An Ancestral Function of Strigolactones as Symbiotic Rhizosphere Signals

Kyoichi **Kodama**[1,13], Mélanie K. **Rich**[2,13], Akiyoshi **Yoda**[3,4,13], Shota **Shimazaki**[1,13], Xiaonan **Xie**[3,4,13], Kohki **Akiyama**[5], Yohei **Mizuno**[1], Aino **Komatsu**[1], Yi **Luo**[1], Hidemasa **Suzuki**[1], Hiromu **Kameoka**[1], Cyril **Libourel**[2], Jean **Keller**[2], Keiko Sakakibara[6], Tomoaki **Nishiyama**[7], Tomomi **Nakagawa**[8], Kiyoshi **Mashiguchi**[9], Kenichi **Uchida**[10], Kaori **Yoneyama**[11], Yoshikazu **Tanaka**[1], Shinjiro **Yamaguchi**[9], Masaki **Shimamura**[12], Pierre-Marc **Delaux**[2*], Takahito **Nomura**[3,4*], Junko **Kyozuka**[1*]

[1] Graduate School of Life Sciences, Tohoku University, Sendai, Japan.
[2] LRSV, Université de Toulouse, CNRS, UPS, Toulouse INP, Auzeville-Tolosane, France.
[3] United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, Tokyo, Japan.
[4] Center for Bioscience Research and Education, Utsunomiya University, Tochigi, Japan.
[5] Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka, Japan.
[6]Department of Life Science, Rikkyo University, Tokyo, Japan.
[7]Research Center for Experimental Modeling of Human Disease, Kanazawa University, Kanazawa, Japan.
[8] National Institute for Basic Biology, Okazaki, Japan.
[9] Institute for Chemical Research, Kyoto University, Kyoto, Japan.
[10] Department of Biosciences, Teikyo University, Tochigi, Japan.
[11] Graduate School of Agriculture, Ehime University, Ehime, Japan.
[12] Graduate School of Integrated Sciences for Life, Hiroshima University, Hiroshima, Japan
[13] These authors contributed equally: Kyoichi Kodama, Mélanie K. Rich, Akiyoshi Yoda, Shota Shimazaki, Xiaonan Xie

[*]Correspondence: junko.kyozuka.e4@tohoku.ac.jp, tnomura@cc.utsunomiya-u.ac.jp, pierre-marc.delaux@lrsv.ups-tlse.fr

Abstract (149 words)

In flowering plants, carotenoid-derived strigolactones (SLs) have dual functions as hormones that regulate growth and development, and as rhizosphere signaling molecules that induce symbiosis with arbuscular mycorrhizal (AM) fungi. Here, we report the identification of bryosymbiol (BSB), a previously unidentified SL from the bryophyte *Marchantia paleacea*. BSB is also found in vascular plants, indicating that it is ancestral in land plants. BSB synthesis is enhanced at AM symbiosis permissive conditions and BSB deficient mutants are impaired in AM symbiosis. In contrast, the absence of BSB synthesis has little effect on the growth and gene expression. We show that the introduction of the SL receptor of Arabidopsis renders *M. paleacea* cells BSB-responsive. These results suggest that BSB is not perceived by *M. paleacea* cells due to the lack of cognate SL receptors. We propose that SLs originated as AM symbiosis-inducing rhizosphere signaling molecules and were later recruited as plant hormone.

INTRODUCTION

Land plants evolved from an algal ancestor more than 450 million years ago[1]. The first land plants faced many challenges, such as UV radiation and nutrient-poor soil. Thus, initial colonization of the terrestrial environment required the evolution of innovations such as the deployment of complex hormonal regulations and the mutualistic symbiosis formed with arbuscular mycorrhizal (AM) fungi, a monophyletic group of symbiotic soil-borne fungi[2, 3]. During AM symbiosis, plants produce and supply carbohydrates and lipids to the fungus, in exchange for mineral nutrients, in particular phosphorus, mined in the soil by the fungal symbiont[4]. Symbiosis with AM fungi is observed in more than 80% of extant land plant species, including the bryophytes that diverged from the vascular plant lineage more than 400 million years ago[3]. In flowering

10 plants, establishment of the AM symbiosis requires the activation of fungal metabolism and stimulation of
11 hyphal branching by plant root-derived strigolactones (SLs)[5]. In addition to their role as rhizosphere
12 signaling molecules, SLs also function as a class of plant hormones and regulate various aspects of growth
13 and development in flowering plants[6, 7]. The dual function, as hormones and as rhizosphere signaling
14 molecules, makes SLs unique among plant hormones.

15 The function and signaling of SLs are well understood in flowering plants, whereas little is known outside
16 this plant lineage[8, 9]. The origin and evolution of their dual function are also largely unknown. One reason
17 why SL research has been hampered is related to their nature. The chemical structure of SLs differs from
18 species to species, many species have multiple SLs and they exist in cells in minute concentrations[10, 11].
19 Phylogenetic studies and genome sequences revealed that genes in the SL biosynthesis pathway are
20 conserved in bryophytes[12, 13]. However, although initial studies reported SLs in some bryophytes, their
21 actual presence is still under debate and, more importantly, their function remains unknown[11, 12, 14, 15].

22 Here we report the identification of an ancestral SL, bryosymbiol (BSB), present in diverse bryophytes such
23 as *Marchantia paleacea*, and vascular plants. In *M. paleacea*, BSB is secreted from the plants and is
24 required for AM symbiosis, but not for development. We show that BSB is not perceived by *M. paleacea*
25 cells due to the absence of cognate SL receptors. Our findings reveal that the ancestral function of SLs is
26 as AM symbiosis-inducing rhizosphere signaling molecules and that this function was already present in
27 the most recent common ancestor of land plants.

28

29 **RESULTS**

30 **Presence of SL biosynthesis genes is specific to AM symbiosis-forming species in *Marchantia***

31 Among the three extant groups of bryophytes, AM symbiosis is observed in liverworts and hornworts, but
32 not in mosses[3, 16]. In liverworts, hyphae of AM fungi enter through rhizoids and produce arbuscules in
33 parenchymatous cells in the midrib region of the thallus (Fig. 1a). We confirmed that AM symbiosis is
34 widely observed in the genus *Marchantia*, including *M. paleacea*, *M. emarginata* and *M. pinnata*, whereas
35 it is absent in *M. polymorpha*, the model liverwort species used for molecular genetic studies (Fig. 1a).

36 To date, more than 30 SLs have been identified in root exudates of various plant species[11]. SL biosynthesis
37 starts from the conversion of all-*trans*-β-carotene to 9-*cis*-β-carotene by DWARF27 (D27)[17] (Fig. 1b). Then,
38 9-*cis*-β-carotene is converted to carlactone (CL) by the consecutive actions of two carotenoid cleavage
39 dioxygenases (CCD7 and CCD8)[18, 19, 20]. Carlactone is converted to carlactonoic acid (CLA), the universal
40 precursor of a variety of species-dependent SLs, by the cytochrome P450, CYP711A, encoded by *MORE*
41 *AXILLARY GROWTH 1* (*MAX1*) homologs[21]. Carlactonoic acid is converted to canonical SLs such as 4-
42 deoxyorobanchol (4DO), orobanchol and 5-deoxystrigol (5DS) by species-specific functions of the P450s,
43 CYP711A and CYP722C[22, 23]. The *M. paleacea* genome contains two orthologs of *D27*, one of *CCD7*, two
44 of *CCD8* and one of *MAX1*[16] (Supplementary Figs. 1 and 2 and Supplementary Table 1). There are no
45 CYP722 family genes in any of the investigated *Marchantia* species[16, 24]. *CCD8* and *MAX1* orthologs exist
46 in all other AM symbiosis-forming *Marchantia* species tested (Supplementary Figs. 1 and 2). Despite the
47 polymorphisms present in the genome sequences, the overall structure of the genes is well conserved and
48 the amino acid sequences in the conserved domains show high similarities (Fig. 1c). In contrast to this, both
49 *CCD8* and *MAX1* orthologs are absent from the genome of *M. polymorpha*, a non-AM symbiosis-forming
50 species, indicative of gene loss[24]. Together, these phylogenetic analyses suggest that AM symbiosis-
51 forming *Marchantia* species produce SLs.

52

53 **Bryosymbiol (BSB), a previously unidentified SL is produced by *M. paleacea***

54 The correlation between the presence of the *CCD8* and *MAX1* genes and the occurrence of AM symbiosis
55 suggested the possibility that AM symbiosis-forming *Marchantia* species produce SLs. Using LC-MS/MS
56 analysis, we detected carlactonoic acid (retention time of 11.86 min) and its unknown analogue (retention
57 time of 10.73 min) in the rhizoid exudates of *M. paleacea* (Figs. 2a and 2b). Next, to determine whether the
58 carlactonoic acid in *M. paleacea* is synthesized through the same pathway as in flowering plants, we
59 generated knock-out mutants of the two *CCD8* genes (named Mpa*ccd8a* Mpa*ccd8b,* or Mpa*ccd8a/8b*) and
60 one *MAX1* (Mpa*max1*) gene using CRISPR/Cas9 (Supplementary Table 2). Carlactonoic acid was not
61 detected in either Mpa*ccd8a/8b* or Mpa*max1* mutants, indicating that carlactonoic acid biosynthesis is
62 dependent on both MpaCCD8 and MpaMAX1 (Fig. 2a).

63 To test for the presence of downstream SL activity, we used a parasitic seed germination assay. This is
64 based on the ability of obligate root parasitic Orobanchaceae plants, such as witchweed (*Striga* spp.) and
65 broomrape (*Orobanche* and *Phelipanche* spp.), to use SLs as host recognition signals in the rhizosphere[25].
66 This assay is sensitive to concentrations of SLs as low as 10 pM. We fractionated the rhizoid exudates of
67 *M. paleacea* by reversed-phase HPLC and found germination stimulation activity in the fraction collected
68 at around 8 min for all three species of parasitic plants (Fig. 2c). Since there is no known SL eluting at this
69 retention time[26], we hypothesized that *M. paleacea* produces an unidentified SL. To identify the nature of
70 this compound, we collected and purified the active fraction from the rhizoid exudate of *M. paleacea* in a
71 large-scale culture. The molecular formula of the purified amorphous solid (0.33 mg) was established to be
72 $C_{19}H_{24}O_6$ based on a protonated ion obtained by HR-ESI-TOF-MS, and its structure was determined by
73 NMR spectroscopy, ESI- and EI-MS spectrometry (Figs. 2d and 2e, Supplementary Figs. 3a-c and
74 Supplementary Table 3). The chemical structure of the identified SL was determined as (*E*)-5-((4-hydroxy-
75 2-oxo-5-(2,6,6-trimethylcyclohex-1-en-1-yl)dihydrofuran-3(2*H*)-ylidene)methoxy)-3-methylfuran-2(5*H*)-

one (Fig. 2f). The CD spectrum indicated that it has a 2′(*R*) configuration like previously identified natural SLs (Supplementary Fig. 3d). We named this newly identified SL bryosymbiol, or BSB. Although further studies are needed to confirm the absolute stereochemistry of BSB, the NOESY correlations suggested the relative stereochemistry at C-4 and C-5 is *anti* and we tentatively assigned BSB to be (4*R*, 5*S*, 2′*R*) (Supplementary Fig. 3a and Supplementary Table 3).

BSB was detected in the exudate and plant extracts of WT *M. paleacea* but not in the Mpa*ccd8a/8b* and Mpa*max1* mutants (Figs. 2d and 3a). In contrast, we detected a higher level of carlactone accumulation in the extract of the Mpa*max1* mutant than that of the WT (Fig. 3b). This suggests that the substrate of MpaMAX1 is likely to be carlactone, similar to that of MAX1 homologs in vascular plants. To determine whether MpaMAX1 directly converts carlactone to BSB, we performed *in vitro* metabolic experiments. When the microsomal fraction of yeast expressing Mpa*MAX1* was incubated with carlactone, the production of carlactonoic acid and BSB were detected (Fig. 3c). This indicates that MpaMAX1 catalyzes two steps, namely the conversion from carlactone to carlactonoic acid and carlactonoic acid to BSB. To further confirm this pathway, we incubated microsomes of yeast expressing Mpa*MAX1* with carlactonoic acid and detected BSB (Fig. 3c). We concluded that MpaMAX1 produces BSB from carlactone via carlactonoic acid. The conversion of carlactonoic acid to BSB is presumed to be via epoxidation and that upon proton abstraction of the carboxyl group, closure of the C ring occurs (Fig. 3d). Stereoselective closure of the C ring may occur, producing BSB (4*R*,5*S*,2'*R*) and its diastereomer (4*S*,5*R*,2'*R*), probably depending on the epoxy configuration. However, the occurrence of 7,8-epoxy-carlactonoic acid, a putative intermediate between carlactonoic acid and BSB (Fig. 3d), could not be confirmed due to the lack of the authentic standard.

To determine whether BSB is a derived trait of *M. paleacea* or more widespread across the plant diversity, we investigated its presence in diverse land plants. First, we analyzed *M. pinnata*, *M. emarginata* and *M.*

99 *polymorpha*. Carlactonoic acid and BSB were detected in exudates from the two AM symbiosis-forming
100 *Marchantia* species but not from *M. polymorpha*, consistent with the presence/absence of *CCD8* and *MAX1*
101 (Figs. 2a, 2d and 2e and Supplementary Figs. 1 and 2). Neither carlactonoic acid nor BSB was detected in
102 the extracts of the model moss *Physcomitrium patens*, which lacks the *MAX1* gene (Figs. 2a and 2d)[27].
103 Since *CCD8* and *MAX1* are present in hornworts[28, 29], we analyzed exudates from the hornwort *Phaeoceros*
104 *carolinianus*. We also analyzed several ferns and seed plants. Carlactonoic acid and BSB (retention time of
105 8.3 min), and/or a likely stereoisomer of BSB (retention time of 7.7 min), were detected in exudates from
106 the hornwort *P. carolinianus*, the ferns *Dryopteris erythrosora* and *Matteuccia struthiopteris*, and the seed
107 plants *Asparagus officinalis* (monocot) and *Arachis hypogaea* (dicot) (Figs. 2a and 2d and Supplementary
108 Fig. 4).

109 Collectively, these results demonstrate that BSB is produced by AM symbiosis-forming *Marchantia* and a
110 variety of vascular plants. Because bryophytes, such as liverworts and hornworts, and vascular plants, such
111 as ferns and flowering plants, diverged more than 400 million years ago, it is inferred that BSB was likely
112 present in the most recent common ancestor of land plants.

113

114 **Enhanced BSB synthesis correlates with an AM symbiosis permissive state**

115 In flowering plants, phosphate starvation stimulates the expression of SL-biosynthesis genes and the actual
116 production of SLs, and correlates with a permissive state for the AM symbiosis to be established[7, 30]. We
117 tested whether BSB synthesis is also enhanced in *M. paleacea* under phosphate-deficient conditions.
118 Expression of all five genes in the SL synthesis pathway, namely, Mpa*D27*, Mpa*CCD7*, Mpa*CCD8A*,
119 Mpa*CCD8B* and Mpa*MAX1*, were increased upon phosphate starvation (Fig. 4a). We also found that the

120 expression of the *CCD8* gene in *Anthoceros agrestis*, a hornwort, was induced by phosphate starvation (Fig.
121 4a).

