emerald.kobe-u.ac.jp). Please
268 note that the transfer of transgenic plants will be governed by an MTA, and will be
269 dependent on appropriate import permits being acquired by the receiver.

270

271 **SUPPLEMENTAL INFORMATION**

272 Figures S1–S4, and Table S1.

273

274 **AUTHOR CONTRIBUTIONS**

275 T.H. and K.I. designed the research, and T.H. performed most of the experiments. K.I.
276 isolated the *kar-1* mutant and H.K. performed linkage analyses. M.K. and K.T.Y. isolated
277 *kar-2* mutants. T.H., Y.Y., and H.T. performed the histology. K.L.Q. and D.U. performed
278 the *in vitro* pull-down and GEF assays. K.I., K.Y., S.Sh., S.Sa., and T.K. performed the
279 whole-genome sequencing. M.S., M.W., and K.T. performed TEM analyses. T.H., K.I., and
280 T.K. performed the gene-targeting experiments. T.H., K.I., Y.Y., D.U., H.F., and T.M.
281 analyzed the data. T.H., Y.Y., and K.I. wrote the article.

282

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291 Glass Foundation and SUNTORY Foundation for Life Sciences (to K.I.).

292

293 **FIGURE LEGENDS**

294 **Figure 1. Phenotype of the *kar* mutants and their complimented lines**

295 (A–Y) Five genotypes are presented, one in each column: (first column) wild type, (second
296 column) *kar-1*, (third column) *kar-2*, (fourth column) *kar-1* complementation line (*kar-1*
297 transformed with *proKAR:KAR*), (fifth column) *kar-2* complementation line (*kar-2*
298 transformed with *proKAR:KAR*). (A–E) Top view of 2-week-old thalli grown from tips of
299 thalli. Scale bars represent 1 mm. (F–J) Surface view of gemma cups in 2-week-old thalli.
300 Scale bars represent 1 mm. (K–O) Toluidine-blue-stained transverse sections of gemma
301 cups in 2-week-old plants. Scale bars represent 100 μ m. (P–Y) Magnified views of
302 toluidine-blue-stained sections of gemma cups in 2-week-old plants. Arrowheads and
303 arrows indicate gemma initials and mucilage papillae, respectively. Scale bars represent 10
304 μ m.

305

306 **Figure 2. Generation of knockout mutants of *KAR***

307 (A) Schematic representation of the structure of the wild-type *KAR* locus (top), the
308 construct designed for gene targeting (middle), and the *KAR* locus disrupted in the
309 gene-targeted lines (bottom). Each primer pair used for genotyping is indicated by
310 arrowheads and marked with (F) for forward or (R) for reverse. Open boxes indicate exons.
311 (B) Genomic PCR analysis of the *KAR^{KO}* lines using the primers indicated in (A). (C, D)
312 Scanning electron microscopy of gemma cups in three genotypes are presented: *kar^{KO}#1*
313 (C), and a representative *kar^{KO}* line transformed with *proKAR:C-KAR*, which contains a
314 citrine-fused *KAR* coding sequence under the endogenous *KAR* promoter. (D). Scale bars
315 represent 100 μ m. (E) Number of gemmae formed in a gemma cup in 3-week-old thalli
316 grown from apical fragments in the wild type, a *kar^{KO}* line, and a representative *kar^{KO}*
317 complemented line (Values are means \pm SD, n = 5).

318

319 **Figure 3. KAR has GEF activity towards MpRop.**

320 (A) Yeast two-hybrid experiments. Clones in the pGBKT7 vector containing the
321 Gal4-binding domain (BD) are noted in the left column and clones in the pGADT7 vector
322 containing the Gal4 activation domain (AD) are noted in the right column. Growth with

323 serial dilutions on the -L, -W dropout media indicates that both pGBKT7 and pGADT7
324 vectors were present. Growth with serial dilutions on the -L, -W, -H dropout media and -L,
325 -W, -H, -A dropout media indicates a physical interaction between the BD and AD fusion
326 proteins. **(B)** Physical interaction of KAR with MpRop. His6-KAR was pulled down with
327 GST or GST-MpRop1 using glutathione agarose. The pull-down samples were separated on
328 a SDS-PAGE gel, then visualized by western blot with an anti-6xHis antibody. **(C)** GEF
329 activity of the full-length KAR (KAR) or the PRONE domain of KAR (KAR-PRONE)
330 toward MpRop. [³⁵S]-GTP γ S binding to 1 μ M GST-MpRop1 was analyzed over time at
331 4°C. Graphs show data from two experiments. Fitting curves were estimated by the
332 one-phase association model in GraphPad Prism software. **(D)** RT-qPCR analysis of *KAR*
333 or *KAR-PRONE* expression in 3-week-old wild type and the respective complementation
334 lines shown in Figure 3E. *MpAPT* was used as a control gene. Data are displayed as means
335 \pm SD ($n = 3$). **(E)** Genetic complementation with the full-length *KAR* and the truncated
336 *KAR* coding sequence containing only the PRONE domain. The histogram shows
337 distribution of the different classes of gemmae in a gemma cup in 3-week-old thalli grown
338 from apical fragments, in WT, a *kar*^{KO} mutant, and two representative *kar* complementation
339 lines with the full-length KAR (*proKAR:C-KAR*) and with the PRONE domain
340 (*proKAR:C-PRONE*) (Values are means \pm s.d., $n=4\sim 5$). Tukey's test was performed for the
341 number of normal gemmae, and letters above the bars indicate significant differences at $p <$
342 0.05. Right panels show pictures of normal gemma of over 500 μ m diameter (normal),
343 abnormal gemma with more than two notches, and small gemma of less than 500 μ m
344 diameter.
345

346 **Figure 4. Expression of KAR and MpRop in vegetative tissues**

347 **(A, B)** Histological GUS activity staining of representative proKAR:GUS **(A)** and
348 proRop:GUS **(B)** transgenic lines. **(C)** RT-qPCR analysis of *KAR* and *MpRop* in
349 1-week-old thalli yet to develop gemma cups (1w thallus), mature gemmae in gemma cups
350 (gemma), gemma cups containing developing gemmae (gemma cups), and midribs. Total
351 RNA was isolated from the respective tissues of Tak-1. *EF1 α* was used as a control gene.
352 Data are displayed as means \pm SD ($n = 3$).
353

354 **STAR METHODS**

355

356 **KEY RESOURCES TABLE**

357

358 (Attached)

359

360 **CONTACT FOR REAGENT AND RESOURCE SHARING**

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362 be fulfilled by the Lead Contact, Kimitsune Ishizaki (kimi@emerald.kobe-u.ac.jp). Please
363 note that the transfer of transgenic lants will governed by an MTA, and will be dependent
364 on appropriate import permits being acquired by the receiver.

365

366 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

367 **Plant Materials and Growth Conditions**

368 Female and male accessions of *M. polymorpha*, Takaragaike-2 (Tak-2) and Takaragaike-1
369 [10], respectively (Ishizaki et al. 2008), were used as the wild type. F₁ spores generated by
370 crossing Tak-2 and Tak-1 plants were used for transformation to generate the *kar-1* mutant
371 and the *kar* knockout lines. Thalli were grown on 1% (w/v) agar medium containing
372 half-strength Gamborg's B5 salts [37] under 50–60 μmol m⁻² s⁻¹ continuous white light
373 with a cold cathode fluorescent lamp (CCFL; OPT-40C-N-L; Optrom, Japan) or white
374 light-emitting diodes (white LED; VGL-1200W; SYNERGYTEC, Japan) at 22°C. For
375 crossing, over 2-week-old thalli were transferred to continuous light conditions with 50–60
376 μmol m⁻² s⁻¹ white LED and 20–30 μmol m⁻² s⁻¹ far red light-emitting diodes
377 (VBL-TFL600-IR730, Valore, Japan).

378

379 **METHOD DETAILS**

380 **Phenotype Analysis and Histology**

381 Two-week-old thalli developed from tips of thalli were dissected into small pieces and
382 transferred to fixative solution with 2% glutaraldehyde in 0.05 M phosphate buffer (pH 7.0)
383 and evacuated with a water aspirator until the specimens sank, then fixed for 2 days at

384 room temperature. The samples were dehydrated in a graded ethanol series and embedded
385 in Technovit 7100 plastic resin. Semi-thin sections (5- μ m thickness) were obtained with a
386 microtome (HM 335E, Leica Microsystems) for light microscopy and stained with
387 toluidine blue O. Sections were observed with an upright microscope (Axio Scope. A1,
388 Carl Zeiss Microscopy).

389 Cultured thalli of *M. polymorpha* were observed using a digital microscope (VHX-5000,
390 KEYENCE). For scanning electron microscopy, thalli were frozen in liquid nitrogen and
391 observed with a scanning electron microscope (VHX-D500, KEYENCE).

392

393 **Electron microscopy**

394 Samples were fixed with 4% paraformaldehyde and 2% glutaraldehyde in 50 mM sodium
395 cacodylate buffer (pH 7.4) for 2 h at room temperature and overnight at 4°C, then
396 post-fixed with 1% osmium tetroxide in 50 mM cacodylate buffer for 3 h at room
397 temperature. After dehydration in a graded methanol series (25, 50, 75, 90, and 100%), the
398 samples were embedded in Epon812 resin (TAAB). Ultrathin sections (100 nm) or
399 semi-thin sections (1 μ m) were cut by a diamond knife on an ultramicrotome (Leica EM
400 UC7, Leica Microsystems, Germany) and placed on a glass slide. The sections were stained
401 with 0.4% uranyl acetate followed by lead citrate solution and coated with osmium under
402 an osmium coater (HPC-1SW, Vacuum Device, Japan). The coated sections were observed
403 with a field-emission scanning electron microscope SU8220 (Hitachi High technology,
404 Japan) with an yttrium aluminum garnet backscattered electron detector at an accelerating
405 voltage of 5 kV.

