1 **COVER PAGE**

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3 **Title:** Cytokinin Signaling is Essential for Organ Formation in *Marchantia polymorpha*

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5 **Running head (short title):** Cytokinin signaling in *Marchantia*

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Title: Cytokinin Signaling is Essential for Organ Formation in *Marchantia polymorpha*

Running head (short title): Cytokinin signaling in *Marchantia*

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Abbreviations：Aux/IAA, AUXIN/INDOLE-3-ACETIC ACID; ARF, AUXIN RESPONSE FACTOR; BA, 6-benzyladenine; CHK, CASE domain-containing histidine kinase receptor; cZ, cis-zeatin; CKX , cytokinin oxidase; CRF, cytokinin response factor; *EF1α*, *ELONGATION FACTOR1α*; GUS, ß-glucuronidase; HPT, histidine-containing; phosphotransfer protein; iP, isopentenyladenine; IPT, isopentenyltransferase; qRT-PCR, quantitative reverse transcription-PCR; RR, response regulator; Tak, Takaragaike; tZ, trans-zeatin.

Abstract

Cytokinins are known to regulate various physiological events in plants. Cytokinin signaling is mediated by the phosphorelay system, one of the most ancient mechanisms controlling hormonal pathways in plants. The liverwort *Marchantia polymorpha* possesses all components necessary for cytokinin signaling; however, whether they respond to cytokinins and how the signaling is fine-tuned remain largely unknown. Here we report cytokinin function in *Marchantia* development and organ formation. Our measurement of cytokinin species revealed that cis-zeatin is the most abundant cytokinin in *Marchantia*. We reduced the endogenous cytokinin level by overexpressing the gene for cytokinin oxidase, Mp*CKX*, which inactivates cytokinins, and generated overexpression and knockout lines for type-A (Mp*RRA*) and type-B (Mp*RRB*) response regulators to manipulate the signaling. The overexpression lines of Mp*CKX* and Mp*RRA*, and the knockout lines of Mp*RRB*, shared phenotypes such as inhibition of gemma cup formation, enhanced rhizoid formation and hyponastic thallus growth. Conversely, the knockout lines of Mp*RRA* produced more gemma cups and exhibited epinastic thallus growth. Mp*RRA* expression was elevated by cytokinin treatment and reduced by knocking out Mp*RRB*, suggesting that Mp*RRA* is upregulated by the MpRRB-mediated cytokinin signaling, which is antagonized by MpRRA. Our findings indicate that when plants moved onto land they already deployed the negative feedback loop of cytokinin signaling, which has an indispensable role in organogenesis.

Introduction

Cytokinin phytohormones are involved in a broad range of physiological events, such as cell proliferation and differentiation, organ growth, shoot initiation and leaf senescence (Brenner et al. 2005, Kim et al. 2006, Dello Ioio et al. 2008, Kieber and Schaller 2014). Cytokinin signaling is mediated by a phosphorelay system (Kieber and Schaller 2014), which, in *Arabidopsis thaliana*, consists of CHASE domain-containing histidine kinase receptors (CHKs), histidine-containing phosphotransfer proteins (HPTs) and response regulators (RRs). Cytokinin perception by CHKs at the plasma and endoplasmic reticulum membrane induces autophosphorylation of CHKs, followed by phosphate transfer to cytosolic HPTs. After moving into the nucleus, HPTs transfer the phosphate to type-B RRs, which are then activated and function as transcription factors to regulate the expression of target genes (Hwang and Sheen 2001, Sakai et al. 2001, Taniguchi et al. 2007). Among these target genes are type-A RRs, which negatively control cytokinin signaling by competing with type-B RRs for phosphate transfer (To et al. 2004, 2007). *Arabidopsis* RRs (ARRs) include 11 type-B members, among which ARR1, ARR10 and ARR12 play a predominant role in cytokinin signaling. The triple mutant largely lacks cytokinin-dependent gene expression, and exhibits retarded shoot growth, aborted primary root growth and seed enlargement (Argyros et al. 2008, Ishida et al. 2008, Mason et al. 2005). Although functional redundancy of RRs gives robustness in cytokinin signaling against various perturbations during plant development (Choi et al. 2014), it also complicates the unraveling of downstream events. For instance, how cytokinins control cell proliferation remains largely unknown. Previous studies demonstrated that *Arabidopsis cyclin D3* (*CYCD3*) is upregulated by cytokinins, and that its overexpression enables callus formation on auxin-treated leaf explants in the absence of exogenous cytokinins (Soni et al. 1995, Riou-

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88   Khamlichi et al. 1999). Therefore, it was hypothesized that *CYCD3* plays a major role in

89   cytokinin-triggered cell proliferation; however, which and how ARRs control *CYCD3*

90   expression and to what extent *CYCD3* contributes to cytokinin action on the cell cycle are still

91   elusive.

92   The liverwort *Marchantia polymorpha* is a model basal land plant (Ishizaki et al.

93   2016, Shimamura 2016). The organization of the thalloid body originates from highly regulated

94   division of the apical cell and its descendants at the notch, which is located at the tip of the

95   thallus. Gemma cups accommodating numerous multicellular clones, gemmae, are produced

96   on the dorsal side of the thallus along the midrib. On the ventral side are many tubular cells

97   called rhizoids, which absorb water and nutrients from the soil. A recent genome and

98   transcriptome project revealed a low redundancy of regulatory genes in *Marchantia* (Bowman

99   et al. 2017); for instance, those related to auxin signaling are one gene for TRANSPORT

100   INHIBITOR RESPONSE 1 (TIR1) receptor, one for AUXIN/INDOLE-3-ACETIC

101   ACID (Aux/IAA) protein, and three for AUXIN RESPONSE FACTOR (ARF) (Flores-

102   Sandoval et al. 2015, Kato et al. 2015). Previous studies demonstrated that an F-box-mediated

103   pathway controls the signaling of auxin, gibberellin, jasmonic acid and strigolactone, and that

104   the phosphorelay system governs cytokinin and ethylene signaling (Wang and Irving 2011).

105   Phylogenetic analyses revealed that the components of the F-box-mediated pathway mostly

106   exist in *Marchantia*, but not in charophyte green algae, suggesting that F-box-mediated

107   hormonal signaling was born when plants moved onto land (Bowman et al. 2017). On the other

108   hand, the phosphorelay system exists in charophytes as well as in *Marchantia*, implying that it

109   is more ancient than the F-box-mediated system (Bowman et al. 2017). *Marchantia* is thus a

110   good model organism to understand basic functions of the major hormonal signaling systems

111   in the earliest divergent lineages of extant land plants.

112   The *Marchantia* genome encodes all essential components of cytokinin signaling,

113   namely two CHKs (MpCHKs), one HPT (MpHPT), and one for each type-A RR (MpRRA) and

114   type-B RR (MpRRB) (Bowman et al. 2017). One of the two MpCHKs was shown to bind to

115   cytokinins *in vitro*, although its binding activity to all tested cytokinins was lower than those of

116   *Arabidopsis* and *Physcomitrella patens* (Gruhn et al. 2014). Flores-Sandoval et al. (2016)

117   reported that microRNA-mediated knockdown of Mp*RRB* and overexpression of Mp*RRA*

118   caused defects in thallus development, generating smaller thalli with ectopic serration on

119   margins and fewer gemma cups. However, a low level of Mp*RRB* transcripts remained in the

120   knockdown lines, and no statistical analysis was performed for the Mp*RRA*-overexpressing

121   lines. Moreover, cytokinin dependency of the observed phenotype was assessed by treatment

122   of the Mp*rrb* knockdown lines with a high dose (40 µM) of 6-benzyladenine (BA); therefore,

123   the involvement of response regulators in cytokinin signaling and their role in *Marchantia*

124   development still remain elusive. Here we investigate biological functions of cytokinins in

125   *Marchantia* by distorting hormone levels and signaling. Our results indicate that cytokinins

126   control gemma cup and rhizoid formation, and thallus growth, through the MpRRB-mediated

127   pathway, which is antagonized by MpRRA, suggesting that cytokinin signaling mediated by

128   the phosphorelay system already participated in crucial developmental processes when plants

129   moved onto land.

130

131   **Results**

132   **cZ-type cytokinins are abundant in *Marchantia***

133 We first examined the response of *Marchantia* to exogenously applied cytokinins. Since

134 gemmae formed in gemma cups are not equal in developmental stages, we grew wild-type (Tak-

135 1) gemmae on cytokinin-free agar medium for 6 days, then individuals were transferred to agar

136 medium containing isopentenyladenine (iP), trans-zeatin (tZ), cis-zeatin (cZ) or BA and grown

137 for 21 days. At a concentration of 10 μM, none of the tested cytokinins affected thallus size,

138 gemma cup number or lobe number, and cZ and BA reduced the number of gemmae produced

139 in gemma cups (Supplementary Fig. S1). At 50 μM, iP severely inhibited thallus growth,

140 thereby making it difficult to count the lobe number (Supplementary Fig. S2B, D); BA also

141 impaired thallus growth, and BA and cZ inhibited gemma cup formation and gemma production,

142 while tZ did not exert any significant effect (Supplementary Fig. S2). Considering that 1 μM

143 cytokinins are sufficient to retard shoot and root growth in *Arabidopsis* (To et al. 2004), the

144 observed phenotypes are likely defects caused by excessive amounts of cytokinins in the culture

145 medium.

146   Cytokinin measurements using one-week-old wild-type (Tak-1) thallus revealed that

147 the total amount of tZ, cZ and iP, including riboside and phosphoriboside precursors and

148 glucosides, was 115 pmol/g fresh weight, which is higher than that in *Arabidopsis* shoots and

149 roots (approximately 50 pmol/g fresh weight) (Ko et al. 2014). Interestingly, cZ-type cytokinins

150 were more abundant than tZ- and iP-types, which are major cytokinin species in *Arabidopsis*

151 (Table 1). Three-week-old thalli contained higher amounts of cZ- and iP-type cytokinins in the

152 apical part than in the basal part (Table 1).

153

154 **Cytokinins are required for thallus growth and gemma cup formation**

155   Since the cytokinin level is relatively high in *Marchantia*, as described above, we examined the

156   phenotype when endogenous cytokinins were decreased by overexpressing cytokinin oxidase

157   (CKX), which inactivates cytokinins (Schmülling et al. 2003). One of the two *Marchantia*

158   CKXs, MpCKX2 (Mapoly0093s0012), which shows 61% identity to *Arabidopsis* CKX1, was

159   overexpressed under the constitutive promoter of *ELONGATION FACTOR1α* (Mp*EF1α*)

160   (Althoff et al. 2014). We isolated twelve independent lines that showed the same phenotype;

161   therefore, the representative lines #7 and #9 with different Mp*CKX2* expression levels were

162   used for further analyses (Fig. 1A). The endogenous cytokinin level was reduced in both lines,

163   although more significantly in #7; in one-week-old thalli, tZ was below the quantification limit

164   in both lines, and cZ was 2.6% and 5.2% in #7 and #9, respectively, of that in wild-type (Table

165   1). Both lines formed smaller thalli that bent upward and excessive rhizoids (Fig. 1B, C). The

166   number of gemma cups per unit thallus area was reduced to less than half in #7, and almost no

167   gemma cups were formed in #9 (Fig. 1D; Supplementary Fig. S3). These results indicate that

168   in *Marchantia*, cytokinins are required for thallus growth and gemma cup formation, and inhibit

169   rhizoid formation.

