

1 ***Lotus japonicus* karrikin receptors display divergent ligand-binding**
2 **specificities and organ-dependent redundancy**

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4 **Samy Carbonnel^{1,2,#,§}, Salar Torabi^{1,2,§}, Maximilian Griesmann¹, Elias Bleek¹, Yuhong**
5 **Tang³, Stefan Buchka¹, Veronica Basso¹, Mitsuru Shindo⁴, François-Didier Boyer⁵,**
6 **Trevor L. Wang⁶, Michael Udvardi³, Mark Waters^{7,8}, Caroline Gutjahr^{1,2,*}**

7

8 ¹LMU Munich, Faculty of Biology, Genetics, Biocenter Martinsried, Großhaderner Str. 2-4,
9 82152 Martinsried, Germany

10

11 ²Technical University of Munich (TUM), School of Life Sciences Weihenstephan, Plant
12 Genetics, Emil Ramann Str. 4, 85354 Freising, Germany

13

14 ³Noble Research Institute, Ardmore, Oklahoma 73401 USA

15

16 ⁴Institute for Materials Chemistry and Engineering, Kyushu University, Kasugakoen-6,
17 Kasuga, Fukuoka, 816-8580, Japan

18

19 ⁵Université Paris-Saclay, CNRS, Institut de Chimie des Substances Naturelles, UPR 2301,
20 91198, Gif-sur-Yvette, France

21

22 ⁶John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK

23

24 ⁷School of Molecular Sciences, The University of Western Australia, 35 Stirling Hwy, Perth,
25 WA 6009, Australia

26

27 ⁸Australian Research Council Centre of Excellence in Plant Energy Biology, The University
28 of Western Australia, 35 Stirling Hwy, Perth, WA 6009, Australia

29

30 [#]Current address: Department of Biology, University of Fribourg, Chemin du Musée 10, 1700
31 Fribourg, Switzerland

32

33 Address for correspondence: caroline.gutjahr@tum.de

34

35 [§]These authors contributed equally to this work.

36

37 **Short title:** Ligand specificity of *Lotus japonicus* KAI2 receptors

38 **Abstract**

39 Karrikins (KARs), smoke-derived butenolides, are perceived by the α/β -fold hydrolase
40 KARRIKIN INSENSITIVE2 (KAI2) and are thought to mimic endogenous, yet elusive plant
41 hormones tentatively called KAI2-ligands (KLs). The sensitivity to different karrikin types as
42 well as the number of *KAI2* paralogs varies among plant species, suggesting diversification
43 and co-evolution of ligand-receptor relationships. In legumes, which comprise a number of
44 important crops with protein-rich, nutritious seed, *KAI2* has duplicated. We report sub-
45 functionalization of KAI2a and KAI2b in the model legume *Lotus japonicus* and demonstrate
46 that their ability to bind the synthetic ligand GR24^{ent-5DS} differs *in vitro* as well as in genetic
47 assays in *Lotus japonicus* and in the heterologous *Arabidopsis thaliana* background. These
48 differences can be explained by the exchange of a widely conserved phenylalanine in the
49 binding pocket of KAI2a with a tryptophan in KAI2b, which occurred independently in KAI2
50 proteins of several unrelated angiosperms. Furthermore, two polymorphic residues in the
51 binding pocket are conserved across a number of legumes and may contribute to ligand
52 binding preferences. Unexpectedly, *L. japonicus* responds to diverse synthetic KAI2-ligands
53 in an organ-specific manner. Hypocotyl development responds to KAR₁, KAR₂ and *rac*-GR24,
54 while root system development responds only to KAR₁. This organ-specificity cannot be
55 explained by receptor-ligand preferences alone, because *LjKAI2a* is sufficient for karrikin
56 responses in the hypocotyl, while *LjKAI2a* and *LjKAI2b* operate redundantly in roots. Our
57 findings open novel research avenues into the evolution and diversity of butenolide ligand-
58 receptor relationships, their ecological significance and the mechanisms controlling diverse
59 developmental responses to different KAI2 ligands.

