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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 (<u>www.plantcell.org</u>) 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 compouds, 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 of SL perception is that the D14 protein is both a receptor and an enzyme that cleaves its substrate (and covalently binds part of the SL) in a signaling mechanism that is still under debate (Yao et al. 2016; de Saint Germain et al. 2016; Shabek et al. 2018; Seto et al. 2019). In all cases, the pocket of the α/β hydrolase appears to be essential for substrate/ligand (SL) interactions (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; Villaecija-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 molecules also act as a growth-limiting signal between neighboring moss plants, as they are 60 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 *PpKA12*-77 LIKE (PpKA12L) 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 (±)-GR24 has been used as a SL analog, but recent reports in angiosperms found that the different enantiomers present in this synthetic mixture do not have the same effect (Scaffidi et 114 115 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 *PpKA12L* 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**

PpCCD8-derived compounds induce the germination of a hemp-specific population of *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 synthese 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

the germination of *P. ramosa* group 2a seeds but not *P. ramosa* group 1 seeds (Figure 1A). In another assay, *P. ramosa* seeds were added to culture plates close to WT or *Ppccd8* plants, with and without (\pm)-GR24 (Figure 1B-C). *P. ramosa* group 2a but not group1 seeds germinated on WT moss plates, while no germination was observed in the vicinity of *Ppccd8* plants. In all cases (WT and *Ppccd8*), the addition of (\pm)-GR24 to the medium restored seed germination. Thus, PpCCD8-derived compounds induce the germination of a specific population of *P. ramosa* seeds, responding to not yet identified GS exuded by hemp.

161

P. patens responds strongly to (+)-GR24 and carlactone application but poorly to (-) 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 µM 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 KAR_1 (Hoffmann et al. 2014). In the present work, we tested the KAR_2 179 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 $10 \,\mu\text{M}$ for both genotypes (Figure 2C).
- 190

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

- 196 of PpCCD8-derived compounds.
- 197

All *PpKAI2L* genes are expressed at relatively low levels and putatively encode proteins 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 *PpKA12L* 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, *PpKA12L-D* from eu-KA12 clade showed the highest transcript levels among 210 *PpKA12L* genes (Supplemental Figure S2A). The data are consistent with those previously 211 reported (Ortiz-Ramirez et al. 2016; Perroud et al. 2018).

212

The PpKAI2L-C, -D, and -E proteins are destabilized by (–)-GR24, as observed for 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°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 μ M), PpKAI2L-E (39 μ M), and PpKAI2L-K (41 μ M). 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 AtKAI2 (Figure 4 and Supplemental Figure S6). None of the PpKAI2L proteins showed as high 258 259 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

The higher cleavage activity of PpKAI2L-H on synthetic SL analogs is explained by the 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 ((\pm) -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 (\pm) -GC242 as a substrate confirmed the relatively high enzyme activity of PpKAI2L-H compared to all the 275 276 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

characterizes the single turnover activity of AtD14 (Supplemental Figure S7B (de Saint
Germain et al. 2016)). The lack of a plateau for PpKAI2L-H suggested that this protein acted
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) residue instead of the phenylalanine found in AtD14 (Phe²⁶), AtKAI2, and all other PpKAI2L 285 286 proteins (Figure 5A). The Phe residue is located at the junction between helix $\alpha 4$ and $\alpha 5$, 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 µM DiFMU, correlating with the protein concentration (Figure 5C and 5D). PpKAI2L-H and PpKAI2L-H^{Leu28Phe} proteins had comparable affinity towards (±)-291 292 GC242 ($K_{1/2}$ = 4,794 µM vs 4,675 µM) but showed different V_{max} values (V_{max} =0,06794 µM.min⁻ 293 ¹ 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.

Thus, poor interactions were observed with (+)-GR24, which was reported to mimick SLs and had the most potent effect on *P. patens* in our phenotypic assays (Figure 2). Strikingly, all eu-KAI2 clade proteins tested showed the strongest affinity for the (–)-GR24 enantiomer (Figure 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

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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 PpKA12L-C, -D, -F, -G, and -J genes were cloned 319 downstream of the AtD14 or AtKA12 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 AtKA12 nor 325 any of the *PpKA12L* genes significantly restored the branching phenotype of Atd14-1 kai2-2. We conclude that none of the tested PpKAI2L genes can fully complement the function of 326 327 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

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

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

351

When either (+)-GR24 or (-)-GR24 was added (Figure 6B), short hypocotyls (similar to WT) were observed in the *pKAI2:AtD14* expressing line, indicating that AtD14-mediated signal transduction of both enantiomers occurs when AtD14 is present in tissues where AtKAI2 is normally active. However, adding GR24 enantiomers to the medium had no such effect on most lines expressing PpKAI2L proteins (Figure 6B and Supplemental Figure S9B). Overall, these assays showed that PpKAI2L-G may mediate KL signaling in Arabidopsis hypocotyls, even 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 ($\Delta hifkgim$) where all the mutations except those in *PpKA12L-J* and *-M* 373 were null (Supplemental Table S1 and see below). For convenience, null mutations are hereafter 374 indicated in bold letters.