122 To obtain a genome-wide view of the phosphate level-dependent gene expression in *M. paleacea*, we
123 performed RNAseq analysis (Fig. 4b). The expression of many genes changed under phosphate-deficient
124 conditions (Supplementary Table 4). In petunia and *Medicago truncatula*, expression of *PDR1*, an SL
125 transporter gene, is also enhanced under low phosphate conditions, implying that increased amounts of SLs
126 are exuded to the rhizosphere under phosphate starvation[31, 32]. Although SL transporters in bryophytes have
127 not yet been identified, ABCG transporter genes, homologs of *PDR1*, were up-regulated under phosphate-
128 deficient conditions in *M. paleacea* (Fig. 4c).

129 We then tested the level of exuded BSB using the parasitic seed germination assay under low phosphate
130 conditions. Consistent with the enhanced expression of SL biosynthesis and putative transporter genes, seed
131 germination frequency was extremely higher in exudates of *M. paleacea* grown under phosphate-deficient
132 conditions (Fig. 4d), implying that the amount of BSB exuded was increased upon phosphate starvation.
133 We finally examined if phosphate starvation affects AM symbiosis in *M. paleacea* as in vascular plants
134 (Fig. 4e). While phosphate-deficient conditions were permissive for AM symbiosis, increasing level of
135 phosphate in the watering solution resulted in a gradual decrease in colonization by the AM fungus,
136 illustrated by an increased zone without AM symbiosis (Fig. 4e).

137 AM symbiosis does not occur during the early stages of *M. paleacea* development and is first observed in
138 three-week-old gemmae. Thus, we also tested the developmental control of BSB synthesis. We found
139 enhanced Mpa*CCD8B* expression under low phosphate conditions in three-week-old gemmae but not in
140 earlier stage gemmae (Fig. 4f). Altogether, these results indicate that BSB synthesis correlates with a
141 permissive state for AM symbiosis in *M. paleacea*.

142

143 **BSB is required for symbiosis with AM fungi in *M. paleacea***

144 The correlation between the presence of BSB and AM symbiosis suggests that bryophytes use BSB as a
145 rhizosphere signaling chemical to promote the establishment of symbiosis with AM fungi. We confirmed
146 that BSB induces hyphal branching in the AM fungus *Gigaspora margarita* with a minimum effective
147 concentration of 10 pg/disk, which is within the range of the effects of previously described natural SLs (1
148 to 100 pg/disk) (Fig. 5a)[33]. To further test the symbiotic function of BSB *in vivo*, the Mpa*ccd8a*, Mpa*ccd8b*
149 and Mpa*ccd8a/8b* mutants were inoculated with spores of the AM fungus *Rhizophagus irregularis*. Six
150 weeks after inoculation, the Mpa*ccd8a* and Mpa*ccd8b* mutant thalli showed colonization similar to the WT.
151 In contrast, almost all thalli of the Mpa*ccd8a/8b* mutants were not colonized (Figs. 5b and 5c and
152 Supplementary Fig. 5). To determine which stages of the symbiotic establishment were affected in the
153 mutants, the number of infection points was scored. While the number of infection points was similar in
154 the WT plants and the Mpa*ccd8a* and Mpa*ccd8b* single mutants, all three alleles of the Mpa*ccd8a/8b* mutant
155 showed no or very few infection sites (Figs. 5c and Supplementary Fig. 5). To further determine whether
156 the lack of infection was due to the absence of stimulation of hyphal branching by BSB, complementation
157 assays were conducted using *rac*-GR24, a synthetic analogue of SL known to induce hyphal branching.
158 Exogenous treatment with 100 nM *rac*-GR24 restored colonization and the number of infection points in
159 the three Mpa*ccd8a/8b* mutant alleles (Figs. 5b and 5c and Supplementary Fig. 5).

160 In flowering plants, D14LIKE (D14L), an ortholog of KARRIKIN INSENSITIVE2 (KAI2), and
161 D3/MAX2-dependent signaling positively regulate AM symbiosis[34, 35]. In contrast to this, AM symbiosis
162 was not affected in *kai2* or *max2* mutants of *M. paleacea* (Fig. 5c and Supplementary Fig. 5). This suggests
163 that KAI2-dependent signaling was recruited to control AM symbiosis in vascular plant lineages.

164 In contrast to the Mpa*ccd8a/8b* mutants, neither colonization nor infection sites were affected in Mpa*max1*
165 mutants, despite the absence of BSB production (Fig. 5c and Supplementary Fig. 5). To understand this
166 discrepancy, we pursued the presence of an active SL other than BSB in the exudates of Mpa*max1* mutants.
167 We fractionated the exudates from Mpa*max1* by reversed-phase HPLC and tested for the presence of SL-
168 like activity using the parasitic seed germination assay. These assays identified a single active fraction at
169 the retention time of 18 min (Fig. 5d), while there was no activity in the corresponding fraction of WT
170 exudates (Fig. 2c). This fraction contains carlactone (Fig. 3b), which is known to induce hyphal branching,
171 although at higher concentrations than BSB[36]. Accumulation of carlactone in the Mpa*max1* mutants may
172 explain the observed normal fungal colonization.

173 From this reverse genetic approach and chemical complementation assays, we conclude that the function
174 of SLs as symbiotic signal in the rhizosphere is an ancestral trait in land plants, which has been maintained
175 in both the vascular plants and the bryophytes for 400 million years.

176

177 **BSB-deficient mutants of *M. paleacea* show no abnormal developmental patterns and gene expression**
178 **profiles**

179 We next examined the possible endogenous functions of BSB in *M. paleacea*. Despite the absence of BSB
180 synthesis, no alteration in growth pattern or morphology was observed in Mpa*ccd8a/8b* and Mpa*max1* loss-
181 of-function mutants throughout their growth, even in plants grown under the low phosphate conditions that
182 stimulate BSB synthesis (Figs. 6a-d). This is consistent with the results of the RNAseq analysis comparing
183 gene expression between WT and Mpac*cd8a/8b*, which shows that only a small number of genes are
184 differentially regulated (Fig. 4b). These data suggest that the absence of endogenous BSB causes little effect
185 on *M. paleacea*. Feedback regulation of SL synthesis genes such as *CCD8* is observed in many seed plants[7,

186 [37], whereas expression of Mpa*CCD8A* and Mpa*CCD8B* genes are not affected by exogenously applied SLs
187 (Fig. 6e).

188 In flowering plants, SL is perceived by D14, encoding an α/β-hydrolase protein[38, 39, 40]. *D14* originated from
189 a gene duplication of *KAI2* before the radiation of seed plants[41]. Although endogenous ligands of KAI2 are
190 unknown, it is thought that D14 and KAI2 interact with other signaling components such as the F-box
191 protein MAX2/D3 upon the perception of their cognate ligands[8]. This leads to the degradation of SMXL
192 proteins that work as regulators of transcription of downstream genes[42, 43]. We previously reported that
193 KAI2, MAX2/D3 and SMXL function in a common proteolysis-dependent signaling pathway in *M.*
194 *polymorpha*[44]. Similarly to *M. polymorpha*, the *M. paleacea* genome contains one MAX2/D3 (Mpa*MAX2*)
195 and two KAI2-like genes (Mpa*KAI2A* and Mpa*KAI2B*) but no *bona fide D14*, as is the case in other non-
196 seed plants. To pursue a possibility that BSB plays an endogenous function in *M. paleacea,* we tested the
197 possibility that MpaKAI2A and/or MpaKAI2B act as the receptors of BSB. Mpa*kai2a* and Mpa*max2*
198 mutants (Supplementary Table 2) showed altered growth patterns, including reduced and upward growth
199 of the early-stage thalli and delayed gemmae cup initiation (Figs. 6a and 6b). Such developmental defects
200 were not observed in the Mpa*ccd8a/8b* or the Mpa*max1* mutants (Figs. 6a and 6b). To further investigate
201 the potential link between MpaKAI2A and MpaKAI2B and SL, their physical interaction with four
202 stereoisomers of the synthetic SL GR24 was investigated by differential scanning fluorimetry (DSF)
203 analysis (Supplementary Figs. 6a and 6b). Only $GR24^{ent\text{-}5DS}$, which has an unnatural D-ring configuration
204 and has been shown to interact with Arabidopsis KAI2[45], was able to weakly interact with MpaKAI2A
205 whereas no sign of interaction was observed between MpaKAI2B and GR24. In flowering plants, SL
206 synthesis is enhanced in SL signaling mutants because of the loss of feedback regulation[46]. However, no
207 such increase was observed in the levels of carlactonoic acid and BSB in exudates of Mpa*kai2a/2b* and
208 Mpa*max2* mutants (Supplementary Figs. 6c-f). Altogether, these phenotypic and biochemical analyses

209 indicate that MpaKAI2A, MpaKAI2B and MpaMAX2 are not involved in the perception of BSB or other
210 metabolites downstream CCD8 or MAX1 in *M. paleacea*.

211

212 **Heterologous expression of AtD14, the SL receptor of Arabidopsis, renders *M. paleacea* cells BSB-**
213 **sensitive**

214 From the previous phylogenetic studies and the experimental work presented above, it can be proposed that
215 BSB does not function as a hormone in bryophytes due to the absence of cognate receptors. In other words,
216 although an SL, BSB, is produced and the known signaling pathway is present in bryophytes, the lack of
217 the upstream receptor results in the two pathways being disconnected. If this hypothesis is valid, mimicking
218 the seed-plant duplication of the KAI2-like gene by adding a cognate SL receptor in *M. polymorpha* and *M.*
219 *paleacea* should lead to SL responsiveness. To experimentally test this hypothesis, we took a gain-of-
220 function approach in which the canonical SL receptor D14 of Arabidopsis (*AtD14*) was fused with the
221 promoter of *M. polymorpha ELONGATION FACTOR1* (*EF1*) gene and introduced into *M. polymorpha* and
222 *M. paleacea.* We examined whether endogenous BSB is recognized by the introduced AtD14, leading to
223 signal transduction. More than 20 independent lines in each species were produced and two lines that show
224 high and low levels of At*D14* expression were selected from each species (named Mp*AtD14*ox and
225 Mpa*AtD14*ox, for M. *polymorpha* and *M. paleacea*, respectively) for further analysis (Fig. 7a and
226 Supplementary Fig. 7a). First, we tested if the introduction of AtD14 enables SL perception and signal
227 transduction in response to *rac*-GR24 in *M. polymorpha* cells that do not produce BSB. The
228 *DIENELACTONE HYDROLASE LIKE PROTEIN1* (*DLP1*) and Mp*SMXL* genes of *M. polymorpha*, whose
229 expression is highly dependent on KAI2-dependent signaling, were used as markers[44]. Expression of
230 Mp*DLP1* and Mp*SMXL* was induced by the application of *rac*-GR24 in the Mp*AtD14*ox lines but not in

231 WT plants (Supplementary Fig. 7b). To verify that AtD14 functions in the same signaling pathway as the
232 endogenous KAI2A, Mp*kai2a* mutation was introduced into the Mp*AtD14*ox lines. By contrast with the
233 Mp*kai2a* mutants, the upward thallus growth phenotype was rescued by application of $GR24^{4DO}$ and
234 $GR24^{5DS}$, which have a 2'(*R*) configuration as natural SLs[45], in the MpAt*D14*ox/Mp*kai2a* lines
235 (Supplementary Fig. 7c).

236 Phenotypes of Mpa*kai2a*, the loss-of-function mutant of *KAI2A* in *M. paleacea*, closely resembled those of
237 Mp*kai2a* (Figs. 6b and 7b). As for *M. polymorpha,* the thallus upward curving was rescued by application
238 of $GR24^{4DO}$ and $GR24^{5DS}$ in the Mpa*AtD14*ox/Mpa*kai2a* (Fig. 7b). These results indicate that introducing
239 AtD14 enables SLs perception and downstream signal transduction through the KAI2-dependent signaling
240 pathway in both *M. paleacea* and *M. polymorpha*.

241 We next directly tested the effect of exogenously applied SLs on the signaling cascade. As is the case in *M.*
242 *polymorpha,* neither $GR24^{4DO}$ nor $GR24^{5DS}$ enhanced expression of the Mpa*DLP1* and Mpa*SMXL* genes in
243 WT *M. paleacea* plants, even at the highest concentration (Figs. 7c and 7d). This implies that the applied
244 SLs are not perceived by WT *M. paleacea* cells, consistent with the disconnection between SL and the
245 KAI2-like signaling pathway. In contrast, in the Mpa*AtD14*ox lines containing the SL receptor gene,
246 expression of Mpa*DLP1* and Mpa*SMXL* was enhanced by the application of either $GR24^{4DO}$ or $GR24^{5DS}$ in
247 a concentration-dependent manner (Figs. 7c and 7d).

248 We showed that BSB synthesis is enhanced under phosphate starvation (Figs. 4a and 4c). Therefore, we
249 hypothesized that enhanced synthesis of BSB may recruit AtD14-dependent signaling under phosphate-
250 deficient conditions. Indeed, expression of Mpa*DLP1* and Mpa*SMXL* was induced in Mpa*AtD14*ox lines
251 under phosphate starvation (Fig. 7e). Although a slight increase in Mpa*SMXL* expression under phosphate
252 depletion was sometimes observed in WT plants, the same trend was detected in Mpa*ccd8a/8b*, suggesting

253 that the slight increase was caused by unknown SL-independent mechanisms (Supplementary Fig. 7d).
254 Besides the activation of marker genes, we tested whether the gain of the cognate SL receptor was sufficient
255 to recruit BSB as an endogenous signal with a physiological effect. Thus, Mpa*kai2a* and
256 Mpa*AtD14*ox/Mpa*kai2a* lines were transferred to a medium with a lower level of phosphate. On this
257 medium, while the upward curving phenotype of the Mpa*kai2a* lines remained fully unaffected, this defect
258 was partially, but significantly, rescued in the Mpa*AtD14*ox/Mpa*kai2a* lines (Fig. 7f). Taken together, these
259 results indicate that endogenous BSB can be perceived by a newly integrated SL receptor and transduce the
260 signal through the MpaMAX2-dependent signaling pathway; that is, BSB is perceived in *M. paleacea* cells
261 when the cognate receptor is present. This strongly supports the hypothesis that the evolution of SLs as an
262 endogenous hormone is only dependent on the evolution of a cognate receptor in the KAI2 clade.

263

264 **DISCUSSION**

265 **The ancestral function of SLs is as symbiotic signaling molecules in the rhizosphere**

266 In flowering plants, SLs play dual roles as a class of phytohormones to control various aspects of growth
267 and development and as rhizosphere signaling chemicals to control symbiosis with AM fungi and parasitism
268 by root parasitic plants[5, 6, 7, 47]. AM symbiosis is found in most extant land plants, which comprise two main
269 monophyletic clades, the non-vascular bryophytes and the vascular tracheophytes and was present in the
270 last common ancestor of land plants more than 400 million years ago[48]. In this study, we showed that the
271 SL, BSB, is produced in a wide range of plant species, including flowering plants, indicating that BSB is
272 an ancestral and conserved form of SLs. In *M. paleacea*, BSB acts as a rhizosphere signaling molecule
273 essential for symbiosis with AM fungi. Thus, it can be concluded that the function of SLs as signaling

274 molecules to communicate with AM fungi was already established in the common ancestor of the
275 bryophytes and vascular plants.