406

407 **Genome sequencing of the *kar-1* and *kar-2* mutants**

408 Genomic DNA was extracted from *kar-1*, *kar-2*, and Tak-2 plants as follows: the tissue was
409 powdered in liquid nitrogen and incubated in 10 mL hexadecyltrimethylammonium
410 bromide (CTAB) buffer (1.5% CTAB, 75 mM Tris-HCl [pH 8.0], 15 mM EDTA, and 1 M
411 NaCl) for 20 min at 56°C. This suspension was mixed with an equal volume of
412 chloroform:isoamyl alcohol (24:1, w/v), incubated for 20 min at room temperature, and
413 centrifuged at 4,000 \times g for 20 min. The aqueous phase was used to repeat the

414 chloroform:isoamyl alcohol extraction, and then mixed gently with 1.5 volumes of CTAB
415 precipitation buffer (1% CTAB, 50 mM Tris-HCl [pH 8.0], and 10 mM EDTA). After
416 centrifugation at 10,000 × g for 30 min at 20°C, the precipitate was dissolved in 1 M
417 sodium chloride containing 10 mg/mL RNaseA and incubated for 30 min at 37°C. The
418 genomic DNA was precipitated with ethanol, dissolved in TE buffer (10 mM Tris-HCl [pH
419 8.0] and 1 mM EDTA), and further purified using a Genomic-tip 100 column (QIAGEN,
420 Germany). The DNA was then sheared on a Covaris sonicator (Covaris, USA),
421 size-selected with Pippin Prep (Sage Science, USA), and used to create the libraries using
422 the TruSeq DNA Sample Preparation Kit (Illumina) with an insert size of ~350 bp. The
423 libraries were sequenced using Illumina HiSeq 2000 with a 2 × 101-nt paired-end
424 sequencing protocol. The sequence reads were mapped to the *M. polymorpha* genome
425 sequence [9] and the plasmid sequence was used for the biolistic transformation [18] by
426 Bowtie2 v.2.2.9 [38] with default parameters, and visualized and assessed using Integrative
427 Genomics Viewer v.2.3.23 [39].

428

429 **Characterization of mutations in *KAR/Mapoly0171s0028***

430 Small pieces (3 × 3 mm) of thalli were taken from individual plants and crushed with a
431 micro-pestle in 100 µl buffer containing 100 mM Tris-HCl, 1 M KCl, and 10 mM EDTA
432 (pH 9.5). Sterilized water (400 µl) was added to each tube and a 1 µl aliquot of the extract
433 was used as a template for PCR using KOD FX Neo DNA polymerase (Toyobo). To
434 identify the mutation in the *kar-1* mutant, the *Mapoly0171s0028* locus was amplified by
435 genomic PCR using the primer set kar-1_gF/kar-1_gR and sequenced. The cDNA of *kar* in
436 the *kar-1* mutant was amplified by RT-PCR using the primer set KAR-cds-F/KAR-cds-sR
437 and sequenced. To confirm the absence of *Mapoly0171s0028* in the *kar-2* mutant, genomic
438 PCR was performed with a KOD FX Neo DNA polymerase using primers
439 KAR-gF/KAR-gR. Primer pairs are shown in Figure S2D and Table S1.

440

441 **Complementation tests**

442 For complementation of the *kar* mutants, the coding sequence of full-length *KAR* and the
443 truncated coding sequence of *KAR* (*KAR-PRONE*), containing just the PRONE domain

444 (residues 132–503) were amplified by RT-PCR using KOD plus neo (TOYOBO) with the
445 primer set KAR-cds-F/KAR-cds-sR and PRONE-L/PRONE-R, respectively. The *KAR* and
446 *KAR-PRONE* coding sequence fragments were cloned into the pENTR/D-TOPO cloning
447 vector (Life Technology) to produce pENTR-KAR and pENTR-PRONE, respectively. The
448 *KAR* promoter region, including about 5 kb upstream of the initiation codon, was amplified
449 from Tak-1 genomic DNA by PCR using KOD-Plus-Neo (TOYOBO) with the primer set
450 KARpro_GW_F/KARpro_GW_302_R. The PCR-amplified product was cloned into the
451 *Xba*I and *Hind*III sites of pMpGWB302 to replace the CaMV35S promoter [40] with the
452 In-Fusion HD cloning kit (Clontech, Mountain View, CA). The entry vector containing the
453 *KAR* coding sequence was introduced into the binary vector by Gateway LR clonase II
454 Enzyme mix (Thermo Fisher Scientific, USA) to generate the *proKAR:KAR* construct. The
455 *proKAR:KAR* vector was introduced into regenerating thalli of *kar-1* and *kar-2* mutants via
456 *Agrobacterium tumefaciens* GV2260 [13].

457 Similarly, the *KAR* promoter region, including about 5 kb upstream of the initiation codon,
458 was amplified from Tak-1 genomic DNA by PCR using KOD-Plus-Neo (TOYOBO) with
459 the primer set KARpro_GW_F/KARpro_GW_305_R. The PCR-amplified product was
460 cloned into the *Xba*I and *Hind*III sites of pMpGWB305, which contains citrine gene in
461 front of the gateway cassette, to replace the CaMV35S promoter [40] with the In-Fusion
462 HD cloning kit (Clontech, Mountain View, CA). The coding sequence fragments of *KAR*
463 and *KAR-PRONE* in the entry vectors pENTR-KAR and pENTR-PRONE were introduced
464 into the binary vectors by Gateway LR clonase II Enzyme mix (Thermo Fisher Scientific,
465 USA) to generate the *proKAR:C-KAR*, and *proKAR:C-PRONE* constructs, respectively.
466 The binary vector was transformed into the *kar*^{KO} line. Transformants were selected with
467 0.5 μM chlorsulfuron and 100 μg/ml cefotaxime.

468

469 **Generation of *KAR*^{KO} and *MpRop*^{KO} plants**

470 To generate the *KAR*-targeting vector, 5'- and 3'-homologous arms (approximately 4.5-kb
471 in length) were amplified from Tak-1 genomic DNA by PCR using KOD FX Neo
472 (TOYOBO) with the primer pairs shown in Table S1. The PCR-amplified 5'- and
473 3'-homologous arms were cloned into the *Pac*I and *Asc*I sites, respectively, of pJHY-TMp1

474 [11] with the In-Fusion HD cloning kit (Clontech, Mountain View, CA). The *KAR*-targeting
475 vector was introduced into F₁ sporelings derived from sexual crosses between Tak-1 and
476 Tak-2 via *Agrobacterium tumefaciens* GV2260 [10]. The transformed plants carrying the
477 targeted insertions were selected by genomic PCR with a KOD FX Neo DNA polymerase
478 and primer pairs shown in Figure 2 and Table S1.

479 To generate the *MpRop*-targeting vector, 5'- and 3'-homologous arms (approximately
480 4.5-kb in length) were amplified from Tak-1 genomic DNA shown in Table S1. The
481 PCR-amplified 5'- and 3 '-homologous arms were cloned into the *PacI* and *AscI* sites,
482 respectively, of pJHY-TMp1 [11] with the In-Fusion HD cloning kit (Clontech, Mountain
483 View, CA). The *MpRop*-targeting vector was transformed into F₁ sporelings derived from
484 sexual crosses between Tak-1 and Tak-2 as described above. The transformed plants
485 carrying the targeted insertions were selected by genomic PCR with a KOD FX Neo DNA
486 polymerase and primer pairs shown in Figure S4B and Table S1.
487

488 **Promoter reporter analyses**

489 The *KAR* genomic sequence, including approximately 5 kb upstream of the initiation codon,
490 was amplified from Tak-1 genomic DNA by PCR using KOD-Plus-Neo (TOYOBO) with
491 the primer set KARpro_F/KARpro_R and was cloned into pENTR/D-TOPO (Thermo
492 Fisher Scientific). Similarly, the *MpRop* genomic region, including about 3 kb upstream of
493 the initiation codon, was amplified from Tak-1 genomic DNA by PCR with the primer set
494 MpRoppro_F/MpRoppro_R and was inserted into pENTR/D-TOPO (Thermo Fisher).
495 These entry vectors were introduced into the Gateway binary vector pMpGWB104 [40]
496 using Gateway LR clonase II Enzyme mix (Thermo Fisher Scientific, USA) to generate
497 *proKAR:GUS* and *proMpRop:GUS* binary constructs, respectively. The *proKAR:GUS* and
498 *proMpRop:GUS* vectors were introduced into regenerating thalli of Tak-1 via
499 *Agrobacterium tumefaciens* GV2260 [13]. Transformants were selected with 0.5 μM
500 chlorsulfuron and 100 μg/ml cefotaxime. For histological GUS activity assays,
501 transformants were incubated in GUS staining solution (0.5 mM potassium ferrocyanide,
502 0.5 mM potassium ferricyanide, and 1 mM X-Gluc) at 37°C and later cleared with 70%
503 ethanol (Jefferson et al., 1987).

504

505 **Yeast Two-Hybrid (Y2H) assay**

506 To construct AD::KAR and AD::MpRop vectors, the *KAR* and *MpRop* coding sequences
507 were amplified by PCR using KOD plus neo (TOYOBO) with primer pairs
508 *KAR_WT_Y2H_pGADT7_F* and *KAR_WT_Y2H_pGADT7_R*, and
509 *Rop_WT_Y2H_pGADT7_F* and *Rop_WT_Y2H_pGADT7_R*, respectively, and subcloned
510 into the *NotI* site of pGADT7-AD in Matchmaker Gold Yeast Two-Hybrid System (Takara
511 Bio, Japan) with the In-Fusion HD cloning kit (Takara Bio). To construct BD::KAR and
512 BD::MpRop vectors, the *KAR* and *MpRop* coding sequences were amplified by PCR using
513 KOD plus neo (TOYOBO) with primer pairs *KAR_WT_Y2H_pGBKT7_F* and
514 *KAR_WT_Y2H_pGBKT7_R*, and *Rop_WT_Y2H_pGBKT7_F* and
515 *Rop_WT_Y2H_pGBKT7_R*, respectively, and subcloned into the *NotI* site of pGBKT7 in
516 Matchmaker Gold Yeast Two-Hybrid System (Takara Bio) with the In-Fusion HD cloning
517 kit (Takara Bio). Indicated combinations of plasmids were co-transformed into yeast strain
518 Y2H Gold (Takara Bio) following the protocol for high-efficiency transformation of yeast
519 with lithium acetate, single-stranded carrier DNA, and polyethylene glycol. Following
520 transformation, colonies were selected for the presence of the plasmids, inoculated in liquid
521 synthetic drop-out (SD) media (lacking the amino acids leucine and tryptophan, with the
522 exception of untransformed strain Y2H Gold, which was grown in YPD), grown to
523 saturation, and plated onto SD media plates lacking the indicated amino acids. SD media
524 plates lacked the amino acids leucine, tryptophan, and histidine (SD -Leu/-Trp/-His).
525 Serial 1:5 dilutions were made in water and 3 µl of each dilution was used to yield one spot.
526 Plates were incubated at 30°C for two (SD -Leu/-Trp) or three (SD -Leu/-Trp/-His) days
527 before taking pictures.

