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RESEARCH ARTICLE

The *Physcomitrium* (*Physcomitrella*) *patens* PpKAI2L receptors for strigolactones and related compounds function via MAX2-dependent and independent pathways

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Short title: Defining strigolactone receptors in *P. patens*

One-sentence summary: The study of moss PpKAI2L receptors for strigolactones and related compounds highlights MORE AXILLARY GROWTH2-dependent and independent pathways for the perception of these compounds.

The authors responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) are Sandrine Bonhomme (Sandrine.Bonhomme@inrae.fr) and Alexandre de Saint Germain (Alexandre.De-Saint-Germain@inrae.fr).

ABSTRACT

In angiosperms, the α/β hydrolase DWARF14 (D14), along with the F-box protein MORE AXILLARY GROWTH2 (MAX2), perceives strigolactones (SL) to regulate developmental processes. The key SL biosynthetic enzyme CAROTENOID CLEAVAGE DIOXYGENASE8 (CCD8) is present in the moss *Physcomitrium patens*, and PpCCD8-derived compounds regulate moss extension. The PpMAX2 homolog is not involved in the SL response, but 13

PpKAI2LIKE (*PpKAI2L*) genes homologous to the *D14* ancestral paralog *KARRIKIN INSENSITIVE2* (*KAI2*) encode candidate SL receptors. In *Arabidopsis thaliana*, AtKAI2 perceives karrikins and the elusive endogenous KAI2-Ligand (KL). Here, germination assays of the parasitic plant *Phelipanche ramosa* suggested that PpCCD8-derived compounds are likely non-canonical SLs. (+)-GR24 SL analog is a good mimic for PpCCD8-derived compounds in *P. patens*, while the effects of its enantiomer (-)-GR24, a KL mimic in angiosperms, are minimal. Interaction and binding assays of seven PpKAI2L proteins pointed to the stereoselectivity towards (-)-GR24 for a single clade of PpKAI2L (*eu-KAI2*). Enzyme assays highlighted the peculiar behavior of PpKAI2L-H. Phenotypic characterization of *Ppkai2l* mutants showed that *eu-KAI2* genes are not involved in the perception of PpCCD8-derived compounds but act in a PpMAX2-dependent pathway. By contrast, mutations in *PpKAI2L-G*, and *-J* genes abolished the response to the (+)-GR24 enantiomer, suggesting that PpKAI2L-G, and -J proteins are receptors for moss SLs.

1 INTRODUCTION

2 Strigolactones (SLs) are butenolide compounds with dual roles in plants: exuded in soil, SLs
3 signal the presence of a host to arbuscular mycorrhizal (AM) fungi (Akiyama et al. 2005;
4 Besserer et al. 2006) and thus favor the establishment of symbiosis; as endogenous compounds,
5 they (or derived compounds) play a hormonal role in developmental programs (Gomez-Roldan
6 et al. 2008; Umehara et al. 2008) for reviews: (Lopez-Obando et al. 2015; Waters et al. 2017).
7 SLs exuded from plant roots also act as signalling molecules in the rhizosphere, inducing
8 parasitic plant seed germination (Cook et al. 1966) for review: (Delavault et al. 2017). SLs have
9 been found in most land plants, including bryophytes, lycophytes, gymnosperms, and
10 angiosperms (Yoneyama et al. 2018b). However, their synthesis and signaling pathways have
11 mainly been described in angiosperms where core enzyme pathways involving DWARF27
12 (D27) isomerase and two CAROTENOID CLEAVAGE DIOXYGENASEs (CCD7 and CCD8)
13 convert carotenoids into carlactone (CL). To date, CL is the reported precursor of all known
14 SLs (Alder et al. 2012) and the substrate for further enzymes such as the cytochrome-P450
15 MORE AXILLARY GROWTH1 (MAX1) (for review: (Al-Babili and Bouwmeester 2015).
16 Depending on the plant species, CL is converted into canonical or non-canonical SLs. These
17 differ in the structure attached to the conserved enol ether-D ring moiety, which is shared by
18 all SLs and essential for biological activity (Yoneyama et al. 2018a; Yoneyama 2020). In
19 angiosperms, SLs are perceived by the α/β hydrolase DWARF14 (D14)/DECREASED
20 APICAL DOMINANCE2 (DAD2)/RAMOSUS 3 (RMS3) (Arite et al. 2009; Hamiaux et al.
21 2012; de Saint Germain et al. 2016), which interacts with the F-box protein MORE AXILLARY
22 GROWTH2 (MAX2) to target SUPPRESSOR OF MAX2-LIKE (SMXL) repressor proteins
23 for proteasome degradation (Soundappan et al. 2015; Waters et al. 2015a). An unusual aspect

24 of SL perception is that the D14 protein is both a receptor and an enzyme that cleaves its
25 substrate (and covalently binds part of the SL) in a signaling mechanism that is still under debate
26 (Yao et al. 2016; de Saint Germain et al. 2016; Shabek et al. 2018; Seto et al. 2019). In all cases,
27 the pocket of the α/β hydrolase appears to be essential for substrate/ligand (SL) interactions
28 (for review: (Bürger and Chory 2020).

29 The evolutionary origins of SLs, and in particular whether their primary role is that of a
30 hormone or rhizospheric signal, are still unclear. The identification and quantification of SLs
31 are a challenge in many species due to the very low amounts of the molecules present in plant
32 tissues or exudates and their high structural diversification (Xie 2016; Yoneyama et al. 2018b).
33 Therefore, the occurrence of SLs in a species was often inferred from the presence of the core
34 biosynthesis enzymes encoded in its genome (Delaux et al. 2012; Walker et al. 2019) or from
35 germination assays using plant exudates on parasitic plant seeds (Yoneyama et al. 2018b).
36 Recently, SLs were proposed to only be produced in land plants (Walker et al. 2019). Evidence
37 of a signaling pathway ancestral to the SL pathway came from the identification of an ancient
38 D14 paralog named KARRIKIN INSENSITIVE2/HYPOSENSITIVE TO LIGHT (KAI2/HTL)
39 during a screening of *Arabidopsis thaliana* mutants (Waters et al. 2012). Like D14, KAI2 is
40 also an α/β hydrolase that interacts with the MAX2 F-box protein in a pathway regulating
41 *Arabidopsis* seed germination and seedling development (Nelson et al. 2011; Waters et al.
42 2012). KAI2 is also involved in stress tolerance, drought tolerance, and AM symbiosis (Gutjahr
43 et al. 2015; Wang et al. 2018; Villacija-Aguilar et al. 2019; Li et al. 2020). However, the
44 endogenous signal perceived by KAI2 remains unknown and is reported thus far as the KAI2-
45 Ligand (KL) (Conn and Nelson 2015).

46 To gain insight into SL signaling evolution, we focused our studies on a model for non-vascular
47 plants, *Physcomitrium (Physcomitrella) patens (P. patens)*. Along with hornworts and
48 liverworts, mosses such as *P. patens* belong to the bryophytes (Bowman et al. 2019).
49 Bryophytes are currently described as a monophyletic group of embryophytes sharing an
50 ancestor with vascular plants (Puttick et al. 2018; Harris et al. 2020). Therefore, comparing
51 signaling pathways between extant vascular plants and extant bryophytes can provide insights
52 into the evolutionary origin of these pathways (Guillory and Bonhomme 2021b). Furthermore,
53 studying extant bryophytes may provide clues for understanding how the first plants have been
54 able to survive out of water, and conquer land, 450 million years ago (Bowman et al. 2019;
55 Blázquez et al. 2020; Harris et al. 2020).

56 In *P. patens*, several D27 homologs, as well as both CCD enzymes (PpCCD7 and PpCCD8)
57 required for SL synthesis are found (Proust et al. 2011), and CL has been detected as the product
58 of PpCCD8 (Decker et al. 2017). The extended phenotype of *Ppccd8* mutant plants indicates
59 that PpCCD8-derived molecules are required for regulating moss filament growth. These
60 molecules also act as a growth-limiting signal between neighboring moss plants, as they are
61 exuded into the medium (Proust et al. 2011). PpCCD8-derived molecules also appear to play a
62 role in rhizoid elongation and gametophore shoot branching (Delaux et al. 2012; Coudert et al.
63 2015). Application of the artificial SL (\pm)-GR24 complemented the *Ppccd8* mutant phenotype,
64 suggesting that PpCCD8-derived molecules are indeed SL-like compounds (Proust et al. 2011).
65 However, the exact nature of PpCCD8-derived molecules is still elusive (Yoneyama et al.
66 2018b), and the absence of *MAX1* homologs in *P. patens* suggests that the biosynthesis pathway
67 in this moss may differ from that of vascular plants. Nevertheless, phylogenetic analysis of
68 *MAX1* homologs highlights the presence of this gene in other mosses and suggests that the
69 biosynthesis pathway is otherwise conserved in land plants (Walker et al. 2019).

70

71 SL signaling also seems to differ between angiosperms and *P. patens*. Indeed, contrary to its
72 angiosperm homolog, PpMAX2 is likely not involved in the response to PpCCD8-derived
73 molecules, as the corresponding mutant does respond to (\pm)-GR24 (Lopez-Obando et al. 2018).
74 The PpMAX2 F-box protein appears to be involved in a light-dependent pathway required for
75 early moss development and the regulation of gametophore number and size. No true homolog
76 for the D14 SL receptor is found in the *P. patens* genome, whereas between 11 and 13 *PpKAI2-*
77 *LIKE* (*PpKAI2L*) candidate genes were described, depending on which version of the *P. patens*
78 genome was searched. These genes, first called *PpD14La* to *PpD14Lk* (Delaux et al. 2012),
79 were renamed *PpKAI2L-A* to *PpKAI2L-M* (Lopez-Obando et al. 2016a) and grouped into four
80 subclades. Hereafter, for simplicity, we renamed the subclades as eu-KAI2 (previous clade (i),
81 including PpKAI2L-A to -E); (FK), including PpKAI2L-F and -K (previous clade (ii)); (HIL),
82 including PpKAI2L-H, -I, -L (previous clade (i.i-i.ii)); (GJM) including PpKAI2L-G, -J, and -
83 M (previous clade iii). A comprehensive phylogenetic assessment placed the *P. patens* clades
84 (FK), (HIL), and (GJM) into a super clade called DDK (D14/DLK2/KAI2) containing
85 spermatophyte (angiosperm and gymnosperm) D14 clades, while the eu-KAI2 clade is highly
86 conserved and common to all land plants (Bythell-Douglas et al. 2017). Nevertheless, moss
87 proteins from the DDK clade were found to be as different from D14 as from KAI2 (Bythell-
88 Douglas et al. 2017).

89 Prediction of PpKAI2L protein structures found various pocket sizes, as observed for D14 and
90 KAI2 from vascular plants (Lopez-Obando et al. 2016a). Larger pocket sizes were predicted
91 for PpKAI2L-F and -K, while smaller pockets were predicted for eu-KAI2 clade proteins.
92 Consequently, these proteins could be receptors with diverse substrate preferences and might
93 bind to either PpCCD8-derived compounds or the elusive KL. Accordingly, in our previous
94 study of PpMAX2, we hypothesized that this F-box protein might be involved in a putative *P.*
95 *patens* KL signaling pathway (Lopez-Obando et al. 2018). However, the involvement of
96 PpKAI2L proteins in the PpMAX2 pathway remains an open question. The crystal structures
97 of PpKAI2L-C, -E, and -H were recently published (Bürger et al. 2019). *In vitro* purified
98 PpKAI2L proteins -C, -D, and -E (eu-KAI2 clade) were destabilized by (-)-5-deoxystrigol, a
99 canonical SL with non-natural stereochemistry, but the binding affinity for the pure enantiomer
100 was not determined. By contrast, PpKAI2L proteins -H, -K, and -L could bind to the karrikin
101 KAR₁ (Bürger et al. 2019). Proteins from the (GJM) clade were not studied, and no evidence
102 for a role of one (or several) PpKAI2L as receptors for PpCCD8-derived molecules was
103 provided, nor were experiments carried out in *P. patens* to validate the results. Moreover, the
104 involvement of PpKAI2L proteins in the putative PpMAX2-dependent KL signaling pathway
105 remains to be explored.

106

107 The aim of the present study was to investigate the nature of the PpCCD8-derived molecules in
108 moss and to identify the moss receptors for these compounds. In an attempt to shed light on the
109 type of SLs derived from PpCCD8, we first tested the activity of *P. patens* as a stimulant for
110 seed germination of the root parasitic plant *Phelipanche ramosa* (*P. ramosa*), for which one SL
111 receptor, PrKAI2d3, has been recently characterized (de Saint Germain et al. 2021b). We then
112 looked for mimics of SL and KL that we could use in assays on *P. patens*. So far, the racemic
113 (\pm)-GR24 has been used as a SL analog, but recent reports in angiosperms found that the
114 different enantiomers present in this synthetic mixture do not have the same effect (Scaffidi et
115 al. 2014). Indeed, (+)-GR24, (also called GR24^{5DS}) with a configuration close to the natural
116 strigol is mostly perceived by D14 and mimics CCD8-derived SLs (e.g. CL). On the other hand,
117 as for (-)-5-deoxystrigol, the configuration of (-)-GR24 (also called GR24^{ent-5DS}) has so far not
118 been found in natural SLs. However, KAI2 perceives the (-)-GR24 analog better than D14
119 proteins, and (-)-GR24 has therefore been described as a KL mimic (Scaffidi et al. 2014; Zheng
120 et al. 2020). We tested both (+)-GR24 and (-)-GR24 isomers in phenotypic assays. Then,
121 refining and supplementing the work of (Bürger et al. 2019), we fully characterized seven

122 PpKAI2L proteins *in vitro* by testing their cleavage activity and binding to pure GR24
123 enantiomers. We showed that stereoselectivity of most of the PpKAI2L proteins for GR24
124 enantiomers is weak, except for the eu-KAI2 clade, which shows preferential affinity for (-)-
125 GR24. We highlighted the stronger (compared to the other PpKAI2L) and non-selective
126 enzyme activity of PpKAI2L-H. We expressed these proteins in the Arabidopsis *d14-1 kai2-2*
127 double mutant to examine conservation of the SL and/or KL perception function. Finally, we
128 used CRISPR-Cas9 technology to generate several *P. patens* multiple mutants affected in all
129 four *PpKAI2L* clades. By coupling analysis of these mutants' phenotypes and responses to pure
130 GR24 enantiomers with our biochemistry results, we provide strong evidence that eu-KAI2
131 clade PpKAI2L proteins could be moss KL receptors that function in a PpMAX2-dependent
132 pathway, while (GJM) clade PpKAI2L proteins would function as moss SL receptors in a
133 PpMAX2-independent pathway.

134

135 RESULTS

136 **PpCCD8-derived compounds induce the germination of a hemp-specific population of** 137 ***Phelipanche ramosa***

138 A recent report (Yoneyama et al. 2018b) indicated that canonical SLs previously identified in
139 *P. patens* tissues (Proust et al. 2011) could be contaminants. However other evidence suggests
140 that *P. patens* does synthesize SL-like products derived from CL. Indeed, PpCCD8 shows
141 carlactone synthase activity (Decker et al. 2017), and both the synthetic SL analog (\pm)-GR24
142 and CL do complement the *Ppccd8* phenotype (Proust et al. 2011; Decker et al. 2017). Still,
143 quantification of SL and related compounds is a challenge in many species (Boutet-Mercey et
144 al. 2018; Yoneyama et al. 2018b; Rial et al. 2019; Floková et al. 2020), and so far no known
145 SL has been identified from *P. patens* (Yoneyama et al. 2018b). Here we tested the ability of
146 *P. patens* exudates to induce the germination of parasitic seeds. Parasitic plants such as
147 *Phelipanche ramosa* can parasitize various host plants in response to specific exuded
148 germination stimulants (GS). Different genetic groups of *P. ramosa* seeds have been identified,
149 depending on the crop grown in the field where the seeds were collected (Huet et al. 2020).
150 Seeds from two populations of *P. ramosa* harvested in hemp (*Cannabis sativa*) (*P. ramosa*
151 group 2a) and oilseed rape (*Brassica napus*) (*P. ramosa* group 1) fields (Stojanova et al. 2019;
152 Huet et al. 2020) were assayed with WT moss exudates (Figure 1). As a control, both groups
153 of seeds were germinated in the presence of (\pm)-GR24 (Figure 1A). WT moss exudates induced

154 the germination of *P. ramosa* group 2a seeds but not *P. ramosa* group 1 seeds (Figure 1A). In
155 another assay, *P. ramosa* seeds were added to culture plates close to WT or *Ppccd8* plants, with
156 and without (\pm)-GR24 (Figure 1B-C). *P. ramosa* group 2a but not group 1 seeds germinated on
157 WT moss plates, while no germination was observed in the vicinity of *Ppccd8* plants. In all
158 cases (WT and *Ppccd8*), the addition of (\pm)-GR24 to the medium restored seed germination.
159 Thus, PpCCD8-derived compounds induce the germination of a specific population of *P.*
160 *ramosa* seeds, responding to not yet identified GS exuded by hemp.

161

162 ***P. patens* responds strongly to (+)-GR24 and carlactone application but poorly to (-)-**
163 **GR24 and KAR₂ in the dark**

164 With the aim of determining which molecules could be used to mimic the yet unknown
165 PpCCD8-derived compounds and moss KL, we tested both enantiomers of GR24 ((+)-GR24
166 and (-)-GR24), the SL precursor CL as racemic form, and the karrikin KAR₂. To quantify the
167 phenotypic response of *P. patens* to SL, we counted the number of caulonemal filaments per
168 plant grown in the dark (Guillory and Bonhomme 2021a) (Figure 2). The number of caulonema
169 filaments decreased following the application of (+)-GR24 in WT and *Ppccd8* in a dose-
170 dependent manner (Figure 2A-B). A dose of 0.1 μ M was sufficient to observe a clear and
171 significant response in both genotypes (Figure 2). No significant changes in caulonema filament
172 number were observed with (-)-GR24, except in WT, for which the 0.1 μ M and 10 μ M doses
173 led to a significant increase (Figure 2A). However, in further assays (see below, Figure 9), this
174 effect of (-)-GR24 was not repeatable.