375

The eu-KAI2 clade quintuple mutants phenocopy *Ppmax2-1* in white light, while mutants 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 $(fk_i, \Delta hfk_j - 1 \text{ and } \Delta hfk_j m)$ 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
- 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

To determine which of the *Ppkai2L* mutants carry mutations in potential receptors for PpCCD8compounds (SL-related) or other (KL-related) compounds, we tested their phenotypic responses to GR24 enantiomers at 0.1 μ M (Figure 9). In the assays reported above on WT and *Ppccd8* in the dark (Figure 2), (–)-GR24 appeared to be a poor KL mimic in moss. However, the stereospecificity of the PpKAI2L proteins for (–)-GR24 led us to pursue our assays with 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 μ M (–)-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 *PpKA12L-J* gene was knocked out (Figure 9D: \mathbf{i} and 460 gim-1, and Supplemental Figure S13C: gimil and $\Delta hfkj-3$ and 13D: gim-3 and gim-5). 461 Interestingly, in the two lines where the *j* mutation was not null but the *PpKA12L-G* gene was 462 knocked out (7x $\Delta hifkgim$ and gim-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.

To further test if PpKAI2L-J and -G could be receptors for PpCCD8-derived SLs, we measured the transcript levels of SL-responsive genes in the corresponding mutants (Figure 10). We previously reported that in WT and *Ppccd8* plants, *PpKUF1LA* gene transcript abundance

469 increases 6 h after plant transfer onto medium containing 3 μ M (±)-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 *PpKUF1LA* only in WT (Figure 476 10). In the quintuple eu-KAI2 mutant, the addition of (+)-GR24 but not (-)-GR24 increased 477 PpKUF1LA 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 (*PpKUF1LA*) 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 Orobanche 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 suceptibility 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 MAX1 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 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 (\pm) -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 μ M. 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 propose (Figure 11 and see below) that (GJM) clade PpKAI2L proteins perceive (-)-GR24. 534

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).

It should be noted that AtKAI2 is degraded following KAR perception, in a MAX2-independent manner (Waters et al. 2015a), and this could be tested on eu-KAI2 clade proteins. As with the two other tested clades, (FK) and (HIL), none of the interaction assays revealed any preferential binding of GR24 isomers, despite the use of pure enantiomers. Indeed, only PpKAI2L-K showed similar binding affinity with both (–)-GR24 and (+)-GR24, but no stereoselectivity. Unfortunately, none of the PpKAI2L proteins from the (GJM) clade could be purified for 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 (±)-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 *PpKA12L-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 KAR_1 by this protein was reported (Bürger et 597 al. 2019). Indeed, the Δh and higher-order mutants ($\Delta hi and \Delta hil$) 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
- association of this role with PpKAI2L-H enzyme activity remains to be discovered.
- 602

The Arabidopsis At*d14-1 kai2-2* mutant complementation assay provides important clues 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 µM 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

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

656

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

In white light (Figure 7), mutants affecting (FK) and (GJM) clades showed phenotypes intermediate between those of WT and *Ppccd8*. In the dark under control conditions (Figure 9), the caulonema numbers of mutants in the (FK) and (GJM) clades were also intermediate between those of WT and *Ppccd8* (see also Table 1). Based on the hypothesis that synthesis 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 *PpKAI2L-J* or *PpKAI2L-G/M* no longer responded to this compound (Figure 9C-D, *j*, and *gjm*-665 666 1, and Supplemental Figure S13D, gjm-3, gjm-5, and gjm-4). By contrast, both Δhfk_j -1 and 667 Δhfk_{j-2} mutants showed a significant response to (+)-GR24 application (Figure 9D and 668 Supplemental Figure S13C), indicating that KO mutations in both *PpKA12L-F* and *PpKA12L*-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 PpKA12L-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 (\pm) -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

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).

- 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

Consequently, the search for proteins that interact with *P. patens* KL and PpCCD8-derived compound receptors should be a priority in the near future. In particular, since SMXL proteins are key members of both the SL and KL pathways in angiosperms and of the KL pathway in *M. polymorpha* (Soundappan et al. 2015; Wang et al. 2015; Khosla et al. 2020; Mizuno et al. 2021), the specific involvement of PpSMXL homologs (four genes) is currently under 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 ($0.2 \,\mu m$). 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 μ M (±)-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 *PpKA12L*-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-\Delta h* mutant, as 781 described in Supplemental Figures S10 and S11.