60 Introduction

61 Karrikins (KARs) are small butenolide compounds derived from smoke of burning vegetation
62 that were identified as germination stimulants of fire-following plants [1]. They can also
63 accelerate seed germination of species that do not grow in fire-prone environments such as
64 *Arabidopsis thaliana*, which enabled the identification of genes encoding karrikin receptor
65 components via forward and reverse genetics. The α/β -fold hydrolase KARRIKIN
66 INSENSITIVE2 (KAI2) is thought to bind KARs, and interacts with the F-box protein MORE
67 AXILLIARY GROWTH 2 (MAX2) that is required for ubiquitylation of repressor proteins via
68 the Skp1-Cullin-F-box (SCF) complex [2-9]. There are six known KARs, of which KAR₁ is
69 most abundant in smoke-water and most active on seed germination of fire-following plants
70 [1, 10, 11], but *Arabidopsis* responds more strongly to KAR₂, which lacks the methyl group at
71 the butenolide ring that is characteristic for KAR₁ [2, 3, 11]. Both KAR₁ and KAR₂ are
72 commercially available and commonly used in research.

73
74 *Arabidopsis* KAI2 regulates several traits in addition to seed germination, including light-
75 dependent hypocotyl growth inhibition, cotyledon and rosette leaf area, cuticle thickness,
76 root hair length and density, root skewing and lateral root density [4, 12-15]. Moreover, the
77 rice orthologs of *KAI2* (*D14-LIKE*) and *MAX2* (*D3*) are essential for root colonization by
78 arbuscular mycorrhiza (AM) fungi, and are involved in regulating mesocotyl elongation [7,
79 16, 17]. These roles of KAI2, unrelated to smoke and seed germination, suggest that karrikins
80 mimic yet-unknown endogenous (and possibly AM fungus-derived) signalling molecules that
81 bind to KAI2 to regulate plant development or AM symbiosis, and are provisionally called
82 KAI2-ligands (KLs) [12, 18].

83

84 Structurally, KARs resemble the apocarotenoid strigolactones (SLs), which were originally
85 discovered in root exudates in the rhizosphere [19], where they act as germination cues for
86 parasitic weeds [19] and as stimulants of AM fungi [20, 21]. In addition to their function in the
87 rhizosphere, SLs function endogenously as phytohormones and repress shoot branching [22,
88 23]. SL signalling also affects secondary growth; and co-regulates lateral and adventitious
89 root formation and rice mesocotyl elongation with the karrikin signalling pathway [7, 15, 24,
90 25]. As with KARs, SLs are perceived by an α/β -fold hydrolase D14/DAD2 that, like KAI2,
91 depends for function on a serine–histidine–aspartate catalytic triad within the ligand binding
92 pocket [26, 27]. As KAI2, D14 interacts with the SCF-complex via the same F-box protein
93 MAX2 [9, 26] to ubiquitylate repressors of the SMXL family and mark them for degradation
94 by the 26S proteasome [28-31].

95 Phylogenetic analysis of the α/β -fold hydrolase receptors in extant land plants revealed that
96 an ancestral *KAI2* is already present in charophyte algae, while the so-called eu-*KAI2* is
97 ubiquitous among land plants. The strigolactone receptor gene, *D14* evolved only in the seed
98 plants likely through duplication of *KAI2* and sub-functionalization [32]. An additional
99 duplication in the seed plants gave rise to *D14-LIKE2* (*DLK2*), an α/β -fold hydrolase of
100 unknown function, which is transcriptionally induced in response to KAR treatment in a *KAI2*-
101 and *MAX2*-dependent manner, and currently represents the best-characterized KAR marker
102 gene in Arabidopsis [4, 33]. Despite their similarity, KAI2 and D14 cannot replace each other
103 in Arabidopsis, as shown by promoter swap experiments [34]. This indicates that their
104 expression pattern does not determine their signaling specificity. Instead, this is reached by
105 ligand-receptor preference, and most likely the tissue-specific presence of their ligands, as

106 well as distinctive interaction with other proteins, such as repressors of the SMXL family to
107 trigger downstream signaling [35].