276 AM symbiosis was an innovation by the first land plants to acquire minerals, in particular phosphate, from
277 the nutrient-poor soil they faced during the colonization of emerged land. In all mutualistic systems, control
278 mechanisms have evolved on both the host and symbiont sides to optimize the benefits of the symbiosis[49].
279 In flowering plants, SL synthesis is regulated by phosphate availability[30, 50, 51]. Upon phosphate starvation,
280 SL biosynthesis and transporter genes are enhanced resulting in an increase in the level of SLs exuded to
281 the rhizosphere[32]. This leads to enhanced AM symbiosis and consequently, to increased phosphate supply.
282 We showed that phosphate-dependent control of SL synthesis occurs in the liverwort *M. paleacea* and the
283 hornworts *Anthoceros agrestis*. These results imply that the control of AM symbiosis through phosphate-
284 dependent SL synthesis was already present in the most common ancestors of bryophytes and vascular
285 plants.

286

287 **Convergent recruitment of SL as hormonal signal**

288 Although we cannot rule out that BSB does play an endogenous role in *M. paleacea*, the absence of
289 observed growth phenotypes in the Mpa*max1* and Mpa*ccd8a/8b* mutant, and the loss of both *CCD8* and
290 *MAX1* in *M. polymorpha*, a non-symbiotic species, suggest that its function is exclusively symbiotic in
291 liverworts. Even more certain is the disconnection between BSB and the KAI2/MAX2 signaling pathway.
292 Contrary to the loss of *CCD8* and *MAX1* in *M. polymorpha*, *KAI2A, KAI2B* and *MAX2* are conserved in *M.*
293 *polymorpha* despite its non-symbiotic characteristics and the developmental phenotypes observed in *M.*
294 *paleacea kai2a* and *max2* mutants resemble the ones observed in *M. polymorpha* but the phenotypes are
295 absent in Mpa*ccd8a/8b* or Mpa*max1* mutant. Loss of *CCD8* is also observed in the water ferns *Azolla*

296 *filiculoides* and *Salvinia cucullata* that have also lost AM symbiosis[52]. Such a correlation between gene and
297 trait losses is indicative of a specific function of the genes, here in AM symbiosis. Thus, it can be inferred
298 that the metabolites from the CCD8/MAX1 SL biosynthesis pathway and KAI2/MAX2 signaling were
299 originally independent. The role of the CCD8-derived metabolites as endogenous plant hormones evolved
300 during seed plant diversification by connecting them to receptors from the KAI2/D14 family. Extensive
301 phylogenetic analysis revealed that D14 evolved through putative neo-functionalization following gene
302 duplications of the KAI2 family members in the common ancestor of seed plants, leading to its function as
303 cognate receptor for SLs as plant hormones regulating multiple developmental traits in flowering plants[13,
304 41, 47]. In this study, we mimicked the evolution of such a hormonal system by introducing the *D14* from
305 Arabidopsis in *M. paleacea* and showed that endogenous BSB and exogenously supplied SL analogs are
306 perceived and trigger signal transduction in these engineered lines through D14-MAX2 dependent signaling.

307 Despite the lack of *MAX1*, molecules with an SL function are synthesized through CCD8 in *P. patens*[15].
308 CCD8-derived metabolites have also been recruited for quorum sensing-like functions in *P. patens*[14]. These
309 metabolites are currently elusive but the absence of *MAX1* in *P. patens* suggests that those are neither
310 carlactonoic acid nor BSB. Interestingly, the *KAI2* genes are highly duplicated in *P. patens* and some of
311 them are involved in the perception of the unknown CCD8-derived metabolites, but their signaling is
312 MAX2-independent[53, 54]. These findings imply that *P. patens* generated both the metabolic pathway and the
313 signaling system independently from other bryophytes after the loss of the *MAX1* gene. Similar acquisition
314 of signaling systems occurred in root parasitic plants, in which the *KAI2* genes duplicated massively[47].
315 Some of the *KAI2* paralogs were identified as extremely sensitive SL receptors that recognize SLs from
316 host plants[47, 55, 56]. Therefore, the generation of SL receptors from KAI2 and of molecules with an SL
317 function occurred repeatedly and independently during the evolution of land plants.

318

319 **A model for the evolution of hormonal networks in plants**

320 The origin of the complex hormonal pathways observed in extant flowering plants has been extensively
321 studied by combining reverse genetics conducted in bryophytes and phylogenetic studies on the entire green
322 lineage[57, 58]. From our work and previous studies, we propose a general model for the evolution of hormonal
323 networks in plants. In a first step, the signaling and biosynthesis pathways evolved independently, following
324 a *ligand first* or a *signaling first* modes of evolution. The *ligand first* mode is captured by the hormone
325 auxin and ABA which are produced by green algae whereas the signaling pathways are restricted to land
326 plants, and highly conserved[59, 60, 61]. The *signaling first* mode has been observed for gibberellin (GA), an
327 essential hormone in vascular plants which is absent from bryophytes whereas the central transcriptional
328 regulator involved in the GA pathway, DELLA, is present in bryophytes where it regulates plant growth
329 and stress responses[62, 63]. With both a biosynthetic pathway and a functional signaling pathway present, we
330 propose that the evolution of a receptor is sufficient to evolve a fully functional hormonal network. In the
331 Zygnematophyceae, which are the closest algal clade relative to land plants, the ortholog of the ABA
332 receptor functions independently of ABA[64, 65]. The ABA-receptor ortholog in land plants acquired the
333 ability to bind ABA which was sufficient to recruit its downstream signaling pathway[24, 66]. Here, we
334 revealed that SL evolved following a *ligand first* mode, and experimentally demonstrated that the gain of a
335 receptor was sufficient to recruit SL as hormonal signal. Why such connections between the product of an
336 existing biosynthetic pathway and a signaling pathway emerge and are selected remains an open question.
337 An attractive view is that SLs being exuded under phosphate deprivation conditions may have been first
338 recruited as a rhizospheric signal in plant communities and later as an endogenous mobile signal. Stepwise
339 recruitment of non-connected biosynthetic and signaling pathways into integrated processes might
340 represent a general mechanism for the evolution of intercellular and inter-organism communication.
341

342 **Methods**

343 **Plant materials and growth conditions**

344 *Marchantia paleacea* ssp. *diptera*, *M. polymorpha*, *M. pinnata*, *M. emarginata* and *Phaeoceros*
345 *carolinianus* were harvested from the wild in Japan (*Marchantia paleacea* ssp. *diptera* was collected in
346 Nara prefecture, the others were collected in Hiroshima prefecture). *Dryopteris erythrosora* and *Matteuccia*
347 *struthiopteris* plants were purchased from a garden store. Seeds of *Asparagus officinalis* (cv. Shower, Takii,
348 Japan) and *Arachis hypogaea* (cv. Chibahandachi, Watanabe Noji, Japan) were used. *M. polymorpha* and
349 *M. paleacea* were grown at 22 ˚C under continuous light on 1/2 Gamborg's B5 medium (pH 5.5) with 1 %
350 agar (Guaranteed Reagent; FUJIFILM WAKO Chemicals, Japan) or on expanded vermiculite fertilised
351 with 1/2,000 hyponex (HYPONEX Japan) every two weeks.

352 *A. agrestis* (Bonn strain) was grown on Half-strength Knop-II media (125 mg/L KNO3, 500 mg/L $Ca(NO_3)_2$
353 $4H_2O$, 125 mg/L $Mg(SO_4)_2$ $7H_2O$, 125 mg/L KH_2PO_4, and 10 mg/L $FeCl_3$ $6H_2O$; pH5.5) containing 1%
354 agar (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

355 For phosphate deficient treatment (Figs. 5a and 7g), Gemmae of *M. paleacea* or thallus of *A. agrestis* were
356 pre-incubated on half-strength Gamborg's B5 medium with 1.0 % agar for 2 weeks at 22℃ under
357 continuous light. After 2 weeks, the thallus was transferred to the medium with or without phosphate and
358 incubated for another week. Resultant plants were harvested and used for expression analysis.

359 For low phosphate treatment (Figs. 5e, 6e-f and 7h), Gemmae of *M. paleacea* were incubated on half-
360 strength Gamborg's B5 medium with 1.0 % agar with reduced phosphorus concentration (5-fold or 10-fold
361 low phosphate concentration) for the appropriate time as described in each legend.

362 To analyze thallus phenotypes, gemmae were grown on half-strength Gamborg's B5 medium with 1.0%
363 agar or the medium with a reduced phosphorus concentration, at 22℃ under continuous light for 21 days.
364 Resultant plants were used for the analysis of thallus phenotypes. To accurately measure the area and

365 rhizoid length, plants were flattened with a coverslip on a glass slide, and images were taken. To measure
366 the rhizoid length, the average lengths of the three longest rhizoids of each thallus were used as a
367 representative rhizoid length for each thallus. To measure the angle between the thallus and the growth
368 medium, images were taken from the side. For all measurements, the images were analyzed using ImageJ[44].
369

370 **Plasmid construction and plant transformation**

371 To construct vectors to generate CRISPR-Cas9 mutants, oligo DNAs containing the target sequences and
372 the complementary sequences were annealed and introduced into pMpGE_En01 (*ccd8a*, *ccd8b*, *kai2a*,
373 *kai2b*) or pMpGE_En03 (*max1*, *max2*). Subsequently, they were inserted into pMpGE010 (*ccd8b-1, -2*,
374 *kai2a-2, kai2b-1, -2*) or pMpGE011 (*ccd8a-1 ,-3*, *max1-1*, *-2*, *kai2a-1*, *max2-1*, *-2*) by Gateway LR reactions.
375 Transformation of *M. polymorpha* was performed as described[67], and slight modifications were applied for
376 the transformation of *M. paleacea*. For transformation of *M. polymorpha*, 10- to 14-day-old thalli grown
377 from gemmae were transferred to fresh media and incubated for 2–3 days before *Agrobacterium* infection.
378 For transformation of *M. paleacea*, 1- to 2-month-old thalli were cut and incubated on fresh media for 5–7
379 days before infection. To generate the Mpa*kai2kai2b* double mutant, *KAI2A* guide RNA inserted in
380 pMpGE011 was introduced to *kai2b-1*. To generate the Mpa*ccd8accd8b* double mutant, *CCD8A* guide
381 RNA inserted in pMpGE011 and *CCD8B* guide RNA inserted in pMpGE010 were introduced to WT thalli
382 at the same time (Mpa*ccd8a-2/8b-3*) or *CCD8B* guide RNA inserted in pMpGE010 was introduced into
383 *ccd8a-1* (Mpa*ccd8a-1/8b-4*).
384

385 **Analysis of CCD8A in *Marchantiopsida* species**

386 Genomic DNA of *Marchantia chenopoda*, *M. emarginata* and *M. pinnata* was extracted as reported
387 previously[68]. 2.5 kb of the CCD8A gene was PCR-amplified using GoTaq polymerase (Promega) according

388 to the manufacturer's protocol and the primers MpCCD8-F and MpCCD8-R (Supplementary Table 5).
389 Purified PCR products were Sanger-sequenced at Eurofins (Germany) using the same primers.
390

391 **SL analysis in exudates and plants**

392 *M. paleacea*, *M. polymorpha*, *M. pinnata*, *M. emarginata* and *P. carolinianus* were grown in 10 cm diameter
393 pots filled with vermiculite under a 15 h light (50 μmol m^{-2} s^{-1})/9 h dark photoperiod at 23 °C in a growth
394 room. *D. erythrosora* and *M. struthiopteris* plants were grown in a glasshouse with temperature control and
395 under a natural photoperiod, at 23 °C in the day and 18 °C at night during spring to summer. *A. officinalis*
396 and *A. hypogaea* seedlings were grown at 25 °C in the day and 20 °C at night. These plants were grown
397 using 1/2,000 Hyponex and tap water. At least two weeks after growing in tap water, 200 ml per pot of
398 water was poured on the plants (*Marchantia* and *Phaeoceros*) or the surface of the vermiculite (other plants),
399 and collected from the bottom of the pot. The exudates were extracted with ethyl acetate. For SL
400 quantification, 5 ng each of [1-$^{13}CH_3$]*rac*-carlactonoic acid and [3a,4,4,5,5,6'-D_6]4DO were added as
401 internal standards prior to the extraction. Endogenous SLs in *M. paleacea* plants were extracted with
402 acetone. SLs were analyzed by LC-MS/MS using QTRAP 5500 (AB Sciex, USA) as reported previously[21].
403 *rac*-CL, *rac*-CLA, [1-$^{13}CH_3$]*rac*-CLA and [3a,4,4,5,5,6'-D_6]4DO were synthesized as described
404 previously[69].
405

406 **Seed germination assay**

407 *Orobanche minor* seeds were collected from mature plants that parasitized red clover (*Trifolium pretense*
408 L.) grown in Tochigi Prefecture, Japan in June 2017. Germination assay of *O. minor* seeds was conducted
409 as reported previously[50]. *Phelipanche ramosa* and *Striga hermonthica* seeds were kindly supplied by Profs.
410 Philippe Delavault (University of Nantes, France) and A. G. T. Babiker (Sudan University of Science and

411 Technology, Sudan), respectively. Seeds of *P. ramosa* and *S. hermonthica* were surface-sterilized in 1%
412 NaOCl containing 0.1% Tween-20 for 5 min. The seeds were then rinsed 10 times with sterile distilled
413 water and air-dried. Twenty to 40 seeds were sown on a 5 mm-glass fiber disc prepared by a hole puncher
414 (Whatman GF/A, UK). Twenty discs were placed in a 5 cm-Petri dish lined with two layers of filter paper
415 wetted with 1.5 ml of distilled water. The Petri dishes were sealed with parafilm, enclosed in polyethylene
416 bags and incubated in the dark at 18°C for 7 days (*P. ramosa*) or at 30°C for 15 days (*S. hermonthica*).
417 After conditioning, the glass fiber discs were blotted to remove excess water. Each group of three discs was
418 transferred to a separate 5 cm-Petri dish lined with two layers of filter paper and wetted with 0.6 ml of test
419 solutions. The Petri dishes were sealed, enclosed in polyethylene bags, and placed in the dark at 20°C for 6
420 days (*P. ramosa*) or at 30°C for 3 days (*S. hermonthica*). The germination rate was determined under a 20×
421 binocular dissecting microscope.
422
423 **Isolation and purification of bryosymbiol (BSB)**
424 *M. paleacea* was grown on vermiculite in a plastic container (with holes in the bottom, 38×25×14 cm,
425 W×L×H) using tap water. Eighteen containers were placed under a 14 h light (60 μmol m^{-2} s^{-1})/10 h dark
426 photoperiod at 24 °C in a growth room. Water was poured on the plants and circulated every 10 min per
427 hour using an aquarium pump. The exudates released into the water were adsorbed on the activated charcoal
428 (2 g per 3 L) in the pump. Fresh activated charcoal was replaced every 2 d. The collected charcoal was
429 eluted with acetone. After evaporation of the acetone *in vacuo*, the aqueous residue was extracted with
430 EtOAc. The EtOAc phase was washed with 0.2 M K_2HPO_4 (pH 8.3), dried over anhydrous $MgSO_4$, and
431 concentrated *in vacuo*. The concentrated samples were kept at 4 °C until use. The crude EtOAc extract
432 (107.1 mg), collected over five weeks, was subjected to silica gel column chromatography (55 g) with
433 stepwise elution of *n*-hexane–EtOAc (100:0 to 0:100, v/v, 10 % step) to give fractions 1–11. Fractions 6