175

176 Recent biochemistry experiments (Bürger et al. 2019) showed that some PpKAI2L proteins
177 could bind to KAR₁ (PpKAI2L-H, K and L), while a previous study concluded that *P. patens*
178 was insensitive to KAR₁ (Hoffmann et al. 2014). In the present work, we tested the KAR₂
179 molecule, described as being more active than KAR₁ in Arabidopsis (Waters et al. 2015a; Yao
180 et al. 2021) (Figure 2A-B). KAR₂ has an unmethylated butenolide group, unlike KAR₁. In WT,
181 no significant effect on caulonema number was observed following the application of
182 increasing doses of KAR₂ (Figure 2A). In *Ppccd8*, we observed an increase in filament number
183 at 10 μ M (Figure 2B). To conclude, the phenotypic effects of KAR₂ on *P. patens* were slight
184 and not clearly dose responsive.

185

186 We also tested CL, described as the natural product of PpCCD8 in *P. patens* (Decker et al.
187 2017). As previously reported (Decker et al. 2017), racemic CL application had a negative
188 effect on caulonema filament number for both WT and *Ppccd8* plants; however, in our assays,
189 the effect was only significant at 10 μ M for both genotypes (Figure 2C).

190

191 Overall, findings from these phenotypic assays suggest that GR24 enantiomers have distinct
192 effects in *P. patens*, as observed in Arabidopsis (Scaffidi et al. 2014). Indeed, the (+)-GR24
193 analog mimics the effects of CL, although it is far more potent, and can thus be used to mimic
194 the effects of PpCCD8-derived compounds. On the other hand, the (-)-GR24 analog and KAR₂
195 have slight phenotypic effects that are not always consistent or even tend to be opposite those
196 of PpCCD8-derived compounds.

197

198 **All *PpKAI2L* genes are expressed at relatively low levels and putatively encode proteins** 199 **with a conserved catalytic triad**

200 Like *D14* and *KAI2* genes, all 13 *PpKAI2L* genes encode a catalytic triad (Ser, His, Asp) (Figure
201 3 and Supplemental Figure S1). To test if the high number of PpKAI2L genes hints at different
202 spatial and temporal expression profiles, we obtained the expression patterns of all *PpKAI2L*
203 genes in *P. patens*, using a cDNA library from various organs/tissues (Supplemental Figure
204 S2). *PpKAI2L* genes transcripts were detected in all tested tissues, at relatively low levels
205 compared to the control genes. Quantitative RT-PCR could not be used to assess the expression
206 of *PpKAI2L-M*, as its predicted transcript is almost identical to that of *PpKAI2L-G*, and the
207 observed transcript levels are attributable to both *PpKAI2L-G* and *-M*. In spores, *PpKAI2L-F*
208 and *-J* had higher transcript levels than other *PpKAI2L* genes. In protonema and gametophores,
209 however, *PpKAI2L-D* from eu-KAI2 clade showed the highest transcript levels among
210 *PpKAI2L* genes (Supplemental Figure S2A). The data are consistent with those previously
211 reported (Ortiz-Ramirez et al. 2016; Perroud et al. 2018).

212

213 **The PpKAI2L-C, -D, and -E proteins are destabilized by (-)-GR24, as observed for** 214 **AtKAI2; PpKAI2L-F, -K, -L, and -H interact weakly with GR24 enantiomers**

215 To investigate whether the PpKAI2L proteins behave similarly to AtD14 or AtKAI2 *in vitro*,
216 we cloned the coding sequences (CDS) of all *PpKAI2L* genes and over-expressed them in *E.*
217 *coli*. After the successful purification and solubilization of seven PpKAI2L proteins (-C,-D,-

218 E,-F,-H,-K and -L), we investigated their interactions with SL analogs and their potential
219 enzymatic activities. Unfortunately, due to low solubility, the six other PpKAI2L proteins could
220 not be purified in sufficient amounts to ensure their quality. We tested the interactions of the
221 purified PpKAI2L proteins with SL analogs via nano Differential Scanning Fluorimetry
222 (nanoDSF) (Figure 4 and Supplemental Figure S3). (+)-GR24 and (-)-GR24 enantiomers
223 destabilized AtD14 (Supplemental Figure S3A), while AtKAI2 was destabilized by (-)-GR24
224 addition only (Supplemental Figure S3B), as previously reported (Waters et al. 2015a). All
225 tested eu-KAI2 clade proteins (PpKAI2L-C -D and -E) were destabilized when (-)-GR24 was
226 added, as was AtKAI2 (Supplemental Figure S3C to 3E), in accordance with the reported
227 stereoselectivity for un-natural (-)-5DS (Bürger et al. 2019). Puzzlingly, the PpKAI2L-C, and
228 -E proteins showed a tendency to be stabilized by (+)-GR24 at high concentrations
229 (Supplemental Figure S3C-E), which was not reported when using (+)-5DS. PpKAI2L-F and
230 PpKAI2L-L also showed a slight increase in T_m following the addition of both (-)-GR24 and
231 (+)-GR24 ($\leq 1^\circ\text{C}$), suggesting a slight stabilization (Figure 4 and Supplemental Figure S3F, 3I).
232 None of the GR24 isomers affected the stability of the PpKAI2L-K or PpKAI2L-H proteins
233 (Supplemental Figure S3G, 3H).

234 Since we sometimes observed opposite effects of different isomers on the stability of the
235 PpKAI2L proteins, we reasoned that their binding affinity had to be assessed for the GR24
236 isomers, rather than for (\pm)-GR24, as previously reported (Bürger et al. 2019). Binding affinities
237 were quantified with K_d affinity calculations following intrinsic tryptophan fluorescence
238 measurements (Figure 4 and Supplemental Figure S4). The affinity for (+)-GR24 could be
239 evaluated for PpKAI2L-K and PpKAI2L-H only (K_d superior to 100 μM), and was weaker than
240 that of AtD14 (23 μM). No K_d value for (+)-GR24 could be determined for AtKAI2, eu-KAI2,
241 or PpKAI2L-F, suggesting a very weak affinity for (+)-GR24 or a lack of binding. With (-)-
242 GR24, comparable affinities were found for AtKAI2 (45 μM), AtD14 (94 μM), PpKAI2L-D
243 (92 μM), PpKAI2L-E (39 μM), and PpKAI2L-K (41 μM). A much weaker affinity for (-)-
244 GR24 was found for PpKAI2L-H (273 μM).

245

246 **PpKAI2L-C, -D, -E, -K, and -L preferentially cleave (-)-GR24, while PpKAI2L-H cleaves**
247 **both (-)-GR24 and (+)-GR24**

248 As all PpKAI2L proteins contain the conserved catalytic triad, and since we found that most of
249 them were able to bind to at least one of the GR24 isomers, we tested their enzyme activity
250 against SL analogs. First, when incubated with the generic substrate for esterases, 4-nitrophenyl
251 acetate (*p*-NPA), AtKAI2 and all tested PpKAI2L proteins showed enzyme activity
252 (Supplemental Figure S5A-B), consistent with a previous report (Bürger et al. 2019). Kinetic
253 constants were in the same range for all proteins but one, and similar to that of AtKAI2. The
254 exception was PpKAI2L-H, which had a higher V_{max} and K_M , highlighting faster catalysis and
255 a better affinity for *p*-NPA than all the others (Supplemental Figure S5C). We further
256 characterized the enzyme activity of PpKAI2L proteins on the GR24 isomers. We compared
257 their substrate bias to that of the pea (*Pisum sativum*) SL receptor RMS3/PsD14 and to that of
258 AtKAI2 (Figure 4 and Supplemental Figure S6). None of the PpKAI2L proteins showed as high
259 an enzyme activity as RMS3/PsD14 (100% cleavage of (+)-GR24 and (-)-GR24 (de Saint
260 Germain et al. 2016)). Only PpKAI2L-H showed a relatively high catalytic activity towards the
261 GR24 isomers, especially towards (-)-GR24 (almost 70%, Supplemental Figure S6). AtKAI2
262 and all other tested PpKAI2L proteins except one selectively cleaved the (-)-GR24 enantiomer,
263 but with low activity (less than 28%, observed for AtKAI2). Finally, PpKAI2L-F showed very
264 low enzyme activity towards both isomers (less than 5%).

265

266 **The higher cleavage activity of PpKAI2L-H on synthetic SL analogs is explained by the** 267 **presence of a specific leucine residue**

268 The higher enzyme activity of PpKAI2L-H (Supplemental Figure S6) and its lack of thermal
269 shift when incubated with GR24 isomers (Supplemental Figure S3H) set this protein apart from
270 other PpKAI2L proteins. To better characterize PpKAI2L-H enzyme activity, we used a pro-
271 fluorescent probe as substrate ((±)-GC242), in which the ABC rings of GR24 are replaced by a
272 coumarine-derived moiety (DiFMU) (de Saint Germain et al. 2016). (±)-GC242 is bioactive on
273 moss, as it reduced the number of caulonema filaments in the dark in a dose-responsive manner
274 (evaluated on the *Ppccd8* mutant, Supplemental Figure S7A). The use of (±)-GC242 as a
275 substrate confirmed the relatively high enzyme activity of PpKAI2L-H compared to all the
276 other PpKAI2L proteins (Supplemental Figure S7B). Indeed, after 2 hours, PpKAI2L-H
277 catalyzed the formation of 1 μM DiFMU, while other PpKAI2L activities were
278 indistinguishable from background noise. However, the PpKAI2L-H enzymatic profile did not
279 show the biphasic curve (a short burst phase, quickly followed by a plateau phase), which

280 characterizes the single turnover activity of AtD14 (Supplemental Figure S7B (de Saint
281 Germain et al. 2016)). The lack of a plateau for PpKAI2L-H suggested that this protein acted
282 as a Michaelian enzyme on the SL analog.

283 To try to understand this singularity, we compared the solvent-exposed residues in the binding
284 pocket of the PpKAI2L proteins and noticed that PpKAI2L-H harbors a leucine²⁸ (Leu²⁸)
285 residue instead of the phenylalanine found in AtD14 (Phe²⁶), AtKAI2, and all other PpKAI2L
286 proteins (Figure 5A). The Phe residue is located at the junction between helix α 4 and α 5, near
287 the catalytic site (Figure 5B), and the crystal structure of PpKAI2L-H (Bürger et al. 2019)
288 indicates that this residue interacts with the D-ring of the SL. Furthermore, a mutant PpKAI2L-
289 H protein where Leu²⁸ is changed to Phe showed a single turnover profile similar to AtD14,
290 both reaching a plateau at 0.4 μ M DiFMU, correlating with the protein concentration (Figure
291 5C and 5D). PpKAI2L-H and PpKAI2L-H^{Leu28Phe} proteins had comparable affinity towards (\pm)-
292 GC242 ($K_{1/2}$ = 4,794 μ M vs 4,675 μ M) but showed different V_{max} values (V_{max} =0,06794 μ M.min⁻¹
293 vs 0,01465 μ M.min⁻¹), suggesting that the Leu²⁸ residue affects the velocity of catalytic
294 activity (Figure 5C and 5D).

295

296 **Moss PpKAI2L proteins, like vascular plant receptors, covalently link GR24 enantiomers**

297 To further investigate whether PpKAI2L proteins play roles as receptors of SLs and related
298 compounds, we examined the covalent attachment of the GR24 isomers to the PpKAI2L
299 proteins (Figure 4 and Supplemental Figure S8). Mass spectrometry analyses revealed 96 Da
300 increments (corresponding to the D ring mass) when AtKAI2 and PpKAI2L-C, -D, -E, -F, or -
301 L were incubated with (-)-GR24. Strikingly, 96 Da increments were also observed when
302 PpKAI2L-E, -F, -L, and -K were incubated with the other isomer (+)-GR24, in contrast to other
303 reports (Bürger et al. 2019). However, for PpKAI2L-E, the intensity peak was much lower with
304 (+)-GR24 than with (-)-GR24, confirming the better affinity for the latter (Figure 4 and
305 Supplemental Figure S8). PpKAI2L-H did not covalently bind the D ring following incubation
306 with either enantiomer, further suggesting that it displays Michaelian enzyme activity.

307 Thus, poor interactions were observed with (+)-GR24, which was reported to mimic SLs and
308 had the most potent effect on *P. patens* in our phenotypic assays (Figure 2). Strikingly, all eu-
309 KAI2 clade proteins tested showed the strongest affinity for the (-)-GR24 enantiomer (Figure
310 4), which is reported to be a good mimic for the putative KL in vascular plants (Scaffidi et al.

311 2014; Zheng et al. 2020). We then carried out *in planta* studies to investigate if these PpKAI2L
312 homologs are necessary for SL or KL perception.

313

314 **None of the *PpKAI2L* genes complement the Arabidopsis *d14-1 kai2-2* double mutant**

315

316 We used cross species complementation assays to test whether any of the PpKAI2L proteins
317 could carry out similar functions in SL and/or KL signaling to that of AtD14 and/or AtKAI2 in
318 Arabidopsis (Figure 6). CDS of the *PpKAI2L-C*, *-D*, *-F*, *-G*, and *-J* genes were cloned
319 downstream of the *AtD14* or *AtKAI2* promoter, and the resulting constructs were expressed in
320 the Arabidopsis double mutant *Atd14-1 kai2-2*, which shows both a hyperbranched phenotype
321 and elongated hypocotyls (Supplemental Figure S9). As controls, the double mutant was
322 transformed with the *AtKAI2* or *AtD14* CDS under the control of endogenous promoters. Only
323 lines expressing *AtD14* under the control of the *AtD14* promoter fully restored rosette branching
324 to WT (Ler) values (Figure 6A). Under the control of the *AtD14* promoter, neither *AtKAI2* nor
325 any of the *PpKAI2L* genes significantly restored the branching phenotype of *Atd14-1 kai2-2*.
326 We conclude that none of the tested *PpKAI2L* genes can fully complement the function of
327 *AtD14* in shoot branching.

328

329 We also examined possible complementation of the AtKAI2 function in the *Atd14-1 kai2-2*
330 mutant by monitoring hypocotyl length under low light conditions, with or without 1 μ M (+)-
331 GR24 or (-)-GR24 in the culture medium (Figure 6B and Supplemental Figure S9). Compared
332 to WT, the double mutant showed longer hypocotyls under control conditions, as did the single
333 *kai2-2* mutant. The addition of (+)-GR24 or (-)-GR24 had no effect on the phenotype of the
334 double mutant (Figure 6B). By contrast, (+)-GR24 treatment led to shorter *kai2-2* hypocotyls,
335 likely due to perception and transduction by the AtD14 protein, which is still active in the single
336 mutant. As expected from the loss of AtKAI2 function, (-)-GR24 addition had no effect on
337 *kai2-2* hypocotyls. Surprisingly, expressing *AtKAI2* under the control of the *AtKAI2* promoter
338 did not fully restore the hypocotyl length of the double mutant under our control conditions,
339 but it did restore the response to (-)-GR24, as anticipated (Figure 6B).

340

341 Under control conditions, almost all double mutant lines expressing AtD14 or one PpKAI2L
342 protein showed long hypocotyls (Figure 6B and Supplemental Figure S9B). Even longer
343 hypocotyl phenotypes (compared to the double mutant) were found under control conditions

344 for one line expressing PpKAI2L-C (#16.2) and one line expressing PpKAI2L-D (#1.4)
345 (Supplemental Figure S9B). This unexpected effect of the introduced α/β hydrolases will be
346 discussed below. By contrast, a line expressing PpKAI2L-G (#7.1) had short hypocotyls under
347 control conditions, suggesting that the expressed protein had indeed complemented the AtKAI2
348 function (Figure 6B). In a separate assay, in addition to shorter hypocotyls, all three lines
349 expressing PpKAI2L-G in the double mutant background showed significantly larger
350 cotyledons, further hinting at the restoration of AtKAI2 function (Supplemental Figure S9C).

351

352 When either (+)-GR24 or (-)-GR24 was added (Figure 6B), short hypocotyls (similar to WT)
353 were observed in the *pKAI2:AtD14* expressing line, indicating that AtD14-mediated signal
354 transduction of both enantiomers occurs when AtD14 is present in tissues where AtKAI2 is
355 normally active. However, adding GR24 enantiomers to the medium had no such effect on most
356 lines expressing PpKAI2L proteins (Figure 6B and Supplemental Figure S9B). Overall, these
357 assays showed that PpKAI2L-G may mediate KL signaling in Arabidopsis hypocotyls, even
358 though it could not fully ensure the function of AtKAI2 in this process.

359

360 **Multiplex editing of *PpKAI2L* genes**

361 Multiplex gene editing using CRISPR-Cas9 allowed us to isolate *P. patens* mutants affected in
362 one or several *PpKAI2L* genes (Lopez-Obando et al. 2016b) (Figure 3 and Supplemental Figure
363 S10). For the eu-KAI2 clade, two triple (*abc*, *cde*) and two quintuple mutants (*abcde*) were
364 chosen for further analysis (Supplemental Table S1). The remaining (HIL), (FK), and (GJM)
365 clades were targeted in separate experiments using combinations of specific CRISPR RNAs
366 (crRNAs) for each gene. As biochemistry experiments suggested a purely enzymatic role for
367 PpKAI2L-H, a single deletion mutant was obtained for the *PpKAI2L-H* gene through
368 homologous recombination, where the full CDS was removed from the moss genome (Δh
369 mutant, Supplemental Figure S10 and S11). This Δh mutant was employed in further
370 transformation experiments with crRNAs from the same (HIL) clade and/or from the (FK) and
371 (GJM) clades, generating the Δhil and $\Delta hfkj$ mutants (Supplemental Table S1). Eventually, a
372 7X mutant was obtained ($\Delta hifkgjm$) where all the mutations except those in *PpKAI2L-J* and *-M*
373 were null (Supplemental Table S1 and see below). For convenience, null mutations are hereafter
374 indicated in bold letters.

