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1 *A Phelipanche ramosa* KAI2 Protein Perceives enzymatically Strigolactones and
2 Isothiocyanates

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22 **Abstract**

23 *Phelipanche ramosa* is an obligate root-parasitic weed threatening major crops in central
24 Europe. For its germination, it has to perceive various structurally diverging host-exuded
25 signals, including isothiocyanates (ITCs) and strigolactones (SLs). However, the receptors
26 involved are still uncharacterized. Here, we identified five putative SL receptors in *P. ramosa*,
27 of which PrKAI2d3 is involved in seed germination stimulation. We established the high
28 plasticity of PrKAI2d3, allowing interaction with different chemicals, including ITCs. The SL
29 perception mechanism of PrKAI2d3 is similar to that of endogenous SLs in non-parasitic
30 plants. We provide evidence that the PrKAI2d3 enzymatic activity confers hypersensitivity to
31 SLs. Additionally, we demonstrated that methylbutenolide-OH binds PrKAI2d3 and
32 stimulates *P. ramosa* germination with a bioactivity comparable to that of ITCs. This study
33 highlights that *P. ramosa* has extended its signal perception system during evolution, a fact to
34 be considered in the development of specific and efficient biocontrol methods.

35 **Introduction**

36 Witchweeds (*Striga* spp.) and broomrapes (*Orobanche* and *Phelipanche* spp.) are obligate
37 root-parasitic plants belonging to the Orobanchaceae family and together comprise the most
38 threatening weeds of the major domesticated crops worldwide¹. At maturity a single plant
39 releases up to 100,000 microscopic seeds, resulting in severe soil pollution². Seed germination
40 of these obligate parasites requires the strict recognition of host-exuded germination
41 stimulants. Broomrapes and witchweeds are all highly sensitive to strigolactones (SLs)
42 secreted by plants into the rhizosphere at picomolar doses³. The structural core of SLs is a
43 tricyclic lactone, referred to as the ABC part in canonical SLs or as a structural variety in non-
44 canonical SLs, invariably connected to an α,β -unsaturated furanone moiety (D ring) via an
45 enol-ether bridge (Supplementary Figure 1a)³. Some germination stimulants are exclusive to
46 specific host-parasite interactions, as illustrated by the unique ability of *Phelipanche ramosa*
47 to germinate upon sensing isothiocyanates (ITCs). These glucosinolate-breakdown products
48 are exuded by rapeseed (*Brassica napus*)^{2,4}, on which *P. ramosa* has adapted in a decade^{1,2}.
49 Broomrapes are increasingly problematic in both intensity and acreage in Europe, North
50 Africa, and Asia, and they are expected to dramatically expand to new territories in the near
51 future⁵. To date, several physical, cultural, chemical, and biological approaches have been
52 explored to control root-parasitic weeds, but no method has been found completely
53 satisfactory⁶.

54 Besides their involvement in germination, SLs have a strong stimulating activity on
55 arbuscular mycorrhizal fungi (Glomeromycotina) by promoting mitochondrial metabolism
56 and hyphal branching⁷, thereby mediating the establishment of the oldest mutualistic
57 interaction of land plants⁸. In addition to their role in rhizosphere signaling, SLs act as
58 hormones *in planta* with pervasive roles throughout the plant development⁹ and, as such, are
59 perceived in vascular plants by the α/β -hydrolase DWARF14 (D14)^{10,11}. Biochemical
60 analyses with recombinant D14 proteins by means of the synthetic SL analog GR24 revealed
61 that the SL signal transduction requires GR24 cleavage^{11,12}. One of the cleavage products, the
62 D ring, may remain covalently attached to the receptor^{13,14}, thereby probably allowing the
63 recruitment of partners for downstream processes^{15,16}. The required SL cleavage for signal
64 transduction is still under debate^{15,16}.

65 In *Arabidopsis thaliana*, D14 belongs to a small gene family, including KARRIKIN
66 INSENSITIVE2/HYPOSENSITIVE TO LIGHT (AtKAI2/AtHTL) that shares the α/β -
67 hydrolase catalytic triad. However, AtKAI2 regulates AtD14-independent processes, such as
68 seed germination, and preferentially perceives (-)-GR24 that mimics non-natural SLs,
69 karrikins¹⁷, and supposedly a still unknown endogenous ligand¹⁸. Interestingly, the *KAI2* gene
70 family has expanded during the evolution of obligate parasitic-plant genomes¹⁹. For example,
71 *Phelipanche aegyptiaca* and *Striga hermonthica* possess five and eleven *KAI2/HTL* genes,
72 respectively. Two *KAI2* paralogs play a role in SL response in *P. aegyptiaca*¹⁹ and six *S.*
73 *hermonthica* *KAI2/HTL* proteins are hypersensitive to SLs^{19,20}, with ShHTL7 exhibiting the
74 same perception mechanism as D14²¹. The remarkable expansion of the *KAI2* gene family in
75 Orobanchaceae along with the capacity of *P. ramosa* to perceive ITCs let us to assume that
76 *KAI2* proteins might also perceive other germination stimulants. Here, we characterize
77 PrKAI2d3 as a *P. ramosa* SL receptor. We demonstrate that it is able to perceive natural SLs
78 by an enzymatically dependent mechanism contributing to its hypersensitivity to SLs. In
79 addition, we show that PrKAI2d3 perceives ITCs as well as a wide range of SL analogs that
80 thus were not found bioactive for hormonal SL functions. Together, these results suggest that
81 PrKAI2 proteins evolved as hypersensitive and plastic receptors, enabling the parasitic plant
82 to detect various host exudate metabolites and contributing to its dramatic success.

83 **Results**

84 **Identification and gene expression profile of PrKAI2 homologs**

85 Iterative nucleotide and amino-acid BLAST analyses were carried out on the recently
86 published transcriptome of *P. ramosa* rapeseed strain²², with known *KAI2* and *D14* sequences
87 of non-parasitic and parasitic plants as queries^{19,20}. Each newly identified sequence was used
88 as a query for new BLAST searches to find all complete sequences. Duplicates were
89 eliminated before alignment, allowing the detection of one *PrD14* and five *PrKAI2* putative
90 orthologs that were amplified and re-sequenced from cDNA isolated from germinated seeds
91 of the same *P. ramosa* strain. Maximum-likelihood analysis indicated that the predicted
92 PrD14 and PrKAI2 proteins (Figure 1a) and genes (Supplementary Figure 2) belonged to
93 clearly distinct D14 and KAI2 clades, respectively, with a conserved catalytic triad and an
94 overall conserved environment of known active-site amino-acid residues (Supplementary
95 Figure 3). Despite a poor separation of non-parasitic and parasitic KAI2 groups, the
96 phylogenetic analysis strongly suggested that among the retrieved transcripts no PrKAI2
97 protein belonged to the intermediate KAI2i subclade, as previously described for other
98 members of the *Orobanche* and *Phelipanche* genera¹⁹. The newly identified PrKAI2 and
99 PrD14 proteins consistently clustered together with *P. aegyptiaca* sequences. Together with
100 the reported low levels of genomic divergence in *Phelipanche*^{23,24}, these findings hint at the
101 detection of *P. aegyptiaca* protein orthologs. Hence, the *P. ramosa* sequences were renamed
102 according to the current nomenclature¹⁹, namely one PrKAI2 protein belonging to the
103 conserved KAI2 (PrKAI2c) clade and four PrKAI2 proteins from the divergent KAI2 clade
104 (PrKAI2d1-d4).

105 **PrKAI2d3 is a putative SL receptor**

106 Sequence homology analysis of the ligand-binding pocket amino-acid residues of the PrKAI2
107 proteins with AtD14 and AtKAI2 showed that PrKAI2d3 possesses the highest similarity with
108 AtD14 (Figure 1b). Predictive models generated *via* the SWISS-MODEL webserver revealed
109 that the binding pocket of PrKAI2c is smaller than that of its putative ortholog AtKAI2
110 (Figure 1c) and that the binding pockets of the PrKAI2d receptors are wider than those of
111 members of the conserved KAI2 clade, probably due to the absence of large hydrophobic
112 residues (Figure 1c). Among all the divergent receptors PrKAI2d3 had the largest predicted
113 binding pocket (Figure 1c). Finally, the expression level of *PrKAI2d3* transcripts in *P. ramosa*
114 seeds was significantly up-regulated upon 1 hour of exogenous treatment with GR24, whereas
115 the transcript levels for PrKAI2c, the putative AtKAI2 ortholog, did not change (Figure 1d).
116 These properties are in agreement with a role of PrKAI2d3 as SL receptor.

117 As no mutants and nor an easy and fast transformation method are available for holoparasitic
118 Orobanchaceae, it is difficult²⁵ to validate the biological function of PrKAI2d3 directly in *P.*
119 *ramosa*. Instead, we assayed the complementation of the *Arabidopsis htl-3* mutant phenotype
120 with PrKAI2d3, a previously successful approach^{19,20}. To assess the role of the catalytic triad
121 in the perception process, *htl-3* was also transformed with a mutated PrKAI2d3^{S98A}-encoding
122 version. We used three p35S::GFP-PrKAI2d3 lines displaying various protein levels and two
123 p35S::GFP-PrKAI2d3^{S98A} lines with a high protein expression (Figure 1e) to phenotype both
124 germination and hypocotyl length.

125 In a thermo-inhibition assay²⁶, almost no germination was detected for all lines upon control
126 treatments. Germination of *Arabidopsis* (accession Columbia-0 [Col-0]) seeds was
127 significantly stimulated upon 10 μ M (-)-GR24, but not upon (+)-GR24, whereas none of the
128 enantiomers had an effect on *htl-3* or *Atd14-1/htl-3* mutants (Figure 1f). Germination of all
129 three p35S::GFP-PrKAI2d3 lines was strongly induced by (+)-GR24. Exogenous (-)-GR24
130 also significantly stimulated germination of these three lines (Figure 1f) and the response
131 amplitude correlated positively with the protein abundance (Figure 1e). Analysis of a fourth
132 p35S::GFP-PrKAI2d3 line showed that even a mild protein expression increased the
133 *Arabidopsis* sensitivity a 100-fold towards (-)-GR24, enabling it to perceive up to picomolar
134 doses of (+)-GR24 and, hence, to have the *P. ramosa* sensitivity (Figure 2a-b, Supplementary
135 Figure 4, Supplementary Table 1). None of the two p35S::GFP-PrKAI2d3^{S98A} lines were
136 sensitive to either (+)- or (-)-GR24.

137 Under red-light conditions, hypocotyls of *htl-3* and *Atd14-1/htl-3* mutants are more elongated
138 than those of Col-0¹⁷ (Figure 1g). Application of (\pm)-GR24 significantly shortened the
139 hypocotyls of Col-0 and the *htl-3* genotypes, but not those of the *Atd14-1/htl-3* plants. This
140 observation corroborates a previous report that seedling photomorphogenesis is redundantly
141 controlled by D14 and KAI2/HTL in *Arabidopsis*¹⁷. Interestingly, only the hypocotyls of the
142 p35S::GFP-PrKAI2d3 and p35S::GFP-PrKAI2d3^{S98A} lines with the highest protein levels
143 were shorter than those of the *htl-3* and *Atd14-1/htl-3* mutants under control conditions.
144 However, the hypocotyls were significantly shortened in all transformed lines after treatment
145 with 1 M (\pm)-GR24 (Figure 1g). In summary, these data indicate that the PrKAI2d3 protein
146 expression rescues the *htl-3* mutant phenotypes, with the catalytic Ser98 being essential for
147 germination, but not for seedling photomorphogenesis.

148 **A structure-activity relationship study reveals that *P. ramosa* efficiently responds to SL**
149 **analogs and mimics with various structures**

150 As *P. ramosa* perceives many high structurally divergent germination stimulants, various
151 receptors might underlie this plasticity^{27,28}. To link the SL structural features with the *P.*
152 *ramosa* seed germination activity, we carried out a structure-activity relationship (SAR) study
153 through a rapid bioassay²⁹.

154 First, we determined the *P. ramosa* sensitivity towards several GR24 analogs with varying
155 stereogenic centers (Figure 2a). The lowest EC₅₀ values were obtained with (+)-GR24 (6.5
156 pM) and (-)-2'-*epi*-GR24 (5.3 pM), of which the stereochemistry corresponds to natural
157 canonical SLs of the strigol-type and orobanchol-type series, respectively (Supplementary
158 Figure 1a). In contrast, *P. ramosa* was approximately 100-fold less sensitive to (-)-GR24 and
159 (+)-2'-*epi*-GR24, of which the stereochemistry is not encountered in natural SLs (Figure 2b,
160 Supplementary Figure 1a).