170   In *Marchantia*, all tissues in the thallus body are derived from four merophytes

171   (dorsal, ventral and two lateral merophytes) that surround the single apical cell at the apical

172   notch (Shimamura 2016). Gemma cups are produced very close to the apical cell; cells that are

173   destined to form the gemma cup do not undergo periclinal divisions, and can thus be identified

174   as a set of cylindrical cells that constitute the gemmiparous area just a few cells above the apical

175   cell (Fig. 1E) (Barnes and Land 1908). When 3D images of the apical notch in 10-day-old thalli

176   were reconstructed, we identified the gemmiparous area in 45% of wild-type notches (n = 22),

177   but only in 10% of the Mp*CKX2*-overexpressing line #7 (n = 10); in line #9, no gemmiparous

178   cells were observed (n = 12) (Fig. 1E). These results suggest that cytokinins are required for

179   the initial process of gemma cup formation at the apical notch.

180

181   **Cytokinin signaling components in *Marchantia***

182   In the previous study, Mapoly0022s0150 (CUFF.5443) and Mapoly0101s0006 (CUFF.16381)

183   were identified as Mp*RRA* and Mp*RRB*, respectively (Bowman et al. 2017). However, we

184   noticed that the nucleotide sequences with Mapoly and CUFF numbers, which correspond to

185   the genomic and cDNA sequences, respectively, were not identical for either type-A or -B RRs,

186   probably due to sequencing errors in Mapoly that arose during the genome project. Since the

187   cDNAs that we obtained from wild-type Tak-1 were the same as those predicted from

188   CUFF.5443 and CUFF.16381, we designated CUFF.5443 and CUFF.16381 as Mp*RRA* and

189   Mp*RRB*, respectively. Mp*RRA* encodes a polypeptide of 243 amino acids, and the receiver

190   domain has 64% identity to that of ARR5 (Fig. 2A). MpRRB shows 71% and 84% identity to

191   ARR2 in the receiver domain and the GARP motif, respectively (Fig. 2B). MpRRB possesses

192   a glutamine- and proline-rich C-terminal region, as do type-B ARRs, although its length is

193   shorter than that in ARR2 (Fig. 2B).

194   The *Marchantia* genome encodes two cytokinin receptors, MpCHK1

195   (Mapoly0075s0066) and MpCHK2 (Mapoly0104s0036), and one HPT, MpHPT

196   (Mapoly0091s0072) (Bowman et al. 2017). MpCHK1 and MpCHK2 have the conserved

197   cytokinin receiver (CHASE) and histidine kinase domains as well as the receiver and receiver-

198   like domains (Fig. 2C). The amino acid sequences in the histidine kinase domain show a higher

199   similarity between MpCHK1 and MpCHK2 (62%) than between MpCHK1 and the *Arabidopsis*

200   cytokinin receptor AHK4 (44%) (Fig. 2C). MpHPT is closely related to AHP1, with 62%

201   identity in the HPt domain that functions as a phospho-transmitter, and has an N-terminal

202   extension (Fig. 2D) (Suzuki et al. 1998). These results indicate that all components of cytokinin

203   signaling in *Marchantia* are well conserved in essential domains as compared to *Arabidopsis*

204   orthologs.

205

206   **Mp*RRA* and Mp*RRB* are expressed in the notch, the midrib and gemma cups**

207   To further understand the functional role of cytokinin signaling in *Marchantia,* we examined

208   the expression pattern of Mp*RRA* and Mp*RRB*. Quantitative reverse transcription-PCR (qRT-

209   PCR) was conducted using total RNA from young thalli without gemma cups, mature thalli

210   with gemma cups, antheridiophores and archegoniophores. As shown in Fig. 3A and B,

211   transcript levels of Mp*RRA* and Mp*RRB* were higher in mature thalli than in younger plants,

212   and Mp*RRB* was highly expressed at the reproductive stage.

213   We then generated ß-glucuronidase (GUS) reporter lines: the 5-kb promoter regions

214   of Mp*RRA* and Mp*RRB* were fused to the *GUS* gene, and introduced into wild-type plants. Ten

215   and seven lines of *pro*Mp*RRA:GUS* and *pro*Mp*RRB:GUS*, respectively, were generated, and

216   both showed the same GUS expression patterns among independent lines; therefore, we

217   hereafter show the results of a representative line for each of Mp*RRA* and Mp*RRB*. In

218   *pro*Mp*RRA:GUS*, GUS signals were not detected in gemmae (Fig. 3C), but appeared in the

219   notch of 5-day-old thalli (Fig. 3D). The signals became stronger in the midrib during thallus

220   growth (Fig. 3E-G), and in 20-day-old plants a high level of GUS expression was also observed

221   in gemma cups and developing gemmae (Fig. 3H). *pro*Mp*RRB:GUS* showed a similar

222   expression pattern to that of *pro*Mp*RRA:GUS*, except that GUS signals in the midrib almost

223   disappeared in 20-day-old thalli (Fig. 3M-R). At the reproductive stage, the promoter activities

224   of Mp*RRA* and Mp*RRB* were detected in whole tissues, but at a reduced level in stalks (Fig. 3I-

225   L, S-V).

226

227   **MpRRB is indispensable for gemma cup formation and restricts the number of rhizoids**

228   To elucidate the physiological function of the MpRRB-mediated pathway, we knocked out

229   Mp*RRB* by inserting the hygromycin phosphotransferase gene using homologous

230   recombination-based gene targeting (Fig. 4A) (Ishizaki et al. 2013), and obtained three

231   independent lines, #194, #307 and #342. Semi-quantitative RT-PCR analysis showed that all

232   three lines lacked the full-length Mp*RRB* transcript (Fig. 4B; Supplementary Fig. S4A). Since

233   they exhibited the same phenotype (Fig. 4C; Supplementary Fig. S4B), we used #307 as a

234   representative line for further analysis. Thalli bent diagonally upward and had no gemma cups

235   (Fig. 4C, D). Moreover, numerous rhizoids were formed on the ventral side as observed in

236   Mp*CKX2*-overexpressing lines (Figs. 1B, 4C). These phenotypes were suppressed by

237   expressing Mp*RRB* under its own promoter (Fig. 4B, C, D). Our results suggest that MpRRB

238   is indispensable for proper development of the thallus and gemma cups, and that rhizoid

239   formation is inhibited by the MpRRB-mediated pathway.

240   We also generated Mp*RRB*-overexpressing lines using the Mp*EF1α* promoter. We

241   transformed 330 pieces of thalli and obtained only one line expressing Mp*RRB* at a higher level

242   (3.2-fold) than wild-type (Supplementary Fig. S5A). Thalli of the overexpression line were

243   heavily curled, making it difficult to evaluate the effect of overexpression on gemma cup and

244   rhizoid formation (Supplementary Fig. S5B). This suggests that constitutive overexpression of

245   Mp*RRB* causes severe developmental defects in *Marchantia*.

246

MpRRA inihibits MpRRB-mediated cytokinin signaling

247

248 We next observed the phenotype of Mp*rra* knockout lines. We obtained three independent lines

249 (#288, #289 and #397) by homologous recombination-based gene targeting (Fig. 5A), and

250 found that all expressed no Mp*RRA* transcript (Fig. 5B; Supplementary Fig. S6A). Because the

251 three lines exhibited the same phenotype (Fig. 5C; Supplementary Fig. S6B), we present the

252 data for the representative line #289. As shown in Fig. 5C and D, thalli curled downward, and

253 the number of gemma cups per unit thallus area was higher than in wild-type. Such phenotypes

254 were suppressed by expressing Mp*RRA* under its own promoter (Fig. 5B, C, D). These data

255 suggest that Mp*RRA* plays an inhibitory role in gemma cup formation. We also observed rhizoid

256 formation on the ventral side of thalli, but found no difference between wild-type and Mp*rra*

257 knockout lines, suggesting that the sensitivity to cytokinins in rhizoid formation is lower than

258 that in gemma cup formation.

259 To further examine the function of MpRRA in *Marchantia* development, we

260 generated twelve independent lines overexpressing Mp*RRA* under the Mp*EF1α* promoter.

261 Among them, we analyzed the representative lines #1 and #4, in which the Mp*RRA* transcript

262 level increased more than 12- and 44-fold, respectively, compared to wild-type (Fig. 6A). Both

263 lines produced thalli bending upward and numerous rhizoids, while gemma cup formation was

264 severely inhibited (Fig. 6B, C). These phenotypes were similar to those observed in the

265 Mp*CKX2*-overexpressing lines and the Mp*rrb* knockout lines (Figs. 1, 4), suggesting that

266 MpRRA has an antagonistic function to MpRRB-mediated cytokinin signaling, which controls

267 proper thallus development, promotes gemma cup formation, and suppresses rhizoid formation.

268 Our qRT-PCR analysis revealed that the Mp*RRA* transcript level was lower in the

269 Mp*CKX2*-overexpressing lines than in wild-type, and was increased by 50 μM tZ treatment of

270   wild-type plants for 2 h (Fig. 7A, B). This suggests that Mp*RRA* is responsive to cytokinins at

271   the mRNA level, as is the case with *Arabidopsis* type-A RRs (Hwang and Sheen 2001, Sakai

272   et al. 2001, Taniguchi et al. 2007). In the Mp*rrb* knockout line, the Mp*RRA* mRNA level was

273   lower than that in wild-type (Fig. 7B), while it was 1.7-fold higher in the Mp*RRB*-

274   overexpressing line (Fig. 7C). Moreover, tZ-triggered induction of Mp*RRA* was completely

275   suppressed in the Mp*rrb* knockout line (Fig. 7B). This and results presented above suggest that

276   cytokinins upregulate Mp*RRA* through MpRRB, whose function is suppressed by MpRRA,

277   thereby generating negative feedback.

278

279   **Discussion**

280   In this study, we demonstrated that Mp*rrb* knockout lines and overexpression lines of Mp*RRA*

281   and Mp*CKX2* shared the phenotypes of upward bending of thalli, formation of numerous

282   rhizoids, and defects in gemma cup formation (Figs. 1B, D, 4C, D, 6B, C). In contrast, Mp*rra*

283   knockout lines exhibited downward bending of thalli and enhanced gemma cup formation (Fig.