375

376 **The eu-KAI2 clade quintuple mutants phenocopy *Ppmax2-1* in white light, while mutants**
377 **in other clades are more similar to WT or *Ppccd8***

378 Our rationale was that a mutant affected in the response to PpCCD8-derived compounds should
379 show a phenotype similar to that of the SL biosynthesis mutant *Ppccd8*. We first performed a
380 phenotypic analysis of the mutants in the light. After four weeks of culture, *Ppccd8* plants were
381 slightly bigger than WT ((Proust et al. 2011), Figure 7), whereas *Ppmax2-1* plants were smaller,
382 with fewer but bigger gametophores ((Lopez-Obando et al. 2018), Figure 7). The diameter of
383 mutants in eu-KAI2 clade genes was significantly smaller than that of *Ppccd8* and WT and
384 slightly larger than that of *Ppmax2-1* (Figure 7A-B). The phenotype of the eu-KAI2 clade
385 mutants, with early and large gametophores, resembled that of *Ppmax2-1*, although not as
386 pronounced (Figure 7A). To the naked eye, three week-old plants from mutants in genes from
387 the (FK), (GJM), and (HIL) clades were indistinguishable from WT (Figure 7A). After a month
388 of growth, however, all mutants affecting genes from the (FK) and (GJM) clades showed a
389 slightly larger diameter, intermediate between that of WT and *Ppccd8* (Figure 7D). Mutants in
390 the (HIL) clade such as Δh , Δhi and Δhil were comparable to WT (Figure 7D). Thus, based on
391 mutant phenotypes in the light, eu-KAI2 clade genes would be involved in a PpMAX2-
392 dependent pathway, while (FK) and (GJM) clade genes would instead be involved in the
393 PpCCD8-derived SLs pathway. Genes from the (HIL) clade would not be involved in either
394 pathway.

395

396 **Like *Ppmax2*, eu-KAI2 clade quintuple mutants are affected in photomorphogenesis**

397 Mutants in eu-KAI2 clade genes showed the typical phenotype of the *Ppmax2-1* mutant in white
398 light. We previously showed that the *Ppmax2-1* mutant is affected in photomorphogenesis
399 under red light (Lopez-Obando et al. 2018). After two months of growth under red light,
400 *Ppmax2-1* gametophores were much more elongated than WT gametophores, whereas *Ppccd8*
401 gametophores were shorter (Figure 8). Among eu-KAI2 mutants, gametophores of both triple
402 mutants *abc* and *cde* were a similar height to WT. Interestingly, the quintuple mutant (*abcde*-
403 *1*) showed significantly elongated gametophores, similar to *Ppmax2-1* (Figure 8A and C). The
404 other tested quintuple mutant (*abcde*-2) also had elongated gametophores under red light,
405 intermediate between WT and *Ppmax2-1* (Supplemental Figure S12). The weak phenotypes of
406 both triple mutants (Figure 8A) suggest a functional redundancy among genes of the eu-KAI2

407 clade, as knockout (KO) mutations for PpKAI2L-A, B, C, and/or D did not result in plants with
408 gametophores as elongated as those of the *Ppmax2-1* mutant.

409 Gametophores from mutants where genes from the (FK) and/or (GJM) clades were mutated
410 (*fkj*, *Δhfkj-1* and *Δhfkjgm*) were similar in height to WT, suggesting that genes from clade (FK)
411 and clade (GJM) have no role in photomorphogenesis in red light (Figure 8B-C and
412 Supplemental Figure S12). (HIL) clade triple mutant *Δhil* showed shorter gametophores under
413 red light, similar to *Ppccd8* (Figure 8A). By contrast, the gametophores of both the single *Δh*
414 and the double *Δhi* mutants were intermediate in height between WT and *Ppccd8* (Figure 8A-
415 B). This suggests a specific role for the (HIL) clade genes, with an opposite impact on
416 gametophore development compared to that of the PpMAX2 pathway.

417 In conclusion, the phenotypes of the *Ppkai2L* mutants in red light allowed us to differentiate
418 the functions of the eu-KAI2 clade genes. These genes are likely to be involved in a PpMAX2-
419 dependent pathway related to photomorphogenesis, whereas genes from the three other clades
420 (DDK superclade) are more likely to play roles independent of PpMAX2.

421

422 **Mutants in the (GJM) clade no longer respond to (+)-GR24 application**

423 To determine which of the *Ppkai2L* mutants carry mutations in potential receptors for PpCCD8-
424 compounds (SL-related) or other (KL-related) compounds, we tested their phenotypic
425 responses to GR24 enantiomers at 0.1 μM (Figure 9). In the assays reported above on WT and
426 *Ppccd8* in the dark (Figure 2), (-)-GR24 appeared to be a poor KL mimic in moss. However,
427 the stereospecificity of the PpKAI2L proteins for (-)-GR24 led us to pursue our assays with
428 this enantiomer.

429 For the eu-KAI2 clade, under control conditions, all mutants showed an equivalent number of
430 filaments to WT, except for the quintuple mutant. The latter tended to have fewer filaments
431 under control conditions, another similar phenotype to *Ppmax2-1* (Figure 9A and Supplemental
432 Figure S13A). The addition of 0.1 μM (-)-GR24 had no significant effect on eu-KAI2 clade
433 mutants or WT (Figure 9A). However, in this assay, both *Ppccd8* and *Ppmax2-1* showed a
434 significant decrease in caulonema filament number in response to 0.1 μM (-)-GR24. In a
435 separate experiment, a dose of 1 μM of (-)-GR24 had opposite effects on WT and *Ppccd8*
436 filament number, with an increase and decrease, respectively (Supplemental Figure S13A, and
437 Figure 2 above), but had no significant effect on the *Ppmax2-1* mutant, although a tendency

438 towards a decrease was observed. At this higher dose, the quintuple eu-KAI2 clade mutant
439 showed a significant decrease in caulonema filament number, like *Ppccd8*, but in contrast to
440 WT. Thus, similar to *PpMAX2* loss of function, mutating eu-KAI2 genes did not abolish a
441 response to the (-)-GR24 enantiomer. The application of (+)-GR24 had a significant negative
442 effect on the number of filaments for the quintuple *abcde* mutant, like for WT and the *Ppccd8*
443 and *Ppmax2-1* mutants (Figure 9B). Thus, mutating any of the eu-KAI2 clade genes did not
444 hamper the response to (+)-GR24, and therefore it likely does not hamper the responses to
445 PpCCD8-derived compounds.

446 We then tested the effects of GR24 enantiomers on *Ppkai2L* mutants from the three other
447 clades: (FK), (GJM), and (HIL). Strikingly, under control conditions, like *Ppccd8*, all the
448 mutants had more filaments than WT, except for mutants in the (HIL) clade, which were similar
449 to WT (Figure 9C to 9E) or tended to have fewer filaments (Supplemental Figure S13). Both
450 the single *j* mutant and the quintuple mutant *gjmil* showed a significant response to (-)-GR24
451 (fewer caulonema), as did *Ppccd8* (Figure 9C and Supplemental Figure S13A-B). No clear
452 response to (-)-GR24 was seen in mutants with KO mutations in *PpKAI2L-F*, *-K*, *-H*, *-G*, *-M*,
453 *-I* or *-L*, like for WT (Figure 9C and Supplemental Figure S13A-B). Finally, we examined the
454 response to (+)-GR24 for mutants of the (FK), (GJM), and (HIL) clades (Figure 9D-E and
455 Supplemental Figure S13C-D). The number of caulonema was clearly reduced in WT and
456 *Ppccd8* (as shown above, Figure 2), and in mutants carrying the Δh mutation, alone or in
457 combination with *f*, *k*, *i* or *l* null mutations. Thus, genes from the (FK) and (HIL) clades do not
458 play a role in the response to (+)-GR24. However, the response to the (+)-GR24 enantiomer
459 was abolished in all mutants where the *PpKAI2L-J* gene was knocked out (Figure 9D: *j* and
460 *gjm-1*, and Supplemental Figure S13C: *gjmil* and $\Delta hfkj-3$ and 13D: *gjm-3* and *gjm-5*).
461 Interestingly, in the two lines where the *j* mutation was not null but the *PpKAI2L-G* gene was
462 knocked out (*7x $\Delta hifk$ gjm* and *gjm-4* mutants), the response to (+)-GR24 was also abolished
463 (Supplemental Figure S13C-D). Thus, based on phenotypic assays of mutant caulonema, both
464 the *PpKAI2L-J* and *-G* genes appear to be necessary for the response to (+)-GR24 and are
465 therefore the best candidates for receptors to PpCCD8-derived SLs.

466 To further test if PpKAI2L-J and -G could be receptors for PpCCD8-derived SLs, we measured
467 the transcript levels of SL-responsive genes in the corresponding mutants (Figure 10). We
468 previously reported that in WT and *Ppccd8* plants, *PpKUFILA* gene transcript abundance
469 increases 6 h after plant transfer onto medium containing 3 μ M (\pm)-GR24 and that this response

470 is enhanced in the dark (Lopez-Obando et al. 2018). We used this marker along with the
471 *Pp3c6_15020* gene (encoding a putative histidine kinase) that was previously found to be
472 upregulated by (\pm)-GR24 (Supplemental Figure S14). Using GR24 enantiomers, we confirmed
473 that the transcript levels of both genes increased following 1 μ M (+)-GR24 addition in WT and
474 *Ppccd8*, but not in *Ppmax2-1*. Strikingly, an increase in transcript levels following (-)-GR24
475 application was observed for both markers in *Ppccd8*, and for *PpKUFILA* only in WT (Figure
476 10). In the quintuple eu-KAI2 mutant, the addition of (+)-GR24 but not (-)-GR24 increased
477 *PpKUFILA* and *Pp3c6_15020* transcript levels. By contrast, in the *j* mutant, the transcript
478 levels of both genes increased following (-)-GR24 addition and were slightly increased
479 (*PpKUFILA*) or unchanged (*Pp3c6_15020*) in response to (+)-GR24 application. In the *gjm-2*
480 mutant, the response marker transcript levels were unchanged following either (+)-GR24 or
481 (-)-GR24 addition. Thus, the transcriptional responses of the tested mutants confirm the notion
482 that eu-KAI2 genes are not involved in the response to (+)-GR24, while this response is
483 impaired in (GJM) clade mutants. Only the *Ppccd8* and *j* mutants showed a clear and significant
484 transcriptional response (for both markers) to (-)-GR24 addition.

485

486 **DISCUSSION**

487 **Are PpCCD8-derived products non-canonical SLs?**

488 *P. patens* exudates were previously reported to induce the germination of *Orobancha ramosa*
489 (old name for *Phelipanche ramosa*) seeds (Decker et al. 2017), but the origin of the tested
490 population was not specified. Here, we further demonstrated that PpCCD8-derived products are
491 germination stimulants of *P. ramosa* group 2a seeds that were harvested in a hemp field but do
492 not induce the germination of *P. ramosa* group 1 seeds collected from an oilseed rape field.
493 Differences in the susceptibility of root parasitic weeds can be attributed to the chemical nature
494 of host plant exudates (Yoneyama et al. 2018b). Our results suggest that the PpCCD8-derived
495 products share similarities with hemp secondary metabolites. So far, no known canonical SLs
496 have been isolated from hemp (Huet et al. 2020). Since *P. patens* likely produces CL (Decker
497 et al. 2017) but lacks a true *MAXI* homolog (Proust et al. 2011), we hypothesize that PpCCD8-
498 derived compounds correspond to non-canonical SLs derived from CL or hydroxyl CLs
499 (Yoneyama 2020). Further supporting this hypothesis, we previously showed that GR5, a non-
500 canonical SL analog, was as bioactive as (\pm)-GR24 on *P. patens* caulonema length (Hoffmann
501 et al. 2014). As mimics of SLs in the present study, we used the (+)- and (-)-GR24 artificial

502 analogs that were available at the time of our study (see below). Of note, both isomers are active
503 on *P. ramosa* group 1 and group 2a seeds. However, the (+)-GR24 isomer, which has a
504 canonical SL structure, has similar germination stimulating activity to (±)-GR24, while the (-)-
505 GR24 isomer is far less active (de Saint Germain et al. 2019). For further identification of
506 PpCCD8-derived SLs in the future, non-canonical SL analogs such as the recently described
507 methyl phenlactonoates (Jamil et al. 2020) may be more appropriate (see also below).

508

509 **Looking for the best mimic of SLs or KL**

510 The (-)-GR24 analog has a non-natural configuration that has never been found in plant
511 exudates, contrary to the (+)-GR24 enantiomer, which bears similarity to 5-deoxystrigol ((+)-
512 5DS) and strigol-type canonical SLs (Scaffidi et al. 2014). In our bioassays of moss phenotypes,
513 CL application decreased the number of caulonema of both WT and *Ppccd8* at 10 μM. A similar
514 (but much stronger) effect was observed with (+)-GR24, which we thus consider to be the best
515 mimic of PpCCD8-derived compounds available so far. It is not surprising that (+)-GR24 is
516 more potent than CL, as the assays were carried out in aqueous medium, and natural SLs were
517 described as being far less stable than synthetic analogs in aqueous medium (Akiyama et al.
518 2010; Boyer et al. 2012). Moreover, we cannot exclude the possibility that CL needs to be
519 transformed into a more bioactive non-canonical SL *in planta* to trigger the effects observed
520 here. By contrast, the effects of (-)-GR24 are weak, not dose responsive, and sometimes
521 contradictory in WT versus *Ppccd8*. Indeed, in several assays, we observed a significant
522 increase in caulonema number in WT (Figure 2A, Supplemental Figure S13A) while this
523 number consistently decreased in *Ppccd8*, mimicking the result of SL application (Figure 9,
524 Supplemental Figure S13A-B).

525

526 Interestingly, we also observed an increase in caulonema number when testing KAR₂, although
527 this increase was only significant at 10 μM for *Ppccd8* (Figure 2B). So far we had not observed
528 any effect of karrikins (KAR₁) on the phenotypes of *P. patens* (Hoffmann et al. 2014), and this
529 is thus the first hint of a possible effect of some karrikins on moss, which needs to be confirmed.
530 We hypothesize that the increase in caulonema filament number is the effect triggered by the
531 as yet unidentified moss KL (see also below and Figure 11). It is puzzling, however, that the
532 effect of KAR₂ is more easily observed in *Ppccd8* (thus in the absence of SLs) than in WT,
533 while the same effect of (-)-GR24 (increasing of filament number) is only seen in WT. We
534 propose (Figure 11 and see below) that (GJM) clade PpKAI2L proteins perceive (-)-GR24,

535 which would explain the apparent contrary effects of this enantiomer. In the absence of
536 endogenous SL (in the *Ppccd8* mutant), (-)-GR24 would thus trigger the SL pathway. This
537 could also explain the transcriptional response to (-)-GR24 observed in *Ppccd8* (Figure 10).
538 Thus, (-)-GR24 is not a very robust mimic of the as yet unidentified moss KL, and KAR₂ is
539 likely not a potent KL mimic either. This conclusion also suggests that *P. patens* KL may be
540 quite different from that of angiosperms. Recently, desmethyl butenolides were reported to be
541 more potent and to function as specific ligands for KAI2, both *in vitro* and in plants, including
542 the lycophyte *Selaginella moellendorffii* (*S. moellendorffii*) and the bryophyte *Marchantia*
543 *polymorpha* (*M. polymorpha*) (Yao et al. 2021). Thus, testing desmethyl butenolides, and in
544 particular the (-)-desmethyl GR24 enantiomer (dGR24^{ent5DS}) on *P. patens* plants and proteins
545 is a priority in the search for a KL mimic.

546

547 **Biochemical characterization suggests that PpKAI2L proteins from the eu-KAI2 clade** 548 **act as KL receptors**

549 Among the analogs examined, we observed the best binding of the (-)-GR24 enantiomer to
550 AtKAI2 and all tested PpKAI2L proteins from the eu-KAI2 clade. This confirms a recent report
551 (Bürger et al. 2019) of the preferential binding of (-)-5DS by PpKAI2L-C, -D, and -E. In
552 addition, we found that the (+)-GR24 enantiomer interacts poorly with these PpKAI2L proteins,
553 but not with AtKAI2, indicating less stringency for moss eu-KAI2 clade proteins. Still, this
554 result suggests that eu-KAI2 proteins share a perception mechanism with AtKAI2, and
555 furthermore, they may recognize KL-like compounds. The moss KL compound(s) may,
556 however, differ from the angiosperm KL, since the expression of PpKAI2L-C or -D did not
557 complement the *kai2-2* hypocotyl phenotype in Arabidopsis, or more likely, the protein partners
558 in Arabidopsis are different (see also below).

559 It should be noted that AtKAI2 is degraded following KAR perception, in a MAX2-independent
560 manner (Waters et al. 2015a), and this could be tested on eu-KAI2 clade proteins. As with the
561 two other tested clades, (FK) and (HIL), none of the interaction assays revealed any preferential
562 binding of GR24 isomers, despite the use of pure enantiomers. Indeed, only PpKAI2L-K
563 showed similar binding affinity with both (-)-GR24 and (+)-GR24, but no stereoselectivity.
564 Unfortunately, none of the PpKAI2L proteins from the (GJM) clade could be purified for
565 interaction assays, which was also reported by (Bürger et al. 2019). In the future,

566 overexpression in *P. patens* or in other heterologous systems (yeast, insect cells) may be a
567 solution for producing these proteins and carrying out biochemistry studies.