161 Second, we investigated whether substitutions occurring on the D-ring alter *P. ramosa*
162 responses²⁷. Surprisingly, both GR24 analogs without a methyl group on the D-ring, (±)-4'-
163 desmethyl-2'-*epi*-GR24 and (±)-4'-desmethyl-GR24, significantly stimulated *P. ramosa* seed
164 germination (EC₅₀ = 0.92 and 0.45 nM), still with 100-fold lower EC₅₀ values than those of
165 (±)-GR24 (Figure 2c). Noteworthy, none of these substituted GR24 analogs could inhibit
166 shoot branching even at 1 μM²⁷. Inversely, *P. ramosa* was less sensitive to (±)-3'-methyl-
167 GR24 (EC₅₀ = 2.6 nM), which harbors two methyl groups on the D-ring (Figure 2c,
168 Supplementary Figure 5, Supplementary Table 2) and is highly bioactive in repressing pea
169 (*Pisum sativum*) shoot branching via RMS3/PsD14^{13,27}.

170 Finally, the effect of the ABC-ring fragment substitution on the *P. ramosa* germination was
171 analyzed. Profluorescent probes were used, in which the ABC-ring was replaced by a
172 coumarine moiety (DiFMU) and one, two, or no methyl groups were added on the D-ring
173 (GC240, GC242, and GC486, respectively)¹³. In another profluorescent probe, the ABC-ring
174 was switched by the fluorescein moiety Yoshimulactone green (YLG)³⁰ (Supplementary
175 Figure 1b). Except for DiFMU, all profluorescent probes had a stimulating activity on the *P.*
176 *ramosa* germination, but still with 1,000- to 10,000-fold lower EC₅₀ values than those of (±)-
177 GR24 (Supplementary Figure 6a-c, Supplementary Table 2).

178 Altogether, these results demonstrate that the stereochemistry of GR24 analogs is crucial for
179 bioactivity and determines the sensitivity to germination-inducing substances. The sensitivity
180 of *P. ramosa* towards desmethyl and 3'-methyl D-ring derivatives highlights differences in
181 contrast to other vascular plants, particularly regarding the signaling via D14 for shoot
182 branching. Additionally, the relative bioactivity of the profluorescent SL probes for
183 germination stimulation in broomrape confirms that ABC-rings are not required for
184 bioactivity²⁸.

185 **SL analogs and mimics interact with PrKAI2d3 according to their germination** 186 **stimulation activity**

187 The questions arising from previous results are whether PrKAI2d3 has the ability to perceive
188 such a large range of structurally divergent compounds and whether its interaction with these
189 molecules is affected by mutations in the catalytic triad. To this end, we expressed and
190 purified the PrKAI2d3 and PrKAI2d3^{S98A} proteins *in vitro* and assessed their abilities to
191 interact with SLs and other chemical mediators.

192 Interactions between PrKAI2d3 and the SL analogs and profluorescent SL probes were
193 analyzed with nano differential scanning fluorimetry (nanoDSF) by recording changes in the
194 tryptophan fluorescence (ratio 350 nm/330 nm). In contrast to “classical” DSF¹¹, nanoDSF
195 does not require a dye and highlights interactions that do not induce conformational changes.
196 Analysis of the initial fluorescence ratios revealed that the four GR24 stereoisomers interacted
197 with PrKAI2d3 according to their bioactivity (Figure 2d). However, only (+)-GR24 and (-)-
198 2'-*epi*-GR24, the most bioactive analogs with natural configurations, induced a 8.5 °C
199 decrease in the PrKAI2d3 melting temperature (Figure 2e), consistent with ligand-mediated
200 protein destabilization.

201 When the GR24 analogs with varying methyl groups on their D-ring were used, all the
202 analogs interacted and destabilized PrKAI2d3, although with an efficiency lower than that of
203 (±)-GR24, and especially that of (±)-3'-Me-GR24 (Figure 2f,g). Similar shifts in melting
204 temperatures of the PrKAI2d3 protein were observed with DSF, but without destabilization of
205 the mutated PrKAI2d3^{S98A} protein (Supplementary Figure 7). The bioactive profluorescent
206 probe GC242, used as a racemic mixture, or separate pure enantiomers also induced a shift in
207 the PrKAI2d3 protein melting temperature (Supplementary Figure 6d-i). These results
208 suggest that the enzymatic activity through Ser98 is required to destabilize the protein.

209 Next, we estimated the PrKAI2d3 affinity for GR24 analogs by means of the tryptophan
210 intrinsic fluorescence assay. The affinity was higher for (+)-GR24 and (-)-2'-*epi*-GR24 ($K_D =$
211 $12.57 \pm 2.85 \mu\text{M}$ and $66.99 \pm 26.94 \mu\text{M}$, respectively) than for (-)-GR24 and (+)-2'-*epi*-
212 GR24 ($K_D = 696 \pm 245 \mu\text{M}$ and $434 \pm 593 \mu\text{M}$, respectively), in accordance with their
213 bioactivity on the *P. ramosa* germination (Figure 2h-k, Supplementary Figure 8), whereas the
214 lower affinity of (\pm)-3'-Me-GR24 than that of (\pm)-4'-desmethyl-2'-*epi*-GR24 and (\pm)-GR24 is
215 consistent with their reduced bioactivity range (Figure 2l). However, protein affinities were
216 significantly lower (micromolar range) than the observed sensitivity of *P. ramosa* (picomolar
217 range). As similar patterns had been reported for *Striga hermonthica*³⁰, we tested this apparent
218 contradiction by investigating the enzymatic activity of the PrKAI2d3 protein.

219 **The PrKAI2d3 enzymatic activity is associated with SL hypersensitivity**

220 First, we tried to visualize the PrKAI2d3 hydrolase activity with a generic substrate, *para*-
221 nitrophenyl acetate (*p*-NPA). Surprisingly, no hydrolytic activity was detected
222 (Supplementary Figure 10) in contrast to other SL receptors with the same probe¹³. When
223 PrKAI2d3 was incubated with (+)-GR24 and (-)-2'-*epi*-GR24, a cleavage activity was
224 unambiguously observed by ultraperformance liquid chromatography (UHPLC)/UV DAD
225 analysis (Figure 3a), but, opposite to AtD14 and AtKAI2, PrKAI2d3 did not cleave (-)-GR24
226 or (+)-2'-*epi*-GR24. Interestingly, a residual cleavage activity of PrKAI2d3^{S98A} still occurred
227 with all GR24 isomers, but without stereoselectivity, possibly the reason for the partial
228 complementation of *htl-3* with PrKAI2d3^{S98A}-overproducing constructs (Figure 1g). These
229 data suggest that SL hydrolysis and the subsequent signal transduction could happen without
230 an intact catalytic triad.

231 As for the interaction assays, we tested the hydrolysis of analogs with substitutions on the D-
232 ring. Desmethyl GR24 isomers were more efficiently hydrolyzed than GR24 by AtKAI2,
233 PrKAI2d3, and PrKAI2d3^{S98A} proteins (Figure 3b). Additionally, these three purified proteins
234 along with AtD14 also displayed a low, but significant, hydrolysis activity towards 3'-Me-
235 GR24. These results indicate that PrKAI2d3 possesses an important hydrolysis capacity
236 towards the natural configuration-mimicking GR24, albeit to a lesser extent than AtD14.

237 An enzymatic kinetic characterization of the purified proteins was carried out with the
238 bioactive profluorescent probes as substrate. Monitoring the DiFMU fluorescence revealed
239 that PrKAI2d3 hydrolyzed (\pm)-GC240, (\pm)-GC242, and (\pm)-GC486 (Figure 3c-e). For all

240 probes, we observed a biphasic time course of fluorescence, consisting of a burst phase (also
241 called initial phase), followed by a plateau phase (or slow phase for AtD14 incubated with
242 (\pm)-GC240). In all cases, the plateau did not reach the maximum of the expected product
243 (20 M), but a concentration product closer to the protein concentration (0.4 M) (Figure 3c-e),
244 implying that PrKAI2d3 might act as a single turnover enzyme towards all GC probes tested,
245 including GC486 that lacks the 3'-methyl group on the D-ring and has a Michaelian cleavage
246 kinetic with AtD14 and RMS3/PsD14 proteins¹³. The S98A substitution in the catalytic triad
247 drastically reduced the (\pm)-GC240 and (\pm)-GC242 cleavage, although the residual activity
248 towards (\pm)-GC486 remained statistically significant. These observations confirmed that the
249 mutation in the catalytic triad does not fully abolish the cleavage activity towards a compound
250 without methyl on the D-ring.

251 Regarding single turnover enzymes, we defined k_{cat} as the rate constant of the presteady-state
252 phase (initial phase) and $K_{1/2}$ as the probe concentration with half maximal velocity (V_{max})
253 (Figure 3g-i). The similar $K_{1/2}$ values of PrKAI2d3 with (\pm)-GC240 and (\pm)-GC242 (5.74 μM
254 and 4.60 μM , respectively) confirmed the low influence of the C3' methyl chain on the
255 substrate affinity, but the differences in the V_{max} (0.072 $\text{M}\cdot\text{min}^{-1}$ and 0.013 $\text{M}\cdot\text{min}^{-1}$,
256 respectively) indicated that the C3' methyl chain reduces the catalytic activity, corresponding
257 with the bioactivities of these probes. Higher $K_{1/2}$ values of PrKAI2d3 for (\pm)-GC240 than
258 that for (\pm)-GC486 (5.74 μM and 1.53 μM , respectively) and V_{max} values (0.072 $\text{M}\cdot\text{min}^{-1}$ and
259 0.209 $\text{M}\cdot\text{min}^{-1}$, respectively) highlighted the importance of the C4' methyl chain in binding
260 and catalytic affinity, supporting the results of the germination bioassays (Supplementary
261 Figure 6a-c).

262 To test the hypothesis that PrKAI2d3 forms a stable intermediate with the D-ring, as
263 previously demonstrated for other SL receptors (AtD14, PsD14/RMS3, ShHTL7, and
264 D14)^{13,14,21,31}, we incubated two bioactive ligands (\pm)-GR24 and probe (\pm)-GC486 with
265 PrKAI2d3 at a pH of 6.8 and recorded mass spectrometry spectra under denaturing
266 conditions. In all cases a mass shift occurred corresponding to the D-ring covalently bound to
267 the protein (Figure 3j-l) and specifically attached to His249 of the catalytic triad (Figure 3m-
268 o).

269 **ITCs interact with PrKAI2d3 and generate a covalent adduct to the catalytic serine**

270 Although PrKAI2d3 perceives structurally diverging SL analogs and mimics that stimulate
271 seed germination of *P. ramosa*, the specificity of the interaction between rapeseed and *P.*
272 *ramosa* correlates with the parasite's ability to perceive ITCs, which structurally differ greatly
273 from SLs⁴. Therefore, we evaluated the ability of PrKAI2d3 to perceive ITCs. Seeds of *P.*
274 *ramosa* were approximately 10,000-fold less sensitive to 2-PEITC and BITC than to (±)-
275 GR24 (Figure 4a-c). Investigation of the putative interactions between PrKAI2d3 and ITCs
276 by nanoDSF revealed a small shift (1.2-2.0 °C) in the PrKAI2d3 melting temperature upon
277 high ITC concentrations, as further confirmed by “classical DSF” (Figure 4d-e,
278 Supplementary Figure 11). These data indicated that ITCs interact with PrKAI2d3 with low
279 affinity, corresponding with a stimulating potential on *P. ramosa* seeds lower than that of
280 bioactive SLs⁴ and analogs. Finally, the apparent melting temperatures of PrKAI2d3 with
281 BITC and 2-PEITC (44.3 °C and 45.1 °C, respectively) varied from values obtained with (±)-
282 GR24 (36.9 °C), suggesting that ITCs might induce a conformational change that differs from
283 the GR24-induced destabilization.

284 As ITCs easily react with nucleophile functions³², we hypothesized that the PrKAI2d3
285 interaction with ITCs may trigger the formation of a covalent adduct. Indeed, a mass shift was
286 detected correlating to the 2-PEITC covalently bound to the protein (Figure 4f). After the
287 PrKAI2d3-2-PEITC complex digestion, the 2-PEITC attachment was localized on a peptide
288 corresponding to the amino acids 87-113 of PrKAI2d3. Tandem mass spectrometry data
289 revealed that the 2-PEITC attachment could be on His97 or on the catalytic Ser98 (Figure 4h).
290 Incubation of 2-PEITC with PrKAI2d3^{S98A} allowed us to conclude that the major site for 2-
291 PEITC attachment is on Ser98 (Figure 4g). These results hint at a perception mechanism for
292 ITC in which the Ser98 hydroxyl group would react with isothiocyanate to generate a
293 PrKAI2d3-attached carbamothioate (Figure 4i). However, no 2-PEITC attachment was
294 detected on His249-containing peptides of the catalytic triad.