284   5C, D). Moreover, our measurement of Mp*RRA* mRNA levels indicated that Mp*RRA*

285   expression is upregulated by cytokinins in an Mp*RRB*-dependent manner (Fig. 7). These data

286   suggest that MpRRB-mediated cytokinin signaling promotes gemma cup formation, inhibits

287   rhizoid formation, controls epinastic thallus growth, and induces Mp*RRA* to give negative

288   feedback as is known in *Arabidopsis*. Therefore, cytokinins have a crucial role in *Marchantia*

289   development, and fine-tuning of the cytokinin signaling involving type-A and type-B RRs is

290   likely an ancestral mechanism controlling plant organ growth. In *Arabidopsis*, six cytokinin

291   response factors (CRFs) function as transcription factors and control cytokinin signaling

292   (Raines et al. 2016). Both RRs and CRFs are regulated by the CHK-HPT phosphorelay,

293   although they work independently (Rashotte et al. 2006). The *Marchantia* genome encodes one

294   CRF (MpCRF, Mapoly0023s0075). Since the Mp*rrb* knockout lines displayed severe

295   morphological defects but not lethality, it is probable that cytokinin signaling is mediated by

296   MpCRF as well as MpRRB. Further studies on downstream events regulated by MpRRB and

297   MpCRF will clarify the intrinsic role of cytokinin signaling in growth, development and

298   responses to internal and external cues.

299   We isolated only one Mp*RRB*-overexpressing line from 330 pieces of thallus, and

300   this line exhibited a much severer phenotype than the Mp*rra* knockout line (Fig. 5;

301   Supplementary Fig. S5). This suggests that an excess amount of MpRRB has a deleterious

302   effect on *Marchantia* development. By contrast, a previous report demonstrated that

303   overexpression of *ARR1*, one of the type-B RRs in *Arabidopsis*, resulted in subtle phenotypic

304   changes, producing hypertrophic cotyledons, and longer cotyledonary petioles and shorter roots

305   as compared with wild-type (Sakai et al. 2001). However, a combination of ARR1

306   overexpression and cytokinin treatment caused disordered cell proliferation in the shoot apex,

307   thereby leading to growth defects (Sakai et al. 2001). This and our results suggest that in

308   *Marchantia*, the endogenous cytokinin level is high enough to activate MpRRB. Indeed,

309   *Marchantia* accumulates a higher level of cytokinins than *Arabidopsis* (Table 1), and only

310   inhibitory effects were observed by treatment with 10 µM or 50 µM cytokinins (Supplementary

311   Figs. S1, S2). Flores-Sandoval et al. (2016) also reported that a high dose of BA inhibited

312   *Marchantia* growth.

313   Our cytokinin measurements showed that cZ-type cytokinins were more abundant

314   than the other types in *Marchantia* (Table 1), although tZ- and iP-types are major cytokinin

315   species in *Arabidopsis* (Sakakibara 2006). In another lineage of basal land plants,

14

316   *Physcomitrella patens*, the most abundant cytokinins are also cZ-types (von Schwartzenberg et

317   al. 2007), and the same applies to cyanobacteria and algae (Stirk et al. 2013, Žižková et al.

318   2017). Indeed, tRNA-isopentenyltransferases (IPTs) involved in biosynthesis of cZ-type

319   cytokinins exist in algae, *Marchantia* and *Physcomitrella*, while adenylate-IPTs, which

320   catalyze the first step of biosynthesis of tZ- and iP-types, are missing (Bowman et al. 2017,

321   Yevdakova and von Schwartzenberg 2007). This suggests that the major pathways for cytokinin

322   biosynthesis have changed during plant evolution. A previous study showed that a *Marchantia*

323   MpCHK exhibited a lower response to all tested cytokinins including cZ than *Arabidopsis*

324   AHK4 or *Physcomitrella* PpCHK4, although it is unclear which of the two *Marchantia*

325   MpCHKs was used for the bacterial complementation assay (Gruhn et al. 2014). The lower

326   cytokinin response of MpCHK may make *Marchantia* insensitive to exogenously applied

327   cytokinins. Besides, 6-day preculture of gemmae prior to cytokinin treatment may be another

328   reason for the insensitivity, because, in the case of auxin treatment, much milder effects were

329   observed in samples with 6-day preculture than in those without preculture (Ishizaki et al. 2012,

330   Flores-Sandoval et al. 2015). While the dorsiventral polarity is established during the 6-day

331   preculture (Bowman 2016), what kind of physiological events occur remains elusive. It is

332   probable that the permeability of cytokinins changes due to alterations in the components of the

333   cell wall and/or the plasma membrane.

334   Previous studies have demonstrated that in contrast to cytokinins, auxin inhibits

335   gemma cup formation, and enhances rhizoid production and epinastic thallus growth in

336   *Marchantia* (Ishizaki et al. 2012, Flores-Sandoval et al. 2015, Eklund et al. 2015). This suggests

337   that cytokinins and auxin have antagonistic functions in the formation of gemma cups and

338   rhizoid, and in thallus growth. During plant evolution, the phosphorelay system controlling

339   cytokinin and ethylene signaling emerged earlier than F-box-mediated hormone pathways,

340   which are represented by auxin signaling (Bowman et al. 2017). Therefore, it is likely that

341   plants exploited auxin signaling to attenuate the cytokinin-mediated pathway in order to

342   regulate organ formation under changing environmental conditions on land. In *Marchantia*,

343   gemma cups and rhizoids are produced very close to the apical cell, and cell fate is determined

344   within a few cell divisions (Shimamura 2016), suggesting that cytokinins and auxin exert their

345   antagonistic functions in the early descendants of stem cells. Further studies will reveal how

346   the two hormones communicate with each other during the initial process of organ formation.

347

348   **Materials and Methods**

349   **Plant materials and growth conditions**

350   The male and female *Marchantia* accessions Takaragaike (Tak)-1 and Tak-2, respectively,

351   were cultured on half-strength Gamborg's B5 agar medium under continuous white light at

352   22ºC. F1 spores were obtained by crossing Tak-1 and Tak-2. For formation of sexual organs,

353   mature thalli were cultured under white light supplemented with far-red light (Ishizaki et al.

354   2016).

355

356   **Cloning of Mp*RR*s and Mp*CKX2***

357   Mp*RR* cDNAs were amplified by RT-PCR using total RNA prepared from Tak-1 thalli and the

358   primers listed in Supplementary Table S1, and were then cloned into the plasmid pDONR221

359   using BP Clonase (Thermo Fisher Scientific, USA) to obtain a Gateway entry clone. Mp*CKX2*

360   cDNA was amplified by RT-PCR using total RNA prepared from Tak-1 thalli and the primers

361   listed in Supplementary Table S1, followed by cloning into the plasmid pENTR/D-TOPO

362   (Thermo Fisher Scientific) to obtain a Gateway entry clone.

363

364   **Quantification of cytokinins**

365   Wild-type (Tak-1) gemmae were grown for one- or three-weeks on half-strength Gamborg's

366   B5 agar medium. For three-week-old thallus, the apical part (0-3 mm from the tip) and the basal

367   part (3-10 mm from the tip) were serparately collected. Approximately 100 mg of each sample

368   was subjected to cytokinin measurement by ultra-performance liquid chromatography (UPLC)-

369   tandem mass spectrometry (AQUITY UPLC System/XEVO-TQS; Waters, USA) with an ODS

370   column (AQUITY UPLC HSS T3, 1.8 μm, 2.1 × 100 mm; Waters) as previously described

371   (Kojima et al. 2009).

372

373   **Generation of overexpression and knockout lines**

374   To obtain overexpression lines of Mp*RRA* and Mp*RRB*, each cDNA in pDONR221 was

375   transferred to the destination vector pMpGWB103 (Ishizaki et al. 2015) by LR Clonase

376   (Thermo Fisher Scientific) to be fused with the Mp*EF1α* promoter. The resultant plasmids were

377   used for transformation of Tak-1 as described by Kubota et al. (2013).

378   To obtain knockout lines of Mp*RRA* and Mp*RRB*, the 5'- and 3'-homologous arms

379   were amplified from Tak-1 genomic DNA by PCR using the primers listed in Supplementary

380   Table S1. The amplified 5'- and 3'-fragments were cloned into the PacI and AscI sites,

381   respectively, of the pJHY-TMp1 vector (Ishizaki et al. 2013) with an In-Fusion HD cloning kit

382   (Takara Bio, Japan). The plasmid was used for transformation of F1 sporelings (Ishizaki et al.

383   2008). Knockout lines were identified as described previously (Ishizaki et al. 2013). To conduct

384 complementation tests of Mp*rr* knockout lines, the promoter and coding sequences of Mp*RR*s

385 were amplified from Tak-1 genomic DNA by PCR using the primers listed in Supplementary

386 Table S1. The amplified fragments were cloned into pDONR221, followed by an LR reaction

387 to transfer them to the destination vector pMpGWB301 (Ishizaki et al. 2015).

388

389 **GUS expression analysis**

390 The 5-kb genomic regions immediately upstream of the start codon of Mp*RR*s were amplified

391 by PCR using Tak-1 genomic DNA and the primers listed in Supplementary Table S1. The

392 amplified fragments were cloned into pDONR221, followed by an LR reaction to transfer them

393 to the destination vector pMpGWB104 (Ishizaki et al. 2015), generating a fusion to the *GUS*

394 gene. After transformation of Tak-1 and Tak-2, hygromycin-resistant plantlets were selected to

395 establish isogenic lines. GUS staining was performed as described by Althoff et al. (2014).

396

397 **RT-PCR**

398 To quantify the Mp*RRA* transcript level, thallus tips were incubated in half-strength Gamborg's

399 B5 liquid medium for 24 h with shaking at 130 rpm, and then tZ was added to the medium to a

400 final concentration of 50 µM, followed by a further 2-h incubation. Total RNA was extracted

401 with a FavorPrep Plant Total RNA Purification Mini Kit (Favorgen Biotech, Taiwan). First-

402 strand cDNAs were prepared from total RNA with ReverTra Ace qPCR RT Master Mix with

403 gDNA Remover (TOYOBO, Japan) according to the manufacturer's instruction. Semi-

404 quantitative RT-PCR was conducted with a Gene Amp PCR System (Thermo Fisher Scientific).

405 qRT-PCR was performed with a THUNDERBIRD SYBR qPCR Mix (TOYOBO) and the

406   LightCycler 480 Real-Time PCR System (Roche, Switzerland). The primers used for RT-PCR

407   are listed in Supplementary Table S1.

408

409   **3D imaging of the apical notch**

410   Ten-day-old thalli were fixed with 4% paraformaldehyde in PBS under vacuum for 2 h. Fixed

411   samples were washed twice with PBS and transferred to ClearSee solution (10% xylitol, 15%

412   sodium deoxycholate and 25% urea) (Kurihara et al. 2015). The cell wall was stained overnight

413   with 0.1% DirectRed23 (Sigma-Aldrich, USA) in ClearSee solution. Stained samples were

414   washed with ClearSee solution for more than an hour, and observed under a confocal laser

415   scanning microscope (Zeiss LSM880). DirectRed23 was excited at 543 nm. 3D images were

416   reconstructed from multiple images of horizontal sections.