568

569 **PpKAI2L-H is the most efficient hydrolase among PpKAI2L proteins**

570 The PpKAI2L-H protein showed stronger cleavage activity towards both GR24 stereoisomers
571 as well as towards the synthetic probe (\pm)-GC242 compared to any other PpKAI2L protein, but
572 also compared to the Arabidopsis AtKAI2 and AtD14 proteins. Mutating the Leu²⁸ residue into
573 a Phe was sufficient to reduce the efficiency of this activity (strong reduction of the k_{cat} and of
574 the V_{max}), but had no effect on the $K_{1/2}$ towards (\pm)-GC242. The efficient cleavage activity of
575 PpKAI2L-H is therefore not likely due to a stronger affinity of this protein for the substrate. It
576 has been hypothesized that the Leu²⁸ residue (like the Phe¹⁸¹ residue), which is unique to
577 PpKAI2L-H, does not particularly enlarge the pocket size of PpKAI2L-H (Bürger et al. 2019).
578 Therefore, our results may highlight the ability of a residue to control the exit of the cleavage
579 reaction product. Indeed, the conserved Phe²⁸ residue in D14/KAI2 proteins may act as a gate
580 keeper, and this could explain the single turnover kinetics observed with some SL analogs (de
581 Saint Germain et al. 2016). We hypothesize that the high velocity of PpKAI2L-H may be linked
582 to the lack of this gate keeper residue, allowing a high substrate turnover. The stronger enzyme
583 activity of PpKAI2L-H could have a specific role in *P. patens*, perhaps as a catabolic enzyme,
584 to regulate the levels of bioactive signaling molecules (Seto et al. 2019).

585 When only the *PpKAI2L-H* gene was mutated (Δh mutant), no striking phenotype was observed
586 (Figure 7), and in particular, the phenotypic response to (+)-GR24, which mimics PpCCD8-
587 derived SLs, was similar to that of WT plants (Figure 9D). In red light, however, the
588 gametophores of Δhi and Δhil mutants were less elongated than WT gametophores, which was
589 also observed in *Ppccd8* (Figure 8). However, in *Ppccd8*, this could be related to the higher
590 number of filaments, leading to the initiation of more (but smaller) gametophores, whereas the
591 number of filaments in the dark was not higher in mutants from the (HIL) clade (Figure 9 and
592 Table 1). In addition, these mutants even tended to have fewer filaments than WT (Figure 9 D-
593 E and Supplemental Figure S13A). Thus, mutants from the (HIL) clade are quite different from
594 *Ppccd8*, and the corresponding genes *PpKAI2L-H -I* and *-L* likely do not encode receptors for
595 PpCCD8-derived compounds. Notably, our results do not support a likely role for PpKAI2L-H
596 in the KL pathway, even though the binding of KAR₁ by this protein was reported (Bürger et
597 al. 2019). Indeed, the Δh and higher-order mutants (Δhi and Δhil) tended to be smaller than WT

598 in white light (Figure 7D and Table 1), but also showed a slight reduction in gametophore height
599 in red light (Figure 8A), in contrast to *Ppmax2-1* and eu-KAI2 clade mutants (Table 1). These
600 findings suggest that (HIL) clade genes play a specific role in *P. patens* development. The
601 association of this role with PpKAI2L-H enzyme activity remains to be discovered.

602

603 **The Arabidopsis *AtD14-1 kai2-2* mutant complementation assay provides important clues** 604 **about PpKAI2L activity**

605 Using the endogenous *AtD14* promoter, we confirmed that expression of the PpKAI2L-C
606 protein does not complement the Arabidopsis D14 function in rosette branching, as previously
607 reported using the 35S promoter (Bürger et al. 2019). We can now extend this observation to
608 PpKAI2L-D, PpKAI2L-F, PpKAI2L-G, and PpKAI2L-J. Using the endogenous *AtKAI2*
609 promoter, we also confirmed that PpKAI2L-C cannot complement the *kai2-2* mutation, as
610 observed by (Bürger et al. 2019). Furthermore, we extended this observation to PpKAI2L-D
611 and PpKAI2L-J. However, expressing moss PpKAI2L-G reduced the hypocotyl size and led to
612 larger cotyledons than in the double mutant *Atd14-1 kai2-2* (Supplemental Figure S9C),
613 suggesting that PpKAI2L-G may be able to perceive and transduce the endogenous KL signal,
614 even though it does not respond to (-)-GR24. Similarly, when expressed in Arabidopsis, one
615 of the two KAI2 homologs from *S. moellendorffi* (SmKAI2b) partially complemented the *kai2*
616 mutant, whereas the other homolog and both the KAI2 homologs from *M. polymorpha* did not
617 restore the *kai2* mutant phenotypes (Waters et al. 2015b). Strikingly, when expressed under the
618 control of the *AtKAI2* promoter in the Arabidopsis *Atd14-1 kai2-2* mutant, the moss PpKAI2L-
619 C or -D proteins exacerbated the defect induced by the *kai2-2* mutation by leading to even more
620 elongated hypocotyls (for each construct, in one line out of two, Supplemental Figure S9B).
621 This suggests a putative interaction of these proteins with the Arabidopsis KAI2/KL pathway,
622 which should be further investigated. Still, it is clear that none of the PpKAI2L proteins fully
623 complements the AtD14 or KAI2 function, likely due to defective interactions with AtMAX2
624 and/or other components of SL/KL pathways.

625

626 **Genetic analysis suggests that genes from the eu-KAI2 clade are involved in the** 627 **PpMAX2-dependent pathway**

628 Mutant phenotypes clearly distinguish the eu-KAI2 clade from the three other clades (Table 1).
629 Indeed, the quintuple *eu-KAI2* mutant shows a phenotype in white light quite similar to that of

630 *Ppmax2-1*. It also has elongated gametophores under red light, and few caulonema filaments in
631 the dark, suggesting that eu-KAI2 and PpMAX2 proteins could be members of the same
632 pathway (Figure 11). As eu-KAI2 proteins preferentially bind to the (-)-GR24 enantiomer, we
633 expected the mutants in this clade to be blind to (-)-GR24 application. This is what we observed
634 when transcriptional response markers were examined, with transcript levels remaining
635 unchanged following (-)-GR24 application in both the *Ppmax2-1* and the quintuple eu-KAI2
636 clade mutant, but increasing in *Ppccd8* (but not in WT, Figure 10 and Table 1). However, as
637 mentioned above, (-)-GR24 does not appear to be a perfect mimic of the unknown moss KL,
638 and other transcriptional response markers need to be found that would better reflect the moss
639 KL response. As for the phenotypic response, the application of 0.1 μ M (-)-GR24 had no effect
640 on caulonema number in the dark in the eu-KAI2 clade mutants or in the WT, but it significantly
641 decreased caulonema number in both *Ppccd8* and *Ppmax2-1* (Figure 9A). Strikingly, a 1 μ M
642 concentration of (-)-GR24, which had the opposite effect on WT (increased caulonema
643 number) and *Ppccd8* (decreased caulonema number), produced no response in the *Ppmax2-1*
644 mutant, while the quintuple eu-KAI2 mutant showed a significant decrease in caulonema
645 number under this treatment (Supplemental Figure S13). Thus, the quintuple eu-KAI2 mutant
646 is still able to perceive (-)-GR24, as is the *Ppmax2-1* mutant. As mentioned above, we
647 hypothesize that other PpKAI2L proteins (presumably members of the (GJM) clade, see below)
648 may perceive (-)-GR24, triggering the PpCCD8-derived compounds pathway (Figure 11). This
649 does not rule out the possibility that eu-KAI2 clade and PpMAX2 proteins function in the same
650 pathway. In addition, (-)-GR24 was reported to have a dual effect, promoting both the KAI2
651 and the D14 pathways in Arabidopsis roots (Villaecija-Aguilar et al. 2019).

652

653 Finally, both phenotypic responses in the dark and transcriptional responses to the (+)-GR24
654 enantiomer were unaffected in the eu-KAI2 clade mutants (Table 1), indicating that eu-KAI2
655 proteins are not receptors for PpCCD8-derived compounds.

656

657 **PpKAI2L- G, -J, and -M mediate PpCCD8-derived (SL-related) responses**

658 In white light (Figure 7), mutants affecting (FK) and (GJM) clades showed phenotypes
659 intermediate between those of WT and *Ppccd8*. In the dark under control conditions (Figure 9),
660 the caulonema numbers of mutants in the (FK) and (GJM) clades were also intermediate
661 between those of WT and *Ppccd8* (see also Table 1). Based on the hypothesis that synthesis
662 and response mutants show similar phenotypes, genes from the (FK) and (GJM) clades are thus

663 the best candidates for PpCCD8-derived compound receptors. When the phenotypic responses
664 of these mutants to (+)-GR24 application were examined, plants with KO mutations in
665 *PpKAI2L-J* or *PpKAI2L-G/M* no longer responded to this compound (Figure 9C-D, *j*, and *gjm-*
666 *l*, and Supplemental Figure S13D, *gjm-3*, *gjm-5*, and *gjm-4*). By contrast, both *Δhfkj-1* and
667 *Δhfkj-2* mutants showed a significant response to (+)-GR24 application (Figure 9D and
668 Supplemental Figure S13C), indicating that KO mutations in both *PpKAI2L-F* and *PpKAI2L-*
669 *K*, or the deletion of *PpKAI2L-H*, do not abolish the response to the PpCCD8-derived
670 compound mimic, not even additively. The absence of a response in higher-order mutants where
671 either *PpKAI2L-J* or *PpKAI2L-G* were knocked out confirms the prominent roles of both genes
672 in the response to (+)-GR24. However, while as expected, the transcript levels of the
673 *Pp3c6_15020* response marker gene did not change in either *j* and *gjm-2* mutants following (+)-
674 GR24 application (Figure 10), the transcript levels of the *PpKUF1LA* gene increased in the *j*
675 mutant, suggesting a response to the SL analog. Thus, while the KO mutation of either
676 *PpKAI2L-J* or *PpKAI2L-G* was sufficient to abolish the phenotypic response in the dark, it did
677 not completely abolish the transcriptional response to (+)-GR24. Presumably, the mutation of
678 both *PpKAI2L-J* and *-G* or even all three genes (*-G-J-M*) is necessary to fully impair this
679 response. Another possibility is that the transcriptional markers, first identified using (±)-GR24
680 (Lopez-Obando et al. 2016a; Lopez-Obando et al. 2018) and Supplemental Figure S14), are not
681 fully specific for assaying the responses to enantiomers. This result could also suggest that the
682 transcriptional response, which was assessed far earlier than the phenotypic response (6 hours
683 versus 15 days), is perhaps more sensitive to a very slight activation of the PpCCD8-derived
684 SL pathway by PpKAI2L proteins.

685

686 **PpKAI2L proteins are likely receptors in two separate pathways, one dependent and the** 687 **other independent of the F-box protein PpMAX2**

688 Our previous study of the F-box protein PpMAX2 indicated that, in contrast to its homolog in
689 angiosperms, it is not involved in the response to PpCCD8-derived compounds (Lopez-Obando
690 et al. 2018). Like MAX2 in angiosperms, however, PpMAX2 plays a role in early gametophore
691 development and photomorphogenesis. We suggested that PpMAX2 could play a role in the
692 moss KL signalling pathway, but we lacked evidence for other actors in this pathway in *P.*
693 *patens*. The present study suggests that eu-KAI2 clade proteins are α/β hydrolases involved in
694 the same pathway as PpMAX2, since mutating these genes resulted in similar light-related
695 phenotypes to those of *Ppmax2*. Specific mimics for the moss KL are, however, still missing,

696 preventing us from obtaining further evidence that eu-KAI2 clade proteins are receptors of the
697 moss KL (Figure 11). Still, these results are consistent with the view that the KL pathway is
698 ancestral relative to the SL pathway, and that the ancestral role of MAX2 in the land plants
699 lineage is the transduction of the KL signal (Bythell-Douglas et al. 2017; Walker et al. 2019).
700 Such a KL pathway has been recently reported in *M. polymorpha*, further supporting this view
701 (Mizuno et al. 2021).

702

703 PpKAI2L-J and PpKAI2L-G proteins are likely receptors of PpCCD8-derived compounds,
704 which we suspect to be non-canonical SLs. Strikingly, these receptors are not particularly more
705 similar to D14 than other PpKAI2L proteins. As hypothesized earlier (Lopez-Obando et al.
706 2016a; Bythell-Douglas et al. 2017), the expansion of the PpKAI2L family in *P. patens* (and
707 not in other bryophytes such as *M. polymorpha*, which contains two *MpKAI2* genes), as in
708 parasitic angiosperms (Conn et al. 2015; Toh et al. 2015), may have allowed the emergence of
709 SL sensitivity (de Saint Germain et al. 2021b). Neofunctionalization of additional KAI2 copies
710 in *P. patens* ancestry towards SL perception is therefore a possible explanation for our findings
711 in this moss and would reveal a parallel evolutionary process, relative to the emergence of D14
712 in seed plants. We can also imagine that these neofunctionalized PpKAI2L proteins lost the
713 ability to interact with MAX2 in *P. patens*, and established a different protein network that
714 potentially integrates new factors such as an alternative F-box protein (Figure 11). The
715 remaining question is therefore to determine how SL signal transduction is achieved
716 downstream of perception by (GJM) clade PpKAI2L proteins.

717

718 Consequently, the search for proteins that interact with *P. patens* KL and PpCCD8-derived
719 compound receptors should be a priority in the near future. In particular, since SMXL proteins
720 are key members of both the SL and KL pathways in angiosperms and of the KL pathway in
721 *M. polymorpha* (Soundappan et al. 2015; Wang et al. 2015; Khosla et al. 2020; Mizuno et al.
722 2021), the specific involvement of PpSMXL homologs (four genes) is currently under
723 investigation by our group.

724

725 **METHODS**

726 **Plant materials and growth conditions**

727 The *Physcomitrium (Physcomitrella) patens* Gransden wild-type (WT) strain was grown as
728 previously described (Hoffmann et al. 2014; Lopez-Obando et al. 2018) under long day (16h)
729 conditions. Unless otherwise stated in the legends, the experiments were always carried out on
730 PpNO₃ medium (minimal medium described by Ashton et al., 1979) under the following control
731 conditions: 25 °C during the day and 23 °C at night, 50% humidity, quantum irradiance of ~80
732 μmol/m²/s using OSRAM L 36W/865 LUMILUX Cool daylight fluorescent tubes. Tissues
733 from young protonema fragments were multiplied prior to every experiment under the same
734 conditions but using medium with higher nitrogen content (PpNH₄ medium, PpNO₃ medium
735 supplemented with 2.7 mM NH₄ tartrate). For red light experiments, plants were grown on
736 PpNO₃ medium in Magenta pots at 25 °C, in continuous red light (~45 μmol μmol/m²/s).

737 **Germination assays of *Phelipanche ramosa* seeds**

738 *P. patens* WT plants were grown on PpNH₄ plates with cellophane disks for two weeks. The
739 plants (and cellophane) were transferred onto low-phosphate PpNO₃ medium (Phosphate buffer
740 was replaced with 1 g/L of MES buffer and the pH adjusted to 5.8) for another two weeks. *P.*
741 *patens* exudates were collected by transferring the plants (still on cellophane disks) onto plates
742 containing 10 mL distilled water and incubating them in the growth chamber with gentle
743 agitation. After 48 h, the exudates were pipeted and filtered (0.2 μm). The exudates (Figure 1A)
744 were diluted twice prior to testing their germination-stimulating activity on preconditioned
745 seeds of parasitic *P. ramosa* plants, as described previously (Pouvreau et al. 2013; Pouvreau et
746 al. 2021). Distilled water was used as a negative control and 0.1 μM (±)-GR24 as a positive
747 control. For each *P. ramosa* group, germination stimulant activities were normalized with the
748 respective negative and positive controls ((GS-GS⁻)/(GS⁺-GS⁻); with GSs: Germination
749 Stimulant activity of a bioassay, GS⁻: mean of GS activities of negative controls and GS⁺: mean
750 of GS activities of positive controls). For germination assays on plates (Figure 1B-C), WT and
751 *Ppccd8* were cultivated as above, and preconditioned *P. ramosa* seeds were placed onto the
752 plates after 10 days of phosphate starvation. Germinated and non-germinated seeds were
753 counted on three plates, with 7-10 microscope fields per plate, five days after adding *P. ramosa*
754 seeds. Results are expressed as percentage of germination.

755 **CRISPR-Cas9 mediated mutagenesis and homologous recombination in *P. patens***

756 *P. patens* mutants were obtained as described in (Lopez-Obando et al. 2016b), using CRISPR-
757 Cas9 technology. *PpKAI2L* coding sequences were used to search for CRISPR RNA (crRNA)
758 contiguous to a PAM motif recognized by *Streptococcus pyogenes* Cas9 (NGG), using the
759 webtool CRISPOR V4 against the *P. patens* genome Phytozome V9 (<http://crispor.tefor.net/>).
760 Guide crRNAs were chosen in each *PpKAI2L* gene, preferably in the first exon, to ideally obtain
761 the earliest nonsense mutation possible. When no guide could be designed in the first exon, it
762 was alternatively chosen to recognize a region in close proximity to the codon for one of the
763 last two residues of the catalytic triad (Figure 3). The same crRNA was used to target *PpKAI2L*-
764 *G* and *-M*. Small constructs containing each crRNA fused to either the proU6 or the proU3
765 snRNA promoters (Collonnier et al. 2017) between the attB1/attB2 Gateway recombination
766 sequences were synthesized by Twist Biosciences. These inserts were then cloned into
767 pDONR207 vectors (Invitrogen). Polyethylene glycol-mediated protoplast transformation was
768 performed with multiple pDONR207-sgRNA as described previously (Lopez-Obando et al.
769 2016b). Mutations in the *PpKAI2L* genes were confirmed by PCR amplification of *PpKAI2L*
770 loci around the recognition sequence of each guide RNA and by sequencing the PCR products.

771 The *PpKAI2L-H* (Δh) deletion mutant was obtained through homologous recombination. The
772 full coding sequence of *PpKAI2L-H* from the ATG to stop was replaced with a resistance
773 cassette. A 550 bp *PpKAI2L-H* 5' CDS flanking sequence was cloned into the pBNRF vector
774 (Thelander et al. 2007) cut with BstBI/XhoI. Then a 500 bp *PpKAI2L-H* 3' CDS flanking
775 sequence was cloned into the BNRF-*PpKAI2L-H* 5' construct digested with BcuI, so that the
776 kanamycin resistance cassette of the vector was flanked by the *PpKAI2L-H* 5' and 3'
777 sequences. *P. patens* WT protoplasts were transformed with the resulting construct as described
778 previously (Lopez-Obando et al. 2016b) and transformants selected on 50 mg/L
779 Geneticin/G418. Transient expression of the CRE recombinase (Trouiller et al. 2006) in a
780 confirmed transformant removed the resistance cassette to obtain the *Ppkai2L- Δh* mutant, as
781 described in Supplemental Figures S10 and S11.