528

529 **Protein purification**

530 The cDNA of MpRop was amplified by RT-PCR using KOD plus neo (TOYOBO) with
531 primer pairs MpRop-cds-F and MpRop-cds-sR and cloned into pENTR/D-TOPO (Thermo
532 Fisher Scientific). The *MpRop* coding sequence in the resultant ENTRY clone and
533 pENTR-KAR and pENTR-PRONE generated above were transferred using Gateway LR

534 clonase II Enzyme mix (Thermo Fisher Scientific, USA) into the bacterial expression
535 vectors pDEST15 or pDEST17, which express GST- or 6xhistidine (6xHis)-tagged protein,
536 respectively. 6xHis-KAR and 6xHis-KAR-PRONE were expressed in the *Escherichia coli*
537 strain Arctic Express RP with 0.25 mM IPTG at 12°C. The cells were harvested by
538 centrifugation and lysed in extraction buffer (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 2
539 mM MgCl₂, 5 mM 2-mercaptoethanol, 1 mM phenylmethane sulfonyl fluoride or
540 phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml leupeptin, 250 µg/ml lysozyme, and 0.2%
541 C₁₂E₁₀) with sonication. The bacterial lysate was centrifuged at 100,000 x g for 1 hr.
542 His-tagged protein was captured from the supernatant using nickel-NTA agarose, washed
543 with wash buffer (20 mM Tris-HCl (pH 7.4), 500 mM NaCl, 5 mM 2-mercaptoethanol, 1
544 mM PMSF, 2 µg/ml leupeptin, and 20 mM imidazole) and eluted with elution buffer (20
545 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM 2-mercaptoethanol, 1 mM PMSF, and 20%
546 glycerol) with 250 mM imidazole. The eluted proteins were dialyzed against elution buffer
547 and frozen at -80°C.

548 Bacterial cells expressing GST or GST-MpRop were lysed in extraction buffer (50 mM
549 Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM MgCl₂, 1 mM EDTA, 5 mM 2-mercaptoethanol,
550 1 mM PMSF, 2 µg/ml leupeptin, 100 µM GDP, 250 µg/ml lysozyme, and 0.2% C₁₂E₁₀)
551 with sonication. The bacterial lysate was centrifuged at 100,000 x g for 1 hr. GST-tagged
552 proteins were captured from the supernatant using glutathione-agarose, washed with wash
553 buffer I (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 5 mM 2-mercaptoethanol,
554 40 µM GDP, 2 mM MgCl₂, 1 mM PMSF, and 2 µg/ml leupeptin), wash Buffer II (50 mM
555 Tris-HCl (pH 7.4), 500 mM NaCl, 40 µM GDP, 2 mM MgCl₂, 1 mM EDTA, 5 mM
556 2-mercaptoethanol, 1 mM PMSF, and 2 µg/ml leupeptin), then wash buffer I again.
557 GST-tagged proteins were eluted with elution buffer (100 mM Tris-HCl (pH 8.8), 200 mM
558 NaCl, 40 µM GDP, 2 mM MgCl₂, 10 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM PMSF,
559 and 20% glycerol) with 20 mM glutathione. The eluted proteins were dialyzed against
560 elution buffer without GDP and frozen at -80°C.

561

562 ***In vitro* pull-down assay**

563 One µg of 6xHis-KAR was incubated with 10 µg of GST or GST-MpROP in Nucleotide

564 Binding Buffer (20 mM HEPES-NaOH (pH 7.4), 5 mM MgCl₂, 1 mM dithiothreitol, 0.1%
565 Triton X-100, and 1 mM EDTA) preloaded with 10 µM GDP, GTPγS, or no nucleotide for
566 4 hours at 4°C. The samples were centrifuged with a table-top centrifuge at full speed for 1
567 min, and the supernatant was incubated with Glutathione-agarose resin for 30 min at 4°C.
568 The resins were then washed for three times with Nucleotide Binding Buffer with the
569 respective nucleotide. The resins were then boiled with SDS loading buffer and separated
570 by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The
571 6xHis-tagged proteins were detected by western blot with a mouse anti-6xHis antibody
572 (Santa Cruz Biotech) as the primary antibody, an HRP-conjugated anti-mouse IgG antibody
573 was used as the secondary antibody. GST and GST-MpRop were detected by Coomassie
574 Brilliant Blue staining.

575

576 **GTPγS binding on MpRop**

577 The GEF enzymatic activity of KAR or KAR-PRONE toward MpRop was analyzed using
578 radio-labelled [³⁵S]-GTPγS, as described in previous studies with slight modifications [26,
579 41]. For [³⁵S]-GTPγS binding, 2 µM GST-MpRop in reaction buffer (50 mM Tris-HCl (pH
580 7.4), 1 mM EDTA, 1 mM DTT, and 5 mM MgCl₂) was mixed with an equal volume of
581 reaction buffer containing 5 µM [³⁵S]-GTPγS to start the exchange reaction on ice. At given
582 time points, 50 µl aliquots were removed and placed into 450 µl of ice-cold wash buffer
583 (20 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 25 mM MgCl₂) with 0.1 mM GTP, then
584 applied to a nitrocellulose membrane filter. The filter was washed three times with 2 to 3
585 ml of ice-cold wash buffer. The amount of [³⁵S]-GTPγS was measured by scintillation
586 counting.

587

588 **RT-qPCR**

589 Total RNA was isolated from the 1-week-old thalli, and mature gemmae, gemma cups, and
590 midribs of 3-week-old thalli of Tak-1 (Fig. 4C) and 1-week-old thalli of the *kar*^{KO}#2 line
591 transformed with *proKAR:Citrine-KAR* or *proKAR:Citrine-PRONE* (Fig. 3D) using the
592 RNeasy Plant mini kit (Qiagen). One µg of total RNA was reverse-transcribed in a 20 µl
593 reaction mixture using ReverTra Ace qPCR RT Master Mix with gDNA remover

594 (TOYOBO). After the reaction, the mixture was diluted with 40 µl of distilled water and 2
595 µl aliquots were used for quantitative PCR (qPCR) analysis. qPCR was performed with the
596 Light Cycler 96 (Roche) using KOD SYBR qRT-PCR Mix (TOYOBO) according to the
597 manufacturer's protocol. The primers used in these experiments are listed in Table S1.
598 Transcript levels of MpEF1a or MpAPT were used as a reference for normalization [42].
599 RT-qPCR experiments were performed using three biological replicates and technically
600 duplicated.

601

602 Phylogenetic Analysis of KAR

603 For phylogenetic analysis of KAR and RopGEFs, peptide sequences were collected from
604 genomic information of *M. polymorpha* in MalpolBase (<http://marchantia.info/>), *A.*
605 *thaliana* in TAIR (<http://www.arabidopsis.org>), *Physcomitrella patens* [43] and *Selaginella*
606 *moellendorffii* [44] in Phytozome (<https://phytozome.jgi.doe.gov/pv/portal.html>), and
607 *Klebsormidium nitens* NIES-2285 in
608 (http://www.plantmorphogenesis.bio.titech.ac.jp/~algaе_genome_project/klebsormidium/kf_download.htm) [45]. A multiple alignment of amino acid sequences of KAR and its
609 homologous RopGEFs was first constructed using the MUSCLE program [46]
610 implemented in MEGA6.06 [47] with default parameters, which was performed using a
611 Maximum Likelihood method by PhyML [48] with the LG+G+I substitution model. One
612 thousand bootstrap replicates were performed in each analysis to obtain the confidence
613 support.

615

616 REFERENCES

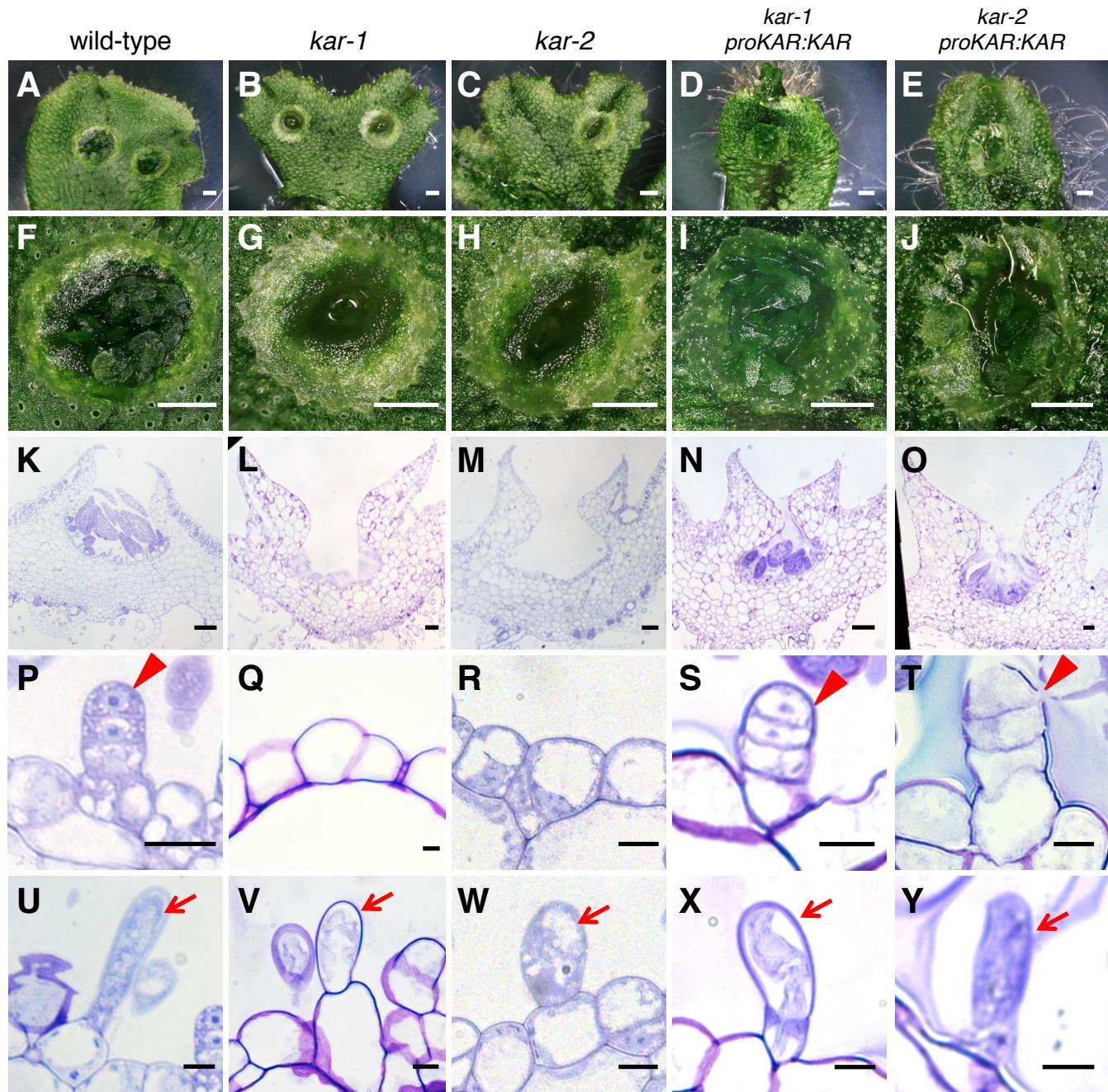
- 617 1. Shimamura, M. (2016). *Marchantia polymorpha*: Taxonomy, phylogeny and
618 morphology of a model system. *Plant Cell Physiol.* *57*, 230-256.
- 619 2. Barnes, C.R., and Land, W.J.G. (1908). Bryological papers. II. The origin of the
620 cupule of Marchantia. *Bot. Gaz.* *46*, 401-409.
- 621 3. Proust, H., Honkanen, S., Jones, Victor A.S., Morieri, G., Prescott, H., Kelly, S.,
622 Ishizaki, K., Kohchi, T., and Dolan, L. (2016). RSL class I genes controlled the
623 development of epidermal structures in the common ancestor of land plants. *Curr.
624 Biol.* *26*, 93-99.
- 625 4. Steward, F.C., Mapes, M.O., and Mears, K. (1958). Growth and organized
626 development of cultured cells. II. Organization in cultures grown from freely
627 suspended cells. *Am. J. Bot.* *45*, 705-708.
- 628 5. Steward, F.C., Mapes, M.O., Kent, A.E., and Holsten, R.D. (1964). Growth and
629 development of cultured plant cells. *Science* *143*, 20-27.
- 630 6. Steeves, T.A., and Sussex, I.M. (1989). Patterns in plant development, 2 Edition,
631 (Cambridge: Cambridge University Press).
- 632 7. Davis, F.T., Geneve, R.L., Wilson, S.E., Hartmann, H.T., Kester, D.E. (2017).
633 Hartmann & Kester's Plant Propagation: Principles and Practices, (Pearson).
- 634 8. Hofmeister, W.F.B. (1862). On the germination, development, and fructification of
635 the higher Cryptogamia, and on the fructification of the Coniferae., (London, Pub.
636 for the Ray society).
- 637 9. Bowman, J.L., Kohchi, T., Yamato, K.T., Jenkins, J., Shu, S., Ishizaki, K., Yamaoka,
638 S., Nishihama, R., Nakamura, Y., Berger, F., et al. (2017). Insights into land plant
639 evolution garnered from the *Marchantia polymorpha* genome. *Cell* *171*, 287-304
640 e215.
- 641 10. Ishizaki, K., Chiyoda, S., Yamato, K.T., and Kohchi, T. (2008).
642 *Agrobacterium*-mediated transformation of the haploid liverwort *Marchantia*
643 *polymorpha* L., an emerging model for plant biology. *Plant Cell Physiol.* *49*,
644 1084-1091.
- 645 11. Ishizaki, K., Johzuka-Hisatomi, Y., Ishida, S., Iida, S., and Kohchi, T. (2013).
646 Homologous recombination-mediated gene targeting in the liverwort *Marchantia*
647 *polymorpha* L. *Sci. Rep.* *3*, 1532.
- 648 12. Ishizaki, K., Nishihama, R., Yamato, K.T., and Kohchi, T. (2016). Molecular
649 genetic tools and techniques for *Marchantia polymorpha* research. *Plant Cell
650 Physiol.* *57*, 262-270.
- 651 13. Kubota, A., Ishizaki, K., Hosaka, M., and Kohchi, T. (2013). Efficient