417

421

422   **Disclosures**

423   The authors declare no conflicts of interest associated with this manuscript.

424

428

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Table 1. Quantification of cytokinins.										
Hormone		One-week-old thallus (pmol/g fresh weight)			Three-week-old thallus (pmol/g fresh weight)					
					Apical part			Basal part		
		Tak-1	Mp*CKX2* [#7]	Mp*CKX2* [#9]	Tak-1	Mp*CKX2* [#7]	Mp*CKX2* [#9]	Tak-1	Mp*CKX2* [#7]	Mp*CKX2* [#9]
tZ-type	**tZ**	1.29 ± 0.28	u.q.	u.q.	0.08 ± 0.03	u.q.	u.q.	0.13 ± 0.03	u.q.	u.q.
	tZR	0.05 ± 0.02	0.03 ± 0.01	0.04 ± 0.00	u.q.	u.q.	u.q.	u.q.	0.02	0.02 ± 0.00
	tZRPs	17.53 ± 1.61	8.54 ± 1.20	8.29 ± 1.38	4.98 ± 1.21	2.36 ± 1.07	2.50 ± 0.36	3.67 ± 0.87	1.46 ± 0.30	0.92 ± 0.18
	tZ7G	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.
	tZ9G	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.
	tZOG	0.23 ± 0.09	u.q.	u.q.	u.q.	u.q.	u.q.	0.17 ± 0.10	u.q.	u.q.
	tZROG	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.
	tZRPsOG	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.
cZ-type	**cZ**	3.82 ± 0.29	0.10 ± 0.03	0.20 ± 0.03	2.91 ± 0.91	0.08 ± 0.02	0.22 ± 0.04	1.50 ± 0.28	0.18 ± 0.15	0.34 ± 0.07
	cZR	0.20 ± 0.06	0.23 ± 0.05	0.42 ± 0.13	0.56 ± 0.18	0.23 ± 0.12	0.44 ± 0.13	0.17 ± 0.05	0.58 ± 0.05	1.11 ± 0.55
	cZRPs	66.69 ± 9.28	52.76 ± 7.80	74.39 ± 9.09	114.85 ± 9.93	58.84 ± 2.57	77.89 ± 13.42	35.39 ± 9.42	33.86 ± 4.99	34.46 ± 3.00
	cZOG	2.98 ± 0.28	0.14	u.q.	1.48 ± 0.31	u.q.	0.11	5.13 ± 2.08	0.57 ± 0.09	0.27 ± 0.05
	cZROG	0.16 ± 0.03	0.13 ± 0.02	0.19 ± 0.04	0.49 ± 0.05	0.59 ± 0.06	0.99 ± 0.05	0.21 ± 0.05	0.49 ± 0.07	0.57 ± 0.06
	cZRPsOG	u.q.	u.q.	u.q.	0.11 ± 0.01	0.06	0.06 ± 0.01	u.q.	0.04	0.03
DZ-type	**DZ**	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.
	DZR	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.
	DZRPs	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.
	DZ9G	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.
iP-type	**iP**	0.51 ± 0.02	u.q.	0.10 ± 0.03	0.58 ± 0.05	u.q.	0.12 ± 0.03	0.22 ± 0.02	0.35	0.07 ± 0.02
	iPR	0.040	u.q.	0.06 ± 0.04	0.13 ± 0.03	u.q.	0.08 ± 0.02	0.06 ± 0.01	u.q.	0.27 ± 0.15
	iPRPs	21.78 ± 4.13	15.58 ± 2.57	29.93 ± 3.31	41.59 ± 3.07	13.24 ± 4.81	46.36 ± 5.15	15.33 ± 2.92	12.13 ± 0.30	27.25 ± 2.90
	iP7G	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.
	iP9G	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.	u.q.

Amounts of cytokinins were measured in 1- and 3-week-old thalli of wild-type (Tak-1) and Mp*CKX2*-overexpressing lines #7 and #9. For 3-week-old thallus, cytokinin content in apical and basal parts was separately measured. Data are presented as mean ± SD (n ≥ 3). u.q., under quantification limit.

552

553

Figure Legends

555 **Fig. 1** Overexpression of Mp*CKX2*. (A) Expression levels of Mp*CKX2*. qRT-PCR was

556 conducted using total RNA from Mp*CKX2*-overexpressing lines #7 and #9. mRNA levels were

557 normalized to that of Mp*EF1α*. Data are presented as mean ± SD (n = 3). Significant differences

558 from wild-type (Tak-1) were determined by Student's *t*-test: **, *P* < 0.01. (B) Thalli and

559 rhizoids of Mp*CKX2*-overexpressing lines. Gemmae were cultured for 21 days. Bars represent

560 5 mm. (C, D) Thallus area (C) and gemma cup number per thallus area (D) of Mp*CKX2*-

561 overexpressing lines. Data are presented as mean ± SD (n = 20). Significant differences from

562 wild-type were determined by Student's *t*-test: **, *P* < 0.01. (E) Vertical longitudinal optical

563 sections of the apical notch in 10-day-old thalli of wild-type (Tak-1) and Mp*CKX2*-

564 overexpressing lines. Gemmiparous cells in the initial stage (wild-type, left) and the subsequent

565 growth stage (wild-type, right) are shown in orange color. An elongated gemmiparous cell

566 observed in the growth stage is indicated by a white arrowhead. Asterisks indicate apical or

567 sub-apical cells. Note that no gemmiparous area is visible in the sections of Mp*CKX2*-

568 overexpressing lines #7 and #9. Bars represent 50 μm.

569

570 **Fig. 2** Protein structures of cytokinin signaling components in *Marchantia*. (A, B) Protein

571 structures of type-A RRs (A) and type-B RRs (B). The receiver domains (gray boxes), GARP

572 domains (black boxes) and glutamine- and proline-rich regions (Q/P-rich) are shown. Three

573 phosphorylation sites in the receiver domain are indicated as D-D-K. The percentage of amino

574 acid identities between the pairs of sequences is shown. (C) Protein structures of CHKs. The

575 CHASE domain, histidine kinase domain (HK), receiver-like domain (RL) and receiver domain

27

576 (R) are shown. (D) Protein structures of HPTs. The HPt domain is shown. The percentage

577 amino acid identity between pairs of sequences is indicated.

578

579 **Fig. 3** Expression patterns of Mp*RR*s. (A, B) Expression levels of Mp*RRA* (A) and Mp*RRB* (B).

580 qRT-PCR was conducted using total RNA from 1-week-old (young) and 3-week-old (mature)

581 thalli, and antheridiophores and archegoniophores. mRNA levels were normalized to that of

582 Mp*EF1α*. Data are presented as mean ± SD (n = 3). (C-V) GUS staining of

583 *pro*Mp*RRA:*Mp*RRA-GUS* (C-L) and *pro*Mp*RRB:*Mp*RRB-GUS* (M-V). Gemmae (C, M), 5-

584 day-old thallus (D), 7-day-old thallus (N), 10-day-old thallus (E, O), the tip region of 10-day-

585 old thallus (F, P), 20-day-old thallus (G, Q), transverse section of a gemma cup on 20-day-old

586 thallus (H, R), young antheridiophore (I, S), mature antheridiophore (J, T), young

587 archegoniophore (K, U), and mature archegoniophore (L, V). The insets in (D) and (N) are

588 enlarged images of the notch region. Arrowheads indicate apical notches. Bars represent 500

589 μm.

590

591 **Fig. 4** Phenotype of Mp*rrb* knockout lines. (A) Schematic representation of the Mp*RRB* locus.

592 Exons are represented by black boxes. The hygromycin phosphotransferase gene (*hpt*) was

593 inserted into the receiver domain by homologous recombination. (B) Mp*RRB* expression in an

594 Mp*rrb* knockout line and two complementation lines. Expression levels were measured by

595 semi-quantitative RT-PCR using total RNA from the knockout line #307 and the

596 complementation lines #10 and #12. Mp*EF1α* was used as a control. (C) Thalli and rhizoids of

597 the Mp*rrb* knockout line and the complementation lines (Mp*rrb^{ko}* Comp). The pieces of thallus

598 tip were cultured for 15 days. Bars represent 5 mm. (D) Gemma cup number per thallus area.

599   Data are presented as mean ± SD (n = 20). Significant differences from wild-type (Tak-2) were

600   determined by Student's *t*-test: **, $P < 0.01$.

601

602   **Fig. 5** Phenotype of Mp*rra* knockout lines. (A) Schematic representation of the Mp*RRA* locus.

603   Exons are represented by black boxes. The hygromycin phosphotransferase gene (*hpt*) was

604   inserted into the receiver domain by homologous recombination. (B) Mp*RRA* expression in

605   Mp*rra* knockout line and complementation lines (Mp*rra^{ko}* Comp). Expression levels were

606   measured by semi-quantitative RT-PCR using total RNA from the knockout line #289 and the

607   complementation lines #10 and #13. Mp*EF1α* was used as a control. (C) Thalli and rhizoids of

608   the Mp*rra* knockout line and the complementation lines. The pieces of thallus tip were cultured

609   for 15 days. Bars represent 5 mm. (D) Gemma cup number per thallus area. Data are presented

610   as mean ± SD (n = 20). Significant differences from wild-type (Tak-2) were determined by

611   Student's *t*-test: **, $P < 0.01$.

612

613   **Fig. 6** Phenotype of Mp*RRA*-overexpressing lines. (A) Mp*RRA* expression in Mp*RRA*-

614   overexpressing lines. qRT-PCR was conducted using total RNA from lines #1 and #4. mRNA

615   levels were normalized to that of Mp*EF1α*. Data are presented as mean ± SD (n = 3). Significant

616   differences from wild-type (Tak-1) were determined by Student's *t*-test: **, $P < 0.01$. (B) Thalli

617   and rhizoids of the Mp*RRA*-overexpressing lines. The pieces of thallus tip were cultured for 15

618   days. Bars represent 5 mm. (C) Gemma cup number per thallus area. Data are presented as

619   mean ± SD (n = 20). Significant differences from wild-type (Tak-1) were determined by

620   Student's *t*-test: **, $P < 0.01$.

621

622 **Fig. 7** Mp*RRA* expression in transgenic lines. (A) Transcript levels of Mp*RRA* in the Mp*CKX2*-

623 overexpressing lines. (B) Transcript levels of Mp*RRA* in the Mp*rrb* knockout line #307 with or

624 without 50 µM tZ treatment. (C) Transcript levels of Mp*RRA* in the Mp*RRB*-overexpressing

625 line #15. mRNA levels were normalized to that of Mp*EF1α*. Data are presented as mean ± SD

626 (n = 3). Significant differences from wild-type (A, C) or the samples with mock treatment (B)

627 were determined by Student's *t*-test: *P < 0.05, ** *P* < 0.01.