782 Phenotypic assays of P. patens

To measure diameters in the light, plants were grown in Petri dishes (6 to 8 plates per genotype), starting from very small pieces of protonema, with 5 plants per plate, on PpNO₃ medium (Ashton et al. 1979) covered with cellophane disks as previously described (Hoffmann et al. 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, quantum irradiance of 15 µmol m⁻²s⁻¹) for approximately two months until capsule maturity. 810 811 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 µL using SsoAdvanced Universal SYBR Green Supermix from BioRad and the 827 following program on QuantStudioTM 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 expression (RE) was calculated as RE = 2 - CTi/2 - CTref. 830

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 AtKA12 CDS were PCR amplified from Col-0 cDNA, and the PpKA12L 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

analysis shown in Figure 6 and Supplemental Figure S9 was performed on segregating T3homozygous 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 µM (+)-GR24, (-)-GR24 or with 0.01% DMSO 867 (control). For the hypocotyl elongation assay, seeds were stratified at 4 $^{\circ}$ C (2 days in the dark), 868 869 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 µmol/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

h per day); daily temperatures fluctuated between 18 °C and 25 °C. Peak PAR levels were

between 700 and 1000 μ mol m⁻² s⁻¹. Plants were watered twice a week with tap water. The

number of rosette branches longer than 5 mm was counted when the plants were 40 days old.

877 Protein expression and purification

AtD14, RMS3, and AtKAI2 with cleavable GST tags were expressed and purified as described in (de Saint Germain et al. 2016). For PpKAI2L protein expression, the full-length CDS from *P. patens* were amplified by PCR using cDNA template and specific primers (see Supplemental Table S2) containing a protease cleavage site for tag removal, and subsequently cloned into the pGEXT-4T-3 expression vector. For PpKAI2L-L, the N-terminal 47 amino acids were removed. The expression and purification of PpKAI2L proteins followed the same method as 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 (±)-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 × 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 4.5.10⁻³ mBar.

904 Enzymatic kinetic assays

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

Direct electrospray ionization – mass spectrometry of PpKAI2L proteins (ESI)-MS under 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 µm 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 µM) 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.

Mass Lynx version 4.1 (Waters) and Peakview version 2.2 (Sciex) software were used for data acquisition and processing, respectively. Deconvolution of multiply charged ions was performed by applying the MaxEnt algorithm (Sciex). The average protein masses were annotated in the spectra, and the estimated mass accuracy was \pm 2 Da. External calibration was performed with NaI clusters (2 µg/µL, isopropanol/H₂O 50/50, Waters) in the acquisition m/z 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

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

949 Accession numbers

950 Sequence data from this article can be found in the GenBank/Phytozome libraries under the951 accession numbers described in Supplemental Table S3.

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

953

Genotypes	Diameter	Gametophore	Caulonema	Response	e to (+)-GR24	Response to (-)-GR24		
		Size	number	Caulonema number	Transcriptional markers	Caulonema Transcriptio number markers		
	(Figure 7)	(Figure 8, Supplemental Figure S12)	(Figure 2, 9, Supplemental Figure S13)	(Figure 2, 9, Supplemental Figure S13)	(Figure 10)	(Figure 2, 9, Supplemental Figure S13)	(Figure 10)	
WT	WT	WT	WT	И	7	71 or =	=	
Ppccd8	>WT	<wt< td=""><td>>WT</td><td>И</td><td>7</td><td>= or א</td><td>Я</td></wt<>	>WT	И	7	= or א	Я	
Ppmax2-1	<wt< td=""><td>>WT</td><td><wt< td=""><td>И</td><td>=</td><td>= or א</td><td>=</td></wt<></td></wt<>	>WT	<wt< td=""><td>И</td><td>=</td><td>= or א</td><td>=</td></wt<>	И	=	= or א	=	
eu-kai2	<wt< td=""><td>>WT</td><td>≤WT</td><td>И</td><td>л</td><td>= or צ</td><td>=</td></wt<>	>WT	≤WT	И	л	= or צ	=	
(fk)*	≥WT	≈WT	≥WT	И	Not tested	=	Not tested	