295 Overall, PrKAI2d3 acts as a receptor for both SL-like molecules and ITCs that are both
296 germination stimulants of *P. ramosa*. These results demonstrate that further research into
297 potential chemical interactors is achievable to design control methods.

298 **Search for small-molecule interactors with PrKAI2d3 proposes D-OH as a simple and**
299 **efficient germination stimulant for *P. ramosa* with a bioactivity similar to that of ITCs**

300 Recently, synthetic inhibitors of D14 and ShHTL7³³, the *S. hermonthica* SL receptor, have
301 been proposed, that include, although all structurally unrelated to SLs, sporidione, KK094,
302 Triton X, and tolfenamic acid (TA) (Figure 5a). We evaluated the putative inhibitory effects
303 of these compounds, along with those of a common serine protease inhibitor,
304 phenylmethylsulfonyl fluoride (PMSF) on the germination of *P. ramosa* seeds treated with
305 10 nM of (±)-GR24 or 100 nM of 2-PEITC. Nonetheless, *P. ramosa* seeds were clearly
306 hyposensitive to each of these compounds when compared to results obtained in *S.*
307 *hermonthica*, with a maximum inhibition obtained in some instances at very high
308 concentrations (Figure 5b,c; Supplementary Table 3). Indeed, all half maximum inhibitory
309 concentrations (IC₅₀) fell above the IC₅₀ of abscisic acid (ABA, 100 nM (GR24) and 34 nM
310 (2-PEITC)], a known inhibitor of *P. ramosa* germination^{34,35}. These data indicate that the use
311 of germination inhibitors intended for *Striga* is reasonably irrelevant and unsuitable for *P.*
312 *ramosa* biological control.

313 Therefore, we looked for specific PrKAI2d3 agonists with the potential for large-scale
314 biocontrol. A serious issue in the search for simple and cheap SL analogs and mimics is their
315 low stability due to the D-ring connection to a leaving group. As alternatives, molecules have
316 been proposed that carry the D-ring only, because it is essential for the high bioactivity of
317 SLs³⁶. Specifically, the effect on the *P. ramosa* germination was evaluated of a set of
318 butenolides containing the D-ring (D-OR) only (Figure 5j, Supplementary Figure 12). D-
319 OsecBu and 3'-Me-D-OH were completely inactive, whereas the *P. ramosa* sensitivity
320 towards D-OAll, D-OMe, D-OEt, and Dihydro-DOEt was low with EC₅₀ in the micromolar
321 range. Inversely, D-OH and 4'-Desmethyl-D-OH were approximately 10-fold more active
322 than 2-PEITC (Supplementary Table 2). Although D-OH had been found bioactive in rice
323 (*Oryza sativa*) at high concentration¹² (50 μM), in pea D-OH and 3'-Me-D-OH had not effect
324 on the branching control by direct injection (100 μM) in the stem (Supplementary Figure 13).

325 To check whether this D-OH sensitivity was a specificity of the *P. ramosa* PrKAI2d3, we
326 reexamined the germination-stimulating activity of D-OH on *S. hermonthica*. In contrast to
327 previous results^{37,38}, D-OH induced *S. hermonthica* germination (EC₅₀ = 1.2 μM). Moreover,
328 in *Arabidopsis* lines expressing a GFP fused to ShHTL7, the known *S. hermonthica* SL
329 receptor^{30,39}, was induced by (±)-DOH (EC₅₀ = 3.1 μM) (Supplementary Figure 14,
330 Supplementary Table 1). In summary, our study validates the perception of D-OH by SL
331 receptors in root-parasitic plants.

332 DSF and intrinsic fluorescence analyses revealed that D-OH and PrKAI2d3 interacted at high
333 concentrations (Figure 5k-l). Incubation of D-OH with PrKAI2d3 at pH 6.8 and the recorded
334 MS spectra under denaturing conditions revealed a mass shift corresponding to the D-ring
335 covalently bound to the protein (Figure 5m). The D-ring attachment could be localized to the
336 His249 of the catalytic triad, similarly as for the SL analogs (Figure 5n). In addition to
337 various SL analogs, and SL-like compounds, and ITCs, D-OH highlights the plasticity of the
338 PrKAI2d3 receptor to interact with different structures with a significant biological activity as
339 a consequence and emphasizes that all SL hydrolysis products are not inactive as germination
340 stimulants of *P. ramosa*.

341 Discussion

342 Obligate parasitic weeds require host-derived signals to germinate and wither their hosts long
343 before they emerge from the soil, arguably making the early stages of the parasitic life cycle a
344 much better target for control strategies than the later ones⁴⁰. An important prerequisite for the
345 design of such methods is an in-depth understanding of how parasites perceive germination
346 stimulants.

347 Here, we demonstrated that PrKAI2d3 provides *P. ramosa* with hypersensitivity to SLs
348 mainly due to its enzymatic activity. The enzymatic data with the GR24 analogs [(+)-GR24
349 and (-)-2'-*epi*-GR24] and GC probes suggest that PrKAI2d3 acts as a single turnover enzyme
350 towards natural SLs, and cleave them to form a covalent complex between the D-ring and the
351 catalytic histidine, as previously described for AtD14 and RMS3¹³. However, this model has
352 recently been challenged¹⁶, because the *Arabidopsis* SL receptor mutated for the catalytic Asp
353 residue, speculatively considered unable to cleave endogenous SLs, had been found to
354 transduce the SL signal; hence, the SL cleavage has been concluded not be required for
355 signaling. Indeed, It is possible that certain SL analogs, can be perceived independently of the
356 enzymatic activity. We showed that the GR24 analogs with non-natural stereochemistry [(-)-
357 GR24 and (+)-2'-*epi*-GR24] are not cleaved by PrKAI2d3. These uncleaved molecules
358 stimulate 100-fold less efficiently the *P. ramosa* seed germination than the GR24
359 stereoisomers with a natural stereochemistry (Figure 3). Moreover, the PrKAI2d3 affinity
360 (K_D) towards the most bioactive SL analogs, (+)-GR24 and (-)-2'-*epi*-GR24, is in the
361 micromolar range, which is several orders lower than the *P. ramosa* seed sensitivity recorded
362 in the germination bioassays. The K_D values for (+)-GR24 and (-)-2'-*epi*-GR24, that reflect a

363 simple binding to the receptor, do not allow explaining the hypersensitivity. Similar
364 differences between *in vitro* and *in vivo* analyses were observed for the *S. hermonthica* SL
365 receptor ShHTL7 characterization³⁰. We propose that the SL hypersensitivity of this SL
366 receptor is obtained by its enzymatic properties.

367 Interestingly, because the enzymatic activity is not completely abolished in the PrKAI2d3^{S98A}
368 protein, a residually significant activity, also present in the DAD2^{S96A} protein⁴¹, might result
369 from the nucleophilic addition of water instead of the attack of the Ser98 hydroxyl group. The
370 partial complementation of the *htl-3* mutant with constructs that overproduce PrKAI2d3^{S98A}
371 corroborates this assumption. A similar proposition could also explain why *Arabidopsis*
372 overexpressing the AtD14 with a mutated Asp catalytic residue is able to transduce a
373 branching inhibitor signal, especially the still untested natural SLs present in *Arabidopsis* (i.e.
374 non-canonical SLs). Noteworthy, *p*-NPA could not efficiently highlight the enzymatic activity
375 of PrKAI2d3, in comparison to the profluorescent SL mimics, pinpointing the weakness of
376 using generic or inappropriate substrates as controls.

377 Moreover, PrKAI2d3 displays a high plasticity that allows binding of modified SLs, such as
378 desmethyl-GR24 isomers or many SL mimics, but in contrast to RMS3 and AtD14, it
379 perceives these compounds similarly as intact SLs. Correspondingly, desmethyl-YLG was
380 bioactive in *Arabidopsis* via AtKAI2⁴². Ligand-mediated protein destabilization is not
381 required to perceive SL analogs, but considerably improves the sensitivity to natural SLs.
382 PrKAI2d3 acts as single-turnover enzyme for compounds with a D-ring without methyl group
383 at the C4' position, but a methyl group at the C-3' position led to low interaction with
384 PrKAI2d3. This result is in accordance with the low germination stimulant activity of 3'-
385 methyl analogs and mimics for *P. ramosa* and corroborates studies conducted in *O. cumana*,
386 *O. minor*, *P. aegyptiaca*, and *S. hermonthica*^{28,43}. In other words, *P. ramosa* might be
387 sensitive to D-ring modified SL and could perceive diverse SL-related compounds as putative
388 SL degradation products⁴⁴.

389 In addition, we established that ITCs, other known germination stimulants, interact with the
390 PrKAI2d3 protein by forming a covalent complex. In *P. ramosa*, PrKAI2d3 can be considered
391 as a germination stimulant receptor for different chemicals, including SLs, SL derivatives,
392 and mimics, but also ITCs with completely different structures. Thus, *P. ramosa* has
393 seemingly optimized its germination capacity by its ability to perceive various chemical
394 mediators emitted by the host plants. The enzymatic activity-dependent ITC perception

395 mechanism suggests that the active catalytic triad might have been conserved, not only to
396 perceive SLs, but also other germination stimulants, not yet characterized for *P. ramosa*.

397 SLs are very unstable in the soil, especially under basic conditions that lead to ABC=CHOH
398 and D-OH derivatives. To date, all SL analogs and mimics possess a D-ring connected to an
399 ABC mimic by a hydrolysable function. The treatment with SL agonists developed until now
400 induces a suicide germination of plant-parasitic seeds⁴⁵. Nevertheless, these agonists are too
401 unstable under field conditions to be effective, even when promising chemicals have been
402 designed and applied for *S. hermonthica*⁴⁶. In addition, cultures of *P. ramosa*-infested
403 rapeseed mostly occur in slightly basic soils⁴⁷, both favoring the formation of ITCs⁴ and
404 complicating the use of SL mimics as a suicidal germination strategy, especially due to their
405 instability. In sharp contrast, *P. ramosa* is highly sensitive to D-OH or DesMe-D-OH. As for
406 D-H, no germination activity was detected, the hemiacetal function seems very important for
407 the interaction with PrKAI2d3. Moreover, the residual bioactivity of D-OR (R ≠ H) might be
408 explained by the hydrolysis of the R group during the bioassay, leading to D-OH. D-OH or
409 DesMe-D-OH are especially interesting for translational research, because both compounds
410 are small, are easily synthesized in comparison with synthetic SLs and SL mimics, and are not
411 subjected to fast degradation. As such, D-OH and DesMe-D-OH seem to be promising
412 chemicals for suicide germination in the field: their lower germination activity than that of SL
413 mimics can be compensated by higher active concentration. Moreover, D-OH has the great
414 advantage of being a natural product. The use of SL mimics, for which the part equivalent to
415 the ABC moiety is in most cases xenobiotic, increases the risk of potential toxicity or
416 pollution for the environment. In contrast to previous studies^{37,38}, we found that D-OH is
417 bioactive as a witchweed seed germination stimulant and that it could possibly be used for *S.*
418 *hermonthica* biocontrol.

419 Here, we identified germination inhibitors and demonstrated the involvement of α/β -
420 hydrolases for germination-stimulating reception, providing an alternative track to fight *P.*
421 *ramosa*⁴⁸. Novel more active inhibitors need to be designed for *P. ramosa* infection control
422 without negative effects on the host plants and arbuscular mycorrhizal fungi. For this purpose,
423 the identification of the SL receptor(s) in these fungi will undoubtedly be an important
424 milestone. The use of specific inhibitors should be combined with other approaches for
425 integrated management strategies⁴⁹, such as chemical suicidal germination with compounds,
426 such as D-OH, to decrease the parasitic-plant seed bank in soil.