Fig. 1

A

B

WT (Tak-1) *pro*Mp*EF1α:* MpCKX2 #7 *pro*Mp*EF1α:* MpCKX2 #9

C

D

E

WT (Tak-1) WT (Tak-1)

*pro*Mp*EF1α:*Mp*CKX2* #7 *pro*Mp*EF1α:*Mp*CKX2* #9

Fig. 2

A

Type-A RR

ARR5

```
1  32      87    139  183 aa
   D     D      K
  25            152
        64%
```

MpRRA

```
        58           185
      D      D     K
 1                        243 aa
        64    119   172
```

B

Type-B RR

ARR2

```
1  34   80   129        213    275                              664 aa
   D    D    K          ■GARP■              Q/P-rich
  28         142
        71%              84%
```

MpRRB

```
        45          159
      D     D    K         ■GARP■           Q/P-rich
 1                                                               542 aa
        51   97   146       215    277
```

C

AHK4

```
1        198      411  472          737   786  910  946 1061 1080 aa
         ▨CHASE▨      ■■HK■■         ▨RL▨    ▨R▨
          25%              44%         33%      37%
```

MpCHK1

```
1        ▨CHASE▨        ■■HK■■         ▨RL▨    ▨R▨       1082 aa
        109  267      404      717   765  873  961  1058
          49%              62%         57%      56%
```

MpCHK2

```
1        ▨CHASE▨        ■■HK■■         ▨RL▨    ▨R▨       1066 aa
        112  268      403      702   751  860  894  1031
```

D

AHP1

```
1 17           140 154 aa
  ▨  HPt  ▨
        62%
```

MpHPT

```
1                        262 aa
         ▨   HPt   ▨
        128       251
```

Fig. 3

*pro*Mp*RRA:GUS*

*pro*Mp*RRB:GUS*

Fig. 4

A

Mp*RRB* locus

Disrupted Mp*RRB* locus

1 kb
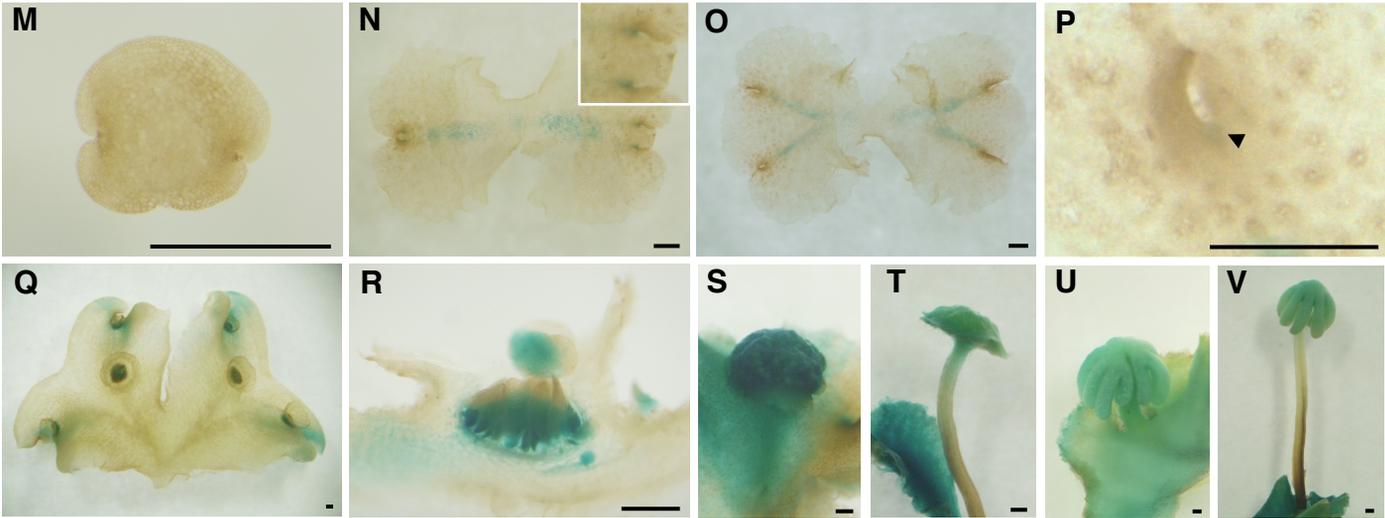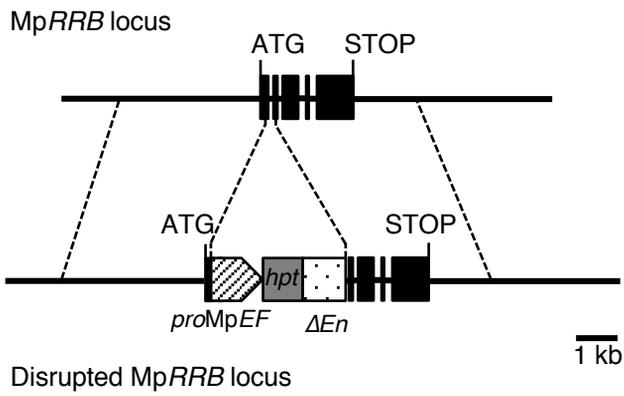
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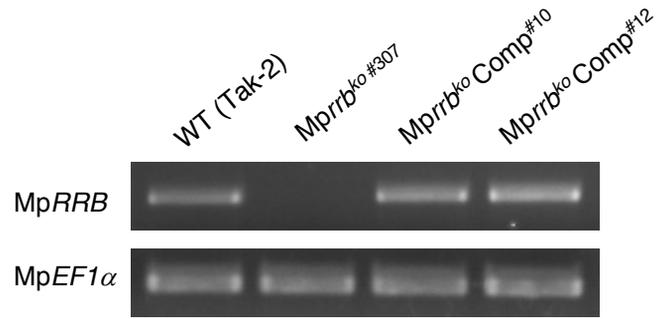
C

| WT (Tak-2) | Mp*rrb*$^{ko\ \#307}$ | Mp*rrb*^{ko} Comp$^{\#10}$ | Mp*rrb*^{ko} Comp$^{\#12}$ |

D

Fig. 5

A

MpRRA locus

ATG STOP

Disrupted MpRRA locus

ATG   STOP

pMpEF   ΔEn

1 kb

B

WT (Tak-2)   Mp*rra*$^{ko\ \#289}$ Mp*rra*^{ko} Comp$^{\#10}$ Mp*rra*^{ko} Comp$^{\#13}$

MpRRA

MpEF1α

C

WT (Tak-2)   Mp*rra*$^{ko\ \#289}$ Mp*rra*^{ko} Comp$^{\#10}$ Mp*rra*^{ko} Comp$^{\#13}$