434 (50% EtOAc, 13.2 mg) containing a novel SL was subjected to silica gel column chromatography (20 g)
435 using *n*-hexane–EtOAc (60:40, v/v). Fractions were collected every 10 ml. Fractions 9–18 were found to
436 contain a novel SL based on LC-MS/MS and GC-MS analyses. Fractions 9–18 were combined (3.17 mg)
437 and purified by HPLC on an ODS column (Kinetex C18, 4.6×250 mm, 5 μm; Phenomenex, USA) with a
438 MeCN/H_2O gradient system (30:70 to 100:00 over 40 min) at a flow rate of 1 ml/min. The column
439 temperature was set to 30 °C. The fraction eluted as a single peak at 28.6 min (detection at 248 nm) was
440 collected. This fraction was further purified by HPLC on a Develosil ODS-CN column (4.6×250 mm, 5
441 μm; Nomura Chemicals, Japan) with isocratic 70% MeCN/H_2O at a flow rate of 0.8 ml/min to afford BSB
442 (0.33 mg, *Rt* 21.1 min, detection at 248 nm).
443
444 **Structural determination of BSB**
445 HR–ESI–TOF–MS (AB Sciex TripleTOF 5600) showed a protonated molecular ion at *m/z* 349.1655
446 $[M+H]^+$ calcd for $C_{19}H_{25}O_6$, *m/z*: 349.1651). EI-GC/MS (JEOL JMS-Q1000GC/K9 on an Agilent DB-5
447 column) gave a molecular ion peak at *m/z* 348 $[M]^+$, with fragment ion peaks at *m/z* 235, 217, 165 (the base
448 peak), 123, and 97 (the D-ring fragment).
449 The ^1H and ^{13}C NMR spectra (JEOL JMN-ECA-500 spectrometer in $CDCl_3$ (δ_H 7.26, δ_C 77.0)) aided with
450 DEPT and HMQC experiments (Supplementary Table S3) revealed 19 carbon signals including two α,β-
451 unsaturated ester carbonyls, two trisubstituted olefins, one tetrasubstituted olefin, three sp^3 oxymethines,
452 three sp^3 methylenes, two methyls, one quaternary sp^3 carbon, and two allylic methyls.
453 The ^1H-^1H COSY and HMBC correlations from H-2′, H-3′, H-6′, and H-7' (Supplementary Fig. 3a-c and
454 Supplementary Table 1) indicated the presence of the conserved enol ether-bridged methylbutenolide (the
455 D-ring). The HMBC correlations of H-6′ to C-2, C-3, and C-4 and of H-5 to C-4 established the presence
456 of α-methylene-β-hydroxy-γ-butyrolactone ring (the C-ring). The ^1H-^1H COSY and HMBC correlations

457 from three methyls at H-12, H-13, and H-14 and from three sp^3 methylenes at H-8, H-9, and H-10 showed
458 a 2,6,6-trimethylcyclohex-1-en-1-yl moiety (the A-ring). The two substructures, the A- and the CD-parts,
459 were connected together at C-5 and C-6 as indicated by the HMBC correlation of H-5 to C-6 and C-11.
460 Therefore, the chemical structure of BSB was determined as (*E*)-5-((4-hydroxy-2-oxo-5-(2,6,6-
461 trimethylcyclohex-1-en-1-yl)dihydrofuran-3(2*H*)-ylidene)methoxy)-3-methylfuran-2(5*H*)-one.
462 The CD spectrum (JASCO J-820W spectropolarimeter in acetonitrile) had a positive and negative Cotton
463 effect around 224 nm (Δε 27.4) and 266 nm (Δε -4.44), respectively (Supplementary Fig. 3d), indicating
464 that BSB has a 2′(*R*) configuration as previously identified natural SLs. The NOESY correlations between
465 H-12/H-13 methyls and H-5 oxymethine, and between H-14 methyl and H-4 oxymethine suggested the
466 relative stereochemistry at C-4 and C-5 is *anti*. Accordingly, we tentatively assigned the stereochemistry to
467 be (4*R*, 5*S*, 2′*R*) (Fig. 2f).
468
469 **Expression of Mpa*MAX1* in yeast**
470 Total RNAs were extracted from whole *M. paleacea* plants with rhizoids using the RNeasy Plant Mini Kit
471 (Qiagen, Germany) and used to synthesize single-stranded cDNAs with the SuperScript III First-Strand
472 Synthesis System (Invitrogen, USA). PCR amplification was performed with PrimeSTAR HS DNA
473 polymerase (TaKaRa Bio, Japan) using the primers MpaMAX1_cDNA_F and MpaMAX1_cDNA_R
474 (Supplementary Table 5). The MpaMAX1 cDNA was cloned into the *BamH I* and *Kpn I* sites of the
475 pYeDP60 vector with the GeneArt Seamless Cloning and Assembly Enzyme Mix (Invitrogen, USA) using
476 the primers MpaMAX1_cloning_F and MpaMAX1_cloning_R (Supplementary Table 5). At least four
477 clones were sequenced to check for errors in the PCR. The accession number of MpaMAX1 was deposited
478 as LC553047. Heterologous expression of MpaMAX1 in yeast (*Saccharomyces cerevisiae*) was carried out
479 as described previously[21, 69]. Microsomes (100 μl) were incubated with *rac*-CL (0.25 to 33 μM) and NADPH

480 (500 μM) at 28 °C for 10 min. The reaction mixtures were extracted with ethyl acetate, dried over anhydrous
481 Na_2SO_4 and then subjected to LC-MS/MS analysis. The product BSB was quantified using the peak area in
482 the transitions of *m/z* 349 to 97 by assuming the same ion intensity as that of 4-deoxyorobanchol. The
483 kinetics parameter was determined using triplicate samples and calculated by the Michaelis-Menten
484 equation using SigmaPlot 14 (Systat Software, USA). The V_{max} of BSB from *rac*-carlactone was 92.8 ± 8.4
485 nM min^{-1} and the Michaelis constant was 4.78 ± 1.22 μM.
486

487 **Mycorrhization assays**

488 Thalli of *Marchantia paleacea*, *Marchantia emarginata*, *Marchantia pinnata* and *Marchantia chenopoda*
489 were grown on a substrate composed of 50 % zeolite and 50 % sand (0.7–1.3 mm) in 7x7x8 cm pots (five
490 thalli per pot). Each pot was inoculated with *ca*. 1,000 sterile spores of *Rhizophagus irregularis* DAOM
491 197198 (Agronutrition, Labège, France) and grown with a 16 h/8 h photoperiod at 22 °C/20 °C. Pots were
492 watered once per week with Long Ashton medium containing 7.5 μM of phosphate[30]. For strigolactone
493 complementation assays, 10 ml of Long Ashton solution with 10^{-7} M GR24 or 0.1 % acetone (control) was
494 added to each pot weekly, starting on the day of the inoculation. Hyphal branching activity of BSB on
495 *Gigaspora margarita* was evaluated as reported previously[33].
496 To establish the mycorrhizal status of the different Marchantia species, thalli were embedded in 6 % agarose
497 and 0.1 mm sections were prepared using a Leica vt1000s vibratome. Sections were incubated overnight in
498 PBS containing 1 μg/ml WGA-FITC (Sigma) and pictures were taken with a Zeiss Axiozoom V16
499 microscope.
500 For the quantitative evaluation of symbiosis, thalli were harvested six weeks post-inoculation (*Marchantia*
501 *paleacea* mutants) or eight weeks post-inoculation (diverse Marchantia species). For the analysis of the
502 mycorrhizal phenotype of the mutants, longitudinal hand sections were cleared in 10 % KOH overnight and

503 ink coloured for 30 min in 5 % Sheaffer ink, 5 % acetic acid. Sections were observed under a microscope
504 and infection points were scored for each thallus.
505
506 **Phylogenetic analysis**
507 Homologues of the reference genes *CCD8* and *MAX1* of *Arabidopsis thaliana* or *Medicago truncatula* were
508 retrieved in a custom database composed of 197 species covering the main land plant lineages
509 (Supplementary Table 1) and including, for Lycophytes, Monilophytes and Bryophytes, predicted coding
510 sequences of transcriptomic data from the 1KP project[16, 57]. Searches were perform using the tblastn v2.9.0+
511 algorithm with default parameters and an e-value threshold of 1^{e-10}.
512 For each gene, their putative homologue coding sequences were aligned using MAFFT v7.313 with default
513 parameters and the resulting alignments trimmed to remove positions with more than 80 % gaps using
514 trimAl v1.4.1. Then, alignments were subjected to Maximum Likelihood (ML) analysis using IQ-TREE
515 v1.6.7 and branch support tested with 10,000 replicates of SH-aLRT and UltraFast Bootstraps. Prior to ML
516 analysis, the best-fitting evolutionary model was tested using ModelFinder and retained according to the
517 Bayesian Information Criteria. Trees were visualized and annotated using the iTOL v5.6.3[70].
518
519 **RNA extraction and expression analysis**
520 RNA was extracted from frozen plant samples using the NucleoSpin RNA Plant kit (MACHEREY-
521 NAGEL). cDNAs were synthesized using SuperScript VILO (Invitrogen). Quantitative PCR was
522 performed using the KOD SYBR® qPCR Mix (Toyobo Life Science) with Light Cycler 480 II (Roche).
523 Primers used to amplify the cDNAs are shown in Supplemental Table 5. The ACTIN gene of each species
524 was used as a standard.
525

526 **RNA-seq analysis**

527 Gemmae of WT, Mpa*ccb8a-1/b-4*, and Mpa*ccb8a-2/b-3* were incubated on half-strength Gamborg's B5
528 medium for 2 weeks at 22℃ under continuous light. After 2 week-culture, the thalli were transferred to the
529 medium with or without phosphate. Three biological replicates were prepared for each genotype. Total
530 RNA was extracted using the NucleoSpin RNA Plant kit (Macherey-Nagel, Germany). mRNA was isolated
531 from the total RNA with an NEB Next poly(A) mRNA Magnetic Isolation Module Kit (New England
532 Biolabs, MA, USA). RNA-seq libraries were synthesized using an NEB Next Ultra RNA Library Prep Kit
533 for Illumina and an NEB Next Adaptor for Illumina Kit (New England Biolabs, MA, USA). Paired-end
534 sequencing was performed using the HiSeqXten (Illumina, CA, USA).

535 The reads were mapped onto the *Marchantia paleacea* genome[48] assembly using HISAT2[71] for Galaxy
536 (https://usegalaxy.org) with default parameters. Read counts were calculated by Feature Counts for Galaxy.
537 To identify differentially expressed genes, q-values were calculated by the TCC R package[72], and genes
538 with q-value < 0.05 and log2-fold change > 1 or log2-fold change < -1 were selected as up-or down-
539 regulated genes. Summary statistics of RNA-seq analysis are available in Supplementary Table 4.

540

541 **Differential Scanning Fluorimetry (DSF) analysis**

542 The coding sequences for MpaKAI2A and MpaKAI2B were amplified from cDNA synthesized from total
543 mRNA of the *Marchantia paleacea* thalli using the following primer sets, MpaKAI2A_cDNA_F and
544 MpaKAI2A_cDNA_R for MpaKAI2A and MpaKAI2B_cDNA_F and MpaKAI2B_cDNA_R for
545 MpaKAI2B (Supplementary Table 5). The PCR products were cloned into the modified pET28 vector using
546 the In-Fusion HD Cloning Kit (Clontech) to generate pET28M-MpaKAI2A and pET28M-MpaKAI2B.
547 *Escherichia coli* strain BL21 (DE3) (Takara) was used for recombinant protein expression. Overnight
548 cultures (3 ml) in LB liquid medium containing 100 μg/ml kanamycin were inoculated into fresh LB

549 medium (1 L) containing 100 μg/ml kanamycin and incubated at 37 °C until the OD600 value reached to
550 0.6 ~ 0.8. Then, IPTG was added to 200 μM, and the cells were further incubated at 25 °C overnight. The
551 cells were collected by centrifugation at 4000 rpm for 15 min. The pellets were resuspended in 30 ml of
552 buffer A (20 mM Tris-HCl (pH 8.0) and 200 mM NaCl). The cells were crushed using a cell disruption
553 device (UD-200, TOMY SEIKO). The soluble fraction was loaded on Ni Sepharose 6B fast flow (Cytiva)
554 pre-equilibrated with buffer A. The bound MpaKAI2A and MpaKAI2B proteins were eluted with 5 ml of
555 buffer A containing 125 mM of imidazole. The purified proteins were concentrated using an Amicon Ultra-
556 4 10 K (Millipore).
557 For DSF analysis, 20 μl reaction mixtures containing 10 μg protein, 0.015 μl Sypro Orange and GR24s with
558 final concentrations of 250 μM, 100 μM or 50 μM, were prepared in 96-well plates. The final acetone
559 concentration in the reaction mixture was 5%. 1xPBS buffer (pH 7.4) containing 5% acetone was used in
560 the control reaction. DSF experiments were performed using a Light Cycler 480 II (Roche). Sypro Orange
561 (Invitrogen) was used as the reporter dye. Samples were incubated at 25 °C for 10 min, then, the
562 fluorescence wavelength was detected continuously from 30 °C to 95 °C. The denaturation curve was
563 obtained using MxPro software (Agilent).
564
565 **Generation of AtD14-overexpressing plants**
566 To generate *AtD14*ox lines, the AtD14 coding sequence cloned into pENTR/D-TOPO vector[40] was inserted
567 into pMpGWB103 or pMpGWB303 by Gateway LR reactions. The MpEFpro: AtD14 vector was
568 introduced into regenerating thalli of Tak-1 or WT of *M. paleacea* as described above.
569 To generate Mp*AtD14*ox/Mp*kai2a* or Mpa*AtD14*ox/Mpa*kai2a* line, Mp*KAI2A* or Mpa*KAI2A* was knocked
570 out in Mp*AtD14*ox or Mpa*AtD14*ox lines. Mp*KAI2A* guide RNA inserted in pMpGE010[44] or Mpa*KAI2A*
571 guide RNA inserted in pMpGE011 were introduced into Mp*AtD14*ox or Mpa*AtD14*ox lines, respectively.

572

573 **Application of GR24**

574 Synthetic strigolactone analog *rac*-GR24 was obtained from Chiralix B.V. (Netherland). The enantiomers
575 of GR24 were obtained by optical resolution from *rac*-GR24 stationary phase (Chiralpak IC-3, 250 × 4.6
576 mm, Daicel, Japan) under normal-phase using hexane/isopropanol (70/30) by HPLC. $GR24^{4DO}$ and
577 $GR24^{5DS}$ were identified by CD analysis. *rac*-GR24, $GR24^{4DO}$, and $GR24^{5DS}$ dissolved in acetone were
578 added to half-strength Gamborg's B5 medium with 1.0 % agar. Thalli grown from gemmae for 2 weeks
579 were incubated on the *rac*-GR24, $GR24^{4DO}$, $GR24^{5DS}$, or mock containing media for 6 hours.

580

581 **Statistical analysis**

582 We used Welch's t test or Tukey's honestly significant difference (HSD) test to evaluate statistical
583 significance. Experimental sample sizes and statistical methods detail are given in the each legend.

584

585 **Data availability**

586 All reads obtained have been deposited in the DDBJ and are available through the Sequence Read Archive
587 (SRA) under the accession numbers DRA012476. The authors declare that all data supporting the findings
588 of this study are available within the manuscript and its supplementary files or are from the corresponding
589 author upon request.