427 **Methods**

428 **Preparation of GR24 isomers, probes, and other ligands.** For general experimental
429 procedures, see the Supplementary Methods. (\pm)-2'-*epi*-GR24 and (\pm)-GR24 were prepared as
430 described⁵⁰ and (\pm)-4-desmethyl-GR24, (\pm)-2'-*epi*-4-desmethyl-GR24, and (\pm)-3'-Me-GR24
431 as described²⁷. (+)-GR24, (-)-GR24, (+)-2'-*epi*-GR24, and (-)-2'-*epi*-GR24 were separated
432 from (\pm)-2'-*epi*-GR24 and (\pm)-GR24 by chiral supercritical fluid chromatography as
433 described^{13,51}. (\pm)-GR24 was purified by semi-preparative HPLC by means of a Interchim
434 puriFlash® 4250 instrument, combined with a fraction collector with integrated ELSD, a
435 PDA and a Phenomenex Luna C18, 250 × 21.2 mm, 5- μ m column (H₂O/CH₃CN: 6/4) or
436 Interchim Uptisphere Strategy SI, 250 × 21.2 mm, 5- μ m column (Heptane/EtOAc: 1/1). 2-
437 Phenethyl isothiocyanate (2-PEITC), benzylisothiocyanate (BEITC), DiFMU, *para*-
438 nitrophenyl acetate (*p*-NPA), Yoshimulactone Green (YLG), abscisic acid (ABA), tolfenamic
439 acid⁵² (TA), TritonX-100⁵³ and phenylmethylsulfonyl fluoride (PMSF) are commercially
440 available. KK094⁵⁴ and soporidine⁵⁵ were kindly provided by T. Asami and P. McCourt
441 (University of Toronto), respectively. Probes (GC486, GC240, and GC242) were prepared as
442 described¹³. For the preparations of D-OH and analogs, see Supplementary Methods.

443 **Expression and purifications.** AtD14 and AtKAI2 were purified and expressed with
444 cleavable GST tags as described¹³. For PrKAI2d3 expression, the coding sequences from
445 *Phelipanche ramosa* were amplified by PCR by means of a seed-derived cDNA template and
446 specific primers (Supplementary Table 4) containing a protease cleavage site for tag removal,
447 and subsequently cloned into the pGEXT-4T-3 expression vector. The PrKAI2d3 and
448 PrKAI2d3^{S98A} proteins were purified and expressed as above.

449 **Site-directed mutagenesis.** Site-directed mutagenesis experiments were done with the
450 QuickChange II XL Site Directed Mutagenesis kit (Stratagene) on pGEX-4T-3-PrKAI2d3
451 (Supplementary Table 4). Mutagenesis was verified by systematic DNA sequencing.

452 **Plant material and growth conditions.** Pea (*Pisum sativum*) branching mutant plants were
453 derived from various cultivars after ethyl methanesulfonate (EMS) mutagenesis and had been
454 described previously⁵⁶. The *rms1-10* (M3T-884) mutant was obtained from the dwarf cv
455 T r se. Plants were grown in a greenhouse under long-day as described⁵⁷.

456 All *Arabidopsis thaliana* (L.) Heynh. mutant plants (Columbia-0 [Col-0] accession
457 background) have been described previously: *htl-3* (ref. ^{58,59}), *Atd14-1/htl-3* (ref. ^{58,59}), and
458 *htl-3* ShHTL7 (kind gift of P. McCourt). For overexpression of the green fluorescent protein
459 (GFP) fusions of PrKAI2d3 and PrKAI2d3^{S98A}, transgenic *Arabidopsis* seeds were generated
460 by the *Agrobacterium* floral dip method⁶⁰ with the *htl-3* mutant as the background accession.
461 Transgenic seeds were selected based on the antibiotic resistance and GFP fluorescence.

462 Two batches of parasitic plant seeds were used. A population of seeds of *Phelipanche ramosa*
463 (L.) Pomel associated to the genetic group 1 (*P. ramosa* 1) was collected from Saint Martin-
464 de-Fraigneau (France) on broomrape-parasitizing winter rapeseed (*Brassica napus* L.) in 2014
465 and 2015 (ref. ⁶¹). Seeds of *Striga hermonthica* (Delile) Benth. (Sudan, 2007) were provided
466 by Lukas Spichal (Olomuc, Czech Republic). Seeds were surface sterilized and conditioned as
467 described²⁹ (darkness; 21 °C and 30 °C for *P. ramosa* and *S. hermonthica*, respectively).

468 **Pea shoot-branching assay.** The compounds to be tested were applied by vascular supply.
469 The control was the treatment with 0.1% dimethylsulfoxide (DMSO) only. Twelve plants
470 were sown per treatment in trays and generally 10 days after sowing the axillary bud at node 3
471 was treated. Compounds in DMSO solution were diluted in water to the indicated
472 concentrations for a treatment with 0.1% (v/v) DMSO. The branches at nodes 1 and 2 were
473 removed to encourage the outgrowth of axillary buds at the nodes above. Nodes were
474 numbered acropetally from the first scale leaf as node 1 and cotyledonary node as node 0.
475 Bud growth at nodes 3 and 4 was measured with digital callipers 8 to 10 days after treatment.
476 Plants with damaged main shoot apex or with a dead white treated-bud were discarded from
477 the analysis. The SL-deficient *rms1-10* pea mutant was used for all experiments.

478 **Cloning and generation of transgenic lines.** For all GFP fusion constructs, cloning was
479 done by Gateway recombination (Thermo Fisher Scientific). The open reading frame (ORF)
480 of PrKAI2d3 was amplified from *P. ramosa* cDNA with iProof™ High-Fidelity DNA
481 Polymerase (Bio-Rad) and the Gateway®-specific primers PrKAI2d3_B2_FW and
482 PrKAI2d3_B3_Rev_STOP. The PCR product flanked by the attB sites was cloned in pDONR
483 P2R-P3 with the BP Clonase II enzyme mix (Invitrogen). The resulting entry vector was used
484 to clone the genes into the destination vector pK7m34GW, under the control of the 35S
485 promoter, and N-terminally fused with GFP with the LR Clonase II Plus enzyme mix
486 (Invitrogen). For the construction of the catalytic site mutant, pDONR P2R-P3-PrKAI2d3 was

487 mutated with the QuikChange II site directed mutagenesis kit (Agilent). The generated clones
488 were checked by sequencing. All primers used for cloning are listed (Supplementary Table 4).

489 **Western Blot.** Total protein was extracted from 5-day-old seedlings, exposed to white light
490 for 3 h, transferred to darkness for 21 h, and exposed to continuous red light for 4 days.
491 Protein concentrations were determined by the Bradford assay (Bio-Rad). Of the protein
492 extracts, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-
493 PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes, 30 μg was detected
494 with horseradish peroxidase (HRP)-conjugated antibodies against GFP (Anti-GFP-HRP,
495 1:10000, Miltenyi Biotec) or anti-tubulin (mouse monoclonal, 1/10000, Sigma-Aldrich) and
496 HRP-conjugated anti-mouse antibodies (rabbit polyclonal, 1/10000, Abcam). The blots were
497 visualized with the Western Lightning Plus Enhanced Chemiluminescence kit (PerkinElmer)
498 and the X-Doc System (Bio-Rad). The Precision Plus Protein™ Dual Color Standards (Bio-
499 Rad) was used as protein size marker.

500 ***Arabidopsis* hypocotyl elongation assays.** *Arabidopsis* seeds were surface sterilized by
501 consecutive treatments of 5 min 70% (v/v) ethanol with 0.05% (w/v) SDS and 5 min 95%
502 (v/v) ethanol and sown on half-strength Murashige and Skoog ($\frac{1}{2}$ MS) media (Duchefa
503 Biochemie) containing 1% (w/v) agar, supplemented with 1 μM (\pm)-GR24 [0.01% (v/v)
504 DMSO] or with 0.01% (v/v) DMSO only (control). Seeds were stratified at 4 °C for 2 days in
505 the dark, then exposed to white light for 3 h, transferred to darkness for 21 h, and exposed to
506 continuous red light for 4 days at 21 °C. Plates were photographed and hypocotyl lengths were
507 quantified using ImageJ (<http://imagej.nih.gov/ij/>).

508
509 ***Arabidopsis* germination assays.** *Arabidopsis* seeds were after-ripened for at least 6 weeks
510 before use. Surface-sterilized seeds were incubated in the incubation solution (1 mM HEPES
511 buffer; pH 7.5) at a ratio of 10 mg of seeds per mL³⁵. Fifty μL of seeds (~ 20-25 seeds) were
512 distributed on 96-well plates and 10 μL of germination stimulants [(10 μM (+)-GR24, 10 μM
513 (-)-GR24, and 0.1% (v/v) DMSO (control) or 10-fold concentrated D-OH] were added. The
514 final volume was adjusted to 100 μL with the incubation solution. Plates were incubated
515 either for 5 days at 25 °C in the dark⁶² or for 7 days at 32 °C-34 °C under constant light
516 illumination at a quantum irradiance of 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (ref. ^{20,26}). A seed was considered
517 germinated when the radicle protruded from the seed coat.

518 **Germination stimulation activity assay on root-parasitic plant seeds.** Germination
519 stimulant activity of chemicals on seeds of parasitic plants were determined as described
520 previously²⁹. Chemicals were suspended in DMSO at 10 mmol L⁻¹, diluted with water at
521 1 mmol L⁻¹ (water/DMSO; v/v; 9/1), and then of 1×10⁻⁵ mol L⁻¹ to 1×10⁻¹² mol L⁻¹ with
522 water/DMSO (v/v; 9/1). For each compound, a concentration range from 10⁻¹³ to 10⁻⁶ mol L⁻¹
523 (water/DMSO; 99/1) was applied to the conditioned parasitic seeds. As negative control, 1%
524 (v/v) DMSO was used (seed germination < 1%) and as positive control (±)-GR24 at a
525 concentration of 1 μmol L⁻¹, inducing 72-87% and 50–65% of seed germination for *P. ramosa*
526 1 and *S. hermonthica*, respectively. To avoid variations related to sterilization events, the
527 germination percentages are reported as a ratio relative to the positive control [1 μmol L⁻¹ (±)-
528 GR24) included in each germination assay. Each dilution and germination assay were
529 repeated at least three times. For each compound tested, dose response curves (germination
530 stimulation activity = f(c); germination stimulant activity relative to 1 μmol L⁻¹ (±)-GR24; c,
531 concentration (mol L⁻¹); half maximal effective concentration (EC₅₀); and maximum
532 germination stimulant activity) were determined with a Four Parameter Logistic Curve
533 computed with SigmaPlot[®] 10.0.

534 **Enzymatic degradation of GR24 isomers by purified proteins.** The ligand (10 μM) was
535 incubated without and with purified AtD14/AtKAI2/PrKAI2d3/PrKAI2d3^{S98A} (5 μM) for
536 150 min at 25°C in 0.1 mL phosphate buffered saline (PBS; 100 mM Phosphate, pH 6.8,
537 150 mM NaCl) in the presence of (±)-1-indanol (100 M) as internal standard. The solutions
538 were acidified to pH = 1 by addition of 2 μL trifluoroacetic acid (TFA) to quench the reaction
539 and centrifugated (12 min, 12,000 tr/min). Thereafter, the samples were subjected to reverse-
540 phase-ultra-performance liquid chromatography (RP-UPLC)-MS analyses by means of UPLC
541 system equipped with a Photo Diode Array (PDA) and a Triple Quadrupole Detector (TQD)
542 mass spectrometer (Acquity UPLC-TQD, Waters). RP-UPLC (HSS C₁₈ column, 1.8 μm, 2.1
543 mm × 50 mm) with 0.1% (v/v) formic acid in CH₃CN and 0.1% (v/v) formic acid in water
544 (aq. FA, 0.1%, v/v, pH 2.8) as eluents [10% CH₃CN, followed by linear gradient from 10% to
545 100% of CH₃CN (4 min)] at a flow rate of 0.6 mL min⁻¹. The detection was done by PDA and
546 with the TQD mass spectrometer operated in Electrospray ionization-positive mode at 3.2 kV
547 capillary voltage. To maximize the signal, the cone voltage and collision energy were
548 optimized to 20 V and 12 eV, respectively. The collision gas was argon at a pressure
549 maintained near 4.5 10⁻³ mBar.

550 **Enzymatic assays with profluorescent probes and *p*-nitrophenyl acetate.** The assays were
551 done as described¹³ with a TriStar LB 941 Multimode Microplate Reader (Berthold
552 Technologies).

553 **Protein melting temperatures.** For the Differential Scanning Fluorimetry (DSF)
554 experiments a CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad) was used with
555 excitation and emission wavelengths of 490 and 575 nm, respectively and Sypro Orange
556 ($\lambda_{\text{ex}}/\lambda_{\text{em}}$: 470/570 nm; Life Technologies) as the reporter dye. Samples were heat-denatured
557 with a linear 25 °C to 95 °C gradient at a rate of 1.3 °C per min after incubation at 25 °C for
558 30 min in the dark. The denaturation curve was obtained by means of the CFX managerTM
559 software. Final reaction mixtures were prepared in triplicate in 96-well white microplates.
560 Each reaction was carried out in 20- μ L sample in PBS (100 mM phosphate, pH 6.8, 150 mM
561 NaCl) containing 6 μ g of protein (so that the final reactions contained 10 μ M protein), 0 to X
562 ligand concentrations in μ M, 4% (v/v) DMSO, and 0.008 μ L Sypro Orange. Plates were
563 incubated in the dark for 30 min before analysis. In the control reaction, DMSO was added
564 instead of the chemical solution. The experiments were repeated three times.