782 **Phenotypic assays of *P. patens***

783 To measure diameters in the light, plants were grown in Petri dishes (6 to 8 plates per genotype),
784 starting from very small pieces of protonema, with 5 plants per plate, on $PpNO_3$ medium
785 (Ashton et al. 1979) covered with cellophane disks as previously described (Hoffmann et al.
786 2014). Thus, between 30 and 40 plants were grown per genotype. After 5 weeks of growth,

787 pictures of the plates were taken, and the ImageJ (<http://imagej.nih.gov/ij/>) manual selection
788 tool was used for each plant, allowing the area and Feret diameter to be measured. Analysis of
789 caulonema growth in the dark was performed in 24-well plates, starting from very small pieces
790 of protonema. For each genotype/treatment combination, 24 plants were grown, dispatched in
791 3 different plates. These plants were grown for ~two weeks under control conditions before
792 being placed vertically (\pm treatment) in the dark for ~10 days. A single picture of each well was
793 taken under Axio Zoom microscope (Zeiss) with a dedicated program. Caulonema were
794 counted and measured using ImageJ (<http://imagej.nih.gov/ij/>) (see also (Guillory and
795 Bonhomme 2021a)).

796 **Chemicals**

797 Racemic and pure enantiomers of GR24 and the (\pm)-GC242 probe were produced by F-D Boyer
798 (ICSN, France) (de Saint Germain et al. 2021a). Racemic CL was kindly provided by A.
799 Scaffidi (University of Western Australia, Perth, Australia). Chemicals were diluted in DMSO
800 or acetone as indicated in the figure legends. KAR₂ was purchased from Chiralix.

801 **RT-qPCR analysis**

802 Freshly ground WT (Gransden) tissues were inoculated in Petri dishes containing PpNO₃
803 medium, overlaid with a cellophane sheet. Protonema tissues were harvested after six days, 10
804 days or 15 days of growth under long-day conditions (see above). To obtain gametophores and
805 spores, plants regenerated from spores were cultivated for two weeks, transferred to Magenta
806 pots containing PpNO₃ medium (nine plants per pot), and cultivated under the same conditions
807 as above. Gametophores were harvested after 35 days. Both protonema and gametophore
808 samples were flash frozen in liquid nitrogen and stored at -80 °C until RNA extraction. At 35
809 days, the remaining pots were transferred to short-day conditions (15 °C, 100% hygrometry,
810 quantum irradiance of 15 $\mu\text{mol m}^{-2}\text{s}^{-1}$) for approximately two months until capsule maturity.
811 Capsules were surface sterilized (5 minutes in a mixture of 90% pure ethanol and 10% sodium
812 hypochlorite solution) and rinsed with sterile water. Each of the four biological replicates
813 consisted of 10-20 capsules from which spores were freed by mechanical disruption and
814 separated from capsule debris by filtering through a 25 μm nylon mesh. Spores were kept in
815 sterile water, flash frozen in liquid nitrogen, and stored at -80 °C until RNA extraction. For all
816 samples except spores, tissues were ground in liquid nitrogen using a mortar and pestle and

817 RNA was extracted from the samples and treated with DNase I using a Plant RNeasy Mini
818 extraction kit (Qiagen) following the manufacturer's instructions. Spores were recovered in 1
819 mL TRIzol reagent (Invitrogen) and crushed manually using fine pestles. RNA was separated
820 from cell debris and protein using chloroform, precipitated with isopropanol, and washed with
821 70% ethanol. The spore RNA pellets were dissolved in RLT buffer from a Qiagen Plant RNeasy
822 Mini kit and treated with DNase I on columns following the manufacturer's instructions. 500
823 ng of each RNA sample was used for reverse-transcription using RevertAid H Minus Reverse
824 Transcriptase from ThermoFisher. The quality of the obtained cDNA extracts was verified by
825 RT-PCR using the reference gene *PpAPT*. Quantitative RT-PCR was carried out in a total
826 volume of 5 μ L using SsoAdvanced Universal SYBR Green Supermix from BioRad and the
827 following program on QuantStudio™ 5 (ThermoFisher Scientific): initial denaturation at 95 °C
828 for 3 minutes, then 45 cycles of 95 °C for 10 seconds and 60 °C for 30 seconds. Using the CTi
829 (for the genes of interest) and CTref (mean for the two reference genes) values obtained, relative
830 expression (RE) was calculated as $RE = 2^{-CTi/2 - CTref}$.

831 **Constructs and generation of transgenic Arabidopsis lines**

832 The expression vectors for transgenic Arabidopsis were constructed using a MultiSite Gateway
833 Three-Fragment Vector Construction kit (Invitrogen). All of the *PpKAI2L* constructs were
834 tagged with a 6xHA epitope tag at their C-terminus. The transgenic lines were resistant to
835 hygromycin. The *AtD14* native promoter (0.8 kb) and *AtKAI2* native promoter (0.7 kb) were
836 amplified by PCR from Col-0 genomic DNA and cloned into *pDONR-P4P1R*, using Gateway
837 recombination (Invitrogen) (see Supplemental Table S2 for primers). The *AtD14* CDS and
838 *AtKAI2* CDS were PCR amplified from Col-0 cDNA, and the *PpKAI2L* CDS were PCR
839 amplified from *P. patens* cDNA and recombined into *pDONR221* (Invitrogen). *6xHA* with a
840 linker (gift from U. Pedmale, Cold Spring Harbor Laboratory) was cloned into *pDONR-P2RP3*
841 (Invitrogen). The suitable combination of promoters, CDS, and *6xHA* were cloned into the
842 *pH7m34GW* final destination vectors using the three fragment recombination system (Karimi
843 et al. 2007) and named pD14:CDS-6xHA or pKAI2:CDS-6xHA. The Arabidopsis *d14-1 kai2-*
844 *2* double mutant in the Landsberg background (gift from M. Waters, University of Western
845 Australia Perth) was transformed following the conventional floral dip method (Clough and
846 Bent 1998), with Agrobacterium strain GV3101. For all constructs, more than 12 independent
847 T1 lines were isolated, and between 2-4 representative single-insertion lines were selected in
848 the T2 generation. Two lines per construct were shown in these analyses. The phenotypic

849 analysis shown in Figure 6 and Supplemental Figure S9 was performed on segregating T3
850 homozygous lines.

851 **Protein extraction and immunoblotting**

852

853 Total protein extract was prepared from 8 to 10 ten-day-old Arabidopsis seedlings in Laemmli
854 buffer and boiled for 5 min. Total protein were separated by 10% SDS-PAGE and transferred
855 onto polyvinylidene difluoride membrane (Bio-Rad) probed with anti-HA primary antibody
856 (1:10000; SIGMA H9658-100UL Lot#128M4789V) and then anti-mouse-IgG-HRP secondary
857 antibody (1:10000; SIGMA A9044-2ML-100UL Lot#029M4799V). As a loading control, a
858 Ponceau staining or a Tubulin detection with anti-Tubulin primary antibody (1:10,000; SIGMA
859 T5168-2ML Lot#04M4760V) and then anti-mouse-IgG-HRP secondary antibody (1:10000;
860 SIGMA A9044-2ML-100UL Lot#029M4799V) was used.

861

862 **Arabidopsis hypocotyl elongation and cotyledon expansion assays**

863 Arabidopsis seeds were surface sterilized by consecutive treatments of 5 min in 70% (v/v)
864 ethanol and 0.05% (w/v) sodium dodecyl sulfate (SDS), and 5 min in 95% (v/v) ethanol. Then
865 seeds were sown on 0.25 X Murashige and Skoog (MS) medium (Duchefa Biochemie)
866 containing 1% agar, supplemented with 1 μ M (+)-GR24, (-)-GR24 or with 0.01% DMSO
867 (control). For the hypocotyl elongation assay, seeds were stratified at 4 °C (2 days in the dark),
868 exposed to white light (125-180 μ mol/m²/s) for 3 h, transferred to the dark for 21 h, and exposed
869 to low light (25-30 μ mol/m²/s) for 10 days at 21 °C. For the cotyledon expansion assay,
870 seedlings were grown in white light (125-180 μ mol/m²/s) for 4 days. Cotyledon area and
871 hypocotyl lengths were quantified using ImageJ (<http://imagej.nih.gov/ij/>)

872 **Quantification of Arabidopsis branching**

873 Experiments were carried out in the summer in a greenhouse under long photoperiods (15–16
874 h per day); daily temperatures fluctuated between 18 °C and 25 °C. Peak PAR levels were
875 between 700 and 1000 μ mol m⁻² s⁻¹. Plants were watered twice a week with tap water. The
876 number of rosette branches longer than 5 mm was counted when the plants were 40 days old.

877 **Protein expression and purification**

878 AtD14, RMS3, and AtKAI2 with cleavable GST tags were expressed and purified as described
879 in (de Saint Germain et al. 2016). For PpKAI2L protein expression, the full-length CDS from
880 *P. patens* were amplified by PCR using cDNA template and specific primers (see Supplemental
881 Table S2) containing a protease cleavage site for tag removal, and subsequently cloned into the
882 pGEXT-4T-3 expression vector. For PpKAI2L-L, the N-terminal 47 amino acids were
883 removed. The expression and purification of PpKAI2L proteins followed the same method as
884 for AtD14 and AtKAI2.

885 **Site-directed mutagenesis**

886 Site-directed mutagenesis of *PpKAI2L-H* was performed using a QuickChange II XL Site
887 Directed Mutagenesis kit (Stratagene), performed on pGEX-4T-3-PpKAI2L-H (see
888 Supplemental Table S2 for primers). Mutagenesis was verified by DNA sequencing.

889 **Enzymatic degradation of GR24 isomers by purified proteins**

890 The ligand (10 μ M) was incubated with and without purified RMS3/AtKAI2/PpKAI2L proteins
891 (5 μ M) for 150 min at 25 °C in PBS (0.1 mL, pH = 6.8) in the presence of (\pm)-1-indanol (100
892 μ M) as an internal standard. The solutions were acidified to pH = 1 by adding trifluoroacetic
893 acid (2 μ L) to quench the reaction and centrifuged (12 min, 12,000 rpm). The samples were
894 then subjected to RP-UPLC-MS analyses. The instrument used for all the analyses was an Ultra
895 Performance Liquid Chromatography system equipped with a PDA and Triple Quadrupole
896 mass spectrometer Detector (Acquity UPLC-TQD, Waters, USA). RP-UPLC (HSS C₁₈ column,
897 1.8 μ m, 2.1 mm \times 50 mm) was carried out with 0.1% formic acid in CH₃CN and 0.1% formic
898 acid in water (aq. FA, 0.1%, v/v, pH 2.8) as eluents [10% CH₃CN, followed by a linear gradient
899 from 10 to 100% of CH₃CN (4 min)] at a flow rate of 0.6 mL/min. The detection was performed
900 by PDA and using the TQD mass spectrometer operated in Electrospray ionization positive
901 mode at 3.2 kV capillary voltage. The cone voltage and collision energy were optimized to
902 maximize the signal, with a cone voltage of 20 V and collision energy of 12 eV. The collision
903 gas was argon at a pressure maintained near $4.5 \cdot 10^{-3}$ mBar.

904 **Enzymatic kinetic assays**

905 Enzyme assays with pro-fluorescent probes and *p*-nitrophenyl acetate were performed as
906 described in (de Saint Germain et al. 2016), using a TriStar LB 941 Multimode Microplate
907 Reader from Berthold Technologies.

908 **nanoDifferential Scanning Fluorimetry (nanoDSF)**

909 Proteins were diluted in Phosphate buffer saline (PBS) (100 mM Phosphate, pH 6.8, 150 mM
910 NaCl) to ~10 μ M concentration. Ligand was tested at the concentration of 200 μ M. The
911 intrinsic fluorescence signal was measured as a function of increasing temperature in a
912 Prometheus NT.48 fluorimeter (NanotemperTM), with 55% excitation light intensity and 1
913 $^{\circ}$ C/minute temperature ramp. Analyses were performed on capillaries filled with 10 μ L of the
914 respective samples. The intrinsic fluorescence signal was expressed as the 350 nm/330 nm
915 emission ratio, which increases as the proteins unfold, and was plotted as a function of
916 temperature. The plots show one of the three independent data collections that were performed
917 for each protein.

918 **Intrinsic tryptophan fluorescence assays**

919 Intrinsic tryptophan fluorescence assays and determination of the dissociation constant K_d were
920 performed as described in (de Saint Germain et al. 2016), using a Spark[®] Multimode
921 Microplate Reader from Tecan.

922 **Direct electrospray ionization – mass spectrometry of PpKAI2L proteins (ESI)-MS under** 923 **denaturing conditions**

924 Mass spectrometry measurements were performed with an electrospray Q-TOF mass
925 spectrometer (Waters) equipped with a Nanomate device (Advion, Inc.). The HD_A_384 chip
926 (5 μ m I.D. nozzle chip, flow rate range 100–500 nL/min) was calibrated before use. For
927 ESI–MS measurements, the Q-TOF instrument was operated in RF quadrupole mode with the
928 TOF data being collected between m/z 400–2990. Collision energy was set to 10 eV, and argon
929 was used as the collision gas. PpKAI2L proteins (50 μ M) in 50 mM ammonium acetate (pH
930 6.8) in the presence or absence of GR24 enantiomers (500 μ M) were incubated for 10 min at
931 room temperature before denaturation in 50% acetonitrile and 1% formic acid. The solutions
932 were directly injected for Mass spectra acquisition or digested before LC-MS/MS analyses.

933 Mass Lynx version 4.1 (Waters) and Peakview version 2.2 (Sciex) software were used for data
 934 acquisition and processing, respectively. Deconvolution of multiply charged ions was
 935 performed by applying the MaxEnt algorithm (Sciex). The average protein masses were
 936 annotated in the spectra, and the estimated mass accuracy was ± 2 Da. External calibration was
 937 performed with NaI clusters (2 $\mu\text{g}/\mu\text{L}$, isopropanol/ H_2O 50/50, Waters) in the acquisition m/z
 938 mass range.

939 Homology modeling

940 Figure 5B showing a superimposition model was prepared using PyMOL (DeLano Scientific)
 941 with the crystal structures of AtD14 (PDB ID 4IH4) and PpKAI2L-H (PDB ID 6AZD).

942 Statistical analyses

943 ANOVA, Kruskal-Wallis, Mann-Whitney, *post-hoc* Dunnett, *post-hoc* Dunn, and Tukey
 944 multiple comparisons tests (details in figure legends) were carried out either in R version 3.6.3
 945 or in GraphPad Prism version 8.4.2. Unless otherwise defined, the statistical significance scores
 946 used were as follow: $*0.01 \leq P < 0.05$, $**0.001 \leq P < 0.01$, $***P < 0.001$. Same letters scores indicate
 947 that $P \geq 0.05$ (non-significant differences). All statistical analyses are described in Supplemental
 948 File S1.

949 Accession numbers

950 Sequence data from this article can be found in the GenBank/Phytozome libraries under the
 951 accession numbers described in Supplemental Table S3.

952 **Table 1** Overview of *Ppkai2L* mutant phenotypes
 953

Genotypes	Diameter (Figure 7)	Gametophore size (Figure 8, Supplemental Figure S12)	Caulonema number (Figure 2, 9, Supplemental Figure S13)	Response to (+)-GR24		Response to (-)-GR24	
				Caulonema number (Figure 2, 9, Supplemental Figure S13)	Transcriptional markers (Figure 10)	Caulonema number (Figure 2, 9, Supplemental Figure S13)	Transcriptional markers (Figure 10)
WT	WT	WT	WT	↘	↗	↗ or =	=
<i>Ppccd8</i>	>WT	<WT	>WT	↘	↗	= or ↘	↗
<i>Ppmax2-1</i>	<WT	>WT	<WT	↘	=	= or ↘	=
<i>eu-kai2</i>	<WT	>WT	≤WT	↘	↗	= or ↘	=
(fk)*	≥WT	≈WT	≥WT	↘	Not tested	=	Not tested

<i>(hil)</i>	≤WT	≤WT	≤WT	↘	Not tested	=	Not tested
<i>(gjm)</i>	≥WT	≤WT	>WT	=	↗ or =	= or ↘	↗ or =

954 * In combination with *j* and/or *h* mutations

955

956

957 **Supplemental Data**

958 **Supplemental Figure S1.** Sequence alignment of *Physcomitrium patens* (Pp) PpKAI2L
959 proteins with AtD14 and AtKAI2 proteins from *Arabidopsis thaliana* (At).

960 **Supplemental Figure S2.** Expression of *PpKAI2L* genes throughout *P. patens* vegetative
961 development.

962 **Supplemental Figure S3.** Nano Differential Scanning Fluorimetry (nanoDSF) analysis of the
963 PpKAI2L protein response to GR24 isomers.

964 **Supplemental Figure S4.** Intrinsic tryptophan fluorescence shows that GR24 isomers bind to
965 PpKAI2L proteins with different affinities.

966 **Supplemental Figure S5.** PpKAI2L hydrolysis activity towards *p*-NPA.

967 **Supplemental Figure S6.** PpKAI2L enzymatic activities confirm stereoselectivity.

968 **Supplemental Figure S7.** Characterization of (±)-GC242 profluorescent probe activity on
969 moss and PpKAI2L enzymatic activities towards (±)-GC242.

970 **Supplemental Figure S8.** Mass spectrometry characterization of covalent PpKAI2L-ligand
971 complexes.

972 **Supplemental Figure S9.** Complementation assays of the *Arabidopsis Atd14-1 kai2-2* double
973 mutant.