- 652 Agrobacterium-mediated transformation of the liverwort *Marchantia polymorpha*
653 using regenerating thalli. Biosci. Biotechnol. Biochem. 77, 167-172.
- 654 14. Sugano, S.S., Shirakawa, M., Takagi, J., Matsuda, Y., Shimada, T., Hara-Nishimura,
655 I., and Kohchi, T. (2014). CRISPR/Cas9-mediated targeted mutagenesis in the
656 liverwort *Marchantia polymorpha* L. Plant Cell Physiol. 55, 475-481.
- 657 15. Nishihama, R., Ishida, S., Urawa, H., Kamei, Y., and Kohchi, T. (2016). Conditional
658 gene expression/deletion systems for *Marchantia polymorpha* using its own
659 heat-shock promoter and Cre/loxP-mediated site-specific recombination. Plant Cell
660 Physiol. 57, 271-280.
- 661 16. Flores-Sandoval, E., Dierschke, T., Fisher, T.J., and Bowman, J.L. (2016). Efficient
662 and inducible use of artificial microRNAs in *Marchantia polymorpha*. Plant Cell
663 Physiol. 57, 281-290.
- 664 17. Ishizaki, K., Mizutani, M., Shimamura, M., Masuda, A., Nishihama, R., and Kohchi,
665 T. (2013). Essential role of the E3 ubiquitin ligase NOPPERABO1 in schizogenous
666 intercellular space formation in the liverwort *Marchantia polymorpha*. Plant Cell 25,
667 4075-4084.
- 668 18. Takenaka, M., Yamaoka, S., Hanajiri, T., Shimizu-Ueda, Y., Yamato, K.T.,
669 Fukuzawa, H., and Ohyama, K. (2000). Direct transformation and plant
670 regeneration of the haploid liverwort *Marchantia polymorpha* L. Transgenic Res. 9,
671 179-185.
- 672 19. Berken, A., Thomas, C., and Wittinghofer, A. (2005). A new family of RhoGEFs
673 activates the Rop molecular switch in plants. Nature 436, 1176-1180.
- 674 20. Zhang, Y., and McCormick, S. (2007). A distinct mechanism regulating a
675 pollen-specific guanine nucleotide exchange factor for the small GTPase Rop in
676 *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. U. S. A. 104, 18830-18835.
- 677 21. Duan, Q., Kita, D., Li, C., Cheung, A.Y., and Wu, H.M. (2010). FERONIA
678 receptor-like kinase regulates RHO GTPase signaling of root hair development.
679 Proc. Natl. Acad. Sci. U. S. A. 107, 17821-17826.
- 680 22. Kessler, S.A., Shimosato-Asano, H., Keinath, N.F., Wuest, S.E., Ingram, G.,
681 Panstruga, R., and Grossniklaus, U. (2010). Conserved molecular components for
682 pollen tube reception and fungal invasion. Science 330, 968-971.
- 683 23. Nibau, C., and Cheung, A.Y. (2011). New insights into the functional roles of
684 CrRLKs in the control of plant cell growth and development. Plant signaling &
685 behavior 6, 655-659.
- 686 24. Oda, Y., and Fukuda, H. (2012). Initiation of cell wall pattern by a Rho- and
687 microtubule-driven symmetry breaking. Science 337, 1333-1336.
- 688 25. Li, Z., Waadt, R., and Schroeder, J.I. (2016). Release of GTP exchange factor

- 689 mediated down-regulation of abscisic acid signal transduction through
690 ABA-Induced rapid degradation of RopGEFs. *PLoS Biol.* *14*, e1002461.
- 691 26. Gu, Y., Li, S., Lord, E.M., and Yang, Z. (2006). Members of a novel class of
692 Arabidopsis Rho guanine nucleotide exchange factors control Rho
693 GTPase-dependent polar growth. *Plant Cell* *18*, 366-381.
- 694 27. Basu, D., Le, J., Zakharova, T., Mallory, E.L., and Szymanski, D.B. (2008). A
695 SPIKE1 signaling complex controls actin-dependent cell morphogenesis through
696 the heteromeric WAVE and ARP2/3 complexes. *Proc. Natl. Acad. Sci. U. S. A.* *105*,
697 4044-4049.
- 698 28. Zhang, C., Kotchoni, S.O., Samuels, A.L., and Szymanski, D.B. (2010). SPIKE1
699 signals originate from and assemble specialized domains of the endoplasmic
700 reticulum. *Curr. Biol.* *20*, 2144-2149.
- 701 29. Meller, N., Merlot, S., and Guda, C. (2005). CZH proteins: a new family of
702 Rho-GEFs. *J. Cell Sci.* *118*, 4937-4946.
- 703 30. Chang, F., Gu, Y., Ma, H., and Yang, Z. (2013). AtPRK2 promotes ROP1 activation
704 via RopGEFs in the control of polarized pollen tube growth. *Molecular plant* *6*,
705 1187-1201.
- 706 31. Fu, Y., Gu, Y., Zheng, Z., Wasteneys, G., and Yang, Z. (2005). *Arabidopsis*
707 interdigitating cell growth requires two antagonistic pathways with opposing action
708 on cell morphogenesis. *Cell* *120*, 687-700.
- 709 32. Gu, Y., Fu, Y., Dowd, P., Li, S., Vernoud, V., Gilroy, S., and Yang, Z. (2005). A Rho
710 family GTPase controls actin dynamics and tip growth via two counteracting
711 downstream pathways in pollen tubes. *J. Cell Biol.* *169*, 127-138.
- 712 33. Etienne-Manneville, S. (2004). Cdc42--the centre of polarity. *J. Cell Sci.* *117*,
713 1291-1300.
- 714 34. Park, H.O., and Bi, E. (2007). Central roles of small GTPases in the development of
715 cell polarity in yeast and beyond. *Microbiol. Mol. Biol. Rev.* *71*, 48-96.
- 716 35. Humphries, J.A., Vejrupkova, Z., Luo, A., Meeley, R.B., Sylvester, A.W., Fowler,
717 J.E., and Smith, L.G. (2011). ROP GTPases act with the receptor-like protein PAN1
718 to polarize asymmetric cell division in maize. *Plant Cell* *23*, 2273-2284.
- 719 36. Facette, M.R., Park, Y., Sutimananapi, D., Luo, A., Cartwright, H.N., Yang, B.,
720 Bennett, E.J., Sylvester, A.W., and Smith, L.G. (2015). The SCAR/WAVE complex
721 polarizes PAN receptors and promotes division asymmetry in maize. *Nature Plants*
722 *1*, 14024.
- 723 37. Gamborg, O.L., Miller, R.A., and Ojima, K. (1968). Nutrient requirements of
724 suspension cultures of soybean root cells. *Exp. Cell Res.* *50*, 151-158.
- 725 38. Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie

- 726 2. Nat. Methods 9.
- 727 39. Robinson, J.T., Thorvaldsdottir, H., Winckler, W., Guttman, M., Lander, E.S., Getz,
728 G., and Mesirov, J.P. (2011). Integrative genomics viewer. Nat. Biotechnol. 29,
729 24-26.
- 730 40. Ishizaki, K., Nishihama, R., Ueda, M., Inoue, K., Ishida, S., Nishimura, Y., Shikanai,
731 T., and Kohchi, T. (2015). Development of Gateway binary vector series with four
732 different selection markers for the liverwort *Marchantia polymorpha*. PLoS One 10,
733 e0138876.
- 734 41. Urano, D., Jones, J.C., Wang, H., Matthews, M., Bradford, W., Bennetzen, J.L., and
735 Jones, A.M. (2012). G protein activation without a GEF in the plant kingdom. PLoS
736 Genet. 8, e1002756.
- 737 42. Saint-Marcoux, D., Proust, H., Dolan, L., and Langdale, J.A. (2015). Identification
738 of Reference Genes for Real-Time Quantitative PCR Experiments in the Liverwort
739 *Marchantia polymorpha*. PLoS One 10, e0118678.
- 740 43. Rensing, S.A., Lang, D., Zimmer, A.D., Terry, A., Salamov, A., Shapiro, H.,
741 Nishiyama, T., Perroud, P.F., Lindquist, E.A., Kamisugi, Y., et al. (2008). The
742 Physcomitrella genome reveals evolutionary insights into the conquest of land by
743 plants. Science 319, 64-69.
- 744 44. Banks, J.A., Nishiyama, T., Hasebe, M., Bowman, J.L., Grabskov, M., dePamphilis,
745 C., Albert, V.A., Aono, N., Aoyama, T., Ambrose, B.A., et al. (2011). The
746 Selaginella genome identifies genetic changes associated with the evolution of
747 vascular plants. Science 332, 960-963.
- 748 45. Hori, K., Maruyama, F., Fujisawa, T., Togashi, T., Yamamoto, N., Seo, M., Sato, S.,
749 Yamada, T., Mori, H., Tajima, N., et al. (2014). *Klebsormidium flaccidum* genome
750 reveals primary factors for plant terrestrial adaptation. Nat Commun 5, 3978.
- 751 46. Edgar, R.C. (2004). MUSCLE: multiple sequence alignment with high accuracy
752 and high throughput. Nucleic Acids Res. 32, 1792-1797.
- 753 47. Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6:
754 Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30,
755 2725-2729.
- 756 48. Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O.
757 (2010). New algorithms and methods to estimate maximum-likelihood phylogenies:
758 assessing the performance of PhyML 3.0. Syst. Biol. 59, 307-321.
- 759

Figure1-4

**Figure 1. Phenotype of the *kar* mutants and their complimented lines**

(A–Y) Five genotypes are presented, one in each column: (first column) wild type, (second column) *kar-1*, (third column) *kar-2*, (fourth column) *kar-1* complementation line (*kar-1* transformed with *proKAR:KAR*), (fifth column) *kar-2* complementation line (*kar-2* transformed with *proKAR:KAR*). (A–E) Top view of 2-week-old thalli grown from tips of thalli. Scale bars represent 1 mm. (F–J) Surface view of gemma cups in 2-week-old thalli. Scale bars represent 1 mm. (K–O) Toluidine-blue-stained transverse sections of gemma cups in 2-week-old plants. Scale bars represent 100 μ m. (P–Y) Magnified views of toluidine-blue-stained sections of gemma cups in 2-week-old plants. Arrowheads and arrows indicate gemma initials and mucilage papillae, respectively. Scale bars represent 10 μ m.

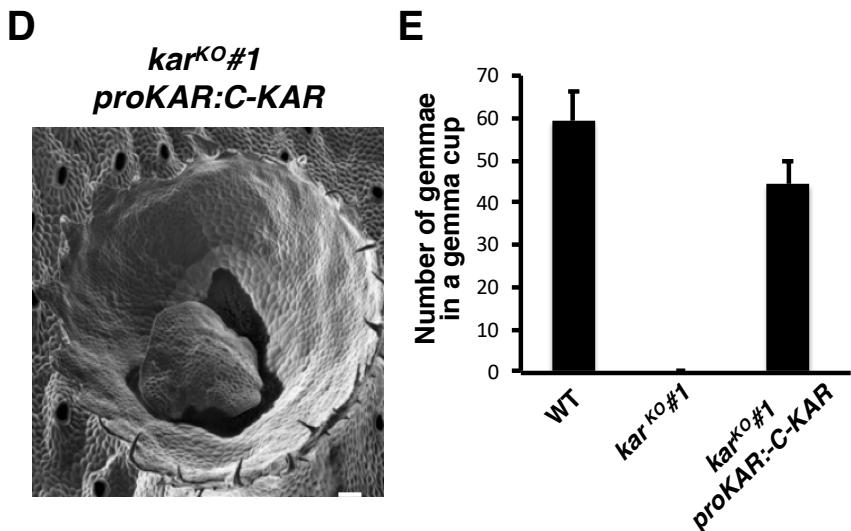
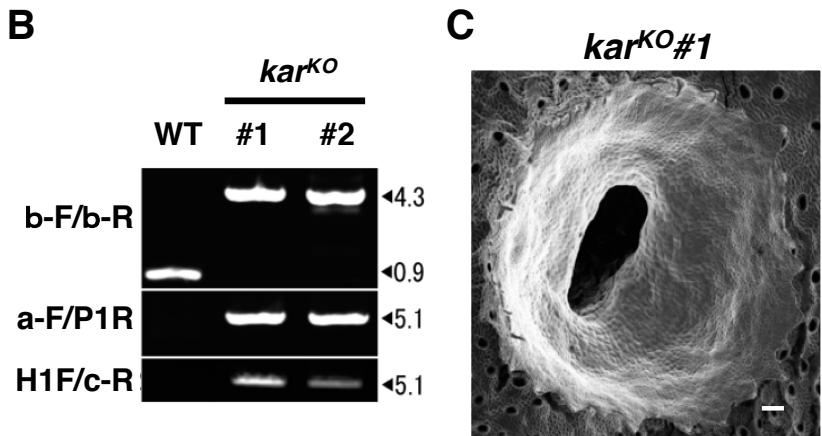
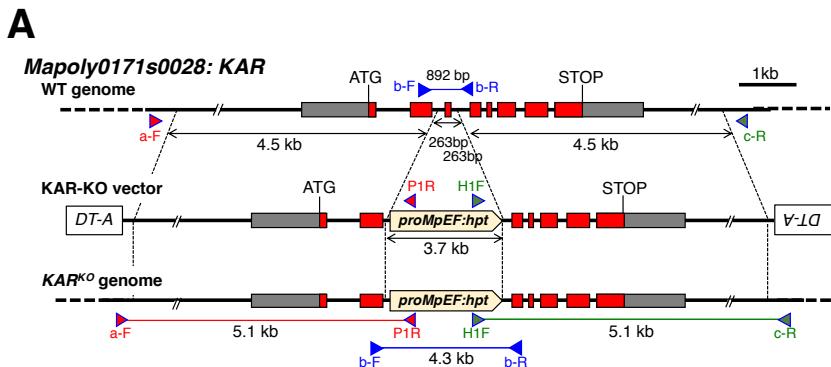


Figure 2. Generation of knockout mutants of *KAR*

(A) Schematic representation of the structure of the wild-type *KAR* locus (top), the construct designed for gene targeting (middle), and the *KAR* locus disrupted in the gene-targeted lines (bottom). Each primer pair used for genotyping is indicated by arrowheads and marked with (F) for forward or (R) for reverse. Open boxes indicate exons. (B) Genomic PCR analysis of the *KAR^{KO}* lines using the primers indicated in (A). (C, D) Scanning electron microscopy of gemma cups in three genotypes are presented: *kar^{KO}#1* (C), and a representative *kar^{KO}* line transformed with *proKAR:C-KAR*, which contains a citrine-fused *KAR* coding sequence under the endogenous *KAR* promoter. (D). Scale bars represent 100 μ m. (E) Number of gemmae formed in a gemma cup in 3-week-old thalli grown from apical fragments in the wild type, a *kar^{KO}* line, and a representative *kar^{KO}* complemented line (Values are means \pm SD, n = 5).

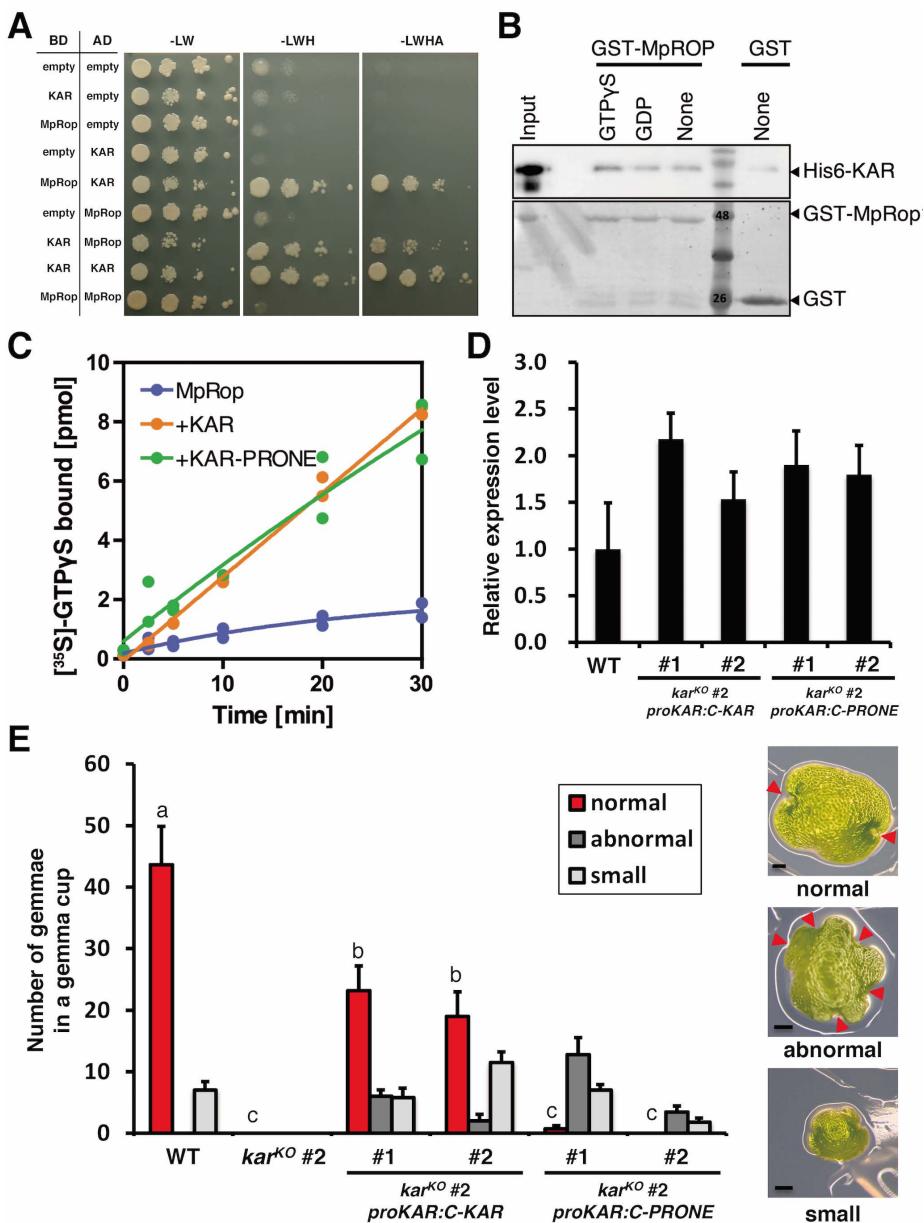


Figure 3. KAR has GEF activity towards MpRop.