565 **nanoDSF.** Proteins were diluted in PBS (100 mM Phosphate, pH 6.8, 150 mM NaCl) to a
566 concentration of \sim 10 μ M. Ligands were tested at the concentration of 200 μ M. The intrinsic
567 fluorescence signal was measured as a function of increasing temperature with a Prometheus
568 NT.48 fluorimeter (NanoTemper Technologies), with 55% excitation light intensity and 1
569 °C/min temperature ramp. Analyses were done on capillaries filled with 10 μ L of the
570 respective samples. Intrinsic fluorescence signals expressed by the 350 nm/330 nm emission
571 ratio that increases as the proteins unfold, were plotted as a function of temperature (Figures
572 **2d**, **2f**, and **4d**). The plots show one of the three independent data collections done for each
573 protein.

574 **Intrinsic tryptophan fluorescence assays and determination of the dissociation constant**
575 **K_D .** The experiments were done as described¹³ by means of the Spark® Multimode
576 Microplate Reader (Tecan).

577 **Direct electrospray ionization (ESI)-MS under denaturing conditions.** Mass spectrometry
578 measurements were carried out with an electrospray quadrupole-time of flight (Q-TOF) mass
579 spectrometer (Waters) equipped with the Nanomate device (Advion). The HD_A_384 chip
580 (5 μ m i.d. nozzle chip, flow rate range 100–500 nL/min) was calibrated before use. For the

581 ESI–MS measurements, the Q-TOF instrument was operated in radio frequency quadrupole
582 mode with the TOF data collected between m/z 400–2990. The collision energy was set to
583 10 eV and argon was used as collision gas. Mass spectra were acquired after denaturation of
584 PrKAI2d3 ± ligand in 50% (v/v) acetonitrile and 1% (v/v) formic acid. The Mass Lynx 4.1
585 (Waters) and Peakview 2.2 (AB Sciex) softwares were used for data acquisition and
586 processing, respectively. Multiply-charged ions were deconvoluted by the MaxEnt algorithm
587 (AB Sciex). The protein average masses were annotated in the spectra and the estimated mass
588 accuracy was ± 2 Da. For the external calibration, NaI clusters (2 µg/µL, isopropanol/H₂O
589 50/50, Waters) were used in the acquisition m/z mass range.

590

591 **Localization of the fixation site of ligands on PrKAI2d3.** PrKAI2d3-ligand mixtures were
592 incubated for 10 min prior overnight Glu-C proteolysis. Glu-C--generated peptide mixtures
593 were analyzed by nanoLC-MS/MS with the Triple-TOF 4600 mass spectrometer (AB Sciex)
594 coupled to the nanoRSLC UPLC system (Thermo Fisher Scientific) equipped with a trap
595 column (Acclaim PepMap 100 C₁₈, 75 µm i.d. × 2 cm, 3 µm) and an analytical column
596 (Acclaim PepMap RSLC C₁₈, 75 µm i.d. × 25 cm, 2 µm, 100 Å). Peptides were loaded at
597 5 µL/min with 0.05% (v/v) TFA in 5% (v/v) acetonitrile and separated at a flow rate of
598 300 nL/min with a 5% to 35% solvent B [0.1% (v/v) formic acid in 100% acetonitrile]
599 gradient in 40 min with solvent A [0.1% (v/v) formic acid in water]. NanoLC-MS/MS
600 experiments were conducted in a Data-Dependent acquisition method by selecting the 20
601 most intense precursors for collision-induced dissociation fragmentation with the Q1
602 quadrupole set at a low resolution for better sensitivity. Raw data were processed with the MS
603 Data Converter tool (AB Sciex) for generation of .mgf data files and proteins were identified
604 with the MASCOT search engine (Matrix Science) against the PrKAI2 sequence with
605 oxidation of methionines and ligand-histidine adduct as variable modifications. Peptide and
606 fragment tolerance were set at 20 ppm and 0.05 Da, respectively. Only peptides were
607 considered with a MASCOT ion score above the identity threshold (25) calculated at 1% false
608 discovery rate.

609

610 **Phylogenetic analysis.** Phylogenetic analyses were done on 32 D14 and KAI2 sequences,
611 containing five sequences of *Phelipanche ramosa* obtained in this study and previously
612 described sequences from *Phelipanche aegyptiaca*, *Striga hermonthica*, *Pisum sativum*,
613 *Arabidopsis thaliana*, and *Oryza sativa*^{13,17,19,20,63,64}. The D14 and KAI2 proteins and the
614 nucleotide data (Supplementary Data files S1 and S2, respectively) were aligned with AFFT⁶⁵

615 and the G-INS-i iterative refinement alignment method⁶⁶. Sequence alignments
616 (Supplementary Data files S3 and S4) were manually trimmed to remove gaps at either end,
617 producing final protein and nucleotide data sets of 262-264 amino acids and 786-792
618 nucleotides, respectively (Supplementary Data files S5 and S6). The α/β hydrolase RbsQ from
619 *Bacillus subtilis* was used as outgroup, because of its high similarity to KAI2 and D14, and a
620 conserved catalytic triad¹⁷. Maximum likelihood (ML) analyses were conducted with
621 RAxML⁶⁷ on 1,000 bootstraps replicates for statistical support of our inferences. The best ML
622 tree for the amino acid sequences was inferred with the PROT-GAMMA model and the WAG
623 substitution matrix. For the nucleotides sequences, the best ML tree was searched with the
624 GTR model and a gamma rate of heterogeneity among sites. The percentage of trees in which
625 the associated taxa clustered together was provided next to the branches. The resulting
626 consensus amino acid and nucleotide trees were drawn to scale with MEGA7 (ref. ⁶⁸), with
627 the branch lengths representing substitutions per sites.

628

629 **Modeling.** Protein sequences of the *P. ramosa* KAI2 proteins were modeled by means of the
630 SWISSMODEL server (<http://swissmodel.expasy.org/>)⁶⁹. Models were generated with the
631 chain A of the Apo form of the *Arabidopsis* KAI2 structure (PDB: 4JYP) as a template⁷⁰.
632 Figures of protein structure were generated with PYMOL. Cavities within homology models
633 were visualized by the surface mode on the setting “cavities and pockets culled” within
634 PYMOL. Pocket sizes were calculated with the CASTp 3.0 server⁷¹ and a probe radius of
635 1.4 Å. The reported pocket sizes were the Connolly' solvent excluded surface volumes of the
636 catalytic pocket.

637

638 **Expression data.** For the complemented *Arabidopsis* lines, seeds were imbibed for 24 h in a
639 growth solution (1 mM HEPES buffer, pH 7.5, 0.1 mM Preservative Plant Mixture in sterile
640 water) and treated for 6 h with 10 μ M (\pm)-GR24 or mock solution. *P. ramosa* seeds were
641 imbibed in the growth solution for 7 days and then treated for 1 h with 1 nM (\pm)-GR24 or
642 mock solution.. Seeds were harvested, snap-frozen in liquid nitrogen, and ground with pestle
643 and mortar to a fine powder. RNA was extracted and purified with the RNeasy Plant mini kit
644 (Qiagen). Genomic DNA was removed by DNase treatment and the samples were purified by
645 ammonium acetate (5 M final concentration) precipitation. The iScript cDNA synthesis kit
646 (Bio-Rad) was used to reverse transcribe RNA. SYBR Green detection was used during qRT-
647 PCR run on a Light Cycler 480 (Roche). Reactions were done in triplicate in a 384-multiwell
648 plate, in a total volume of 5 μ L and a cDNA fraction of 10%. Cycle threshold values were

649 obtained and analyzed with the 2- $\Delta\Delta$ CT method⁷². The values from three biological and three
650 technical repeats were normalized against those of the seed-specific housekeeping gene
651 *At4g12590* for *Arabidopsis*⁷³. For *P. ramosa*, *PrCACS* was used to normalize the expressions.
652 The normalized values were analyzed according to the published model⁷⁴ with the mixed
653 model procedure (SAS Enterprise).

654

655 **Statistical analyses.** As deviations from normality had been observed for the axillary bud
656 length, hypocotyl length, and germination after SL treatments, the Kruskal–Wallis test was
657 used to assess the significance of one treatment with one compound in comparison to a
658 treatment with another by means of the R Commander software (version 1.7–3) (ref.⁷⁵).

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934

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948 synthesized the profluorescent probes; V.Se. and F.-D.B. synthesized the chemicals; A.d.S.G.,
949 A.J., and E.B. produced and purified the proteins; A.d.S.G. and A.J. characterized the proteins
950 and did the kinetic experiments; A.J., G.B., J.-B.P., and F.-D.B. performed the plant

951 experiments; D.C. performed the mass experiments; A.d.S.G., V.St., and F.-D.B. performed
952 the HPLC analyses and separations; A.d.S.G., A.J., G.B., J.-B.P., L.B, D.C., K.G., P.S., S.W.,
953 S.G., P.D., and F.-D.B. analyzed the data; A.d.S.G., A.J., G.B., J.-B.P., and F.-D.B. wrote the
954 paper. All authors critically revised the manuscript.

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956 **Additional information**

957 **Supplementary information** is available for this paper at www.....

958 **Materials and Correspondence and requests for materials** should be addressed to F.-D.B.

959

960 Legend to Figures

961 **Figure 1. Identification of PrKAI2 putative SL receptors in *P. ramosa*.** (a) Phylogenetic
962 analysis of KAI2 and D14 amino acid sequences. The phylogenetic tree was constructed with
963 the maximum likelihood method and 1,000 bootstraps replicates by means of RAxML. The
964 scale bar represents 0.05 substitutions per site. Clades were designated as described¹⁹ (b)
965 Amino acid sequence alignment of the active PrKAI2 protein sites. Amino acids that differed
966 from AtKAI2 and those similar to AtD14 are in orange and red, respectively; whereas,
967 AtHTL and PrKAI2d3 are in blue and yellow, respectively. A fully expanded alignment can
968 be found in Supplementary Figure 3. (c) Visual representation of the ligand pockets of the *P.*
969 *ramosa* KAI2 genes. The KAI2 protein sequences were modeled with the chain A of the
970 karrikin-bound *Arabidopsis* KAI2 structure as a template (PDB: 4JYP). The protein structures
971 were generated with PYMOL and the cavities within the homology models were visualized
972 with the surface mode on the setting “cavities and pockets culled” within PYMOL. (d)
973 Expression of the KAI2 genes in *P. ramosa*. Primers for *PrKAI2c* and *PrKAI2d3* were used in
974 a qRT-PCR experiment with *EF101* as housekeeping gene on seeds treated for 1 h with (±)-
975 GR24⁷⁶ ($P < 0.001$, Student’s *t* test). (e-g) Cross-species complementation assays of the *htl-3*
976 mutant with *P. ramosa* KAI2d3 and the catalytic site mutant S98A. (e) Protein levels of
977 PrKAI2d3 of 4-day-old seedlings transformed with *p35S::GFP:PrKAI2d3* or
978 *p35S::GFP:PrKAI2d3^{S98A}* detected with anti-GFP (top) and anti-tubulin (bottom) antibodies
979 as loading control. The experiment was repeated twice with comparable results and one
980 representative repeat is shown. (f) Seed germination after 5 days of growth at 25°C in the
981 dark, with DMSO (control), 10 μM (+)-GR24, or 10 μM (-)-GR24 treatments. Transgenes
982 were expressed in the null *htl-3* mutant background (Col-0 accession) under control of the
983 35S promoter. (one representative experiment of 18 wells per condition with an average of 19
984 seeds/well shown). (g) Hypocotyl lengths of 4-day-old seedlings grown under continuous red
985 light at 21 °C ($n = 25$) with 10 μM (±)-GR24 treatments. Graphs represent means of three
986 biological repeats ± SE. Statistical groups indicated by letters were determined by Kruskal-
987 Wallis test with Dunn’s post test, $P < 0.001$ (f) and $P < 0.05$ (g).