974 **Supplemental Figure S10.** Mutations obtained in all 13 *PpKAI2L* genes.

975 **Supplemental Figure S11.** The *Ppkai2L-Δh* mutant.

976 **Supplemental Figure S12.** Gametophore heights of *Ppkai2L* mutants in red light.

977 **Supplemental Figure S13.** Phenotypic response of *Ppkai2L* mutants to (-)-GR24 and (+)-
978 GR24 application: caulonema number in the dark.

979 **Supplemental Figure S14.** Transcriptional response of WT and *Ppccd8* to (±)-GR24
980 application: use of the *Pp3c6_15020* marker gene.

981 **Supplemental Table S1.** Mutants used in this study.

982 **Supplemental Table S2.** Oligonucleotides used in this study.

983 **Supplemental Table S3.** List of gene sequences used in this study.

984 **Supplemental File S1.** Results of statistical tests.

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1002

1003 **AUTHOR CONTRIBUTIONS**

1004 SB, AdSG, ML-O and CR designed the project. ML-O, AG, F-DB, DC, BH, PLB, J-BP,
1005 AdSG and SB conducted experiments. AG, ML-O, F-DB, DC, PLB, J-BP, PD, CR, AdSG
1006 and SB analyzed the data. AG and SB wrote the manuscript, with essential contributions from
1007 ML-O, F-DB, PD, CR and AdSG.

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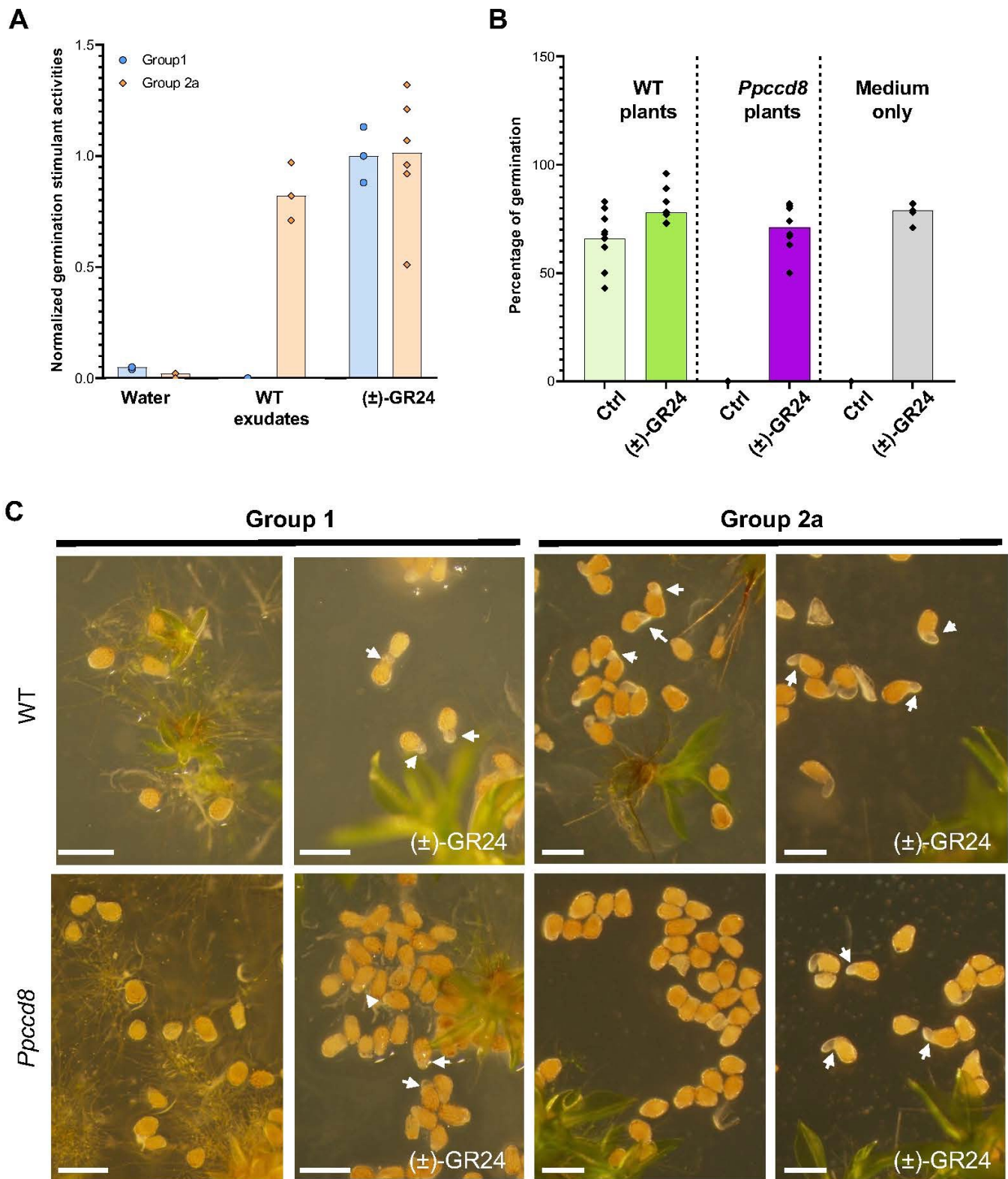


Figure 1. PpCCD8-derived compounds are germination stimulants (GS) of a specific group of *Phelipanche ramosa*.

(A) Germination Stimulant activities of *P. patens* exudates on *P. ramosa* group 1 and 2a seeds relative to 0.1 μ M (\pm)-GR24. (n = 6). **(B)** Percentage of germination of seeds of *P. ramosa* group 2a on plates with *P. patens* WT, *Ppccd8* mutant plants, or with culture medium only, with or without 0.1 μ M (\pm)-GR24 (n = 6). **(C)** Seeds from *P. ramosa* group 1 (left) and group 2a (right) on plates with WT (top) or *Ppccd8* mutant plants (bottom), with or without 0.1 μ M (\pm)-GR24. Arrows indicate germinating seeds. Scale bar = 0.5 mm.

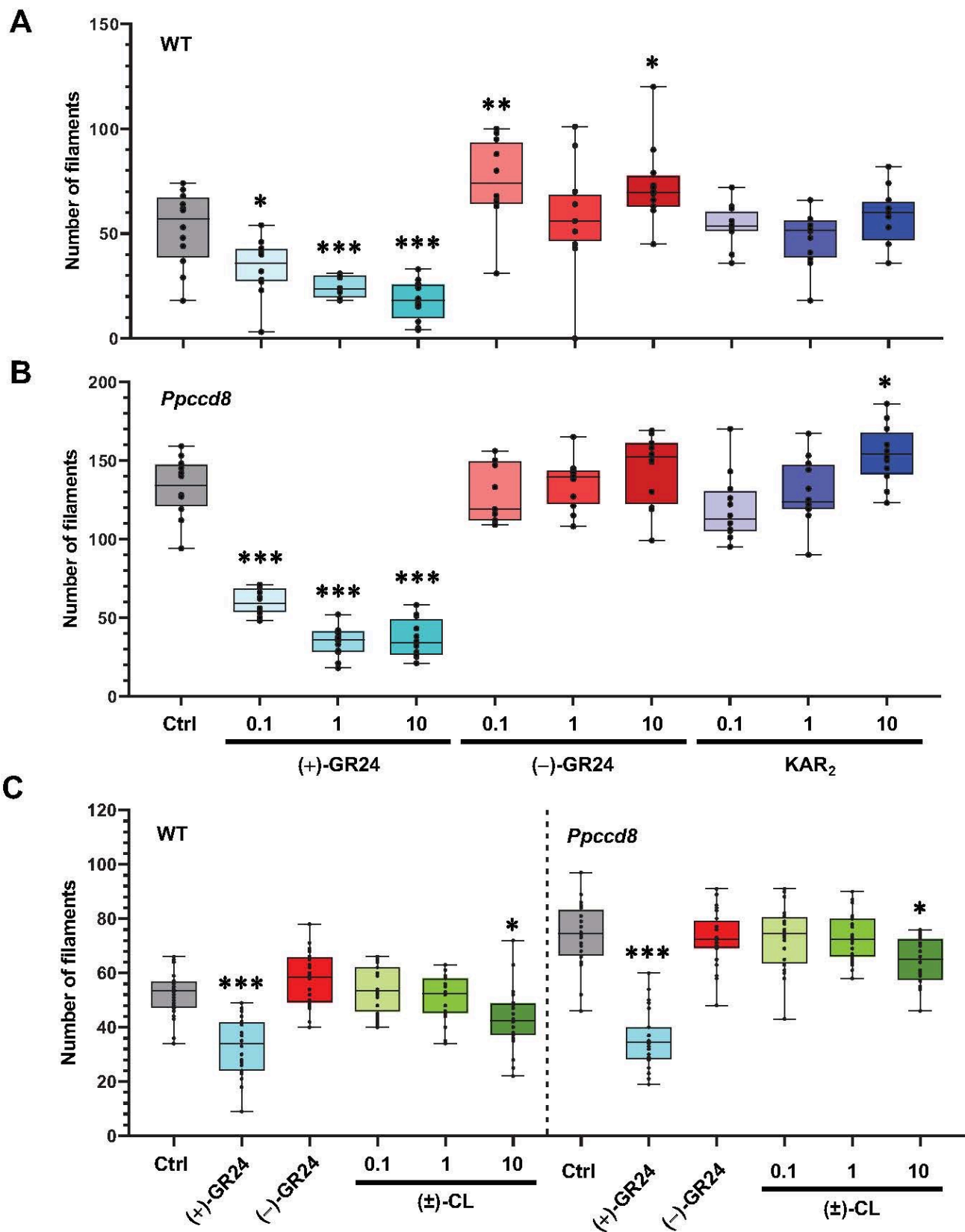


Figure 2. Phenotypic response to (+)- and (-)-GR24 enantiomers and natural compounds: number of caulonema filaments.

Caulonema filaments were counted for WT (**A**) and the *Ppccd8* SL synthesis mutant (**B**) grown for 10 days vertically in the dark, following application of increasing concentrations (0.1, 1 and 10 μ M) of (+)-GR24 (blue boxes), (-)-GR24 (red boxes) and KAR₂ (dark blue boxes). Control (Ctrl): 0.01% DMSO. (**C**) Caulonema filament numbers of WT and *Ppccd8* mutant grown for 10 days vertically in the dark, with increasing concentrations (0.1, 1 and 10 μ M) of (\pm)-CL (green boxes). Control (Ctrl): 0.01% DMSO. (+)-GR24 (blue boxes) and (-)-GR24 (red boxes) were applied at 1 μ M. Significant differences between control and treated plants within a genotype based on an ANOVA, followed by a Dunnett *post-hoc* test for multiple comparisons: *** P <0.001; ** P <0.01; * P <0.05; For each genotype/treatment combination, n = 24 plants grown in three different well-plates. Whiskers refer to minimum and maximum values, bars inside the boxplot to the median.

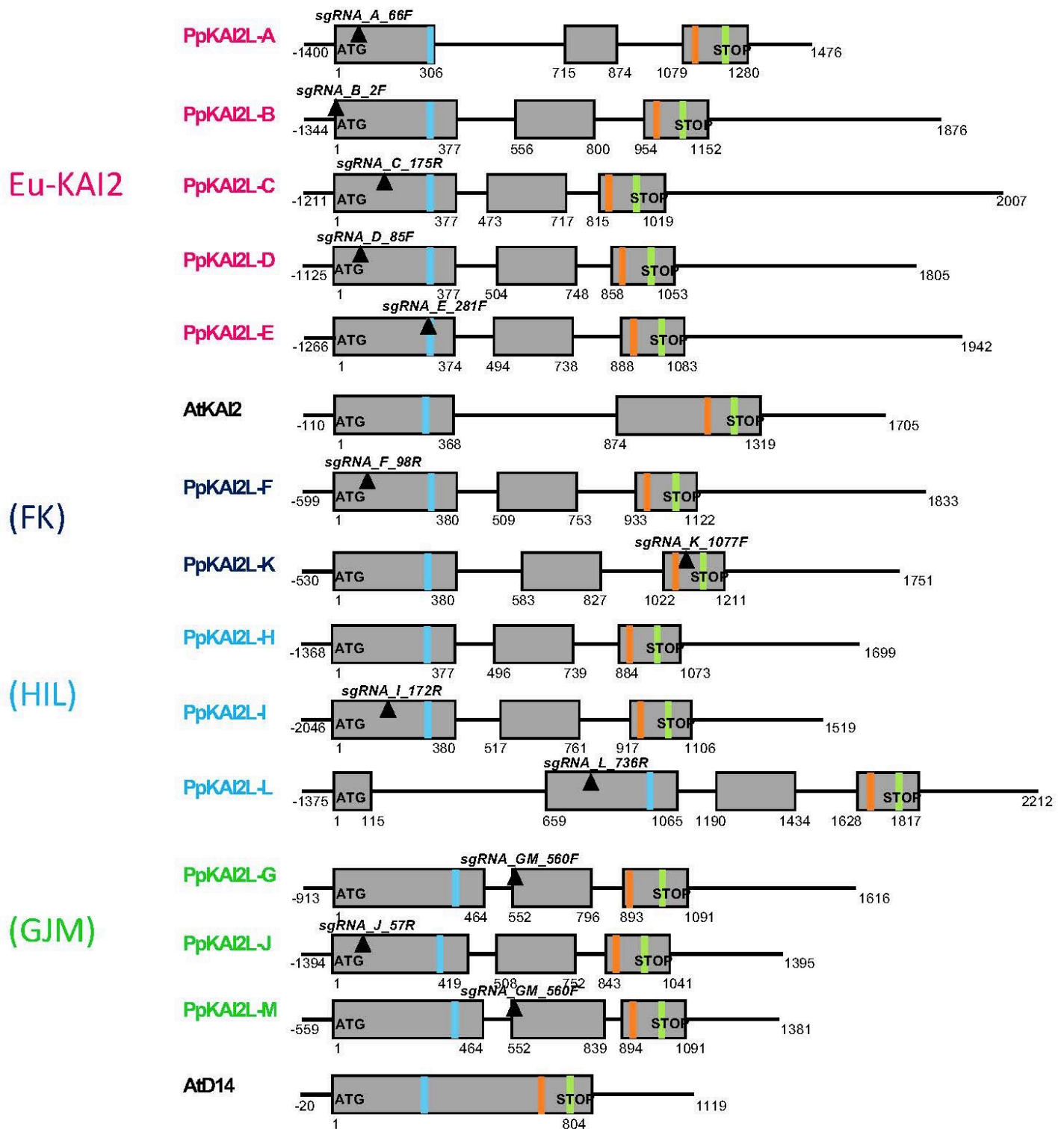
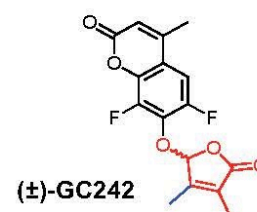
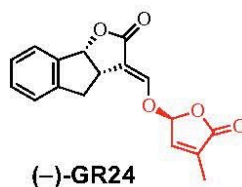
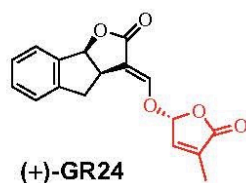


Figure 3. Gene models of the *PpKAI2L* gene family showing the catalytic triad and position of crRNAs.

Genes are presented as organized in subclades. Exons are displayed as grey boxes, introns and UTRs are depicted as thin black lines. Start and Stop codons are written in bold, while plain text indicates the start/end position for each feature, relative to the start codon. Only 5'-UTR regions are not represented true to scale. Transcript versions that were used are V3.1 (downloaded from the Phytosome website in September 2019) for all *PpKAI2L* genes except for *PpKAI2L-B*, *PpKAI2L-H* and *PpKAI2L-M* (V3.2). Regions targeted by crRNAs are indicated as black inverted triangles, with their names written in bold italic. Light blue, orange and light green bands represent respectively the codons for the S, D and H residues of the catalytic triad (see Supplemental Table S3 for reference sequences).

A**B**

Related figure	Protein stability assay ^a			Binding affinity determination (K _d) ^b		GR24 cleavage assay ^c		SL probe cleavage assay ^d	Covalent adduct detection ^e	
	Supplemental Figure S3	Supplemental Figure S3		Supplemental Figure S4		Supplemental Figure S7		Supplemental Figure S8	Supplemental Figure S9	
Ligand	Mock	(+)-GR24	(-)-GR24	(+)-GR24	(-)-GR24	(+)-GR24	(-)-GR24	(±)-GC242	(+)-GR24	(-)-GR24
AtKAI2	46.7 °C	46.6 °C	42.4 °C	No binding detected	45 ± 9 μM	1.1%	28.0%	Residual activity	No	Yes
PpKAI2L-C	49.7 °C	50.5 °C	42.9 °C	Not tested	Not tested	5.3%	21.3%	Residual activity	No	Yes
PpKAI2L-D	49.9 °C	49.5 °C	39.9 °C	No binding detected	92 ± 10 μM	0.3%	16.2%	Residual activity	No	Yes
PpKAI2L-E	53.2 °C	54.0 °C	42.3 °C	No binding detected	39 ± 10 μM	1.3%	20.5%	Residual activity	No	Yes
PpKAI2L-F	44.0 °C	44.6 °C	45.0 °C	No binding detected	No binding detected	4.1%	4.9%	Residual activity	No	Small amount
PpKAI2L-K	44.9 °C	No signal	No signal	107 ± 11 μM	41 ± 43 μM	2.2%	19.9%	Residual activity	Yes	Yes
PpKAI2L-H	No signal	No signal	No signal	104 ± 16 μM	273 ± 47 μM	26.2%	69.2%	Michaelian kinetic	No	No
PpKAI2L-L	43.2 °C	44.1 °C	44.3 °C	Not tested	Not tested	8.3%	21.4%	Residual activity	Yes	Yes
AtD14	53.0 °C	46.7 °C	47.2 °C	23 ± 9 μM	94 ± 3 μM	100.0% ¹	100.0% ¹	Single turnover kinetic	Yes ²	Yes ²

^a Protein stability assays were performed by nanoDSF. Protein denaturation temperature (T_m) is indicated in the absence (mock) or presence of ligand ((+)-GR24 and (-)-GR24). A lower temperature (in purple) indicates protein destabilization and a higher temperature (in green) indicates a protein stabilization. A change of protein stability was considered significant when at least 0.5°C difference was observed, according to the manufacturer recommendation.