108 In *Arabidopsis* and rice, in which KAR/KL signalling has so far been mostly studied, *KAI2* is
109 a single copy gene. However, *KAI2* has multiplied and diversified in other species. For
110 example, the *Physcomitrella patens* genome contains 11 genes encoding KAI2-like proteins
111 [36]. Of these some preferentially bind KAR and others the SL 5-deoxystrigol *in vitro* and this
112 preference is determined by polymorphic amino acids in a loop that determines the rigidity of
113 the ligand-binding pocket [37]. The genomes of parasitic plants of the Orobanchaceae also
114 contain several *KAI2* copies. Some of these have evolved to perceive strigolactones, some
115 can restore KAR-responses in *Arabidopsis kai2* mutants, and others do not mediate
116 responses to any of these molecules in *Arabidopsis* [12, 38, 39]. Thus, in plant species with
117 an expanded KAI2-family there is scope for a diverse range of ligands and ligand-binding
118 specificities, as well as for diverse protein interaction partners. Apart from discriminating
119 KARs from SLs, it was very recently reported that *KAI2* genes have diversified in the genome
120 of the fire follower *Brassica tournefortii* to encode KAI2 receptors, with different ligand
121 preferences towards KAR₁ and KAR₂ [40]. Of these, *BtKAI2a* mediates stronger responses
122 to KAR₂, while *BtKAI2b* mediates stronger responses to KAR₁, when expressed in the
123 heterologous *Arabidopsis* background. This binding preference is determined by two valine
124 (*BtKAI2a*) to leucine (*BtKAI2b*) substitutions at the ligand binding pocket [40]. Also among
125 plants with a single copy *KAI2* gene, the responsiveness to karrikin molecules can differ
126 significantly: *Arabidopsis* plants respond more strongly to KAR₂ than to KAR₁ [4, 15]. In
127 contrast, rice roots did not display any transcriptional response to KAR₂, not even for the

128 marker gene *DLK2* [16]. It is yet unclear what determines these differences in KAR₂
129 responsiveness among plant species.

130 Legumes comprise a number of agronomically important crops and they are special among
131 plants as most species in the family can form nitrogen-fixing root nodule symbiosis with
132 rhizobia in addition to arbuscular mycorrhiza. Given the possible diversity in KAI2-ligand
133 specificities among plant species, we characterized the karrikin receptor machinery in a
134 legume, using *L. japonicus* as a model. We found that *KAI2* has duplicated prior to the
135 diversification of legumes and that *L. japonicus* KAI2a and KAI2b differ in their binding
136 preferences to synthetic ligands *in vitro* and in the heterologous *Arabidopsis kai2* and *kai2*
137 *d14* mutant backgrounds. We demonstrate that these ligand binding preferences can be
138 explained by substitution of a highly conserved phenylalanine to a tryptophan in the binding
139 pocket of *LjKAI2b*. This tryptophan occurs rarely also in other unrelated angiosperm species,
140 and seems to have arisen several times independently. Two additional polymorphic residues
141 that are conserved in the KAI2a and KAI2b clades across several legumes may also
142 contribute to ligand binding preference. In addition, we found a surprising organ-specific
143 responsiveness to synthetic KAI2-ligands, with *L. japonicus* hypocotyl development
144 responding to KAR₁, KAR₂ and the strigolactone/karrikin analog *rac*-GR24, and root system
145 development responding only to KAR₁. These responses depend only on *LjKAI2a* in
146 hypocotyls, while *LjKAI2a* and *LjKAI2b* operate redundantly in roots. Together these findings
147 suggest that a diversity of mechanisms may influence KAR/KL responses including receptor-
148 ligand binding specificity or organ-specific interaction of KAI2 with other proteins.

149

150

151 **Results**

152 ***KAI2* underwent duplication prior to diversification of the legumes**

153 To characterize the karrikin and the strigolactone perception machinery in *L. japonicus* we
154 retrieved *KAI2*, *D14* and *MAX2* by protein BLAST using Arabidopsis *KAI2*, *D14* and *MAX2*
155 as templates. A phylogenetic tree revealed that *LjD14* (*Lj5g3v0310140.4*) is a single copy
156 gene whereas *LjKAI2* has duplicated (Fig 1), resulting in two paralogs *LjKAI2a*
157 (*Lj2g3v1931930.1*) and *LjKAI2b* (*Lj0g3v0117039.1*). The *KAI2* duplication event must have
158 occurred prior to the diversification of the legumes or at least before the separation of the
159 Millettoids and the ‘Hologalegina’ clade [41] because a similar duplication pattern as in *L.*
160 *japonicus* (Hologalegina) is also detected in pea, *Medicago truncatula* (both Hologalegina)
161 and soybean (Millettoid). The Millettoid soybean genome additionally contains a third, more
162 distantly related *KAI2* copy (*KAI2c*).

163 The F-box protein-encoding gene *LjMAX2* also underwent duplication likely as a result of
164 whole genome duplication, because the two *LjMAX2* copies are in two syntenic regions of
165 the genome (S1A Fig). However, only one *LjMAX2* copy (*Lj3g3v2851180.1*) is functional. The
166 other copy *ΨMAX2-like* (*Lj0g3v0059909.1*) appears to be a pseudogene, as it contains an
167 early stop codon, thus encoding a putative truncated protein of 216 instead of 710 amino
168 acids (S1B Fig). It appears that an insertion of one nucleotide into *ΨMAX2-like* created a
169 frameshift, as manual deletion of thymine 453 restores a correct nucleotide and amino acid
170 sequence (S1B Fig).