988 **Figure 2. PrKAI2d3 shows a stereoselectivity towards GR24 analogs mimicking the SL**
989 **natural stereoconfigurations and perceives SL analogs without a methyl group on the D-**
990 **ring.** (a) Structures of GR24 analogs. (b) and (c) Germination stimulation activity on *P.*
991 *ramosa* seeds (EC_{50}) with four stereoisomers and with GR24 analogs harboring variation on

992 the D-ring (\pm SE), respectively. **(d-g)** Thermostability of PrKAI2d3 at 10 μ M in the absence
993 of ligands (black line) or in the presence of various ligands at 100 μ M analyzed by nanoDSF.
994 The panels **(d)** and **(f)** show the changes in fluorescence (ratio $F_{350\text{nm}}/F_{330\text{nm}}$) with temperature
995 and **(e)** and **(g)** the first derivatives for the $F_{350\text{nm}}/F_{330\text{nm}}$ curve against the temperature gradient
996 from which the apparent melting temperatures (T_m) was determined for each sample. The
997 experiment was carried out twice. **(h)** and **(i)** SL analogs binding PrKAI2d3 based on intrinsic
998 tryptophan fluorescence. Plots of fluorescence intensity *versus* probe concentrations. The
999 change in intrinsic fluorescence was monitored (Supplementary Figure 8) and used to
1000 determine the apparent K_D values. The plots represent the mean of two replicates and the
1001 experiments were repeated at least three times. The analysis was done with GraphPad Prism
1002 5.0 Software.

1003 **Figure 3. The PrKAI2d3 enzymatic activity improves the SL biological activity.** **(a)** and **(b)**
1004 Hydrolysis activity of GR24 isomers and analogs by various proteins. (+)-GR24, (-)-GR24,
1005 (+)-2'-*epi*-GR24 and (-)-2'-*epi*-GR24 **(a)** and (\pm)-GR24, (\pm)-4'-desmethyl-GR24, (\pm)-4'-
1006 desmethyl-2'-*epi*-GR24, and (\pm)-3'-Me-GR24 **(b)** at 10 μ M were incubated with PrKAI2d3,
1007 PrKAI2d3^{S98A}, AtD14, and AtKAI2 at 5 μ M for 150 min at 25 °C. UPLC-UV (260 nm)
1008 analysis was used to detect the remaining amount of GR24 isomers and analogs. Bars
1009 represent the mean value of the hydrolysis rate calculated from the remaining GR24 isomers
1010 and analogs taking into account the hydrolysis in the buffer alone (without protein sample),
1011 quantified with indanol as internal standard. Error bars represent the SD of three replicates
1012 (means \pm SD, $n = 3$). nd, no hydrolysis detected. The asterisks indicate statistical significance
1013 from the PrKAI2d3 protein sample as $***P \leq 0.001$; and $P > 0.05$, as measured by Kruskal-
1014 Wallis test. **(c-e)** Enzymatic kinetics for PrKAI2d3, PrKAI2d3^{S98A}, AtD14 and AtKAI2
1015 proteins incubated with (\pm)-GC486 **(c)**, (\pm)-GC240 **(d)**, and (\pm)-GC242 **(e)**. Progress curves
1016 during hydrolysis of the probes, monitored (λ_{em} 460 nm) at 25 °C with the use of 400 nM
1017 protein and 20 μ M probes. The traces represent one of the three replicates and the
1018 experiments were repeated at least twice. **(f-h)** Hyperbolic plot of the PrKAI2d3 presteady-
1019 state kinetics reaction velocity with (\pm)-GC486 **(f)**, (\pm)-GC240 **(g)**, and (\pm)-GC242 **(h)**. The
1020 initial velocity was determined with profluorescent probe concentrations from 0.3 μ M to
1021 40 μ M and with proteins at 400 nM. Error bars represent SE of the mean of three replicates
1022 and the experiments were repeated at least three times. **(i)** Kinetics constants of probes
1023 towards PrKAI2d3. $K_{1/2}$ and k_{cat} are presteady-state kinetics constants for PrKAI2d3 with
1024 different profluorescent probes and represent the mean \pm SE of three replicates. **(j-o)** Mass

1025 spectrometry characterization of covalent PrKAI2d3-ligand complexes. On the left,
1026 deconvoluted electrospray mass spectra of PrKAI2d3 prior and after addition of different
1027 ligands (\pm)-GR24 and (\pm)-GC486. Peaks with an asterisk correspond to PrKAI2d3 covalently
1028 bound to a ligand (PrKAI2d3-ligand). The mass increments were measured for different
1029 PrKAI2d3-ligand complexes: 96.3 Da (\pm)-GR24) and 82.4 Da (\pm)-GC486). Ligand-modified
1030 amino acids were identified by nanoLC-MSMS analyses after Glu-C proteolysis. On the right,
1031 fragmentation spectra of unmodified and different ligand-modified peptides. Labeled peaks
1032 correspond to the b and y fragments of the double-charged precursor ion displayed at the top.
1033 The histidine residue modified by different ligands is underlined.

1034 **Figure 4. The isothiocyanate germination stimulants are perceived by PrKAI2d3. (a)**
1035 **Structure of isothiocyanate. (b) and (c) Modeled curves of dose response germination**
1036 **stimulant activities (b) and EC₅₀ (half maximal effective concentration) (c). Data presented \pm**
1037 **SE. (d) and (e) Thermostability of PrKAI2d3 at 10 μ M in the absence of ligand or in the**
1038 **presence of various ligands at 100 μ M analyzed by nanoDSF. Changes in fluorescence (ratio**
1039 **F_{350nm}/F_{330nm}) with temperature (d) and first derivatives for the F_{350nm}/F_{330nm} curve against the**
1040 **temperature gradient from which the apparent melting temperatures (T_m) were determined. for**
1041 **each sample (e). The experiment was carried out twice. (f-h) Mass spectrometry**
1042 **characterization of covalent PrKAI2d3-ligand complexes. Deconvoluted electrospray mass**
1043 **spectra of PrKAI2d3 (f) and PrKAI2d3^{S98A} (g) after addition of the 2-PEITC ligand, Peaks**
1044 **with an asterisk correspond to PrKAI2d3 covalently bound to a ligand (PrKAI2d3-ligand).**
1045 **The mass increments were measured for the PrKAI2d3-2-PEITC complex, 162.7 Da. Ligand-**
1046 **modified amino acids were identified by nanoLC-MSMS analyses after Glu-C proteolysis. (h)**
1047 **Fragmentation spectra of unmodified and different ligand-modified peptides. Labeled peaks**
1048 **correspond to the b and y fragments of the double-charged precursor ion displayed at the top.**
1049 **The PEITC ligand-modified serine residue is underlined. (i) Perception mechanism of PEITC.**

1050 **Figure 5. Identification of antagonists and agonists for the *P. ramosa* seed germination.**
1051 **(a) Chemical structures of inhibitors of D14 protein and abscisic acid (ABA). (b) and (c)**
1052 **Comparison of the inhibitor effect on *P. ramosa* germination stimulation (GS) induced by**
1053 **GR24 (10 nM) or 2-PEITC (100 nM). Maximum of inhibition (b) and IC₅₀ (c). (d) Structure**
1054 **of D-OH rings and derivatives. (e) and (f) GS activity of D-OH and its derivatives on *P.***
1055 ***ramosa* seeds. Maximum of GS activity (e) and EC₅₀ (f). (g) D-OH binding of PrKAI2d3,**
1056 **based on intrinsic tryptophan fluorescence. Plots of fluorescence intensity *versus* probe**

1057 concentrations. The change in intrinsic fluorescence was monitored and used to determine the
1058 apparent K_D values. The plots represent the mean of two replicates and the experiments were
1059 repeated at least three times. The analysis was done with GraphPad Prism 5.0 Software. **(h)**
1060 Biochemical analysis of the interaction between PrKAI2d3 at 10 μ M and D-OH at various
1061 concentrations by DSF. Each line represents the average protein melt curve for two technical
1062 replicates and the experiment was carried out twice. **(i)** and **(j)** Mass spectrometry
1063 characterization of covalent PrKAI2d3-D-OH complexes. **(i)** Deconvoluted electrospray mass
1064 spectrum of PrKAI2d3 after addition of the D-OH ligand. Peaks with an asterisk correspond
1065 to PrKAI2d3 covalently bound to D-OH. The mass increments were measured for the
1066 PrKAI2d3-D-OH complex, 114.2 Da. Ligand-modified amino acids were identified by
1067 nanoLC-MSMS analyses after Glu-C proteolysis. **(j)** Fragmentation spectra of unmodified
1068 and different ligand-modified peptides. Labeled peaks correspond to the b and y fragments of
1069 the double-charged precursor ion displayed at the top. The D-OH ligand-modified histidine
1070 residue is underlined.

1071 **Supplementary information**

1072 Supplementary Figure 1. **Chemical structures. (a)** Natural strigolactones. **(b)** Profluorescent
1073 probes. GC series and Yoshimulactone (YLG).

1074 Supplementary Figure 2. **Phylogenetic analysis of KAI2 and D14 nucleotide sequences.**
1075 The phylogenetic tree was constructed with the maximum likelihood method and 1,000
1076 bootstraps replicates by means of RAxML. Scale bar = 0.1 substitutions per site.

1077 Supplementary Figure 3. **Sequence alignment of *Phelipanche ramosa* (Pr) and *P.***
1078 ***aegyptiaca* (Pa) KAI2 protein with D14 and KAI2 proteins from *Arabidopsis* (At), rice**
1079 **(Os), pea (RMS), and the bacterial RbsQ.** The three amino acid residues corresponding to
1080 the catalytic triad are marked with asterisks. Amino residues highlighted in the Figure 1b are
1081 indicated with a blue arrowhead. Amino acid numbers are indicated for AtD14. Note that the
1082 rice OsD14 protein has a non-conserved 50-amino-acid N-terminal extension omitted in the
1083 alignment.

1084 Supplementary Figure 4. **Dose response germination stimulant activities of (+)-GR24 and**
1085 **(-)-GR24 with *Arabidopsis* lines.** Dose response germination stimulation activities and
1086 modeled curves of (+)-GR24 **(a)** and (-)-GR24 **(b)** on seeds of *Arabidopsis* Col-0 and
1087 *htl-3/p35S::PrKAI2d3*. **(c)** Half maximal effective concentration (EC₅₀). **(d)** Maximum
1088 germination stimulation activities. Data are indicated ± SE. **(e)** Relative accumulation of
1089 *PrKAI2d3* transcripts in *Arabidopsis* Col-0, *htl-3*, and *p35S::GFP-PrKAI2d3 #4 (htl-3)* seeds
1090 imbibed for 24 h. **(f)** Accumulation of PrKAI2d3 proteins in *Arabidopsis p35S::GFP-*
1091 *PrKAI2d3 #4 (htl-3)* and *htl-3* 5d-old seedlings.

1092 Supplementary Figure 5. **Germination assay on *P. ramosa* parasitic-plant seeds of GR24**
1093 **isomers [(+)-GR24, (-)-GR24, (+)-2'-*epi*-GR24, and (-)-2'-*epi*-GR24] and methyl**
1094 **variation on the GR24 D-ring [(±)-Desmethyl-GR24, (±)-4'-Desmethyl-2'-*epi*-GR24, and**
1095 **(±)-3'-Me-GR24]. (a) and (c)** Dose response germination stimulation activities and modeled
1096 curves. **(b) and (d)** Maximum germination stimulation activity relative to (±)-GR24 (1 μM).
1097 Data are indicated ± SE.

1098 Supplementary Figure 6. **Germination assay on *P. ramosa* parasitic-plant seeds of various**
1099 **profluorescent ligands and biochemical analysis of the interaction between PrKAI2d3**

1100 **and various ligands by DSF. (a)** Dose response germination stimulation activities and
1101 modeled curves. **(b)** Maximum germination stimulant activity relative to (\pm)-GR24 (1 μ M).
1102 **(c)** Half maximal effective concentration (EC_{50}) (mol L^{-1}). Data are indicated \pm SE. **(d-i)**
1103 Melting temperature curves of PrKAI2d3 with (\pm)-GR24 **(d)**, (\pm)-GC486 **(e)**, (\pm)-GC240 **(f)**,
1104 DiFMU **(g)**, (+)-GC242 **(h)**, and ($-$)-GC242 **(i)** at varying concentrations assessed by DSF.
1105 Each line represents the average protein melt curve for three technical replicates and the
1106 experiment was carried out twice.

1107 Supplementary Figure 7. **Biochemical analysis of the interaction between PrKAI2d3 (a-d,**
1108 **i-k) or PrKAI2d3^{S98A} (e-h, l-n) and various ligands by DSF.** Melting temperature curves of
1109 PrKAI2d3 or PrKAI2d3^{S98A} at 10 μ M with (+)-GR24 **(a, e)**, ($-$)-GR24 **(b, f)**, (+)-2'-*epi*-GR24
1110 **(c, g)**, ($-$)-2'-*epi*-GR24 **(d, h)**, (\pm)-GR24 **(i, l)**, (\pm)-4'-desmethyl-2'-*epi*-GR24 **(j, m)**, or (\pm)-3'-
1111 Me-GR24 **(k, n)** at varying concentrations assessed by DSF. Each line represents the average
1112 protein melt curve for three technical replicates and the experiment was carried out twice.