(1111)	≤WT	≤WT	≤WT	И	Not tested	=	Not teste
(gjm)	≥WT	≤WT	>WT	=	7 or =	= or א	7 or =
in combinau	on with J and/or	In mutations					
с I							
Supplem	ental Data	L					
				_			
Supplem	ental Figu	ire S1. Sec	quence alig	nment of	Physcomitriun	n patens (1	Pp) PpKA
proteins v	with AtD14	and AtKA	12 proteins f	rom <i>Arabi</i>	dopsis thaliand	a (At).	
						_	
Supplem	ental Figu	ire S2. Exp	pression of	PpKAI2L	genes through	out P. pate	ens vegeta
developm	ient.						
~ •			5:00				
Supplem	ental Figu	re S3. Nand	Differentia	I Scanning	g Fluorimetry (nanoDSF)	analysis o
PpKAI2L	protein res	sponse to G	R24 isomer	s.			
Sumplass	ontol Fig-	no SA Tratais	ania torrata -1	oon floorer		hat CD24	
Supplem	ental Figu	re 54. Intri	nsic tryptopl	han Huores	scence shows the	nat GK24 19	somers bii
РрКАІ2L	<i>p</i> roteins w	with differen	it affinities.				
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Supplem	ental Figu	re 55. rpm	AIZE IIYUIOI	ysis activi	ty towards <i>p</i> -in	FA.	
Supplem	ental Figu	re S6. PpK/	AI2L enzvm	atic activi	ties confirm ste	ereoselectiv	itv.
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Supplem	ental Figu	ire S7. Cha	aracterization	n of (±)-C	C242 profluor	rescent pro	be activit
moss and PpKAI2L enzymatic activities towards (±)-GC242.							
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Supplem	ental Figu	re S8. Mas	s spectrome	etry charac	cterization of c	ovalent Pp	KAI2L-li
complexe	es.						
Supplem	ental Figu	re S9. Com	plementatio	n assays of	f the Arabidops	sis At <i>d14-1</i>	<i>kai2-2</i> do
mutant.							
Supplem	ental Figu	re S10. Mu	tations obtai	ned in all	13 PpKAI2L ge	enes.	
C 1							
Supplem	ental Figu	re S11. The	e Ppkai2L-∆l	h 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 (\pm) -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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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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WT

Ppccd8



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 (±)-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 (±)-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(±)-GR24. Arrows indicate germinating seeds. Scale bar = 0.5 mm.





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 (±)-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 Phytozome 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).



B

	Protein stability assay ^a Supplemental Figure S3			Binding affinity determination (Ka) ^b Supplemental Figure S4		GR24 cleavage assay^c Supplemental Figure S7		SL probe cleavage assay ^d Supplemental Figure S8	Covalent adduct detection ^e Supplemental Figure S9	
Related figure										
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	$39 \pm 10 \mu M$	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	$107 \pm 11 \mu M$	$41\pm43\mu\text{M}$	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 ^z	Yes²

^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.

° PpKAl2L (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.

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 PpKAl2L 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. PpKAl2L-H enzymatic activity reveals special feature.

(A) Sequence alignment of active site amino acid residues in PpKAl2L proteins. Amino acids that differ from AtKAl2 are colored in red. A fully expanded alignment can be found in Supplemental Figure S1. (B) Superimposition of the AtD14 and PpKAl2L-H structure showing the position of Phe²⁸ and Leu²⁸ residues. Zoom onto helices α 4 and α 5. (C) Enzyme kinetics for PpKAl2L-H, PpKAl2L-H^{Leu28Phe} and AtD14 incubated with (±)-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 (±)-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 At*d14-1 kai2-2* mutant (in Ler background), transformed using the *AtD14* promoter (**A**) or *AtKAl2* promoter (**B**) controlling the expression of At*D14*, *AtKAl2* (controls) or *PpKAl2L* CDS (as noted below the graph). Ler (WT), *kai2-2* and At*d14-1 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:PpKAl2L-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 ½ MS medium with DMSO (control, grey bars) 1 μ M (+)-GR24 (turquoise bars) or 1 μ M (–)-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 ≤ P < 0.05; *** P ≤ 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-KAl2 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 $\Delta hfkgjm$). 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 15 and 25 leaves. Whiskers refer to minimum and maximum values, bars inside the boxplot to the median. Letters indicate statistical significance 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 μ M (-)-GR24 (in red, A) or 0.1 μ M (+)-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: $\Delta hfkj-1$, following application of 0.1 μ M (-)-GR24 (in red, C) or 0.1 μ M (+)-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<0.5.



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 **g**jm-2 ((GJM) clade), after a 6-hour treatment in the dark with DMSO (control, grey), 1 μ M (+)-GR24 (turquoise) or 1 μ M (-)-GR24 (red). Box plots of at least four biological repeats are shown (n ≥ 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 doted arrow indicates non-demonstrated effect. Thickness of black arrows indicate the strength of the compound's effect.