(A) Yeast two-hybrid experiments. Clones in the pGBKT7 vector containing the Gal4-binding domain (BD) are noted in the left column and clones in the pGADT7 vector containing the Gal4 activation domain (AD) are noted in the right column. Growth with serial dilutions on the -L, -W dropout media indicates that both pGBKT7 and pGADT7 vectors were present. Growth with serial dilutions on the -L, -W, -H dropout media and -L, -W, -H, -A dropout media indicates a physical interaction between the BD and AD fusion proteins. (B) Physical interaction of KAR with MpRop. His6-KAR was pulled down with GST or GST-MpRop1 using glutathione agarose. The pull-down samples were separated on a SDS-PAGE gel, then visualized by western blot with an anti-6xHis antibody. (C) GEF activity of the full-length KAR (KAR) or the PRONE domain of KAR (KAR-PRONE) toward MpRop. [35 S]-GTP γ S binding to 1 μ M GST-MpRop1 was analyzed over time at 4° C. Graphs show data from two experiments. Fitting curves were estimated by the one-phase association model in GraphPad Prism software. (D) RT-qPCR analysis of *KAR* or *KAR-PRONE* expression in 3-week-old wild type and the respective complementation lines shown in Figure 3E. MpAPT was used as a control gene. Data are displayed as means \pm SD ($n = 3$). (E) Genetic complementation with the full-length *KAR* and the truncated *KAR* coding sequence containing only the PRONE domain. The histogram shows distribution of the different classes of gemmae in a gemma cup in 3-week-old thalli grown from apical fragments, in WT, a *kar*^{KO} mutant, and two representative *kar* complementation lines with the full-length KAR (*proKAR:C-KAR*) and with the PRONE domain (*proKAR:C-PRONE*) (Values are means \pm s.d., $n=4\sim 5$). Tukey's test was performed for the number of normal gemmae, and letters above the bars indicate significant differences at $p < 0.05$. Right panels show pictures of normal gemma of over 500 μ m diameter (normal), abnormal gemma with more than two notches, and small gemma of less than 500 μ m diameter.

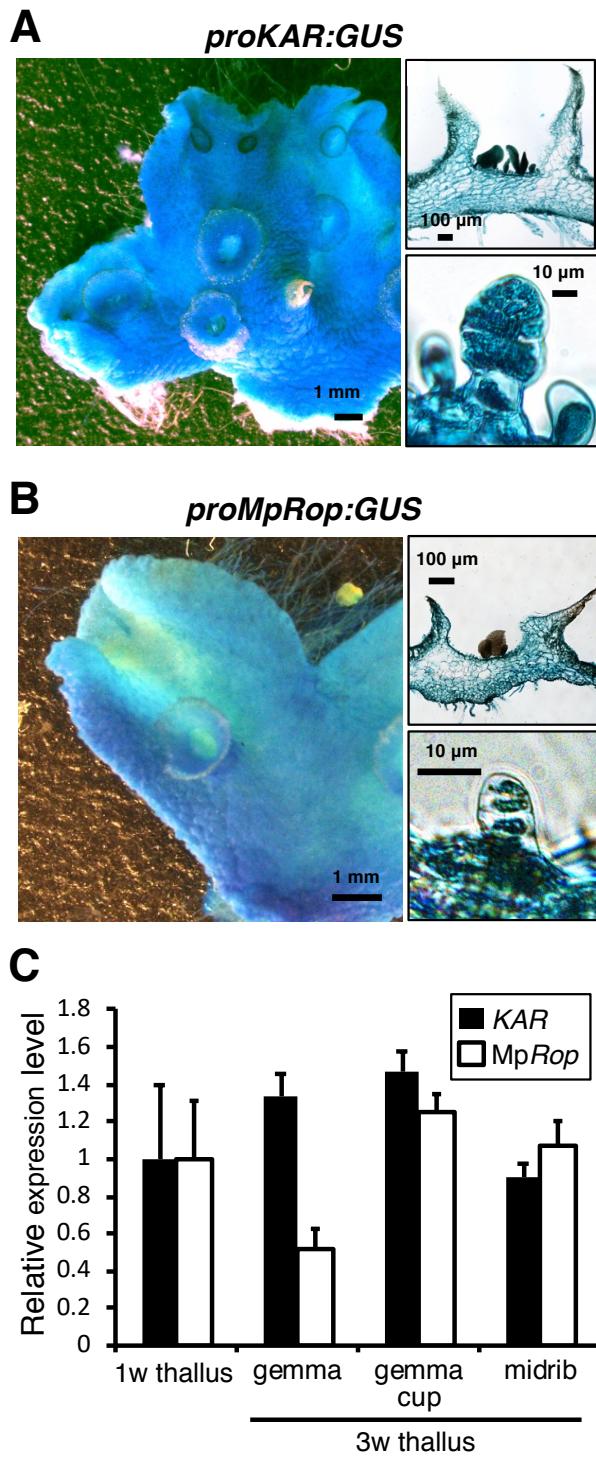


Figure 4. Expression of KAR and MpRop in vegetative tissues

(A, B) Histological GUS activity staining of representative *proKAR:GUS* (A) and *proRop:GUS* (B) transgenic lines. (C) RT-qPCR analysis of *KAR* and *MpRop* in 1-week-old thalli yet to develop gemma cups (1w thallus), mature gemmae in gemma cups (gemma), gemma cups containing developing gemmae (gemma cups), and midribs. Total RNA was isolated from the respective tissues of Tak-1. *EF1α* was used as a control gene. Data are displayed as means \pm SD ($n = 3$).

Figure S1-S4 and Table S1

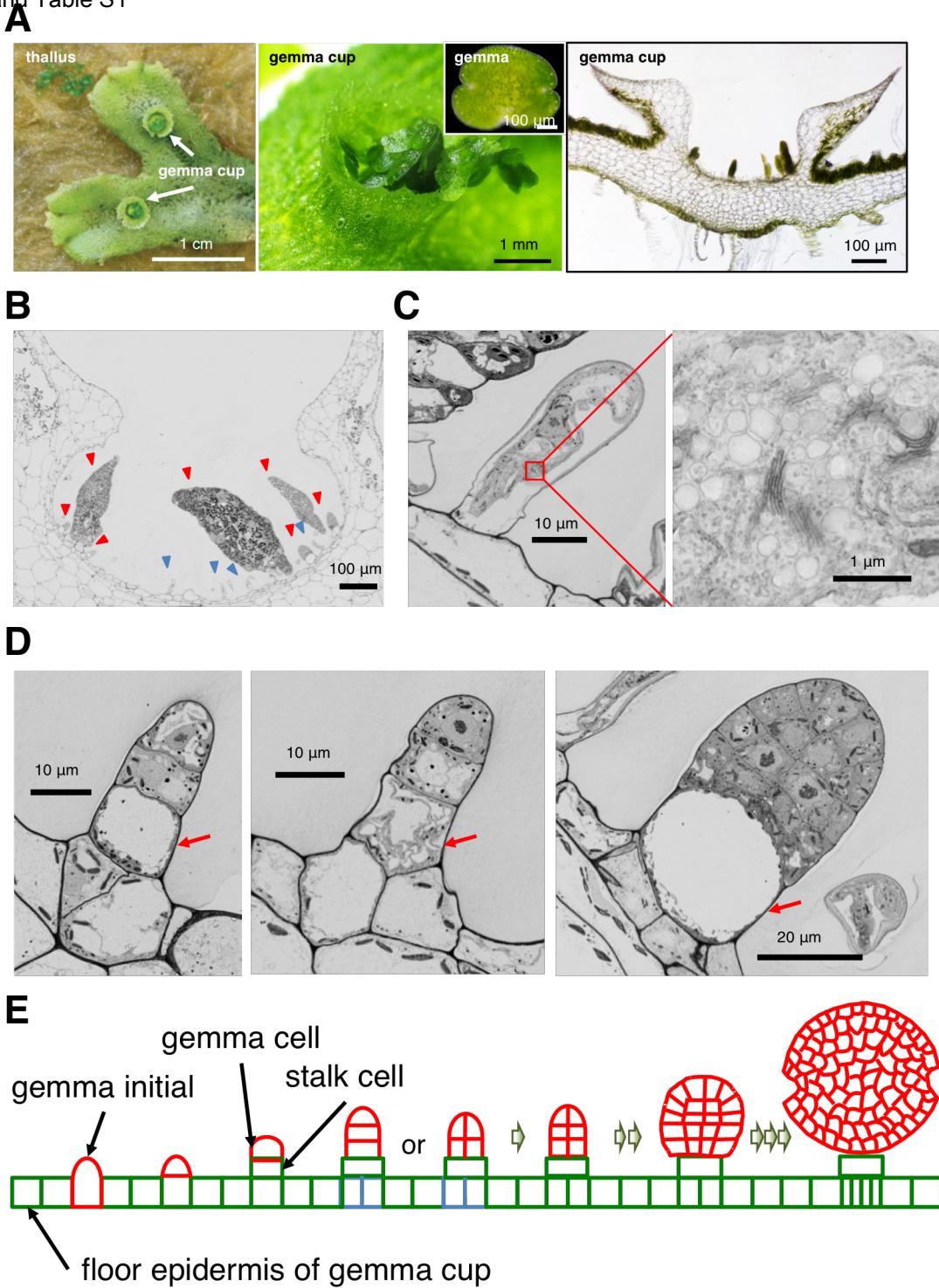


Figure S1. Gemma development in *M. polymorpha*, Related to Figure 1.

(A) Optical observation of gemma and gemma cup in *M. polymorpha*. Gemma cups formed on the dorsal surface of gametophyte body, thallus. Top view of thallus (left), a close-up view of gemma cup (middle), and a transverse section of a gemma cup (right). (B-D) Electron microscopy in the basal floor of gemma cup. (B) Transverse section of a gemma cup. Red arrow-heads indicate developing gemmae. Blue-arrow heads indicate mucilage papillae. (C) Close up view of mucilage papillae observed at the floor epidermis of gemma cup. The right image shows an enlarged image of indicated cytosolic region in the left image. (D) Close up view of developing gemmae. Red arrows indicate basal stalk cells. (E) Schematic model of gemma development in the floor epidermis of gemma cup.