590

591 **Acknowledgements**

592 We thank Philippe Delavault (University of Nantes) and A.G.T. Babiker (Sudan University of Science and
593 Technology) for *Phelipanche ramosa* and *Striga hermonthica* seeds, respectively. We are grateful to the
594 genotoul bioinformatics platform Toulouse Occitanie (Bioinfo Genotoul, doi:

595 10.15454/1.5572369328961167E12) for providing computing resources. Researches conducted by Ju.K.
596 lab and Ta.N. lab were supported by a Grants-in-Aid from the Ministry of Education, Culture, Sports,
597 Science, and Technology, Japan (20H05684 and 17H06475 to Ju.K., 19K05838 to Ta.N., 18K05452 to
598 X.X.) and Canon Foundation to Ju.k. Research conducted in P.-M.D. lab was supported by the Agence
599 Nationale de la Recherche (ANR) grant EVOLSYM (ANR-17-CE20-0006-01) and was supported by the
600 project Engineering Nitrogen Symbiosis for Africa (ENSA) currently supported through a grant to the
601 University of Cambridge by the Bill & Melinda Gates Foundation (OPP1172165) and the UK Government's
602 Department for International Development (DFID). The Laboratoire de Recherche en Sciences Végétales
603 (LRSV) belongs to the TULIP Laboratoire d'Excellence (ANR-10-LABX-41).

604

605 **Author contributions**

606 Ju.K., P.-M.D., M.K.R. and Ta.N. designed the research and wrote the article. K.K., M.K.R., S.S., Y.M.
607 and A.K. produced mutants and analyzed phenotypes. K.K., S.S., Y.M., A.K., Y.L., H.S. and H.K.
608 examined gene expression. S.S., M.K.R., To.Na. and P.-M.D. analyzed A.M. symbiosis. C.L. and Je.K.
609 conducted phylogenetic analysis. S.S., A.Y., K.Y., K.M., S.Y., Y.K. and Y.T. analyzed BSB and
610 enzyme activity. X.X., K.U. and K.A. determined the chemical structure of BSB. K.S., M.S.,
611 To.Na. and To.Ni. prepared materials.

612

613 **Additional information**

614 **Supplementary information**

615 Supplementary Figure 1: Phylogenetic analysis of *CCD8* genes.

616 Supplementary Figure 2: Phylogenetic analysis of *MAX1* genes.

617 Supplementary Figure 3: 1D and 2D NMR spectra ($CDCl_3$), and CD spectra of bryosymbiol.

618 Supplementary Figure 4: Strigolactones in hornwort, moss, ferns and seed plants.

619 Supplementary Figure 5: Mycorrhizal phenotype of strigolactone biosynthesis and signalling mutants.

620 Supplementary Figure 6: Evaluation of the MpaKAI2-SLs interaction.

621 Supplementary Figure 7: Effects of SLs and phosphorus on gene expressions in *AtD14* line in

622 *M. polymorpha* and in *M. paleacea*.

623 Supplementary Table 1: Species used in the analysis shown in Supplementary Figs 1 and 2.

624 Supplementary Table 2: Production of CRISPR mutants.

625 Supplementary Table 3: NMR spectroscopic data for BSB.

626 Supplementary Table 4 (Excel file): Results of RNAseq analysis.

627 Supplementary Table 5: Oligonucleotide primers used in this study.

628

629 **Correspondence** and requests for materials should be addressed to Ju.K., Ta.N., or P.-M.D.

630

631 **Figure Legends**

632 **Figure 1. Presence of SL biosynthesis genes is specific to AM symbiosis-forming species in *Marchantia.***

633 **(a)** *Marchantia* species, namely *Marchantia polymorpha, Marchantia paleacea*, *Marchantia emarginata*

634 and *Marchantia pinnata*, used in this study. Upper panels show plant morphology. Scale bars: 1 cm. Middle

635 and lower panels are transverse sections after colonization by *Rhizophagus irregularis*. The lower panel

636 shows WGA-FITC staining of the fungi. Arbuscules are formed in the midrib region in the thallus (arrows).

637 Scale bars: 0.5 mm. **(b)** Biosynthesis pathway for strigolactones in seed plants and lycophytes. **(c)**

638 Conservation of the CCD8A genes in mycorrhizal Marchantia species. Amplified sequences of CCD8A are

639 aligned against the *Marchantia paleacea* gene model. Red lines indicate polymorphisms. The predicted

640 amino acid sequences in the conserved domains are shown below the gene model. Polymorphic amino acids

641 are colored.

642

643 **Figure 2. Bryosymbiol (BSB), a previously unidentified SL is produced by *M. paleacea*.**

644 **(a)** Detection of carlactonoic acid. Carlactonoic acid in the exudates of different *Marchantia* species and

645 the hornwort *Phaeoceros carolinianus*, protonema extract of the moss *Physcomitrium patens*, root exudates

646 of ferns *Dryopteris erythrosora* and *Matteuccia struthiopteris*, and seed plants *Asparagus officinalis* and

647 *Arachis hypogaea* were analyzed by LC-MS/MS. Multiple reaction monitoring (MRM) chromatograms of

648 carlactonoic acid (blue, 331.10/113.00; red, 331.10/69.00; *m/z* in negative mode) by LC-MS/MS are shown.

649 **(b)** Identification of carlactonoic acid. Product ion spectra derived from the precursor ion (*m/z* 331 in

650 negative mode) of the peaks detected in the exudates of *M. paleacea* and authentic carlactonoic acid (CLA)

651 are shown. **(c)** Germination stimulation activity of the exudate of *M. paleacea* on root parasitic plants. The

652 exudate was separated by reversed-phase HPLC every 30 seconds. All the fractions were tested for seed

653 germination activity on *Orobanche minor* (blue), *Striga hermonthica* (orange) and *Phelipanche ramosa*

654 (gray). Data are means ± SE (approx. 30 seeds per disk, n = 3). **(d)** Detection of BSB. BSB in the exudates
655 of *Marchantia* species and the hornwort *P. carolinianus*, protonema extract of the moss *P. patens*, root
656 exudates of ferns *D. erythrosora* and *M. struthiopteris*, and seed plants *A. officinalis* and *A. hypogaea* were
657 analyzed by LC-MS/MS. MRM chromatograms of BSB (blue, 349.00/97.00; red, 349.00/165.00; green,
658 349.00/235.00; *m/z* in positive mode) are shown. **(e)** Product ion spectra of BSB. Product ion spectra
659 derived from the precursor ion (*m/z* 349 in positive mode) of peaks detected in *Marchantia* species are
660 shown. **(f)** Chemical structure of bryosymbiol (BSB). The absolute stereochemistry at C-4 and C-5 could
661 be interchangeable.
662
663 **Figure 3. MpaMAX1 produces BSB from carlactone via carlactonoic acid.**
664 **(a and b)** Detection of BSB and carlactone in plant tissues of *Marchantia*. Whole plants of WT and the
665 Mpa*max1* mutant (two alleles) were extracted and analyzed by LC-MS/MS. MRM chromatograms of BSB
666 (a) and carlactone (b) (blue, 303.00/97.00; red, 303.00/189.00; green, 303.00/207.00; *m/z* in positive mode)
667 are shown. **(c)** Detection of carlactonoic acid and BSB in feeding experiments with recombinant MpaMAX1.
668 *Rac*-carlactone (CL) or *rac*-carlactonoic acid (CLA) was incubated with the microsomes of MpaMAX1-
669 expressed yeast. Yeast microsomes with an empty vector were used as a control. MRM chromatograms of
670 carlactonoic acid and BSB in extracts of the microsomes are shown. **(d)** Proposed mechanism of the
671 conversion to BSB from carlactone. MpaMAX1 oxidizes C-19 to produce carlactonoic acid and catalyzes
672 the α- or β-epoxidation of Δ7,8. Upon proton abstraction of the carboxyl group, the stereoselective closure
673 of the C ring occurs, producing BSB isomers depending on the epoxy configuration.
674
675 **Figure 4. Enhanced BSB synthesis correlates with an AM symbiosis permissive state.**

676 **(a)** Increased expression of the SL biosynthesis genes, Mpa*D27*, Mpa*CCD7*, Mpa*CCD8A*, Mpa*CCD8B* and
677 Mpa*MAX1*, of *M. paleacea*, and Aa*CCD8* of *Anthoceros agrestis* under phosphate starvation. Plants were
678 grown on half-strength Gamborg's B5 medium with (+P) or without (-P) phosphate. **(b)** Identification of
679 DEGs under phosphate starvation and in Mpa*ccd8a/8b* mutants by RNAseq analysis. Venn diagrams of
680 genes down-regulated in -P condition compared to +P condition and up-regulated in Mpa*ccd8a/8b*
681 compared to WT (left) and up-regulated in -P condition compared to +P condition and down-regulated in
682 Mpa*ccd8a/8b* compared to WT (right). **(c)** Heatmap of gene expression under phosphate deficiency in *M.*
683 *paleacea.* **(d)** Increase in the stimulation activity of the exudate of *M. paleacea* on seed germination of
684 *Orobanche minor* under phosphate starvation. Exudates from *M. paleacea* grown on +P and -P medium
685 were used for analysis. **(e)** Low phosphate allows mycorrhizal colonization. Plants inoculated with
686 *Rhizophagus irregularis* were watered with Long Ashton solution containing increasing phosphate
687 concentration and the distance (mm) between the notch and the colonized area of the thalli was measured
688 ($n \geq 15$). Higher distance signifies arrest of the colonization. Stars show statistical significance (ANOVA,
689 post hoc Tukey). **(f)** Developmental stage-dependent induction of Mpa*CCD8B* expression in response to
690 low phosphate conditions. Gemmae of 1-3 weeks after inoculation (wai) were transferred to media
691 containing different concentrations of phosphate.
692 Values in (a) and (d) are means ± SD ($n = 3$, $n = 4$, respectably), and significant differences detected with
693 Welch's t-test are indicated (a and d). Values in (f) are means ± SD ($n = 3$) and The HSD test was used for
694 multiple comparisons. Statistical differences (P-values < 0.05) are indicated by different letters.
695
696 **Figure 5. BSB is required for symbiosis with AM fungi in *M. paleacea*.**
697 **(a)** Hyphal branching of *Gigaspora margarita* induced by BSB (100 pg per disk) using the paper disk
698 diffusion method. Arrows indicate the direction of growth of primary, secondary and tertiary hyphae.

699 Asterisks indicate paper disks. **(b)** Upper and bottom panels are transverse sections of colonization by
700 *Rhizophagus irregularis.* The bottom panels show WGA-FITC staining of the fungi. Scale bars: 0.5 mm.
701 **(c)** The number of infection points per thalli (n ≥ 13). Letters show different statistical groups (ANOVA,
702 post hoc Tukey). **(d)** Germination stimulation activity on root parasitic plants of the exudates of the
703 Mpa*max1* mutant. The exudates of the two alleles were separated by reversed-phase HPLC. Every 30 sec
704 all the fractions were tested for seed germination activity on *Orobanche minor*. Data are means ± SE
705 (approx. 30 seeds per disk, n = 3).
706
707 **Figure 6. BSB-deficient mutants of *M. paleacea* show no abnormal developmental patterns and gene**
708 **expression profiles.**
709 **(a and b)** Growth of BSB-deficient (Mpa*ccd8a-2/8b-3*, Mpa*ccd8a-1/8b-4* and Mpa*max1-1* and Mpa*mx1-*
710 *2*) and KAI2 signaling (Mpa*kai2a-1*, Mpa*kai2b-1*, Mpa*kai2a-3* Mpa*kai2b-1* and Mpa*max2-1*) mutants of
711 *M. paleacea* grown in soil for 28, 42 and 60 days (a) and in media for 20 days (b). **(c and d)** Thallus area
712 and rhizoid length of BSB-deficient mutants of *M. paleacea* grown on half-strength Gamborg's B5 medium
713 containing 560 µM (c) and 56 µM (d) phosphorus (NaH_2PO_4) for 14 days. **(e)** Expression of Mpa*CCD8B*
714 in WT plants after $GR24^{4DO}$ and $GR24^{5DS}$ treatments.
715 Values in (c)and (d) are means ± SD (n ≧ 7) and in (e) are means ± SD (n = 3). The HSD test was used
716 for multiple comparisons and statistical differences (P-values < 0.05) are indicated by different letters.
717
718 **Figure 7. Heterologous expression of *AtD14*, the SL receptor of Arabidopsis, renders *M. paleacea* cells**
719 **BSB-responsive.**
720 **(a)** Expression of *AtD14* introduced into *M. paleacea*. **(b)** Complementation of Mpa*kai2a* phenotypes in
721 Mpa*AtD14*ox/Mpa*kai2a* (*M. paleacea*) lines by addition of 1 µM $GR24^{4DO}$ or 1 µM $GR24^{5DS}$. **(c and d)**

722 Induction of Mpa*DLP1* and Mpa*SMXL*, marker genes of KAI2-dependent signaling, in response to SL
723 treatment. Expression of Mpa*DLP1* and Mpa*SMXL* in WT and Mpa*AtD14ox* lines in *M. paleacea* after
724 $GR24^{4DO}$ (c) or $GR24^{5DS}$ (d) treatment are shown. **(e)** Induction of Mpa*DLP1* and Mpa*SMXL* expression by
725 phosphate starvation in Mpa*AtD14*ox lines of *M. paleacea.* **(f)** Complementation of Mpa*kai2a* phenotypes
726 in Mpa*AtD14*ox/Mpa*kai2a* (*M. paleacea*) lines grown on media with a reduced phosphorus level.
727 Values in (a) and (c) to (e) are means ± SD (n = 3), and values in (b) and (f) are means ± SD (n = 10). The
728 HSD test was used for multiple comparisons. Statistical differences (P-values < 0.05) are indicated by
729 different letters.

References

1. Delwiche CF, Cooper ED. The evolutionary origin of a terrestrial flora. *Curr Biol* **25**, R899-910 (2015).
2. Rensing SA. Great moments in evolution: the conquest of land by plants. *Curr Opin Plant Biol* **42**, 49-54 (2018).
3. Delaux PM, Schornack S. Plant evolution driven by interactions with symbiotic and pathogenic microbes. *Science* **371**, eaba6605 (2021).
4. Smith S, Read D. Mycorrhizal Symbiosis, 3rd Edition. *Academic Press* (2008).
5. Akiyama K, Matsuzaki K, Hayashi H. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature* **435**, 824-827 (2005).
6. Gomez-Roldan V*, et al.* Strigolactone inhibition of shoot branching. *Nature* **455**, 189-194 (2008).
7. Umehara M*, et al.* Inhibition of shoot branching by new terpenoid plant hormones. *Nature* **455**, 195-200 (2008).
8. Waters MT, Gutjahr C, Bennett T, Nelson DC. Strigolactone signaling and evolution. *Annu Rev Plant Biol* **68**, 291-322 (2017).

9. Machin DC, Hamon-Josse M, Bennett T. Fellowship of the rings: a saga of strigolactones and other small signals. *New Phytol* **225**, 621-636 (2020).

10. Al-Babili S, Bouwmeester HJ. Strigolactones, a novel carotenoid-derived plant hormone. *Annu Rev Plant Biol* **66**, 161-186 (2015).

11. Yoneyama K*, et al.* Which are the major players, canonical or non-canonical strigolactones? *J Exp Bot* **69**, 2231-2239 (2018).

12. Delaux PM*, et al.* Origin of strigolactones in the green lineage. *New Phytol* **195**, 857-871 (2012).

13. Walker CH, Siu-Ting K, Taylor A, O'Connell MJ, Bennett T. Strigolactone synthesis is ancestral in land plants, but canonical strigolactone signalling is a flowering plant innovation. *BMC Biol* **17**, 70 (2019).