D

Fig. 6

A

B

| | WT (Tak-1) | *pro*Mp*EF1α:* Mp*RRA* #1 | *pro*Mp*EF1α:* Mp*RRA* #4 |

C

Fig. 7

A

B

C
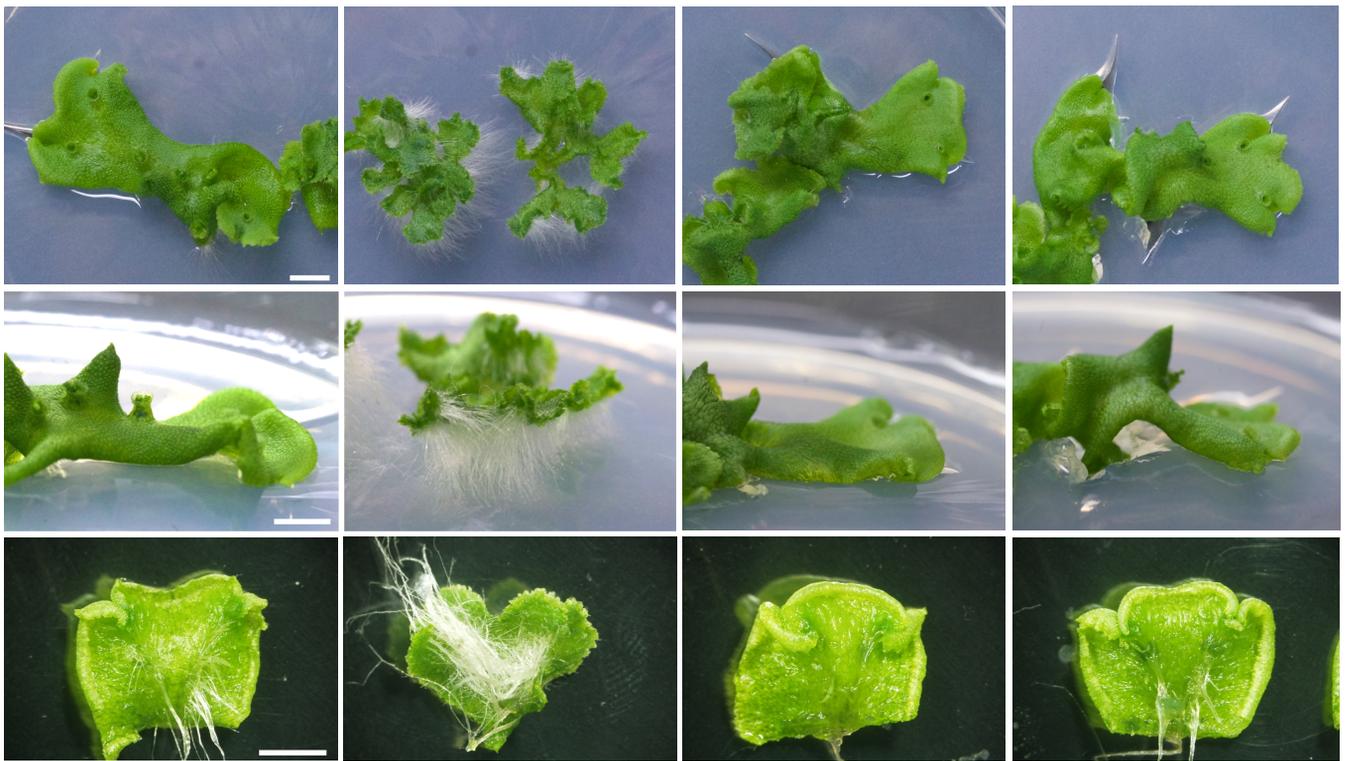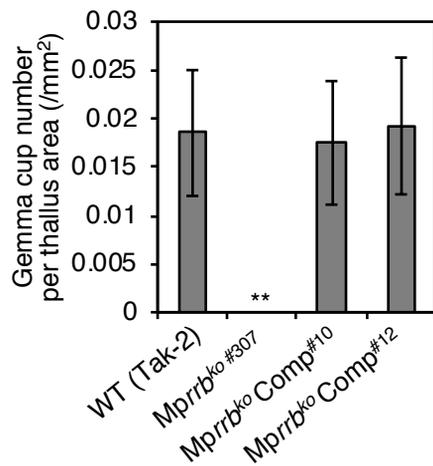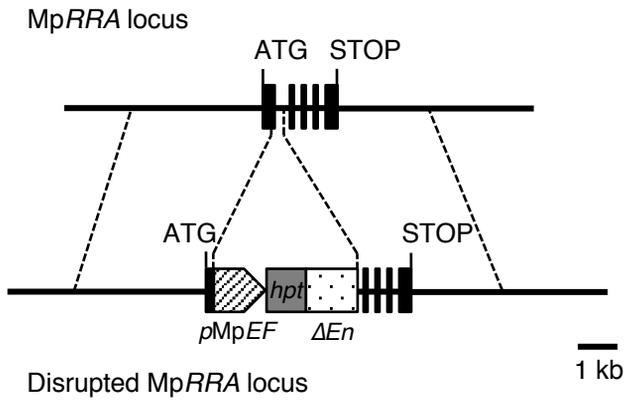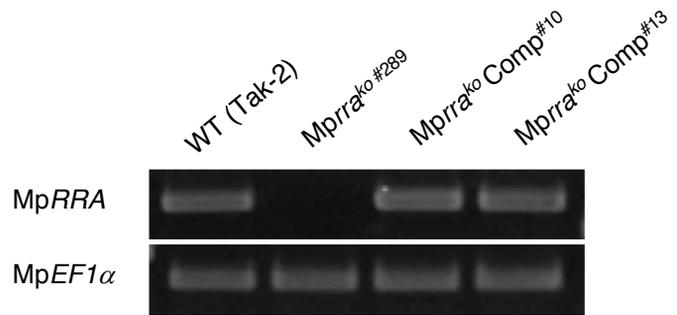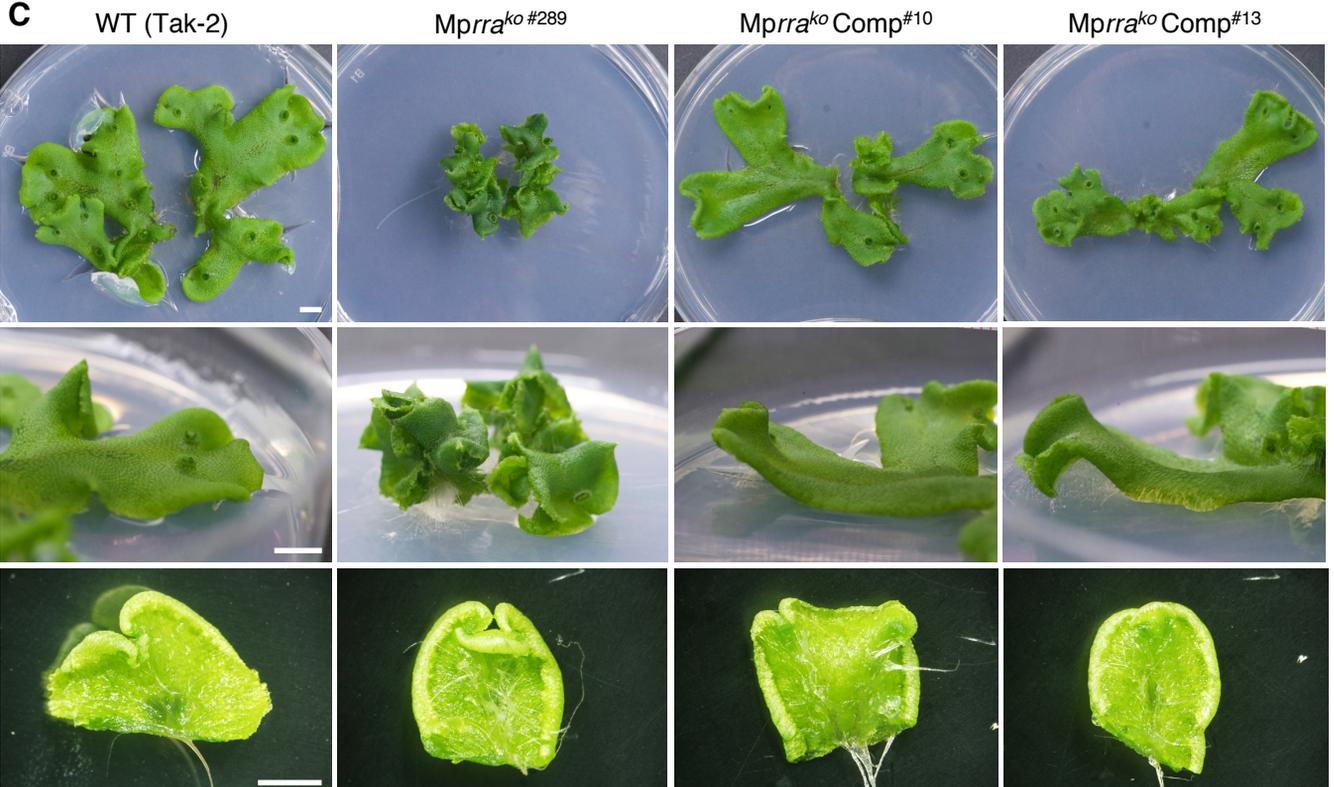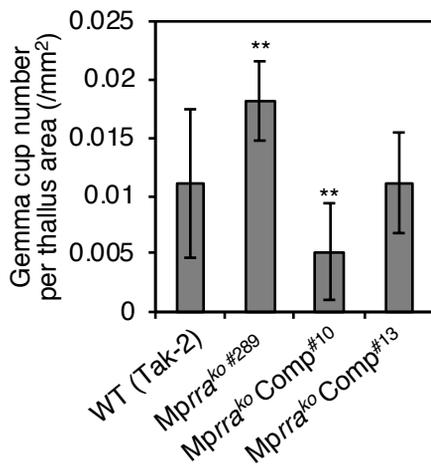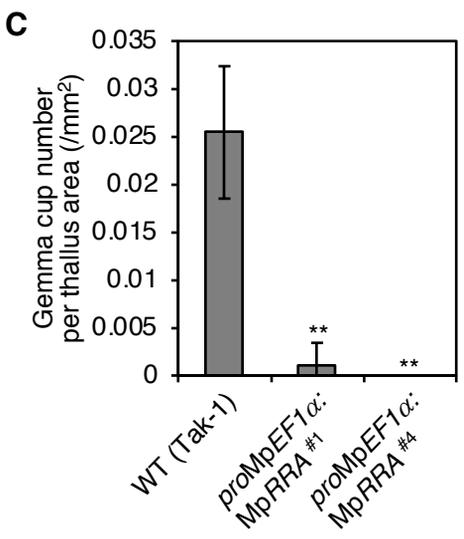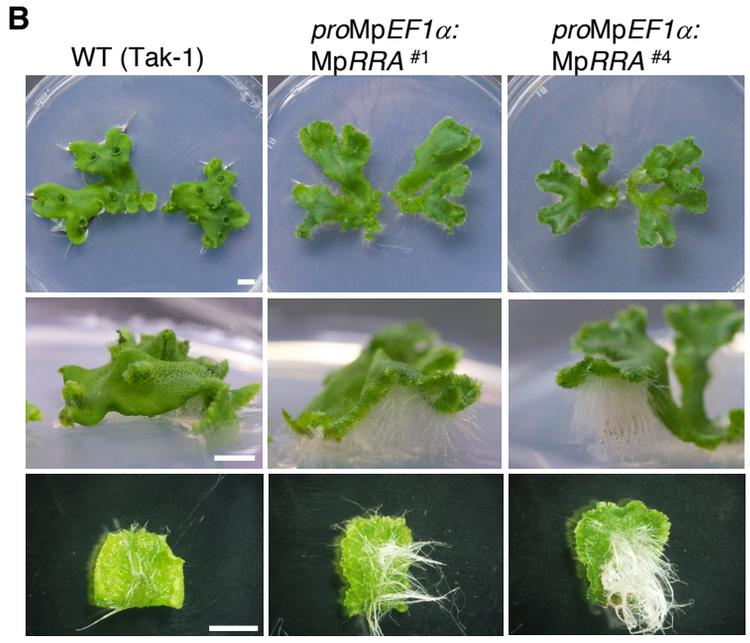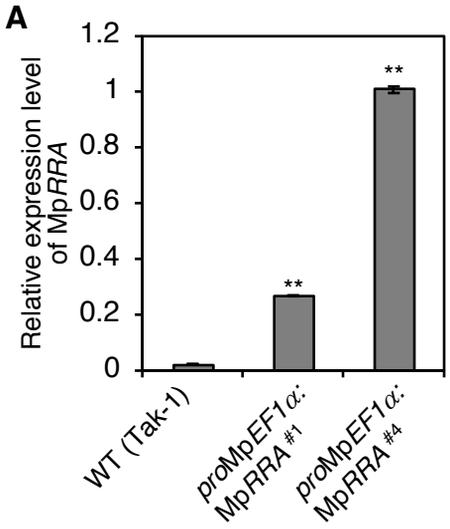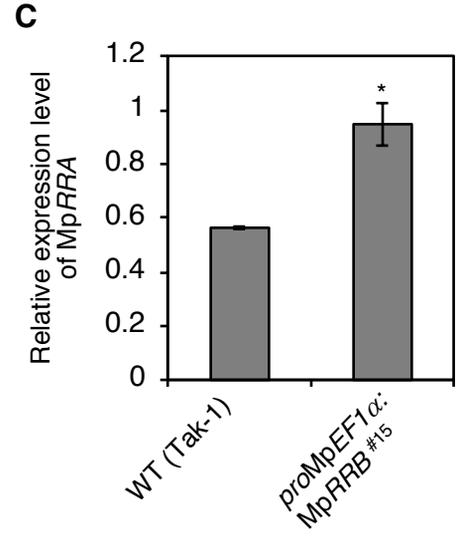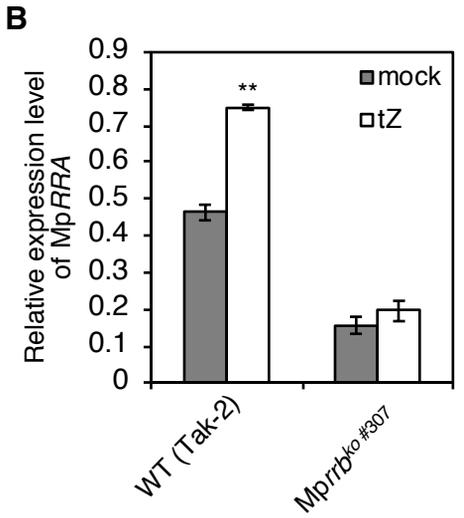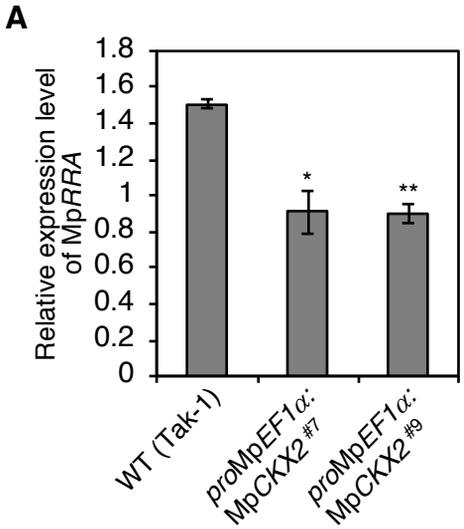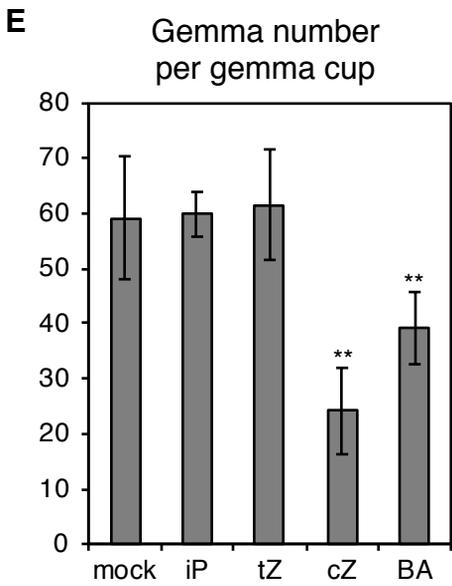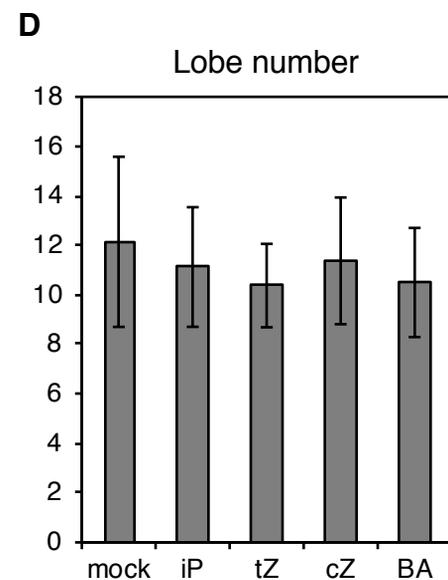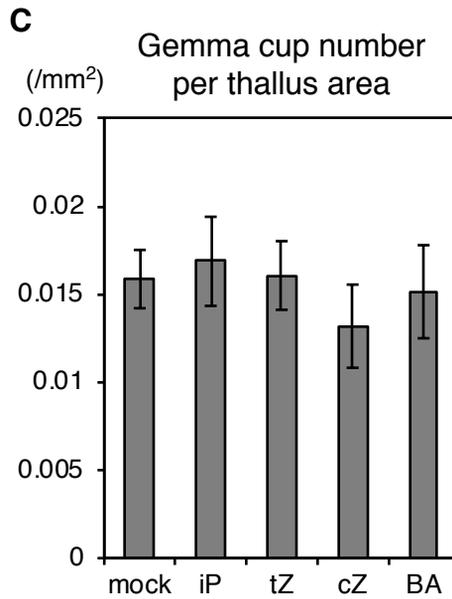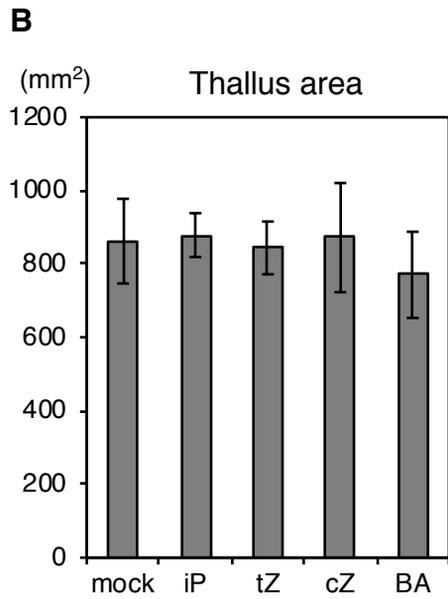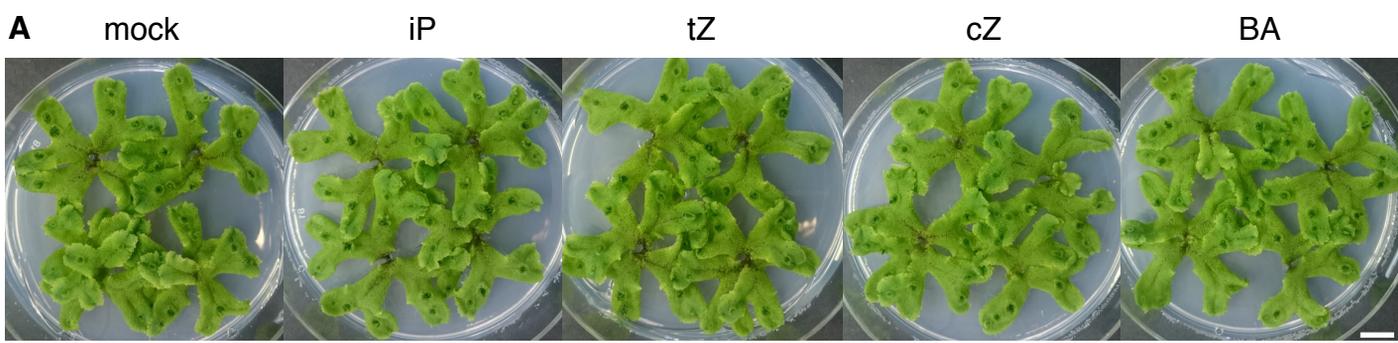
Fig. S1 Treatment of wild-type *Marchantia* (Tak-1) with 10 µM cytokinins. Six-day-old plants were transferred to agar medium containing 10 µM cytokinins, iP, tZ, cZ or BA, and cultured for 21 days. (A) Thalli of cytokinin-treated plants. Bar represents 1 cm. (B-E) Thallus area (B), gemma cup number per thallus area (C), lobe number (D) and gemma number per gemma cup (E) of cytokinin-treated plants. Data are presented as mean ± SD (n ≥ 7). Significant differences from the samples without cytokinin treatment (mock) were determined by Student's *t*-test: **, $P < 0.01$.
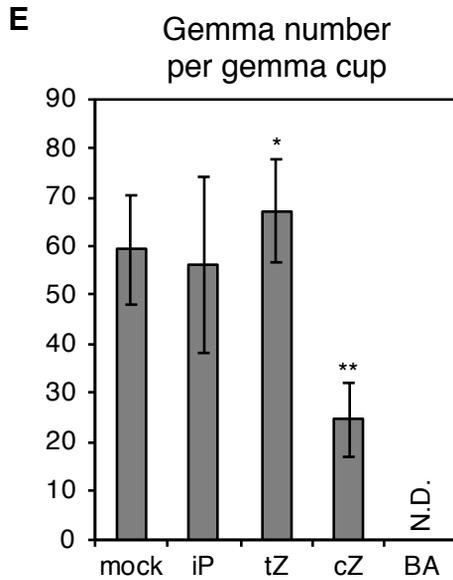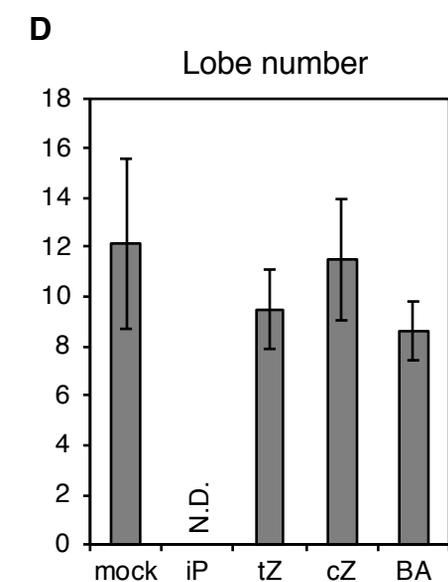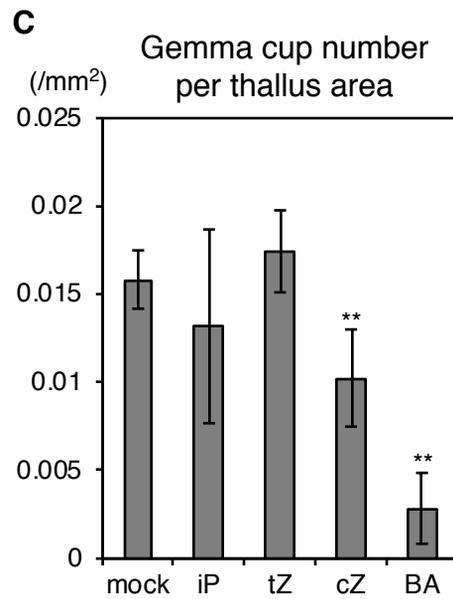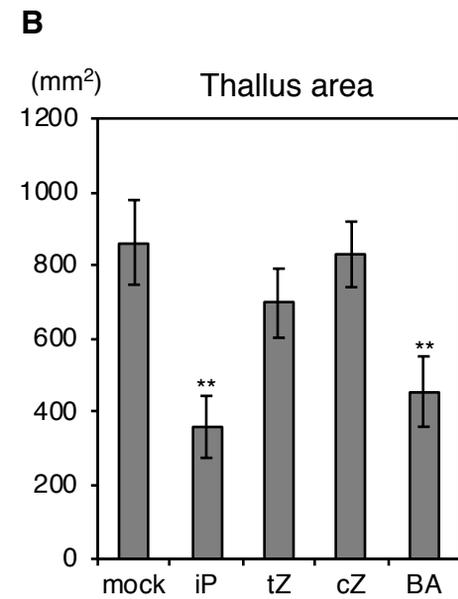