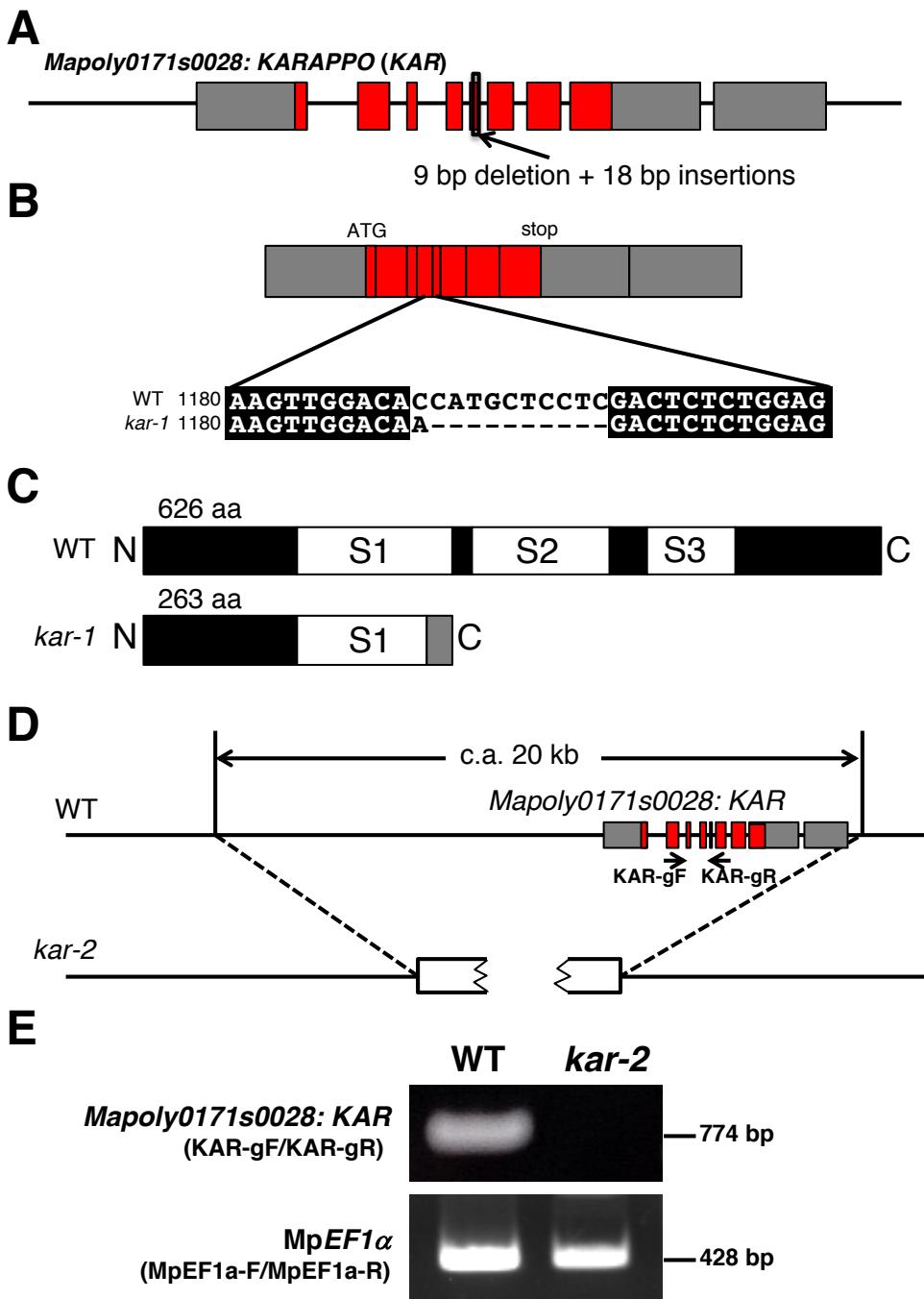


Figure S2. Molecular characterization of *kar-1* and *kar-2*, Related to Figure 1 to 2.

Whole genome analysis revealed respective mutations in *Mapoly0171s0028* locus in *kar-1* and *kar-2*. (A) Schematic representation of the *Mapoly0171s0028* genomic locus in wild-type and *kar-1*. Gray boxes indicate exons of untranslated region. Red boxes indicate exons of protein coding region. A small deletion found in the *Mapoly0171s0028* locus of *kar-1* genome. (B) cDNA sequences of *Mapoly0171s0028* in *kar-1*. (C) Schematic representation of deduced gene products of *Mapoly0171s0028* in wild-type and *kar-1*. (D) Whole genome analysis revealed c.a. 20 kb deletion in *kar-2*. Broken open boxes indicate partial fragments of pMT plasmid (Takenaka et al. 2000), which was introduced by particle bombardment protocol. A series of genomic PCR suggested that the 5' and 3' region of *Mapoly0171s0028* is not adjacent to each other in *kar-2* (data not shown), suggesting occurrence of a genomic rearrangement accompanied with the physical DNA delivery. (E) Genomic PCR of *Mapoly0171s0028* in wild-type and *kar-2*. *MpEF1 α* ; *M. polymorpha* Elongation Factor alpha gene (*MpEF1 α*) was used as a positive control.

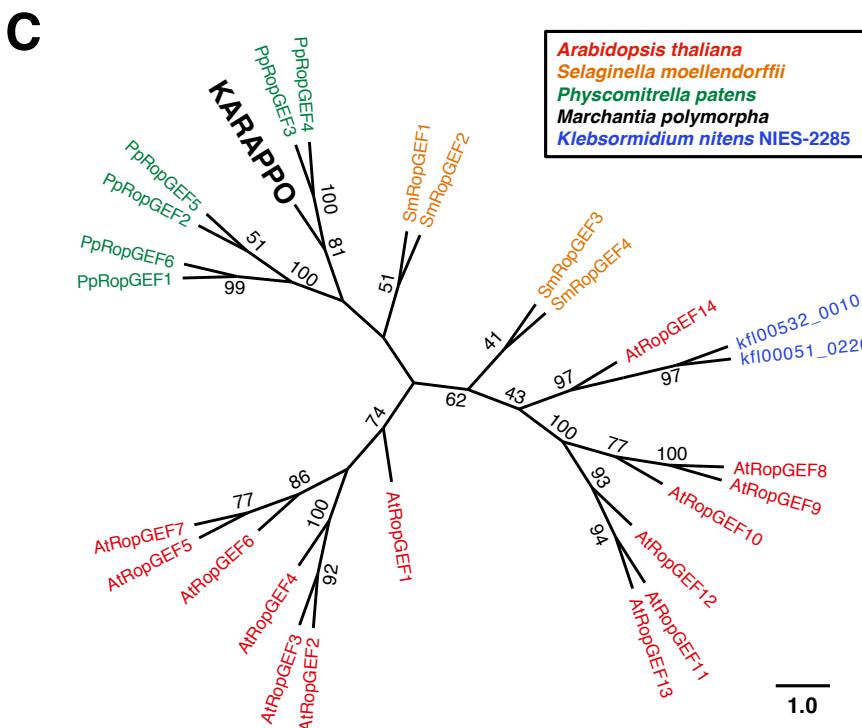
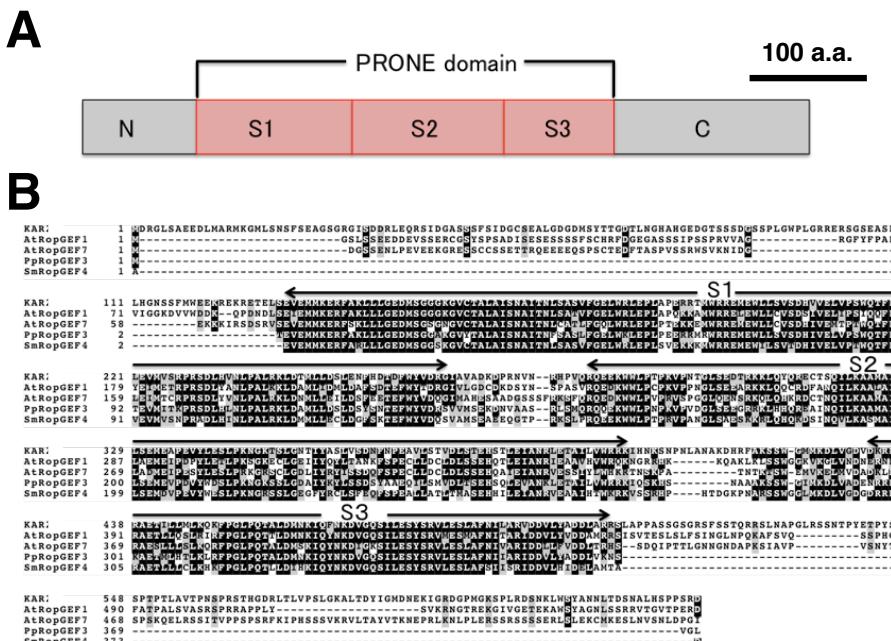


Figure S3. KAR encodes a highly conserved PRONE domain of RopGEF, Related to Figure 2 to 3.

(A) A domain structure of the *KAR* gene product. (B) Multiple alignment of the full amino acid sequences of KAR and representative RopGEFs in the moss *Physcomitrella patens*, the lycophyte *Selaginella moellendorffii* and *Arabidopsis thaliana* RopGEFs. Lines above aligned sequences indicate highly conserved regions in a PRONE domain composed of three subdomains (S1, S2, and S3), which has been in *Arabidopsis* to be essential for catalytic activity as guanine nucleotide exchange factor of ROP (Gu *et al.*, 2006; Oda *et al.*, 2012). (C) Unrooted Maximum-Likelihood tree of KAR and the other related RopGEF proteins across various plant lineages. The numbers on the branches show bootstrap values calculated from 1000 replicates. The scale bars are evolutionary distance at the ratio of amino acid substitutions.