171 We hypothesized that *L. japonicus* (and other legumes) retained two intact *KAI2* copies
172 because they may have functionally diverged, perhaps through changes in their expression

173 pattern and/or sequence, possibly resulting in a divergent spatial distribution, ligand affinity
174 and/or ability to interact with other proteins. We examined transcript accumulation of *LjKAI2a*
175 and *LjKAI2b*, as well as *LjD14* and *LjMAX2* in different organs of *L. japonicus* (S2 Fig).
176 Overall, both *LjKAI2a* and *LjKAI2b* transcripts accumulated to higher levels than those of
177 *LjD14* and *LjMAX2*. *LjKAI2a* transcripts accumulated approximately 100-fold more in aerial
178 organs than *LjKAI2b*, whereas *LjKAI2b* accumulated 10-fold more than *LjKAI2a* in roots of
179 adult plants, which were grown in a sand-vermiculite mix in pots under long day conditions
180 (16h light / 8h dark). However, 1-week-old seedlings grown on water-agar in Petri dishes
181 under short-day conditions (8h light / 16h dark), displayed 10-fold higher transcript levels of
182 *LjKAI2a* than *LjKAI2b* in both roots and hypocotyls (S2B Fig). Thus, *LjKAI2a* and *LjKAI2b* are
183 regulated in an organ-specific, age- and/or environment-dependent manner, suggesting that
184 their individual expression involves at least partially different transcriptional regulators.

185 Fusions of the four corresponding proteins with T-Sapphire or mOrange in transiently
186 transformed *Nicotiana benthamiana* leaves showed similar subcellular localization as in
187 *Arabidopsis* and rice [16, 17, 42, 43]. T-Sapphire-MAX2 was detected exclusively in the
188 nucleus, while the α/β -hydrolases (D14, KAI2a and KAI2b) fused to mOrange localized to the
189 nucleus and cytoplasm (S3A Fig). Western blot analysis confirmed that the mOrange signal
190 observed in the cytoplasm resulted from the full-length fusion protein and not from free
191 mOrange (S3B Fig).

192

193 ***L. japonicus* KAI2a, KAI2b and D14 can replace their orthologs in Arabidopsis**

194 To examine whether both *LjKAI2a* and *LjKAI2b* function in a canonical manner, we employed
195 a well-established hypocotyl elongation assay in *Arabidopsis* [12, 34], after transgenically

196 complementing the *Arabidopsis thaliana kai2-2* mutant [4] with *LjKAI2a* and *LjKAI2b* driven
197 by the *AtKAI2* promoter. Both restored inhibition of hypocotyl elongation in the *kai2-2* mutant
198 (Fig 2A). *LjD14* driven by the *AtKAI2* promoter was unable to restore hypocotyl growth
199 inhibition, but it restored repression of shoot branching of the *Arabidopsis d14-1* mutant [4],
200 when driven by the *Arabidopsis D14* promoter. As expected, *LjKAI2a* and *LjKAI2b* could not
201 do the same (Fig 2B and 2C). Together with the phylogenetic analysis (Fig 1), these results
202 demonstrate that *L. japonicus KAI2a* and *KAI2b* are both functional orthologs of the
203 *Arabidopsis* karrikin/KL receptor gene *KAI2*, whereas *L. japonicus D14* is the functional
204 orthologue of the *Arabidopsis* strigolactone receptor gene *D14*. Furthermore, the *L. japonicus*
205 *KAI2* genes are not interchangeable with *D14* [34].

206 207 ***Lotus japonicus* KAI2a and KAI2b differ in their ligand binding specificity**

208 To explore whether *L. japonicus* *KAI2a* and *KAI2b* can mediate hypocotyl responses to
209 karrikins, we quantified hypocotyl length of the *Atkai2-2* lines transgenically complemented
210 with *LjKAI2a* or *LjKAI2b* after treatment with KAR_1 and KAR_2 (Fig 3A and 3B). Two
211 independent lines complemented with *LjKAI2a* displayed a similar reduction in hypocotyl
212 growth in response to both KAR_1 and KAR_2 . However, the two lines expressing *LjKAI2b*
213 responded more strongly to KAR_1 than to KAR_2 , contrasting with the common observation,
214 that *Arabidopsis* hypocotyl growth tends to be more responsive to KAR_2 [4, 44]. We wondered
215 if the preference towards a specific *KAR* compound is also observed with *KAI2* from other
216 species. To this end, we tested the karrikin response in a line resulting from a cross of the
217 *kai2* mutant *htl-2* with an *Arabidopsis* line transgenic for the cDNA of the rice *D14L/KAI2* [16].
218 In contrast to *LjKAI2b*, *OsD14L/KAI2* mediated a stronger response to KAR_2 than to KAR_1