^b Affinity of the protein for the different ligands evaluated by intrinsic fluorescence assay.

^c PpKAI2L (5 μM) enzymatic activity towards (+)-GR24 and (-)-GR24 at 10 μM, evaluated by calculating the remaining percentage of GR24 isomers in comparison to the amount of ligand incubated in the same condition without protein, after 150 min.

^d Enzymatic kinetic profile for each PpKAI2L protein (330 nM), evaluated by recording (±)-GC242 (4 μM) probe cleavage.

^e Covalent PpKAI2L-ligand complexes were investigated by mass spectrometry. "Yes" indicates a detected mass increment of 96.3 Da.

¹ For this assay RMS3 was used instead of AtD14.

² These data are not shown here, but were previously reported (de Saint Germain et al. 2016).

Figure 4. Biochemical characterization of the PpKAI2L proteins.

(A) Chemical structures of the (+)-GR24 and (-)-GR24 enantiomers, and the (±)-GC242 profluorescent probe. (B) Summary of biochemical assays for testing interactions between PpKAI2Ls and SL analogs and probe.

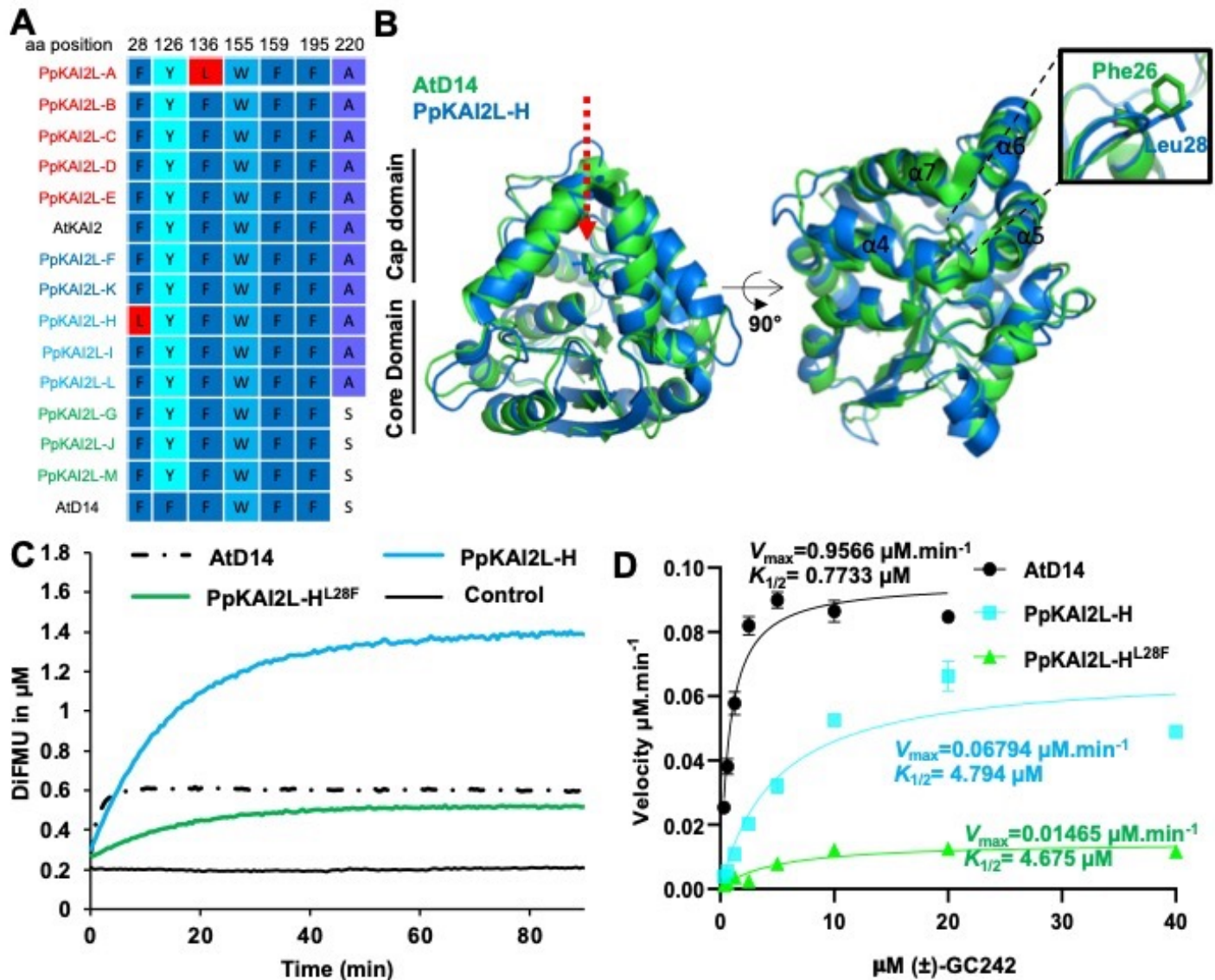


Figure 5. PpKAI2L-H enzymatic activity reveals special feature.

(A) Sequence alignment of active site amino acid residues in PpKAI2L proteins. Amino acids that differ from AtKAI2 are colored in red. A fully expanded alignment can be found in Supplemental Figure S1. (B) Superimposition of the AtD14 and PpKAI2L-H structure showing the position of Phe²⁸ and Leu²⁸ residues. Zoom onto helices $\alpha 4$ and $\alpha 5$. (C) Enzyme kinetics for PpKAI2L-H, PpKAI2L-H^{Leu28Phe} and AtD14 incubated with (\pm)-GC242. Progress curves during probe hydrolysis, monitored at 25 °C (λ_{em} 460 nm). Protein catalyzed hydrolysis with 330 nM of protein and 20 μM of probe. These traces represent one of the three replicates and the experiments were repeated twice. (D) Hyperbolic plot of pre-steady state kinetics reaction velocity with (\pm)-GC242. Initial velocity was determined with pro-fluorescent probe concentrations from 0.310 μM to 40 μM and protein at 400 nM. Points are the mean of three replicates and error bars represent SE. Experiments were repeated at least three times.

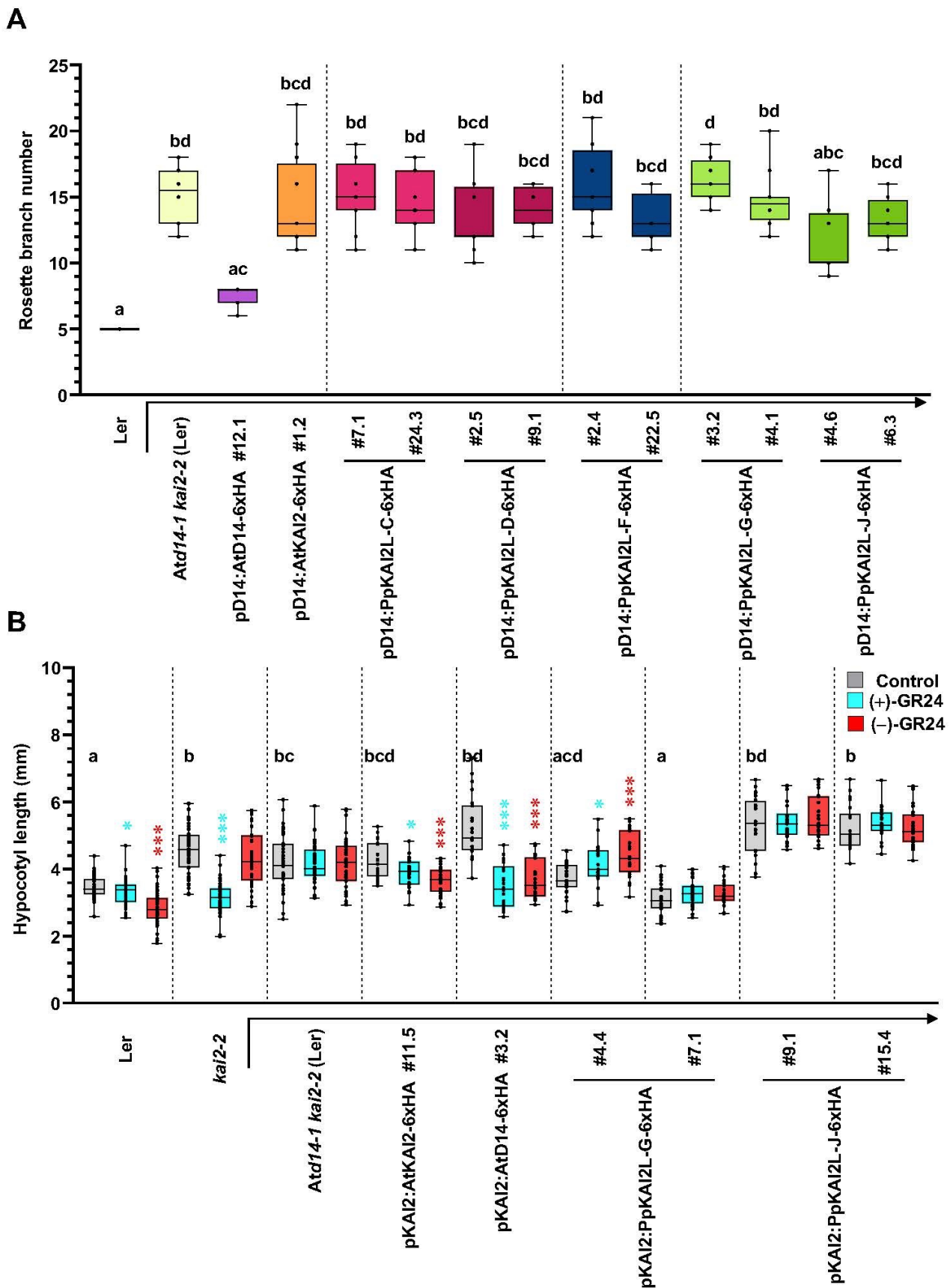


Figure 6. Complementation assays of the Arabidopsis *Atd14-1 kai2-2* double mutant with *PpKAI2L* genes.

Complementation assays of the *Atd14-1 kai2-2* mutant (in Ler background), transformed using the *AtD14* promoter (A) or *AtKAI2* promoter (B) controlling the expression of *AtD14*, *AtKAI2* (controls) or *PpKAI2L* CDS (as noted below the graph). Ler (WT), *kai2-2* and *Atd14-1 kai2-2* mutants are shown as controls. (A) Number of rosette axillary branches per plant. Results are mean of $n = 12$ plants per genotype, except for Ler and lines pD14:AtD14 #12.1 and pD14:PpKAI2L-C-#24.3 ($n = 11$). Different letters indicate significantly different results between genotypes based on a Kruskal-Wallis test ($P < 0.05$, Dunn *post hoc* test). (B) Hypocotyl length under low light, on $\frac{1}{2}$ MS medium with DMSO (control, grey bars) 1 μM (+)-GR24 (turquoise bars) or 1 μM (-)-GR24 (red bars). Results of $n=20$ to 24 seedlings. Whiskers refer to minimum and maximum values, bars inside the boxplot to the median. Different letters indicate significantly different results between genotypes in control conditions based on a Kruskal-Wallis test ($P < 0.05$, Tukey *post hoc* test). Asterisks in turquoise and red give the statistical significance of response to (+)-GR24 and (-)-GR24 respectively (Mann-Whitney tests, * $0.01 \leq P < 0.05$; *** $P \leq 0.001$).

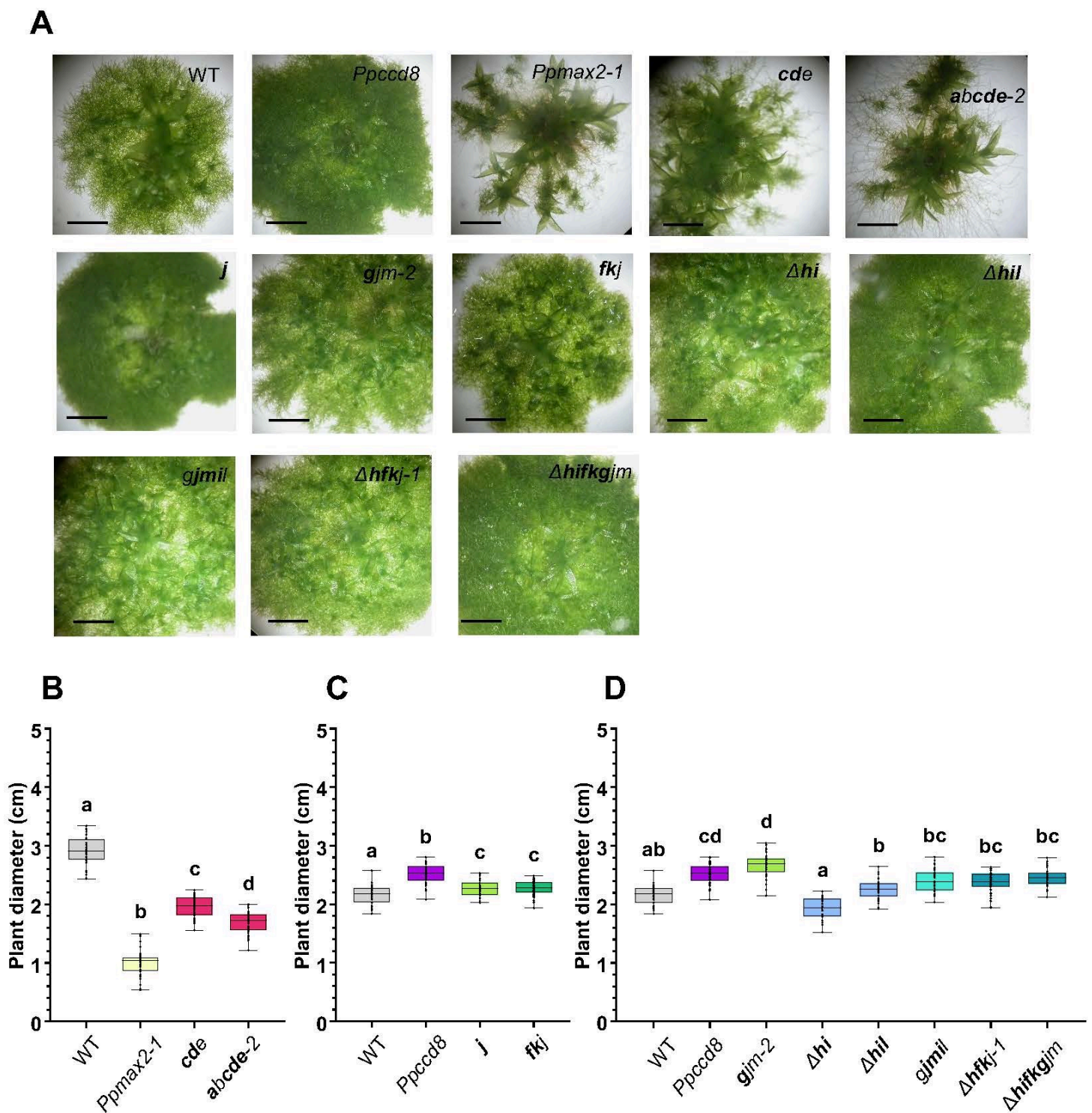


Figure 7. Phenotype of the *Ppkai2L* mutants in light.

(A) 3-week-old plants. Scale bar = 2 mm (B) and (C): 30-day-old plants, (n > 40); (D): 28-day-old plants, (n = 30). (B), (C) and (D): All plants were grown on cellophane disks. Whiskers refer to minimum and maximum values, bars inside the boxplot to the median. Letters indicate statistical significance of comparisons between all genotypes based on a Kruskal-Wallis test followed by a Dunn *post hoc* test ($P < 0.05$). Mutant genotypes carry mutations as described in Supplemental Figure S10 and Supplemental Table S1. Bold letters indicate null mutations.