Fig. S2 Treatment of wild-type *Marchantia* (Tak-1) with 50 μM cytokinins. Six-day-old plants were transferred to agar medium containing 50 μM cytokinins, iP, tZ, cZ or BA, and cultured for 21 days. (A) Thalli of cytokinin-treated plants. Bar represents 1 cm. (B-E) Thallus area (B), gemma cup number per thallus area (C), lobe number (D) and gemma number per gemma cup (E) of cytokinin-treated plants. Data are presented as mean ± SD (n ≥ 7). Significant differences from the samples without cytokinin treatment (mock) were determined by Student's *t*-test: **, $P < 0.01$. N.D., not determined.
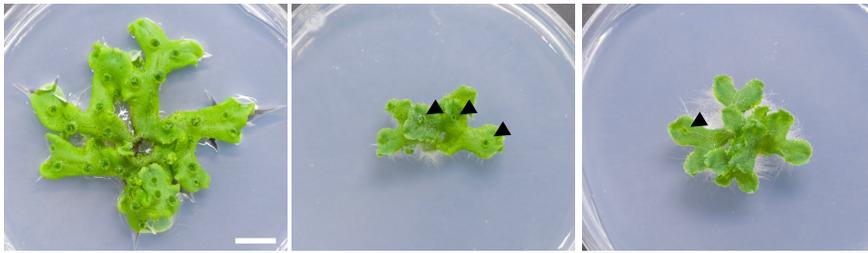
Fig. S3 Mp*CKX2*-overexpressing lines #7 and #9. Gemmae were grown for 28 days. Arrowheads indicate gemma cups. Bar represents 1 cm.
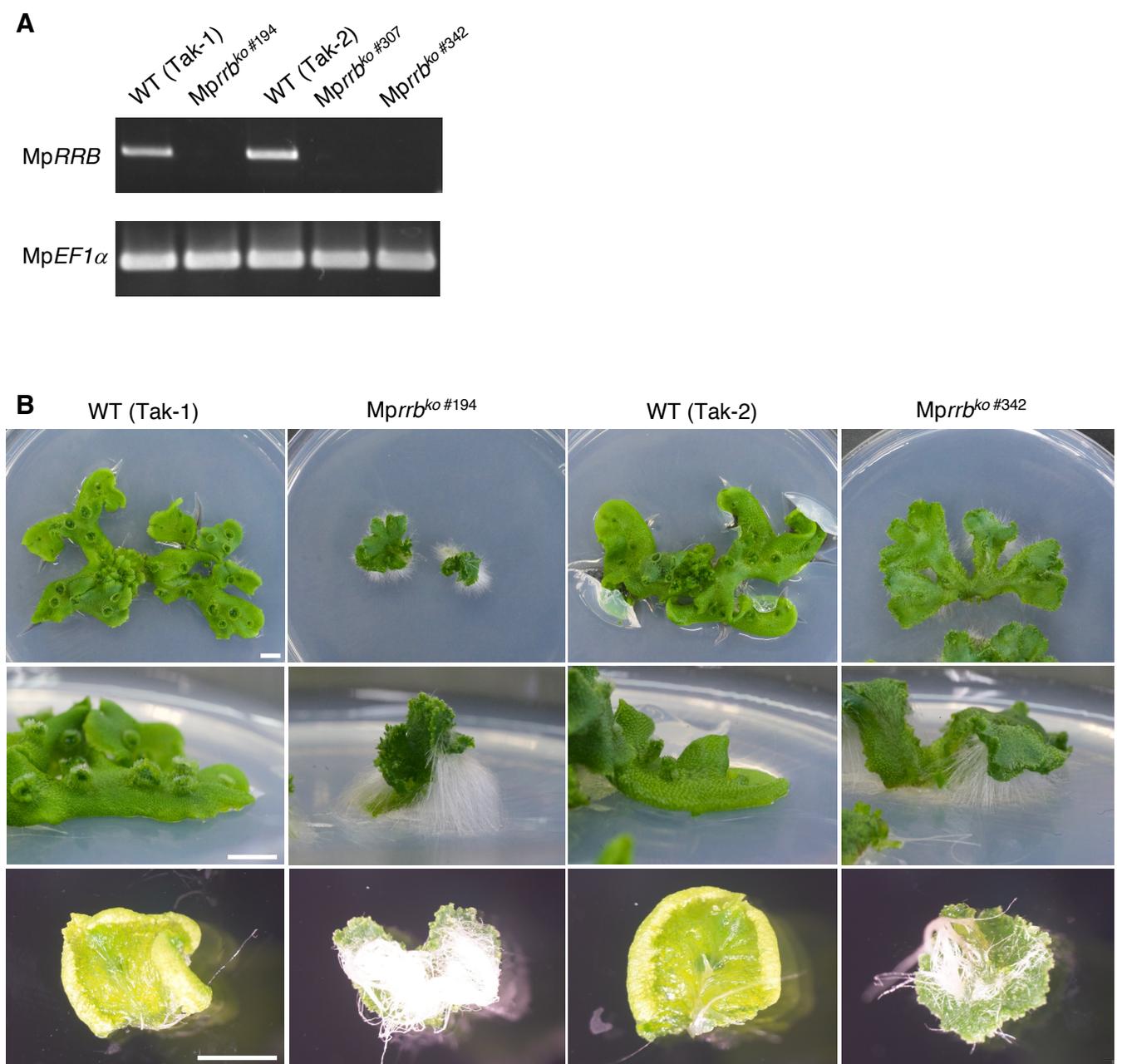
Fig. S4 Mp*rrb* knockout lines #194 and #342. (A) Mp*RRB* expression in Mp*rrb* knockout lines. Semi-quantitative RT-PCR was performed using total RNA from the knockout lines #194, #307 and #342. Mp*EF1a* was used as a control. (B) Thalli and rhizoids of the Mp*rrb* knockout lines. The pieces of thallus tip were cultured for 15 days. #194 and #342 have Tak-1 and Tak-2 backgrounds, respectively. Bars represent 5 mm.
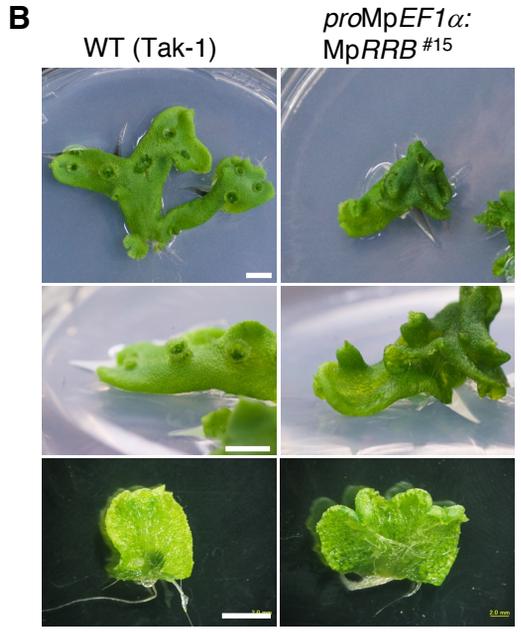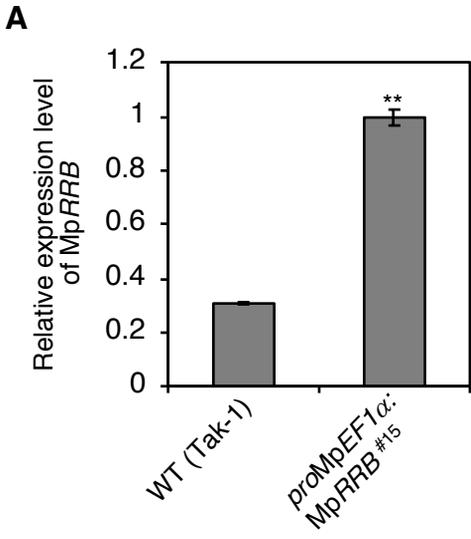
Fig. S5 Phenotype of the Mp*RRB*-overexpressing line. (A) Mp*RRB* expression in the Mp*RRB*-overexpressing line #15. qRT-PCR was conducted using total RNA, and mRNA level was normalized to that of Mp*EF1a*. Data are presented as mean $\pm$ SD (n = 3). A significant difference from wild-type (Tak-1) was determined by Student's *t*-test: **, $P < 0.01$. (B) Thalli and rhizoids of the Mp*RRB*-overexpressing line #15. The pieces of thallus tip were cultured for 15 days. Bars represent 5 mm.