14. Proust H*, et al.* Strigolactones regulate protonema branching and act as a quorum sensing-like signal in the moss *Physcomitrella patens*. *Development* **138**, 1531-1539 (2011).

15. Decker EL*, et al.* Strigolactone biosynthesis is evolutionarily conserved, regulated by phosphate starvation and contributes to resistance against phytopathogenic fungi in a moss, Physcomitrella patens. *New Phytol* **216**, 455-468 (2017).

16. Radhakrishnan GV*, et al.* An ancestral signalling pathway is conserved in intracellular symbioses-forming plant lineages. *Nat Plants* **6**, 280-289 (2020).

17. Lin H*, et al.* DWARF27, an iron-containing protein required for the biosynthesis of strigolactones, regulates rice tiller bud outgrowth. *Plant Cell* **21**, 1512-1525 (2009).

18. Schwartz SH, Qin X, Loewen MC. The biochemical characterization of two carotenoid cleavage enzymes from Arabidopsis indicates that a carotenoid-derived compound inhibits lateral branching. *J Biol Chem* **279**, 46940-46945 (2004).

19. Alder A*, et al.* The path from β-carotene to carlactone, a strigolactone-like plant hormone. *Science* **335**, 1348-1351 (2012).

20. Seto Y*, et al.* Carlactone is an endogenous biosynthetic precursor for strigolactones. *Proc Natl Acad Sci U S A* **111**, 1640-1645 (2014).

21. Abe S*, et al.* Carlactone is converted to carlactonoic acid by MAX1 in Arabidopsis and its methyl ester can directly interact with AtD14 in vitro. *Proc Natl Acad Sci U S A* **111**, 18084-18089 (2014).

22. Zhang Y*, et al.* Rice cytochrome P450 MAX1 homologs catalyze distinct steps in strigolactone

biosynthesis. *Nat Chem Biol* **10**, 1028-1033 (2014).

23. Wakabayashi T*, et al.* Direct conversion of carlactonoic acid to orobanchol by cytochrome P450 CYP722C in strigolactone biosynthesis. *Sci Adv* **5**, eaax9067 (2019).

24. Bowman JL*, et al.* Insights into land plant evolution garnered from the *Marchantia polymorpha* genome. *Cell* **171**, 287-304 e215 (2017).

5. Yoneyama K, Awad AA, Xie X, Yoneyama K, Takeuchi Y. Strigolactones as germination stimulants for root parasitic plants. *Plant Cell Physiol* **51**, 1095-1103 (2010).

26. Xie X, Yoneyama K, Nomura T, Yoneyama K. Evaluation and quantification of natural strigolactones from root exudates. *Methods Mol Biol* **2309**, 3-12 (2021).

27. Rensing SA*, et al.* The *Physcomitrella* genome reveals evolutionary insights into the conquest of land by plants. *Science* **319**, 64-69 (2008).

28. Li FW*, et al.* Anthoceros genomes illuminate the origin of land plants and the unique biology of hornworts. *Nat Plants* **6**, 259-272 (2020).

29. Zhang J*, et al.* The hornwort genome and early land plant evolution. *Nat Plants* **6**, 107-118 (2020).

30. Balzergue C, Puech-Pages V, Becard G, Rochange SF. The regulation of arbuscular mycorrhizal symbiosis by phosphate in pea involves early and systemic signalling events. *J Exp Bot* **62**, 1049-1060 (2011).

31. Banasiak J, Borghi L, Stec N, Martinoia E, Jasinski M. The full-size ABCG transporter of Medicago truncatula Is involved in strigolactone secretion, affecting arbuscular mycorrhiza. *Front Plant Sci* **11**, 18 (2020).

32. Kretzschmar T*, et al.* A petunia ABC protein controls strigolactone-dependent symbiotic signalling and branching. *Nature* **483**, 341-344 (2012).

33. Akiyama K, Ogasawara S, Ito S, Hayashi H. Structural requirements of strigolactones for hyphal branching in AM fungi. *Plant Cell Physiol* **51**, 1104-1117 (2010).

34. Choi J*, et al.* The negative regulator SMAX1 controls mycorrhizal symbiosis and strigolactone biosynthesis in rice. *Nat Commun* **11**, 2114 (2020).

35. Gutjahr C*, et al.* Rice perception of symbiotic arbuscular mycorrhizal fungi requires the karrikin receptor complex. *Science* **350**, 1521-1524 (2015).

36. Mori N, Nishiuma K, Sugiyama T, Hayashi H, Akiyama K. Carlactone-type strigolactones and their synthetic analogues as inducers of hyphal branching in arbuscular mycorrhizal fungi. *Phytochemistry* **130**, 90-98 (2016).

37. Nelson DC*, et al.* F-box protein MAX2 has dual roles in karrikin and strigolactone signaling in *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A* **108**, 8897-8902 (2011).

38. Hamiaux C*, et al.* DAD2 is an α/β hydrolase likely to be involved in the perception of the plant branching hormone, strigolactone. *Curr Biol* **22**, 2032-2036 (2012).

39. Yao R*, et al.* DWARF14 is a non-canonical hormone receptor for strigolactone. *Nature* **536**, 469-473 (2016).

40. Seto Y*, et al.* Strigolactone perception and deactivation by a hydrolase receptor DWARF14. *Nat Commun* **10**, 191 (2019).

41. Bythell-Douglas R*, et al.* Evolution of strigolactone receptors by gradual neo-functionalization of KAI2 paralogues. *BMC Biol* **15**, 52 (2017).

42. Jiang L*, et al.* DWARF 53 acts as a repressor of strigolactone signalling in rice. *Nature* **504**, 401-405 (2013).

43. Zhou F*, et al.* D14-SCF(D3)-dependent degradation of D53 regulates strigolactone signalling. *Nature* **504**, 406-410 (2013).

44. Mizuno Y*, et al.* Major components of the KARRIKIN INSENSITIVE2-dependent signaling pathway are conserved in the liverwort *Marchantia polymorpha*. *Plant Cell*, koab106 (2021).

45. Scaffidi A*, et al.* Strigolactone hormones and their stereoisomers signal through two related receptor proteins to Induce different physiological responses in Arabidopsis. *Plant Physiol* **165**, 1221-1232 (2014).

46. Mashiguchi K*, et al.* Feedback-regulation of strigolactone biosynthetic genes and strigolactone-regulated genes in Arabidopsis. *Biosci Biotechnol Biochem* **73**, 2460-2465 (2009).

47. Conn CE*, et al.* Convergent evolution of strigolactone perception enabled host detection in parasitic plants. *Science* **349**, 540-543 (2015).

48. Rich MK*, et al.* Lipid exchanges drove the evolution of mutualism during plant terrestrialization. *Science* **372**, 864-868 (2021).

49. Foster KR, Schluter J, Coyte KZ, Rakoff-Nahoum S. The evolution of the host microbiome as an

ecosystem on a leash. *Nature* **548**, 43-51 (2017).

50. Yoneyama K, Yoneyama K, Takeuchi Y, Sekimoto H. Phosphorus deficiency in red clover promotes exudation of orobanchol, the signal for mycorrhizal symbionts and germination stimulant for root parasites. *Planta* **225**, 1031-1038 (2007).

51. Umehara M, Hanada A, Magome H, Takeda-Kamiya N, Yamaguchi S. Contribution of strigolactones to the inhibition of tiller bud outgrowth under phosphate deficiency in rice. *Plant Cell Physiol* **51**, 1118-1126 (2010).

52. Li FW*, et al.* Fern genomes elucidate land plant evolution and cyanobacterial symbioses. *Nat Plants* **4**, 460-472 (2018).

53. de Saint Germain A*, et al.* An histidine covalent receptor and butenolide complex mediates strigolactone perception. *Nat Chem Biol* **12**, 787-794 (2016).

54. Lopez-Obando M*, et al. Physcomitrella patens* MAX2 characterization suggests an ancient role for this F-box protein in photomorphogenesis rather than strigolactone signalling. *New Phytol* **219**, 743-756 (2018).

55. Toh S*, et al.* Structure-function analysis identifies highly sensitive strigolactone receptors in *Striga*. *Science* **350**, 203-207 (2015).

56. Tsuchiya Y*, et al.* Probing strigolactone receptors in Striga hermonthica with fluorescence. *Science* **349**, 864-868 (2015).

57. One Thousand Plant Transcriptomes I. One thousand plant transcriptomes and the phylogenomics of green plants. *Nature* **574**, 679-685 (2019).

58. Blazquez MA, Nelson DC, Weijers D. Evolution of plant hormone response pathways. *Annu Rev Plant Biol* **71**, 327-353 (2020).

59. Hartung W. The evolution of abscisic acid (ABA) and ABA function in lower plants, fungi and lichen. *Funct Plant Biol* **37**, 806-812 (2010).

60. Hauser F, Waadt R, Schroeder JI. Evolution of abscisic acid synthesis and signaling mechanisms. *Curr Biol* **21**, R346-355 (2011).

61. Mutte SK, Kato H, Rothfels C, Melkonian M, Wong GK, Weijers D. Origin and evolution of the nuclear auxin response system. *Elife* **7**, e33399 (2018).

62. Briones-Moreno A*, et al.* Evolutionary analysis of DELLA-associated transcriptional networks.

Front Plant Sci **8**, 626 (2017).

63. Hernández-García J*, et al.* Coordination between growth and stress responses by DELLA in the liverwort *Marchantia polymorpha*. *Curr Biol*, S0960-9822(0921)00805-00808 (2021).

64. Sun Y*, et al.* A ligand-independent origin of abscisic acid perception. *Proc Natl Acad Sci U S A* **116**, 24892-24899 (2019).

65. Sun Y, Pri-Tal O, Michaeli D, Mosquna A. Evolution of abscisic acid signaling module and its perception. *Front Plant Sci* **11**, 934 (2020).

66. Jahan A*, et al.* Archetypal roles of an abscisic acid receptor in drought and sugar responses in liverworts. *Plant Physiol* **179**, 317-328 (2019).

67. Kubota A, Ishizaki K, Hosaka M, Kohchi T. Efficient *Agrobacterium*-mediated transformation of the liverwort *Marchantia polymorpha* using regenerating thalli. *Biosci Biotechnol Biochem* **77**, 167-172 (2013).

68. Edwards K, Johnstone C, Thompson C. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res* **19**, 1349 (1991).

69. Yoneyama K*, et al.* Conversion of carlactone to carlactonoic acid is a conserved function of MAX1 homologs in strigolactone biosynthesis. *New Phytol* **218**, 1522-1533 (2018).

70. Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS. ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat Methods* **14**, 587-589 (2017).

71. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods* **12**, 357-360 (2015).

72. Sun J, Nishiyama T, Shimizu K, Kadota K. TCC: an R package for comparing tag count data with robust normalization strategies. *BMC Bioinformatics* **14**, 219 (2013).

Figure 1

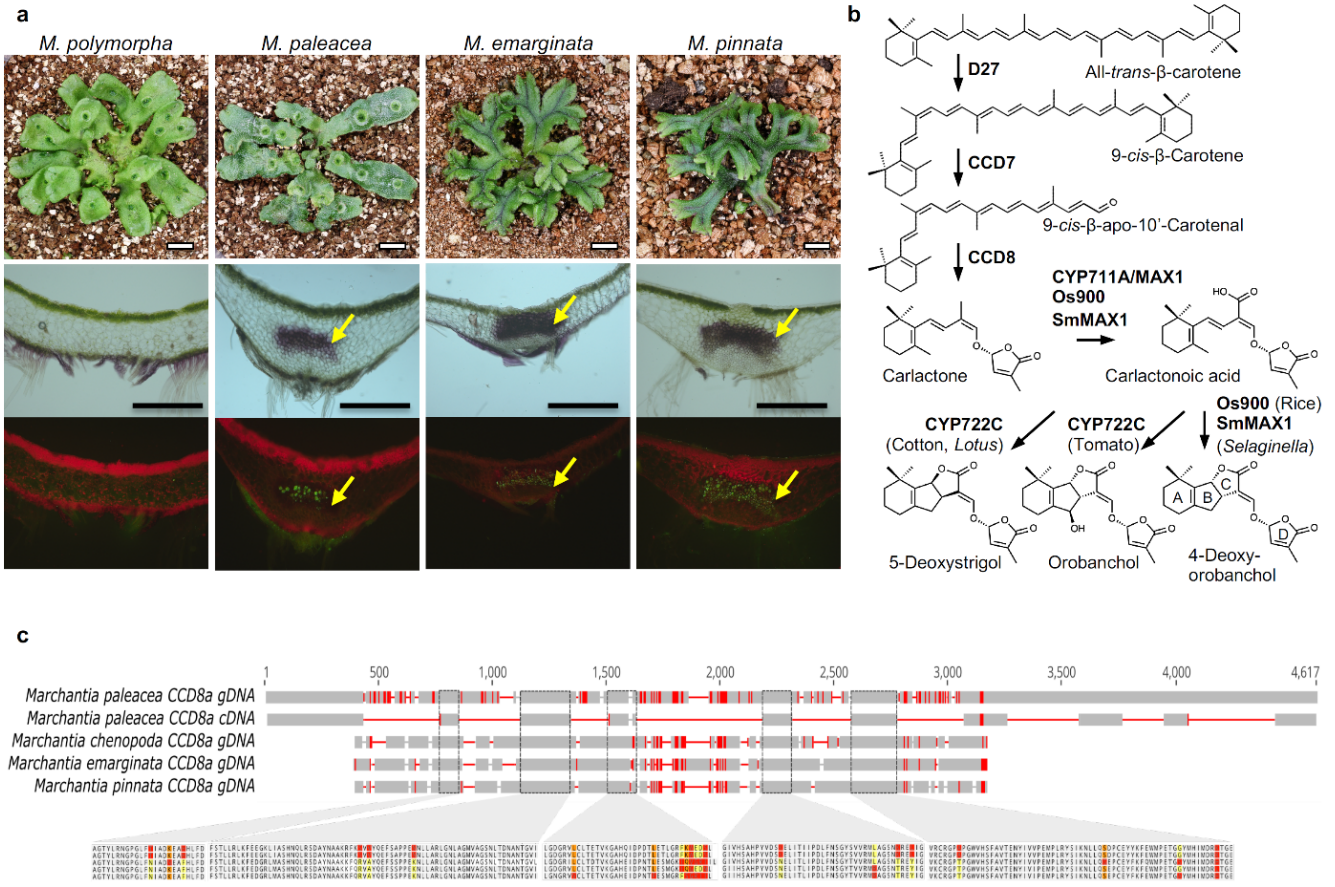

Figure 1. Presence of SL biosynthesis genes is specific to AM symbiosis-forming species in *Marchantia.*
(a) *Marchantia* species, namely *Marchantia polymorpha, Marchantia paleacea*, *Marchantia emarginata* and *Marchantia pinnata*, used in this study. Upper panels show plant morphology. Scale bars: 1 cm. Middle and lower panels are transverse sections after colonization by *Rhizophagus irregularis*. The lower panel shows WGA-FITC staining of the fungi. Arbuscules are formed in the midrib region in the thallus (arrows). Scale bars: 0.5 mm. **(b)** Biosynthesis pathway for strigolactones in seed plants and lycophytes. **(c)** Conservation of the CCD8A genes in mycorrhizal Marchantia species. Amplified sequences of CCD8A are aligned against the *Marchantia paleacea* gene model. Red lines indicate polymorphisms. The predicted amino acid sequences in the conserved domains are shown below the gene model. Polymorphic amino acids are colored.