1113 Supplementary Figure 8. **Intrinsic tryptophan fluorescence of PrKAI2d3 in the presence**
1114 **of SL analogs.** Changes in intrinsic fluorescence emission spectra of PrKAI2d3 in the
1115 presence of various concentrations of (+)-GR24 **(a)**, ($-$)-GR24 **(b)**, (+)-2'-*epi*-GR24 **(c)**, ($-$)-
1116 2'-*epi*-GR24 **(d)**, (\pm)-GR24 **(e)**, (\pm)-4'-desmethyl-2'-*epi*-GR24 **(f)**, or (\pm)-3'-Me-GR24 **(g)**.
1117 Proteins (10 μ M) were incubated with increasing amounts of ligand (0–800 μ M from top to
1118 bottom). The observed relative changes in intrinsic fluorescence were plotted as a function of
1119 the SL analog concentration, transformed to a saturation degree, and used to determine the
1120 apparent K_D values relevant to Figure 2**h,i**. The plots represent the mean of two replicates and
1121 the experiments were repeated at least three times. The analysis was done with GraphPad
1122 Prism 8.0 Software.

1123 Supplementary Figure 9. **Intrinsic tryptophan fluorescence of PrKAI2d3 in the presence**
1124 **of profluorescent SL probes.** Changes in intrinsic fluorescence emission spectra of
1125 PrKAI2d3 in the presence of various concentrations of (\pm)-GC486 **(a)**, (\pm)-GC240 **(b)**,
1126 DiFMU **(c)**, YLG **(d)**, (\pm)-GC242 **(i)**, (+)-GC242 **(j)**, or ($-$)-GC242 **(k)**. Proteins (10 μ M)
1127 were incubated with increasing amounts of ligand (0–800 μ M from top to bottom). The
1128 observed relative changes in intrinsic fluorescence were plotted as a function of the SL analog
1129 concentration and transformed to saturation. Plots of fluorescence intensity *versus* (\pm)-GC486
1130 **(e)**, (\pm)-GC240 **(f)**, DiFMU **(g)**, and YLG concentrations used to determine the apparent K_D

1131 values. The plots represent the mean of two replicates and the experiments were repeated at
1132 least three times. The analysis was done with GraphPad Prism 8.0 Software.

1133 Supplementary Figure 10. **PrKAI2d3 hydrolysis activity.** Progress curves during the 4-
1134 nitrophenyl acetate (*p*-NPA) (1 mM) hydrolysis by PrKAI2d3, PrKAI2d3^{S98A}, AtKAI2, and
1135 AtD14 (4 μM). The *p*-NPA release was monitored (A_{405}) at 25 °C.

1136 Supplementary Figure 11. **Biochemical analysis of the interaction between PrKAI2d3 and**
1137 **ITCs by DSF.** Melting temperature curves of PrKAI2d3 at 10 μM with (±)-GR24 (**a**), 2-
1138 PEITC (**b**), or BITC (**c**) at varying concentrations assessed by DSF. Each line represents the
1139 average protein melt curve for three technical replicates and the experiment was carried out
1140 twice.

1141 Supplementary Figure 12. **Germination inhibition by various chemicals and stimulation**
1142 **by D-analogs.** Dose response germination stimulation (GS) activities with 10 nM GR24 (**a**)
1143 and 100 nM 2-PEITC (**b**) and modeled curves (**c**) and (**d**). Maximum of germination
1144 stimulation activity relative to (±)-GR24 (1 μM). Data are indicated ± SE.

1145 Supplementary Figure 13. **Bud outgrowth inhibition activity assay for D derivatives after**
1146 **direct stem infusion.** Data are means ± SE ($n = 12$), 8 days after treatment of the pea plants
1147 *rms1-10* and Tèrese as control. * $P < 0.5$, *** $P < 0.001$, Kruskal-Wallis rank sum test,
1148 compared to control values (CTL0).

1149 Supplementary Figure 14. **Perception of the germination stimulant D-OH by ShHTL7.**
1150 Dose response germination stimulation activities and modeled curves of (±)-GR24 and D-OH
1151 on seeds of *S. hermonthica* (**a**), *Arabidopsis* Col-0 (**c**), and *Arabidopsis htl-3*, ShHTL7 (34 °C,
1152 continuous light for *Arabidopsis* (**d**). (**b**) Seed germination of *Arabidopsis* Col-0, *htl-3*, *htl-*
1153 *3/ShHTL7*, and *max2* with GR24 and D-OH (10 μM). (**e**) Half maximal effective
1154 concentration (EC_{50}). (**f**) Maximum germination stimulation activities. Data are indicated ±
1155 SE.

1156 Supplementary Table 1. EC_{50} and maximum germination percentage of (+)-GR24, and (-)-
1157 GR24 and of (±)-GR24 and D-OH in *Arabidopsis* lines.

1158 Supplementary Table 2. EC_{50} and maximum of germination stimulant activity of compounds
1159 for *P. ramosa*.

- 1160 Supplementary Table 3. IC₅₀ and maximum of inhibition of putative inhibitors of PrKAI2d3
1161 for *P. ramosa*.
- 1162 Supplementary Table 4. Oligonucleotides used.

A *Phelipanche ramosa* KAI2 Protein Perceives enzymatically Strigolactones and Isothiocyanates

Alexandre de Saint Germain,^a Anse Jacobs,^{b-e} Guillaume Brun,^{f,g} Jean-Bernard Pouvreau,^f Lukas Braem,^{b-e} David Cornu,^h Guillaume Clavé,ⁱ Emmanuelle Baudu,^a Vincent Steinmetz,ⁱ Vincent Servajean,ⁱ Susann Wicke,^g Kris Gevaert,^{d,e} Philippe Simier,^f Sofie Goormachtig,^{b,c} Philippe Delavault^f and François-Didier Boyer^{i*}

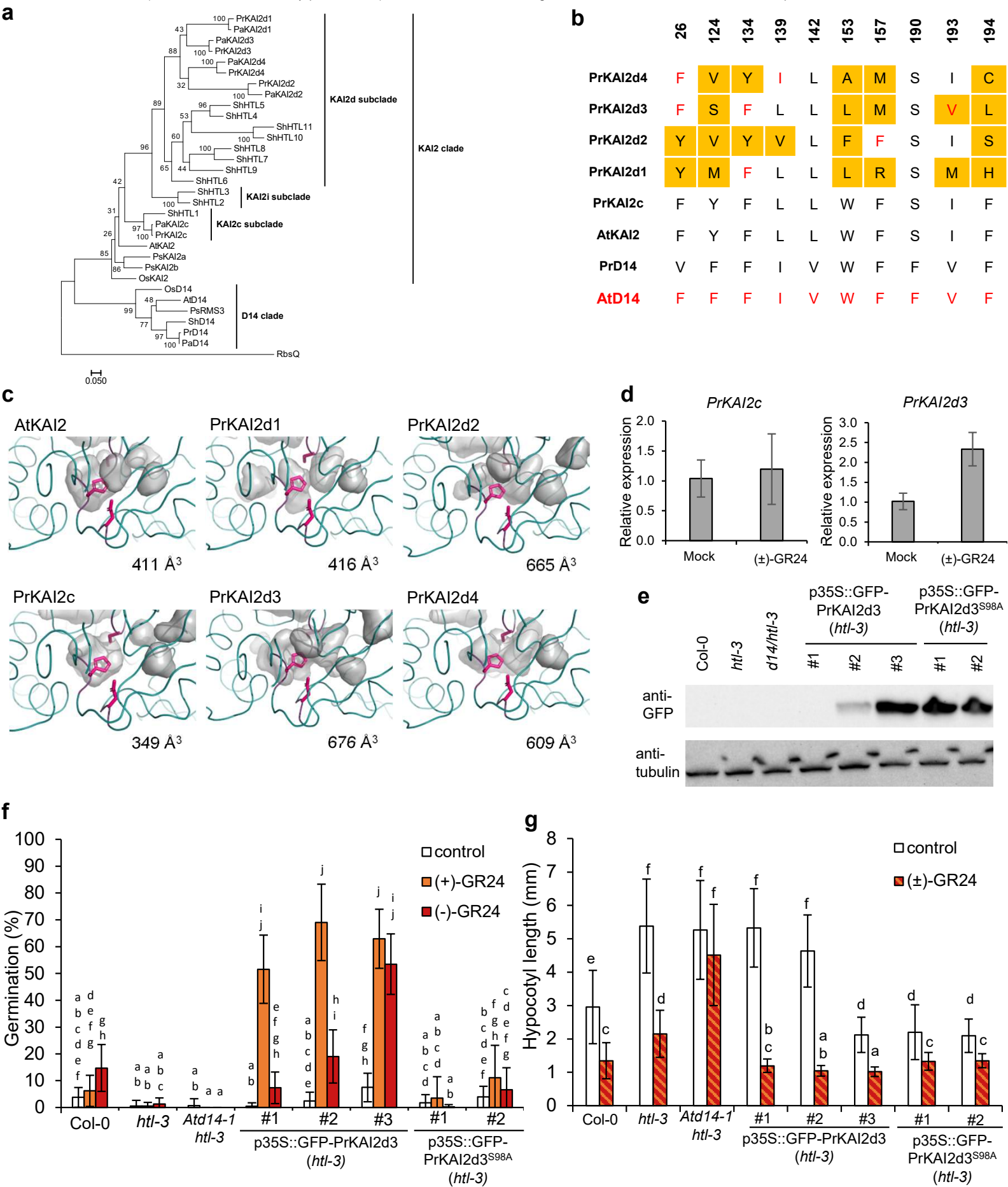
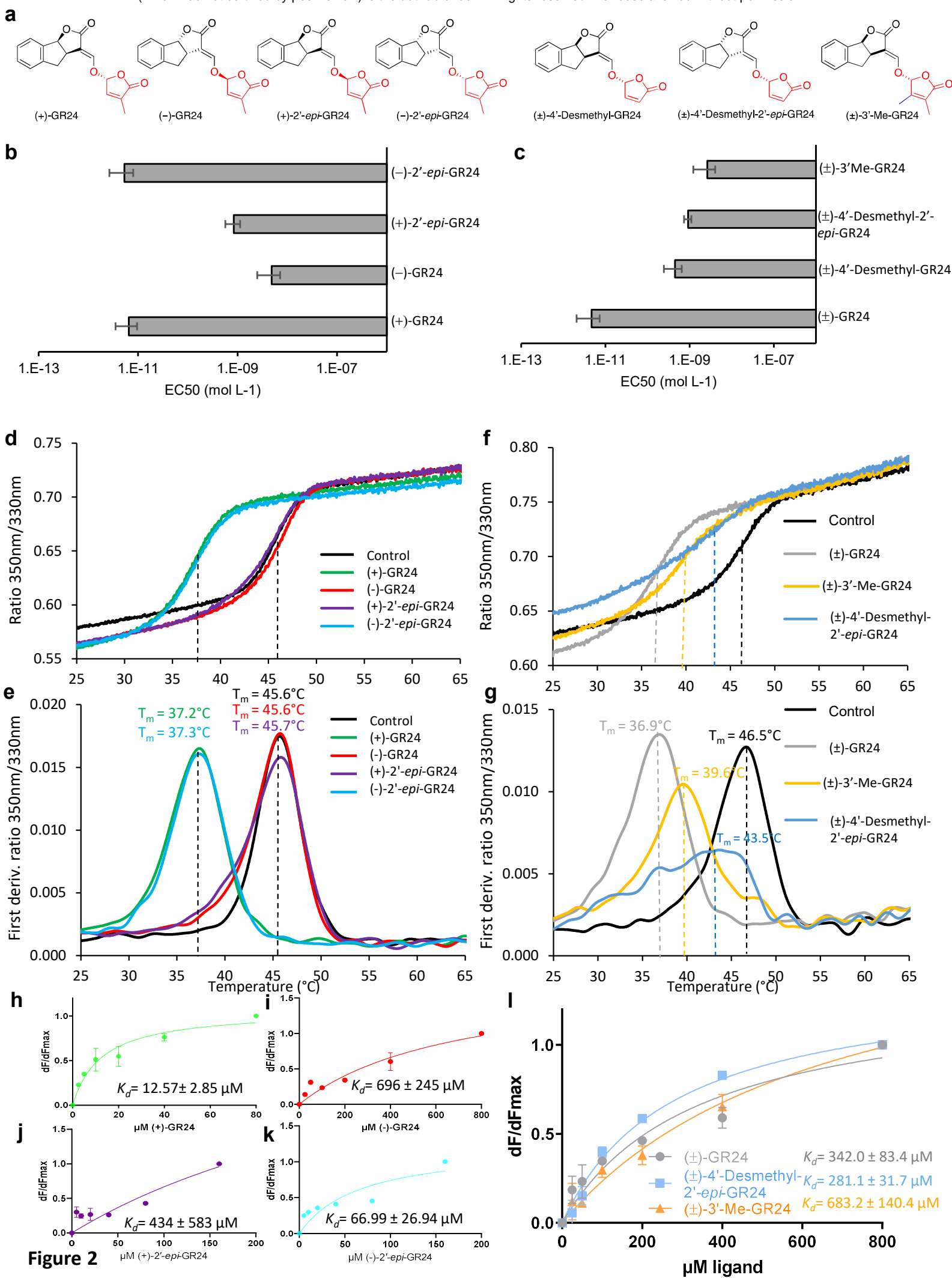