A
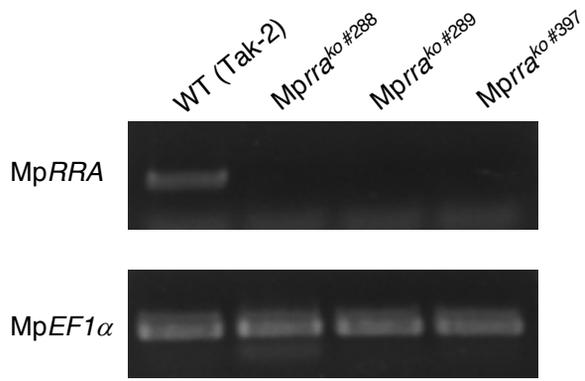
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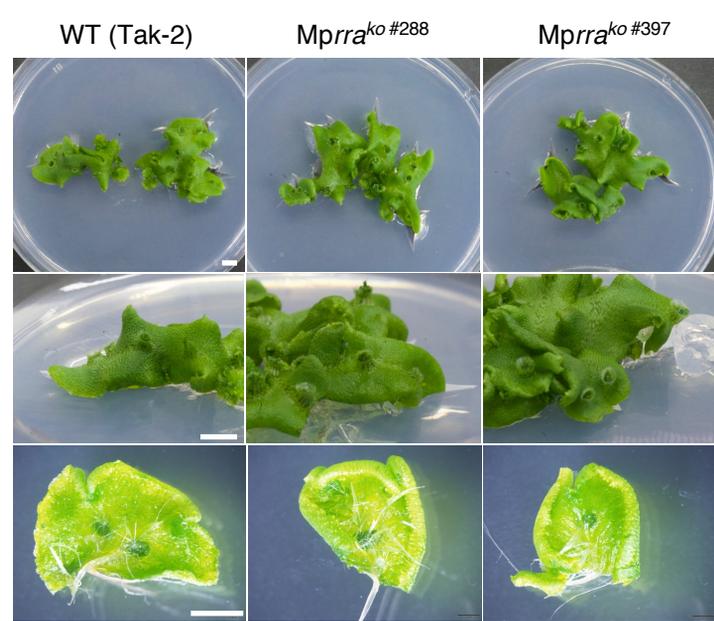
Fig. S6 Mp*rra* knockout lines #288 and #397. (A) Mp*RRA* expression in Mp*rra* knockout lines. Semi-quantitative RT-PCR was performed using total RNA from the knockout lines #288, #289 and #397. Mp*EF1a* was used as a control. (B) Thalli and rhizoids of the Mp*rra* knockout lines. The pieces of thallus tip were cultured for 15 days. Bars represent 5 mm.

Supplementary Table S1. Primers used in this study

Experiment	Gene		Primer sequence (5' to 3')
Cloning of genes	MpRRA	cds	ATGCATGGGCGTTTCGAAGG
			TCAGGCAACGGTAAGCTCACTTA
	MpRRB	cds	ATGATGAAGTCATTTGATACAGC
			TCACTGACCCTGACCAGGCCTAT
	MpRRA	promoter - stop codon	ATATCTACTTTTCTTGCAATAGAA
			TCAGGCAACGGTAAGCTCACTTA
	MpRRB	promoter - stop codon	ACTAGTCCTCTTACTTGGCT
			TCACTGACCCTGACCAGGCCTAT
	MpCKX2	cds	ATGATGCTGCAATTACTGAAATATC
			TCATAATAGGAACGGGAATGTC
Cloning of promoters	MpRRA	promoter	ATATCTACTTTTCTTGCAATAGAA
			GCAGGGGAGCTGCCTGCG
	MpRRB	promoter	ACTAGTCCTCTTACTTGGCT
			GGACTCGCAACTAGACGTTC
Gene targeting	MpRRA	PacI site	ctaaggtagcgattaGACAAAGGAATGCAGTGCCGAATT
			gcccgggcaagcttaCTCTCTACGGTGTCCACCGAATT
		AscI site	taaactagtggcgcgGTTTGGCTTTCATCGAGTTTCTTGT
			ttatccctaggcgcgCAGAGGGCCGCAACCAATT
	MpRRB	PacI site	ctaaggtagcgattaTAGGCCTACGGCCCTACTCT
			gcccgggcaagcttaGCACTTTCAAACCAATGGGAGAAAA
		AscI site	taaactagtggcgcgCCCGTCATTAGTAAGTTATTTCCCCTTCT
			ttatccctaggcgcgCAGGAAGGCGATGATACACAAAATC
Semi-quantitative RT-PCR	MpRRA		ATGCATGGGCGTTTCGAAG
			TCAGGCAACGGTAAGCTCA
	MpRRB		ATGATGAAGTCATTTGATACAGCCA
			TCACTGACCCTGACCAGG
	MpEF1a		TCACTCTGGGTGTGAAGCAG
			GCCTCGAGTAAAGCTTCGTG
Quantitative RT-PCR	MpRRA		TCCCTGAAGGAAGTTCCAGTC
			CTTCACGTCCGCTAGTTGCAC
	MpRRB		GGTTTGAAAGTGCTCGTTGTCG
			AGGGAGAGAGCATCTACGGC
	MpCKX2		CCGTGGATTTTGGCCACATC
			TCACATTGGAGCTCGAGGC
	MpEF1a		AAGCCGTCGAAAAGAAGGAG
			TTCAGGATCGTCCGTTATCC