•

Figure 2

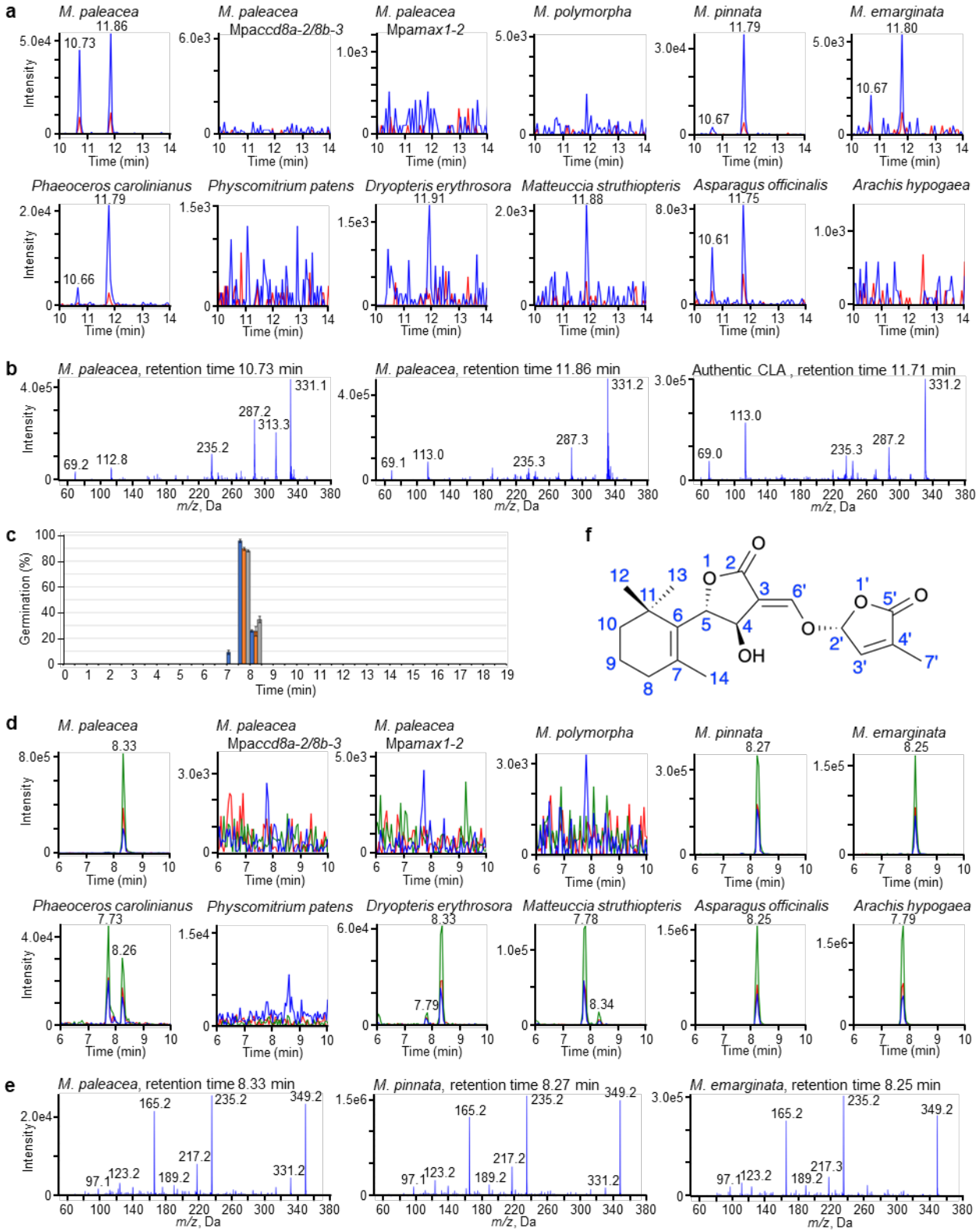
a
M. paleacea
M. paleacea Mpaccd8a-2/8b-3
M. paleacea Mpamax1-2
M. polymorpha
M. pinnata
M. emarginata
Phaeoceros carolinianus
Physcomitrium patens
Dryopteris erythrosora
Matteuccia struthiopteris
Asparagus officinalis
Arachis hypogaea
Intensity
Time (min)
b
M. paleacea, retention time 10.73 min
M. paleacea, retention time 11.86 min
Authentic CLA , retention time 11.71 min
m/z, Da
c
Germination (%)
Time (min)
f
d
e
M. paleacea, retention time 8.33 min
M. pinnata, retention time 8.27 min
M. emarginata, retention time 8.25 min

Figure 2. Bryosymbiol (BSB), a previously unidentified SL is produced by *M. paleacea*.
(a) Detection of carlactonoic acid. Carlactonoic acid in the exudates of different *Marchantia* species and the hornwort *Phaeoceros carolinianus*, protonema extract of the moss *Physcomitrium patens*, root exudates of ferns *Dryopteris erythrosora* and *Matteuccia struthiopteris*, and seed plants *Asparagus officinalis* and *Arachis hypogaea* were analyzed by LC-MS/MS. Multiple reaction monitoring (MRM) chromatograms of carlactonoic acid (blue, 331.10/113.00; red, 331.10/69.00; *m/z* in negative mode) by LC-MS/MS are shown. **(b)** Identification of carlactonoic acid. Product ion spectra derived from the precursor ion (*m/z* 331 in negative mode) of the peaks detected in the exudates of *M. paleacea* and authentic carlactonoic acid (CLA) are shown. **(c)** Germination stimulation activity of the exudate of *M. paleacea* on root parasitic plants. The exudate was separated by reversed-phase HPLC every 30 seconds. All the fractions were tested for seed germination activity on *Orobanche minor* (blue), *Striga hermonthica* (orange) and *Phelipanche ramosa* (gray). Data are means ± SE (approx. 30 seeds per disk, n = 3). **(d)** Detection of BSB. BSB in the exudates of *Marchantia* species and the hornwort *P. carolinianus*, protonema extract of the moss *P. patens*, root exudates of ferns *D. erythrosora* and *M. struthiopteris*, and seed plants *A. officinalis* and *A. hypogaea* were analyzed by LC-MS/MS. MRM chromatograms of BSB (blue, 349.00/97.00; red, 349.00/165.00; green, 349.00/235.00; *m/z* in positive mode) are shown. **(e)** Product ion spectra of BSB. Product ion spectra derived from the precursor ion (*m/z* 349 in positive mode) of peaks detected in *Marchantia* species are shown. **(f)** Chemical structure of bryosymbiol (BSB). The absolute stereochemistry at C-4 and C-5 could be interchangeable.

Figure 3

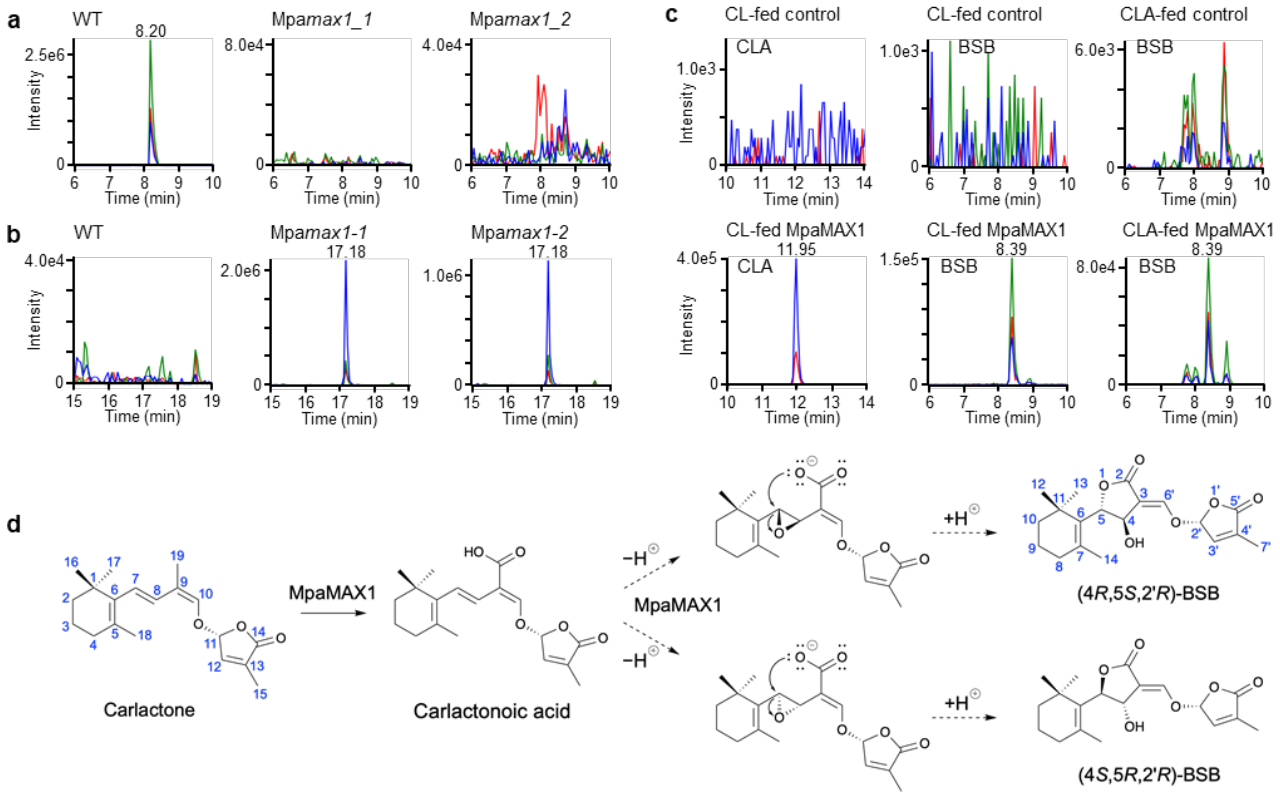

Figure 3. MpaMAX1 produces BSB from carlactone via carlactonoic acid.
(a and b) Detection of BSB and carlactone in plant tissues of *Marchantia*. Whole plants of WT and the Mpa*max1* mutant (two alleles) were extracted and analyzed by LC-MS/MS. MRM chromatograms of BSB (a) and carlactone (b) (blue, 303.00/97.00; red, 303.00/189.00; green, 303.00/207.00; *m/z* in positive mode) are shown. **(c)** Detection of carlactonoic acid and BSB in feeding experiments with recombinant MpaMAX1. *Rac*-carlactone (CL) or *rac*-carlactonoic acid (CLA) was incubated with the microsomes of MpaMAX1-expressed yeast. Yeast microsomes with an empty vector were used as a control. MRM chromatograms of carlactonoic acid and BSB in extracts of the microsomes are shown. **(d)** Proposed mechanism of the conversion to BSB from carlactone. MpaMAX1 oxidizes C-19 to produce carlactonoic acid and catalyzes the α- or β-epoxidation of Δ7,8. Upon proton abstraction of the carboxyl group, the stereoselective closure of the C ring occurs, producing BSB isomers depending on the epoxy configuration.

Figure 4

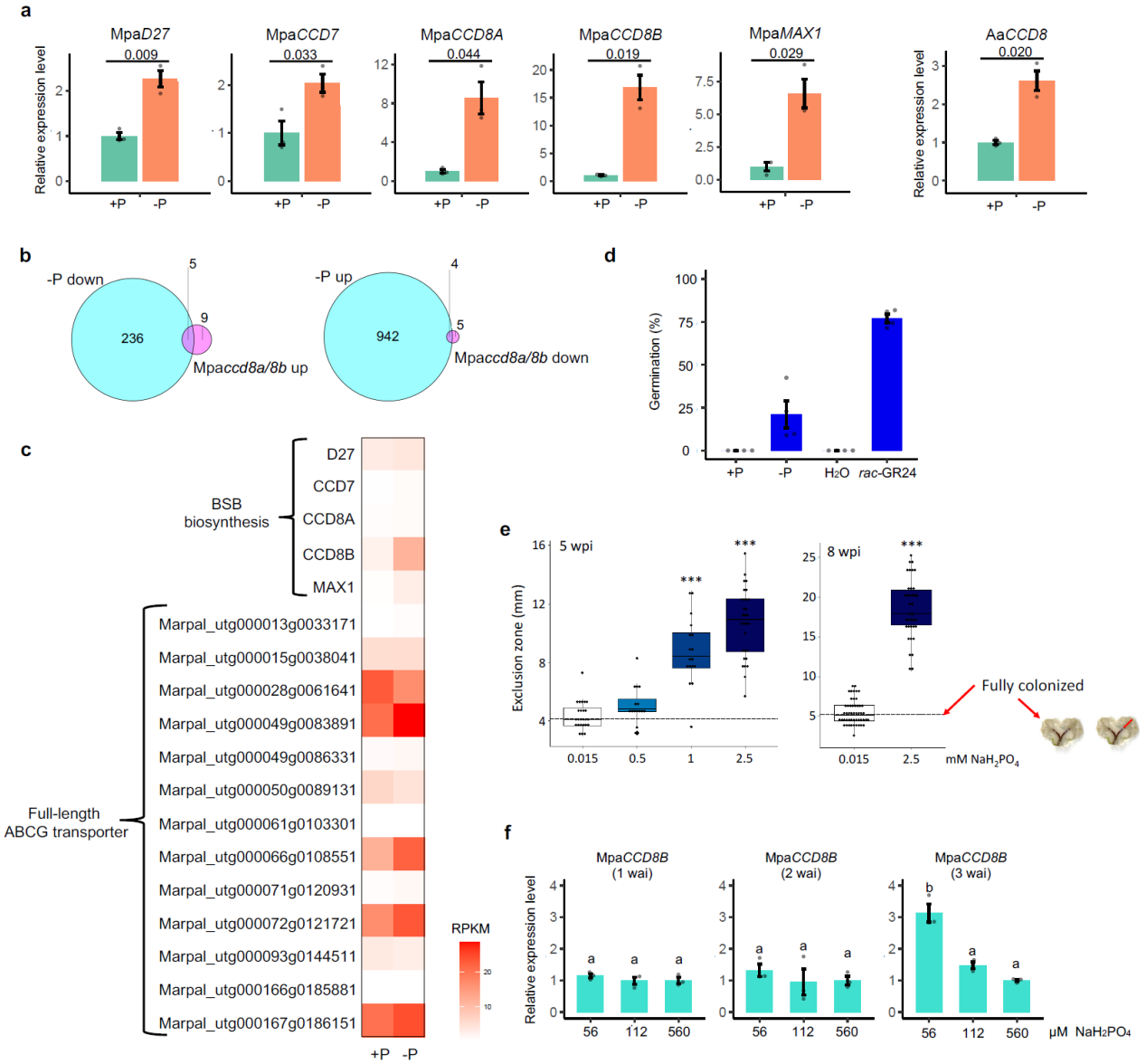

Figure 4. Enhanced BSB synthesis correlates with an AM symbiosis permissive state.
(a) Increased expression of the SL biosynthesis genes, Mpa*D27*, Mpa*CCD7*, Mpa*CCD8A*, Mpa*CCD8B* and Mpa*MAX1*, of *M. paleacea*, and Aa*CCD8* of *Anthoceros agrestis* under phosphate starvation. Plants were grown on half-strength Gamborg's B5 medium with (+P) or without (-P) phosphate. **(b)** Identification of DEGs under phosphate starvation and in Mpa*ccd8a/8b* mutants by RNAseq analysis. Venn diagrams of genes down-regulated in -P condition compared to +P condition and up-regulated in Mpa*ccd8a/8b* compared to WT (left) and up-regulated in -P condition compared to +P condition and down-regulated in Mpa*ccd8a/8b* compared to WT (right). **(c)** Heatmap of gene expression under phosphate deficiency in *M. paleacea*. **(d)** Increase in the stimulation activity of the exudate of *M. paleacea* on seed germination of *Orobanche minor* under phosphate starvation. Exudates from *M. paleacea* grown on +P and -P medium were used for analysis. **(e)** Low phosphate allows mycorrhizal colonization. Plants inoculated with *Rhizophagus irregularis* were watered with Long Ashton solution containing increasing phosphate concentration and the distance (mm) between the notch and the colonized area of the thalli was measured (n ≥ 15). Higher distance signifies arrest of the colonization. Stars show statistical significance (ANOVA, post hoc Tukey). **(f)** Developmental stage-dependent induction of Mpa*CCD8B* expression in response to low phosphate conditions. Gemmae of 1-3 weeks after inoculation (wai) were transferred to media containing different concentrations of phosphate.
Values in (a) and (d) are means ± SD (n = 3, n=4, respectably), and significant differences detected with Welch's t-test are indicated (a and d). Values in (f) are means ± SD (n = 3) and The HSD test was used for multiple comparisons. Statistical differences (P-values < 0.05) are indicated by different letters.