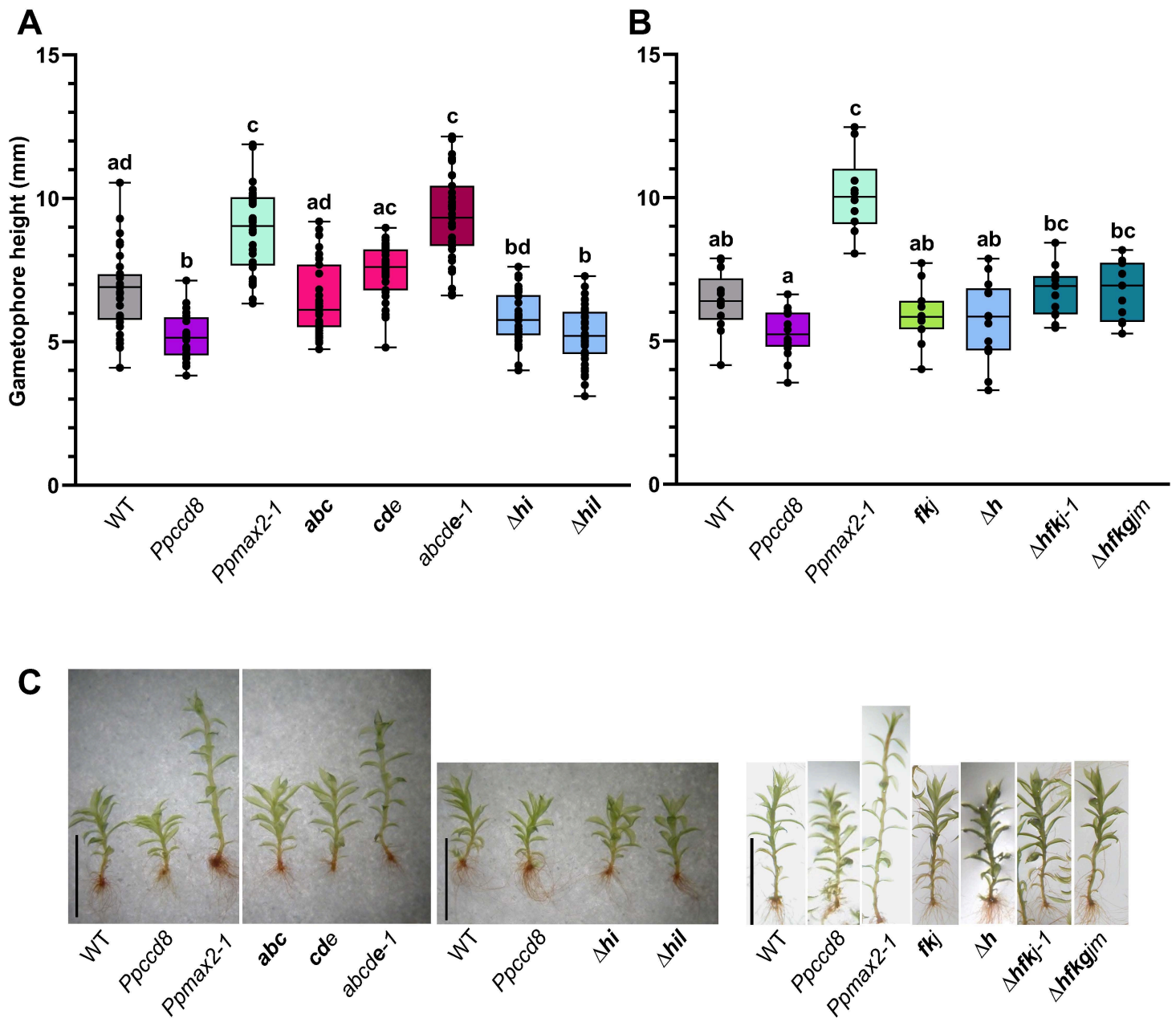


Figure 8. *Ppkai2L* mutant gametophores in red light.

(A) Gametophore height of *Ppkai2L* mutants affecting eu-KAI2 clade genes (*abc*; *cde*; *abcde-1*) and clade (HIL) genes (Δhi and Δhil), compared to that of WT, *Ppccd8* and *Ppmax2-1* mutants, following two months growth under red light. Box plots of $n = 32-36$ gametophores, grown in 3 Magenta pots, harboring between 15 and 25 leaves. Whiskers refer to minimum and maximum values, bars inside the boxplot to the median. Letters indicate statistical significance of comparisons between all genotypes based on a Kruskal-Wallis test followed by a Dunn *post hoc* test ($P < 0.05$). **(B)** Gametophore height of *Ppkai2L* mutants affecting clade (HIL) gene (Δh), both clades (FK) and (JGM) (*fkj*) and all three clades (HIL) (FK) and (JGM) ($\Delta hfkj-1$ and $\Delta hfkjlm$). Box plots of $n = 11-15$ gametophores, grown in 3 Magenta pots, harboring between 15 and 25 leaves. Whiskers refer to minimum and maximum values, bars inside the boxplot to the median. Letters indicate statistical significance of comparisons between all genotypes based on a Kruskal-Wallis test followed by a Dunn *post hoc* test ($P < 0.05$). **(C)** Examples of gametophores following a 2-month growth under red light, from WT, *Ppccd8*, *Ppmax2-1*, and *Ppkai2L* mutants as shown in (A) and (B). Scale bar = 5 mm. Mutant genotypes carry mutations as described in Supplemental Figure S10 and Supplemental Table S1. Bold letters indicate null mutations.

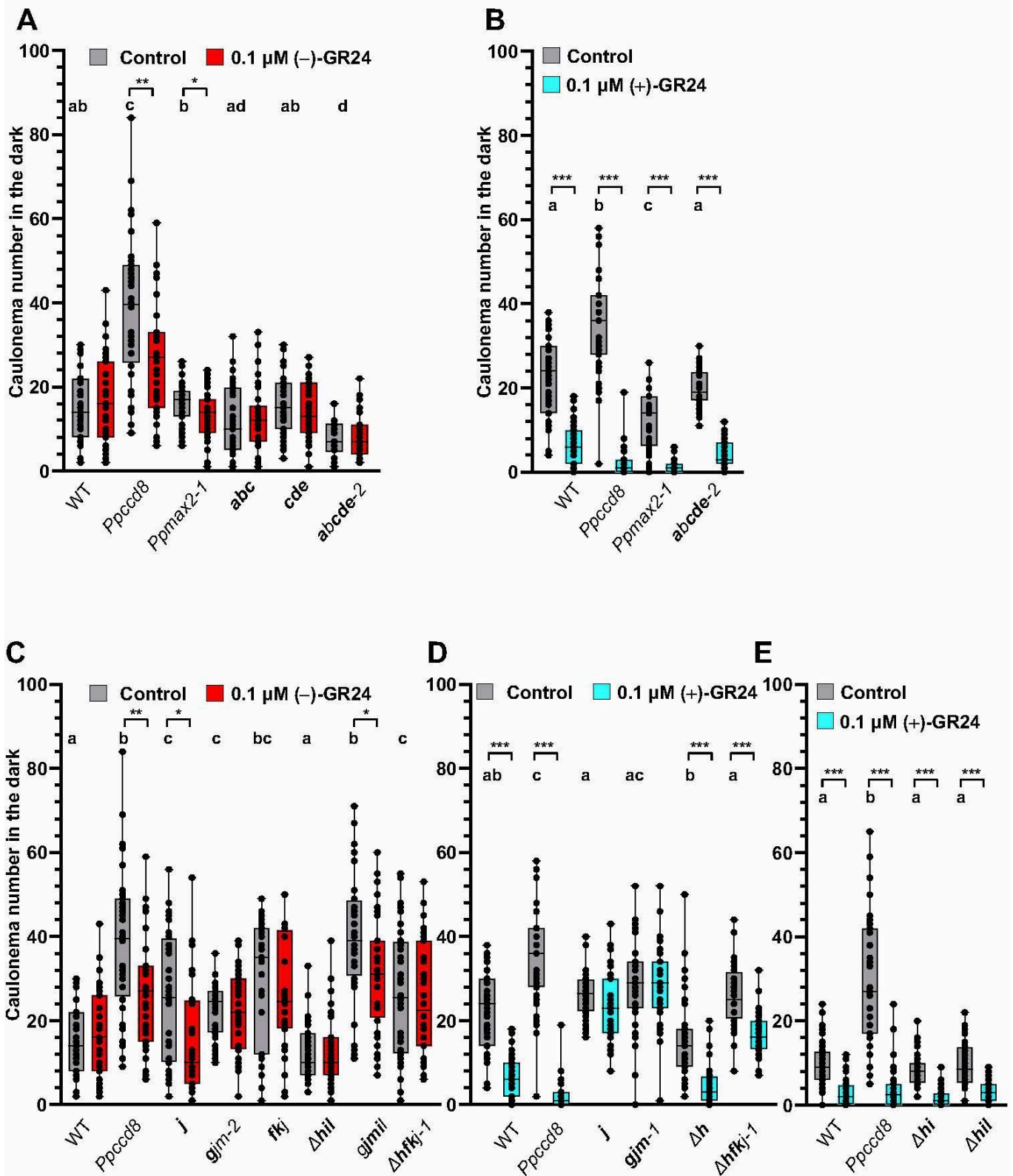


Figure 9. Phenotypic response of *Ppkai2L* mutants to (-)-GR24 and (+)-GR24 application: caulonema number in the dark.

(A-B) Caulonema numbers from mutants affecting eu-KAI2 clade genes following application of 0.1 μM (-)-GR24 (in red, A) or 0.1 μM (+)-GR24 (in turquoise, B). DMSO was applied as a control treatment (dark grey). WT and both the *Ppccd8* and *Ppmax2-1* mutants were used as control genotypes. (C-E) Caulonema numbers from mutants affecting (GJM) clade genes: *j*; *gjm-2*; *gjm-1*; (FK) and (GJM) clade genes: *fkj*, (HIL) clade genes: *Δh*, *Δhi*, *Δhil*, (HIL) and (GJM) clade genes: *gjmil*, or all 3 clades genes: *Δhfkj-1*, following application of 0.1 μM (-)-GR24 (in red, C) or 0.1 μM (+)-GR24 (in turquoise, D, E). 0.01% DMSO was applied as a control treatment (dark grey). WT and the *Ppccd8* mutant were used as control genotypes. Mutant genotypes carry mutations described in Supplemental Figure S10 and Supplemental Table S1. Bold letters indicate null mutations. For each genotype/treatment combination, caulonema were counted after two weeks in the dark, from 24 individuals (n=24). Whiskers refer to minimum and maximum values, bars inside the boxplot to the median. Letters indicate statistical significance of comparisons between all genotypes in control conditions, based on a Kruskal-Wallis test followed by a Dunn *post hoc* test ($P < 0.05$). Significant differences between control and treated plants within a genotype based on a Kruskal-Wallis test, followed by a Dunn *post-hoc* test for multiple comparisons: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

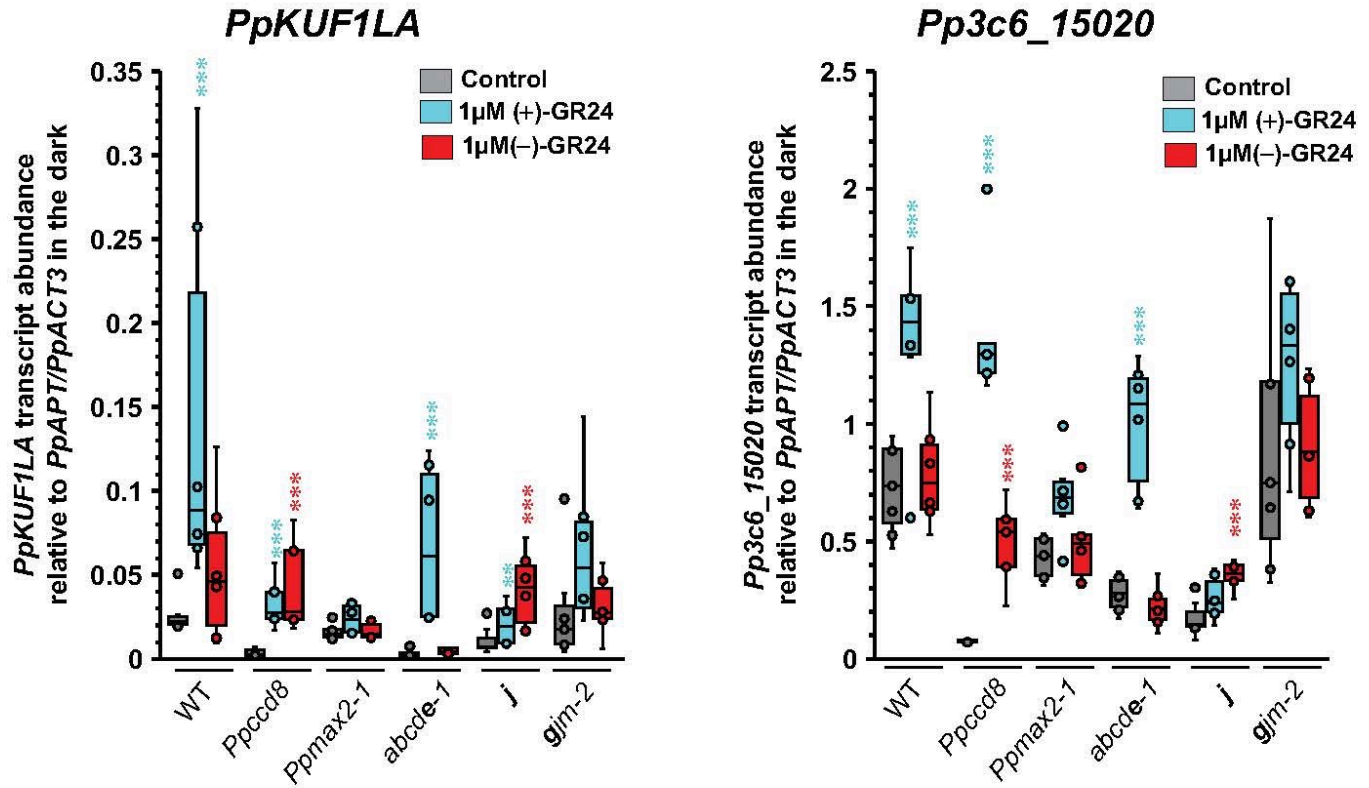


Figure 10. *Ppkai2L* mutant transcriptional response to (+)- and (-)-GR24.

Transcript abundance analysis of the SL-responsive genes *PpKUF1LA* and *Pp3c6_15020*, in WT, *Ppccd8*, *Ppmax2-1* and *Ppkai2L* mutants *abcde-1* (eu-KAI2 clade), *j* and *gjm-2* ((GJM) clade), after a 6-hour treatment in the dark with DMSO (control, grey), 1 μM (+)-GR24 (turquoise) or 1 μM (-)-GR24 (red). Box plots of at least four biological repeats are shown ($n \geq 4$), relative to mean (*PpAPT-PpACT3*) transcript abundance. Whiskers refer to lower and upper quartiles, bars inside the box to the median. Kruskal-Wallis test followed by a Dunn *post hoc* test (Asterisks indicate significant differences between the treatment and the corresponding DMSO control ** $P < 0.01$ and *** $P < 0.001$).

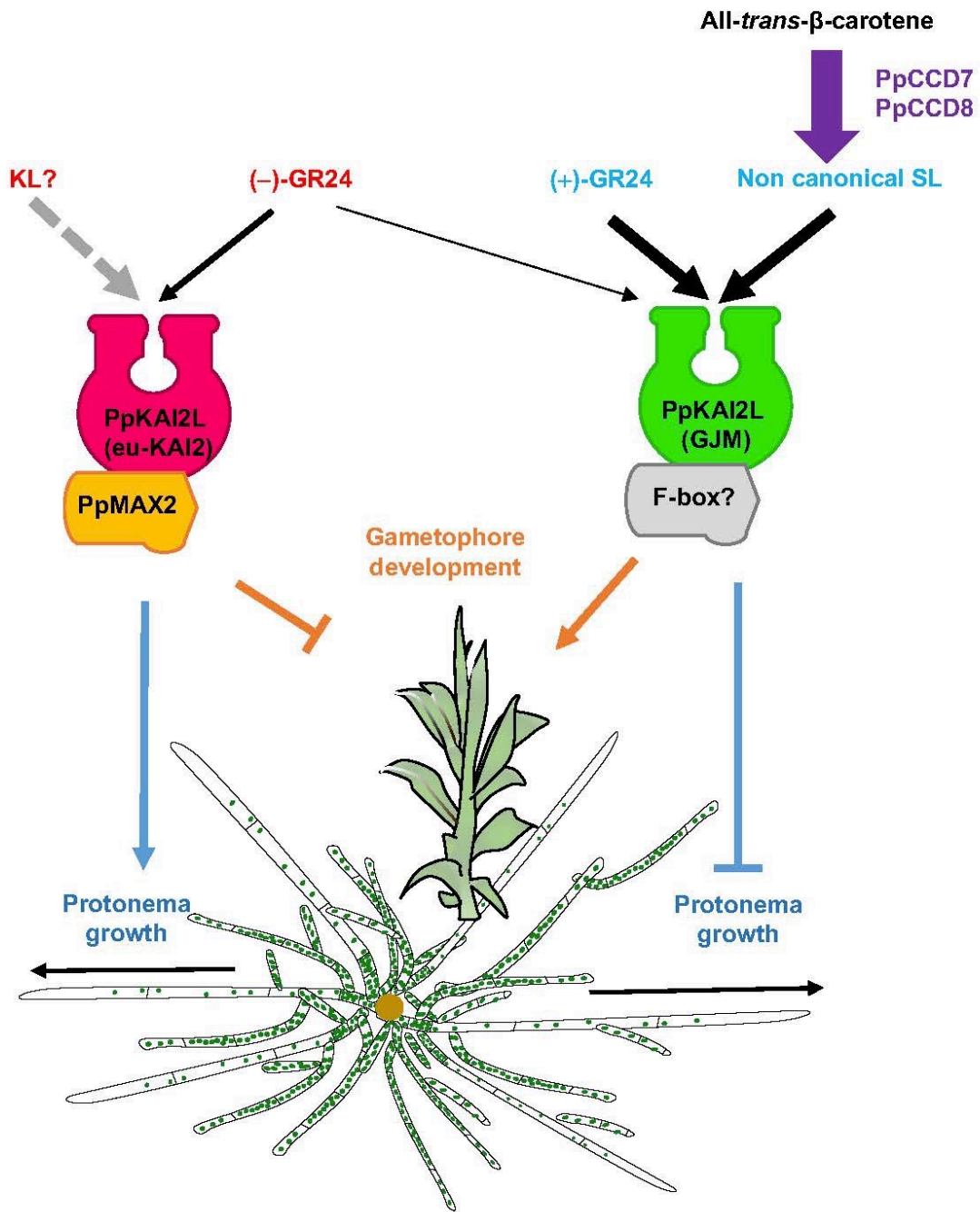


Figure 11. Current model for SL (PpMAX2-independent) and KL (PpMAX2-dependent) perception in *P. patens*.

Eu-KAI2 clade PpKAI2L proteins perceive KL compounds which promote protonema growth and inhibit gametophore development through a PpMAX2-dependent pathway. (GJM) clade PpKAI2L proteins perceive PpCCD8-derived compounds (noted non-canonical SL) which inhibit protonema growth while promoting gametophore development, in a PpMAX2-independent manner. (+)-GR24 mimics PpCCD8-derived compound effects and is perceived by (GJM) clade PpKAI2L proteins. (-)-GR24 is perceived by both eu-KAI2 clade and (GJM) clade PpKAI2L proteins, making it a poor mimic of moss KL. Grey dotted arrow indicates non-demonstrated effect. Thickness of black arrows indicate the strength of the compound's effect.