Figure 1



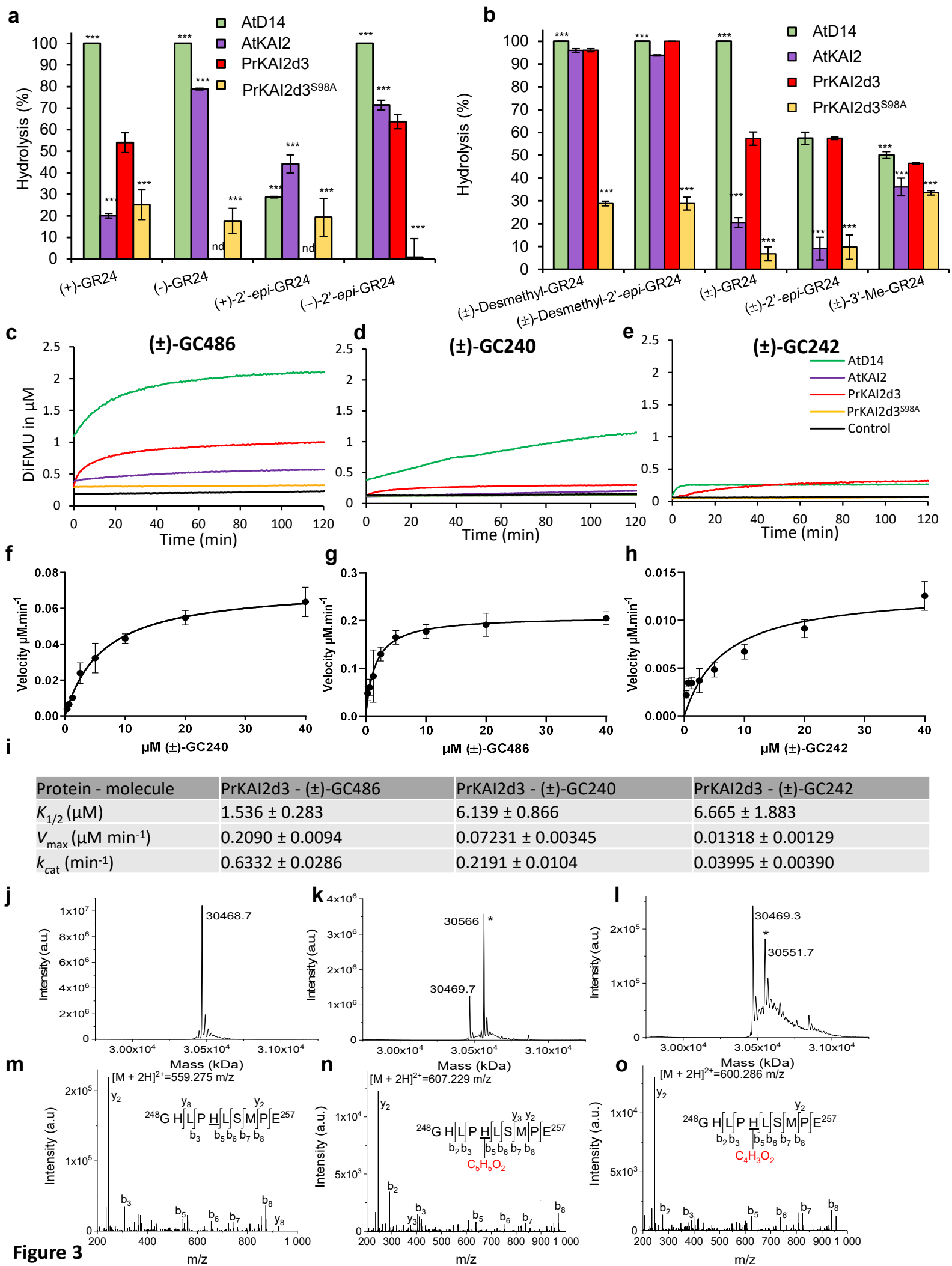


Figure 3

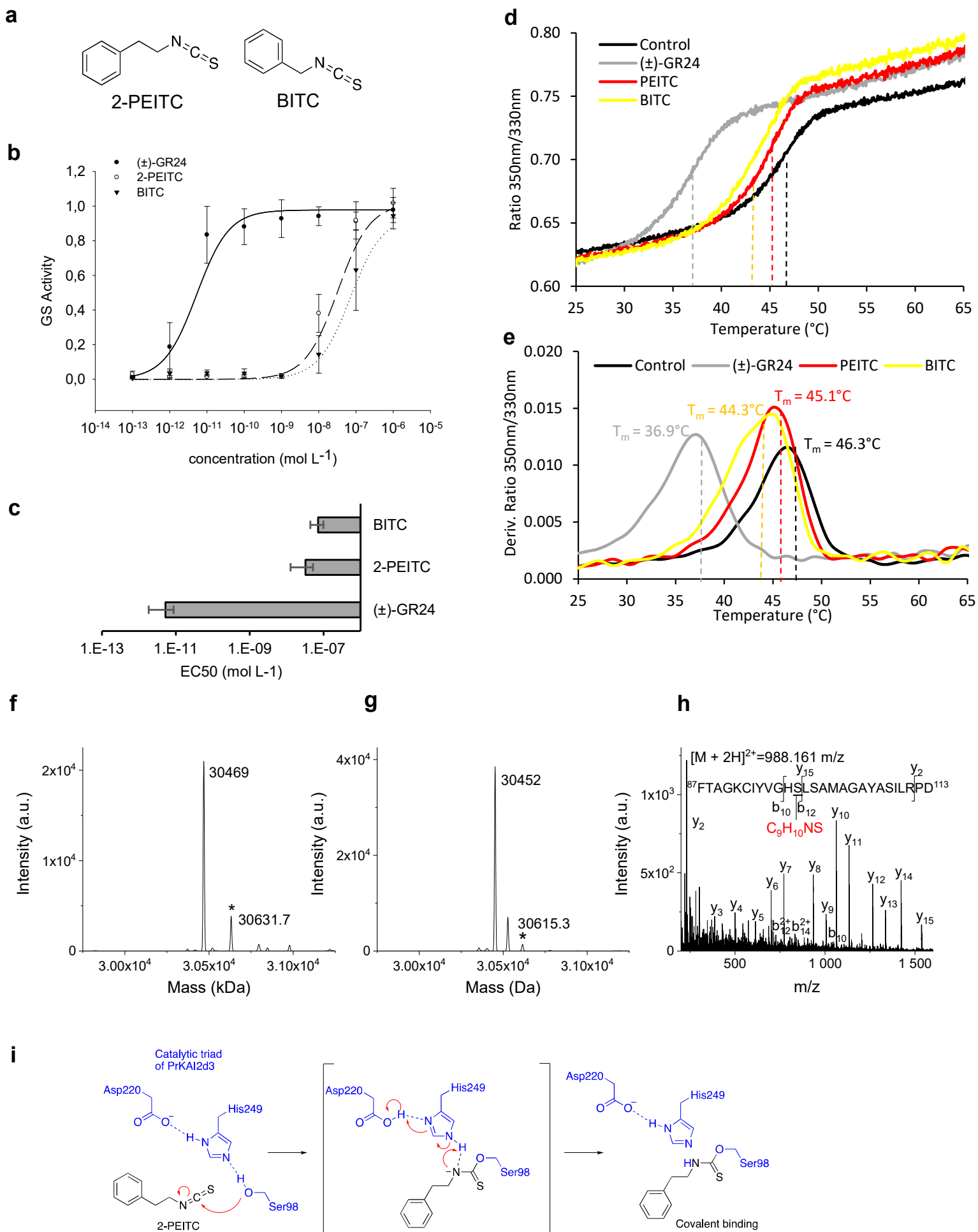


Figure 4

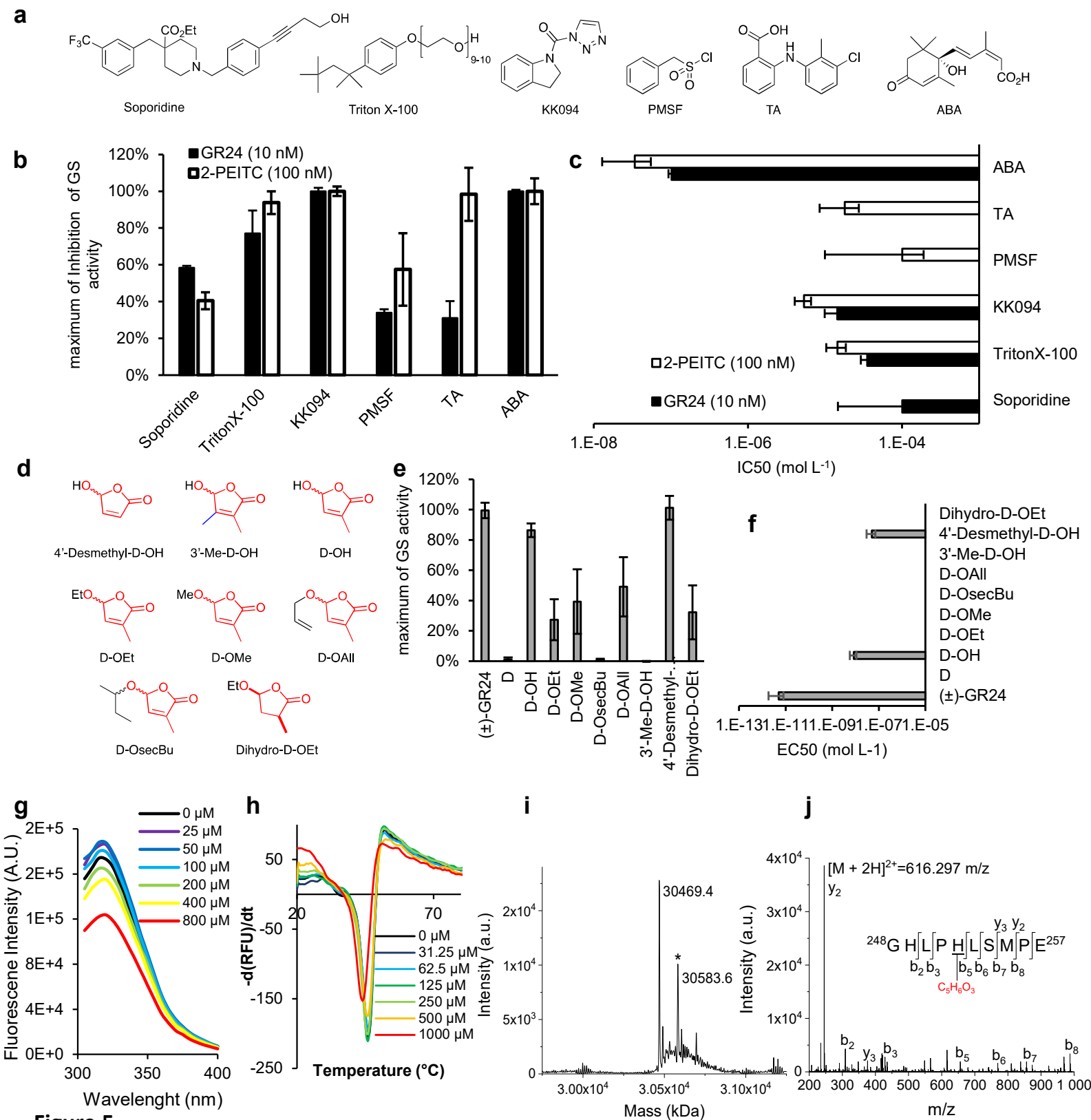


Figure 5