Figure 5

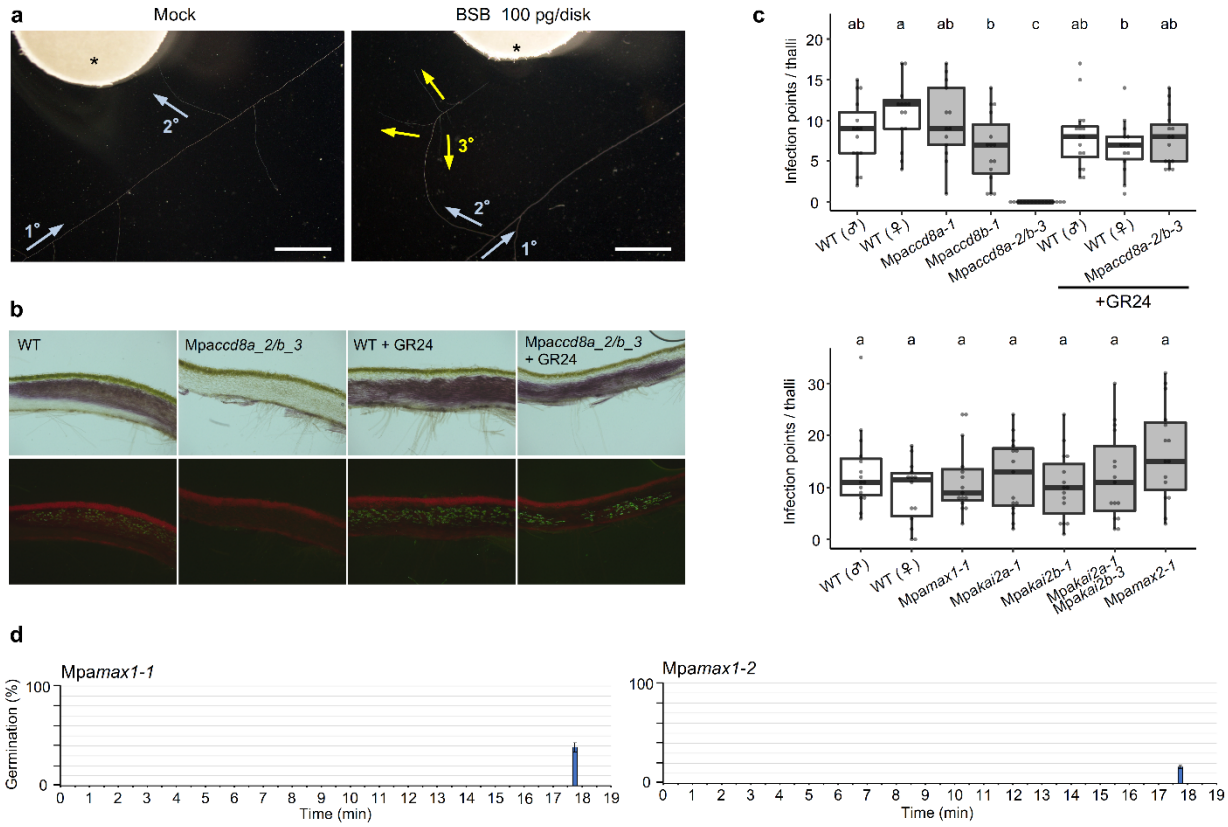

Figure 5. BSB is required for symbiosis with AM fungi in *M. paleacea*.
(a) Hyphal branching of *Gigaspora margarita* induced by BSB (100 pg per disk) using the paper disk diffusion method. Arrows indicate the direction of growth of primary, secondary and tertiary hyphae. Asterisks indicate paper disks. **(b)** Upper and bottom panels are transverse sections of colonization by *Rhizophagus irregularis*. The bottom panels show WGA-FITC staining of the fungi. Scale bars: 0.5 mm. **(c)** The number of infection points per thalli (n ≥ 13). Letters show different statistical groups (ANOVA, post hoc Tukey). **(d)** Germination stimulation activity on root parasitic plants of the exudates of the Mpa*max1* mutant. The exudates of the two alleles were separated by reversed-phase HPLC. Every 30 sec all the fractions were tested for seed germination activity on *Orobanche minor*. Data are means ± SE (approx. 30 seeds per disk, n = 3).

Figure 6

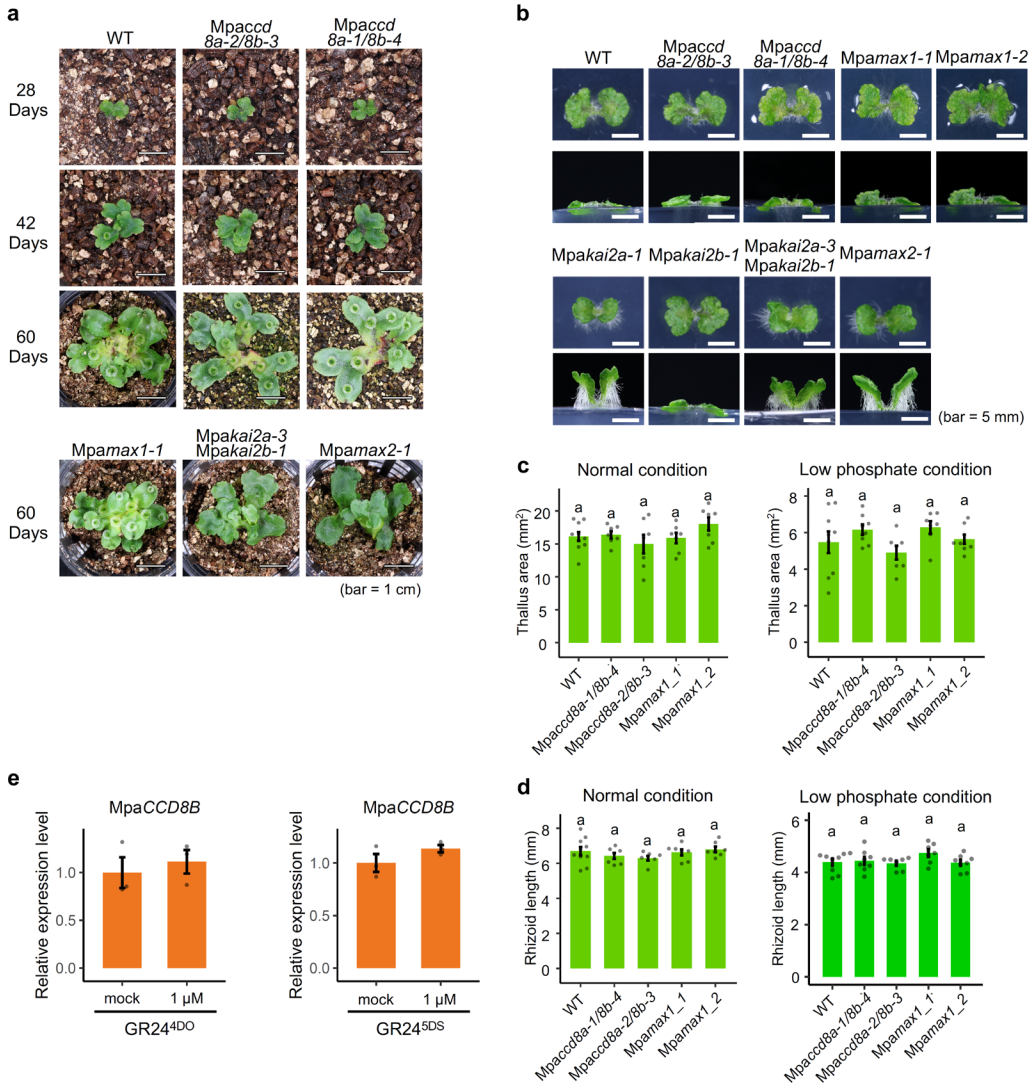

Figure 6. BSB-deficient mutants of *M. paleacea* show no abnormal developmental patterns and gene expression profiles.
(a and b) Growth of BSB-deficient (Mpa*ccd8a-2/8b-3*, Mpa*ccd8a-1/8b-4* and Mpa*mx1-2*) and KAI2 signaling (Mpa*kai2a-1*, Mpa*kai2b-1*, Mpa*kai2a-3* Mpa*kai2b-1* and Mpa*max2-1*) mutants of *M. paleacea* grown in soil for 28, 42 and 60 days (a) and in media for 20 days (b). (c and d) Thallus area and rhizoid length of BSB-deficient mutants of *M. paleacea* grown on half-strength Gamborg's B5 medium containing 560 μM (c) and 56 μM (d) phosphorus (NaH_2PO_4) for 14 days. (e) Expression of Mpa*CCD8B* in WT plants after $GR24^{4DO}$ and $GR24^{5DS}$ treatments.
Values in (c)and (d) are means ± SD (n ≧ 7) and in (e) are means ± SD (n = 3). The HSD test was used for multiple comparisons and statistical differences (P-values < 0.05) are indicated by different letters.

Figure 7

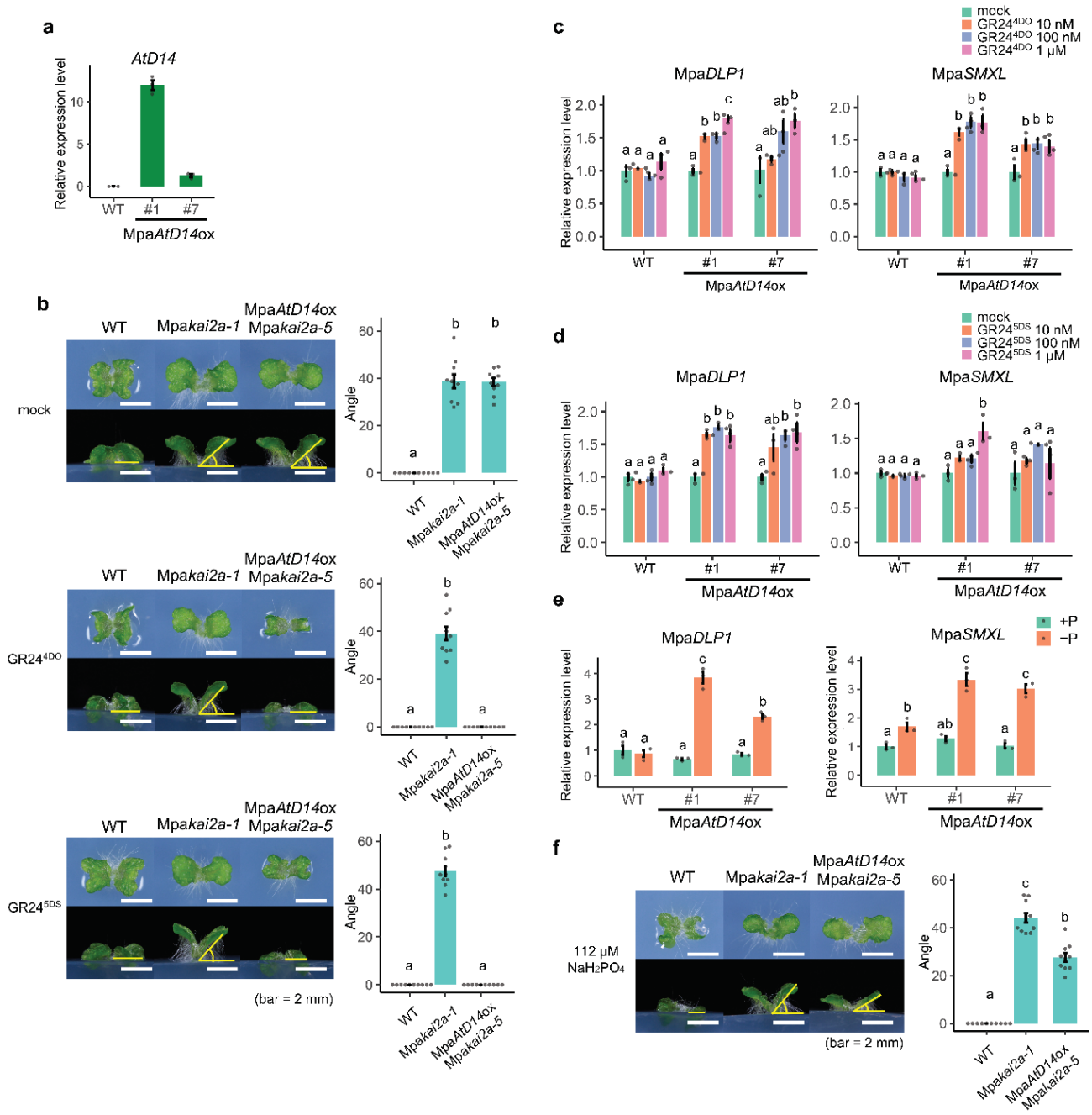

Figure 7. Heterologous expression of *AtD14*, the SL receptor of Arabidopsis, renders *M. paleacea* cells BSB-responsive.
(a) Expression of *AtD14* introduced into *M. paleacea*. **(b)** Complementation of Mpa*kai2a* phenotypes in Mpa*AtD14*ox/Mpa*kai2a* (*M. paleacea*) lines by addition of 1 μM GR24^{4DO} or 1 μM GR24^{5DS}. **(c and d)** Induction of Mpa*DLP1* and Mpa*SMXL*, marker genes of KAI2-dependent signaling, in response to SL treatment. Expression of Mpa*DLP1* and Mpa*SMXL* in WT and Mpa*AtD14ox* lines in *M. paleacea* after GR24^{4DO} (c) or GR24^{5DS} (d) treatment are shown. **(e)** Induction of Mpa*DLP1* and Mpa*SMXL* expression by phosphate starvation in Mpa*AtD14*ox lines of *M. paleacea.* **(f)** Complementation of Mpa*kai2a* phenotypes in Mpa*AtD14*ox/Mpa*kai2a* (*M. paleacea*) lines grown on media with a reduced phosphorus level.
Values in (a) and (c) to (e) are means ± SD (n = 3), and values in (b) and (f) are means ± SD (n = 10). The HSD test was used for multiple comparisons. Statistical differences (P-values < 0.05) are indicated by different letters.