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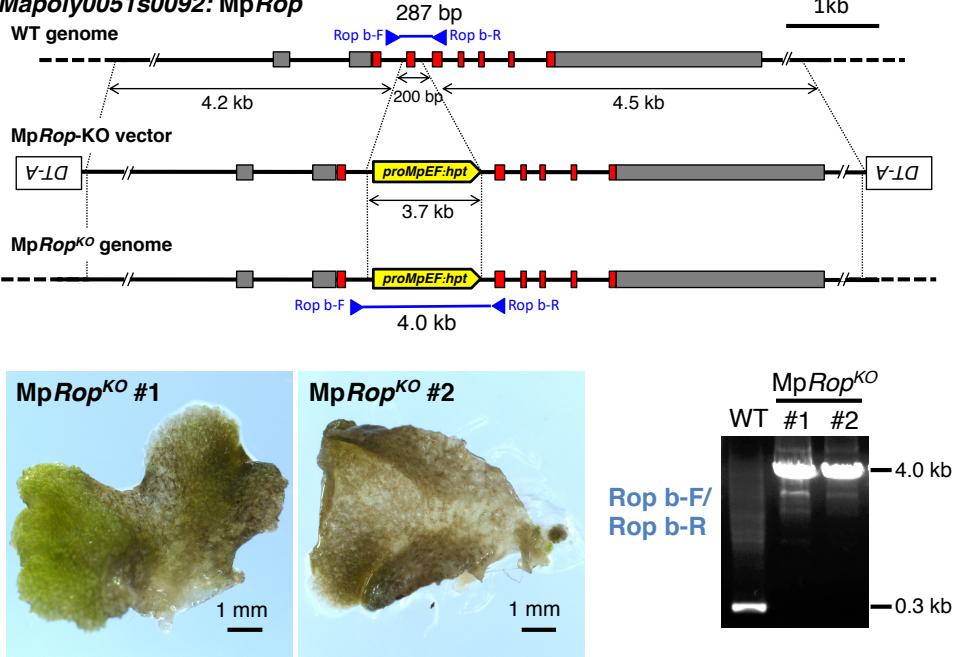
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AtRop2  1  M*SRFIKCVTVGDAVGKTCMLISYTSNTPTDYPVTFDNFSANVVVUDGNTVNLLGLNDTAGQEDYNRLRPLSRYRGADVFLAFLSLKASYENISKWIPELRHYA
AtRop7  1  M*SRFIKCVTVGDAVGKTCMLISYTSNTPTDYPVTFDNFSANVVVUDGNTVNLLGLNDTAGQEDYNRLRPLSRYRGADVFLAFLSLKASYENISKWIPELRHYA
SmRop1 1  M*SRFIKCVTVGDAVGKTCMLISYTSNTPTDYPVTFDNFSANVVVUDGNTVNLLGLNDTAGQEDYNRLRPLSRYRGADVFLAFLSLKASYENISKWIPELRHYA
FpRop2 1  M*SRFIKCVTVGDAVGKTCMLISYTSNTPTDYPVTFDNFSANVVVUDGNTVNLLGLNDTAGQEDYNRLRPLSRYRGADVFLAFLSLKASYENISKWIPELRHYA

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MpRop  111 VPIILVGKTKLDLRDKKFIDBPGAAPIITIQQEELKIGAMVIECSSKTQONVQAVFDAAIKVVLQPFQK-----KKKKQKTCV-----S-----
AtRop2  110 VPILVGKTKLDLRDKKFIDBPGAAPIITIQQEELKIGAMVIECSSKTQONVQAVFDAAIKVVLQPFQK-----KKKNKNRCAF-----S-----
AtRop7  111 VPILVGKTKLDLRDKKFIDBPGAAPIITIQQEELKIGAMVIECSSKTQONVQAVFDAAIKVVLQPFQK-----KKRSPICFFF-----S-----
SmRop1 111 VPILVGKTKLDLRDKKFIDBPGAAPIITIQQEELKIGAMVIECSSKTQONVQAVFDAAIKVVLQPFQK-----KKAKRRCCTV-----S-----
FpRop2 111 VPILVGKTKLDLRDKKFIDBPGAAPIITIQQEELKIGAMVIECSSKTQONVQAVFDAAIKVVLQPFQK-----KKKKQKTCV-----XXXXX

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B*Mapoly0051s0092: MpRop***C**

	RAC/ROP	RopGEF (KAR)	SPK1	REN	RopGAP	RopGDI	RIC	ICR
<i>Arabidopsis</i>	11	14	1	3	5	3	11	5
<i>Selaginella</i>	2	4	1	1	2	2	1	0
<i>Physcomitrella</i>	4	6	6	2	6	3	1	0
<i>Marchantia</i>	1	1	1	1	1	2	0	0
<i>Klebsormidium</i>	1	2	1	1	1	0	?	0
<i>Chlamydomonas</i>	0	0	0	0	0	0	0	0
<i>Ostreococcus</i>	1	0	1	0	0	0	0	0

Figure S4. RopGTPase and its related gene families in *M. polymorpha*, Related to Figure 4

(A) Multiple alignment of the full-amino acid sequences of MpROP and representative RopGTPases in *P. patens*, *S. moellendorffii*, and *A. thaliana*. (B) Generation of knockout mutants of MpRop. Schematic representation of the structure of the MpRop locus in wild type, the construct designed for gene targeting, and the MpRop locus disrupted in the gene-targeted lines (upper). Phenotype of two independent disruptants of MpRop gene showed severe impairment of thallus growth, and had died in the early stage of thallus development (lower left). A genotyping of MpRop indicates successful disruption of the cds structure occurred in MpRop knockouts (lower right). (C) Relative sizes of Rop signalling gene families in Viridiplantae. Total number of all homologous genes in the indicated gene families are indicated.

Table S1. Oligonucleotide primers used in this study.

Name	Sequence (5'→3')	Usage
kar-1_gF	CCGTATTTGGAGAGTTGTGGA	genomic PCR of <i>kar-1</i>
kar-1_gR	ACCGACTGCAGCTTGTGTT	genomic PCR of <i>kar-1</i>
KAR-gF	CGGAAATTCTGCCTTCATGT	genomic PCR of <i>kar-2</i> and wild type
KAR-gR	CTCCAGCCTCCACAACCTCTC	genomic PCR of <i>kar-2</i> and wild type
MpEF1a-F	TCACTCTGGGTGTGAAGCAG	genomic PCR of <i>kar-2</i> and wild type
MpEF1a-R	GCCTCGAGTAAAGCTTCGTG	genomic PCR of <i>kar-2</i> and wild type
KAR_5IF_F	CTAAGGTAGCGATTAATTAAATGAGCTTACCATATAATCACCGAGT	Construction of the targeting vector for KAR
KAR_5IF_R	GCCCGGGCAAGGTTAATTAAAGGTGGAACATTCTACATCGACAT	Construction of the targeting vector for KAR
KAR_3IF_F	TAAACTAGTGGCCGCACCGGATACGAGTTCTTTCTT	Construction of the targeting vector for KAR
KAR_3IF_R	TTATCCCTAGGCCGCCTGGTAATCTCCAGAACCTCTGA	Construction of the targeting vector for KAR
a-F	TAGACTCGTAGTCTGGCCCTCAC	For checking of gene targeting site
b-F	GATGGGGACATGAGCTACACCAC	For checking of gene targeting site
b-R	CGAACCAACACGACGTCAGATT	For checking of gene targeting site
c-R	GTTGTGTGATTGTCGGGTAGAG	For checking of gene targeting site
P1R	GAAGGCTTCTGATTGAAGTTCTTTCTG	For checking of gene targeting site
H1F	GTATAATGTATGCTATACGAAGTTATGTTT	For checking of gene targeting site
KARpro_GW_F	GGCCAGTGCCAAGCTTACAACTGTGGCTCGACGAAC	Construction of KARpro:KARcds
KARpro_GW_302_R	TGTTGATAACTCTAGATCCATAGCCGAGCCACGTACAG	Construction of KARpro:KARcds
KARpro_GW_305_R	CCATGCTCATTCTAGATCCATAGCCGAGCCACGTACAG	Construction of KARpro:Citrine-KARcds
KAR-cds-F	caccATGGATCGAGGGCTCTGTCTG	Construction of entry clone containing KAR coding region
KAR-cds-sR	CTAATCAGCACTGGAGGGCTGT	Construction of entry clone containing KAR coding region
PRONE_F	CACCATGGAGGTCGAAATGATGAAGGA	Construction of KAR-PRONE entry clone
PRONE_R	TCAAAGGAACGCCTGGCTAAGTC	Construction of KAR-PRONE entry clone
MpRop_cds_F	CACCATGAGTACTCCAGGTTAT	Construction of MpRop entry clone
MpRop_cds_sR	TCACAGGATGGAACATGT	Construction of MpRop entry clone
KAR_WT_Y2H_pGADT7_F	GAGGCCAGTGAATTCatggatcgagggctctgt	Constraction of Y2H vector
KAR_WT_Y2H_pGADT7_R	ACCCGGGTGGAATTCCtaatcacgactggagggtgt	Constraction of Y2H vector
KAR_WT_Y2H_pGBK7_F	ATGGAGGCCGAATTCatggatcgagggctctgt	Constraction of Y2H vector
KAR_WT_Y2H_pGBK7_R	GATCCCCCGGAATTCCtaatcacgactggagggtgt	Constraction of Y2H vector
ROP_WT_Y2H_pGADT7_F	GAGGCCAGTGAATTCatgagtagttccaggttat	Constraction of Y2H vector
ROP_WT_Y2H_pGADT7_R	ACCCGGGTGGAATTCtcacaggatgaaacatgtct	Constraction of Y2H vector
ROP_WT_Y2H_pGBK7_F	ATGGAGGCCGAATTCatgagtagttccaggttat	Constraction of Y2H vector
ROP_WT_Y2H_pGBK7_R	GATCCCCCGGAATTCtcacaggatgaaacatgtct	Constraction of Y2H vector
KARpro_F	CACCATACAACGTGGCTCGACGAAC	Construction of KARpro:GUS
KARpro_R	ATCCATAGCCGACCCACCTACAG	Construction of KARpro:GUS
MpRoppro_F	CACCTCCCTCGAGGATTTTCGAA	Construction of MpRoppro:GUS
MpRoppro_R	AGTACTCATGTTCACTCCT	Construction of MpRoppro:GUS
PRONEcds_qRT-PCR_F	AAGGAGAGGTTGCCCAAGC	RT-qPCR in Figure 3D
PRONEcds_qRT-PCR_R	TCTACCTCGAGGGCTTCAA	RT-qPCR in Figure 3D
MpAPT-F	CGAAAGCCAAGAACGCTACC	RT-qPCR in Figure 3D
MpAPT-R	GTACCCCCGGTTGCAATAAG	RT-qPCR in Figure 3D
KAR-cds-F	CACCATGGATCGAGGGCTCTGTCTG	RT-qPCR in Figure 4C
KAR-gR	CTCCAGCCTCCACAACCTCTC	RT-qPCR in Figure 4C
MpRop_cds_F	CACCATGAGTACTCCAGGTTAT	RT-qPCR in Figure 4C
MpRop_cds_sR	TCACAGGATGGAACATGT	RT-qPCR in Figure 4C
MpEF1a-F	TCACTCTGGGTGTGAAGCAG	RT-qPCR control in Figure 4C
MpEF1a-R	GCCTCGAGTAAAGCTTCGTG	RT-qPCR control in Figure 4C
MpRop_5IF_F	ctaaggtagcgatTAAGGAGCTGGTTGAAGCCGAC	Construction of the targeting vector for MpRop
MpRop_5IF_R	gccccggcaagcttATCAGTCAACAAGATCAAGGC	Construction of the targeting vector for MpRop
MpRop_3IF_F	ttaaactagtggcgcgCACTAATTTCATCGTAT	Construction of the targeting vector for MpRop
MpRop_3IF_R	ttatccctaggcgcgCACTGATCTTCACTCTCGTC	Construction of the targeting vector for MpRop
Rop b-F	GCCTCCAGTCGCCGTTCCGG	For checking of gene targeting site
Rop b-R	AGGAAACATCGGATGCCGGG	For checking of gene targeting site