219 (Fig 3C). Thus, the differential responsiveness of transgenic Arabidopsis lines to KAR₁ and
220 KAR₂ does not result from a general incompatibility of a heterologous KAI2 protein with the
221 Arabidopsis background, but suggests different ligand affinities of the transgenic receptors to
222 the karrikins or their possible metabolised products [34, 40].

223 The two enantiomers of the synthetic strigolactone *rac*-GR24, namely GR24^{5DS} and GR24^{ent-}
224 ^{5DS}, trigger developmental and transcriptional responses via D14 as well as KAI2,
225 respectively, in Arabidopsis [15, 45]. For some KAI2-mediated responses, GR24^{ent-5DS} was
226 shown to be more active than karrikin [46] and it has been hypothesized that karrikin may
227 need to be metabolized *in planta*, to yield a high affinity KAI2 ligand, while this may not be
228 necessary for GR24^{ent-5DS} [34, 46, 47]. We examined whether *LjKAI2a* and *LjKAI2b* can
229 mediate hypocotyl growth responses to GR24^{5DS} and GR24^{ent-5DS} in the *Arabidopsis thaliana*
230 *d14-1 kai2-2* double mutant background (Fig 3D). Lines expressing *LjKAI2a* responded to
231 both enantiomers with reduced hypocotyl elongation, but displayed a much stronger
232 response to the preferred KAI2 ligand GR24^{ent-5DS}. Unexpectedly, the lines expressing
233 *LjKAI2b* did not significantly respond to either of the two enantiomers. This contrasting
234 sensitivity to GR24 enantiomers together with the differences in response to KAR₁ and KAR₂
235 suggests that *LjKAI2a* and *LjKAI2b* differ in their binding pocket, resulting in divergent affinity
236 to the synthetic ligands.

237

238 **Replacement of a conserved phenylalanine by a tryptophan at the binding pocket of** 239 **KAI2b explains rejection of GR24^{ent-5DS}**

240 We used differential scanning fluorimetry (DSF) to examine whether purified recombinant
241 *LjKAI2a* and *LjKAI2b* (S4A Fig) display a different ligand affinity *in vitro*, employing GR24^{5DS}

242 and GR24^{ent-5DS} as model ligands (Fig. 4, S4 Fig). The DSF assay has been widely used for
243 deducing GR24^{5DS} and GR24^{ent-5DS} binding to D14 and KAI2 proteins respectively, by means
244 of thermal destabilization [26, 34, 40, 48-50]. However, DSF is unfortunately not suitable for
245 the characterization of karrikin binding [34, 40]. Neither *LjKAI2a* nor *LjKAI2b* were
246 destabilized in the presence of GR24^{5DS} (S4B Fig). GR24^{ent-5DS} induced a significant thermal
247 destabilization of *LjKAI2a* at a concentration > 50 μ M. In contrast, it did not cause any
248 significant thermal shift of *LjKAI2b* (Fig 4B), thus recapitulating the difference in hypocotyl
249 growth response between Arabidopsis lines expressing *LjKAI2a* and *LjKAI2b* (Fig 3D).

250 We found 16 conserved amino acid differences between the KAI2a and the KAI2b clade of
251 the investigated legumes (Fig. S5), which may contribute to functional diversification of KAI2a
252 and KAI2b. Modelling of *LjKAI2a* and *LjKAI2b* on the KAR₁-bound *AtKAI2* crystal structure
253 (4JYM) [5] revealed that only three of these were located at the binding pocket, namely
254 L160/M161, S190/L191 and M218/L219 (for the KAI2a/KAI2b comparison, Fig 4A). Out of
255 these three, L219 was conserved between *LjKAI2b* and the KAI2 proteins from Arabidopsis
256 and rice, which displayed an opposite response pattern to KAR₁ and KAR₂ compared to
257 *LjKAI2b*, when expressed in the Arabidopsis background (Fig. 3). Therefore, we concluded
258 that the M218/L219 polymorphism likely does not play a major role in determining the
259 differential ligand-preference between *LjKAI2a* and *LjKAI2b* and discounted it as a candidate.
260 However, we also found that in *LjKAI2b* exclusively, a highly conserved phenylalanine inside
261 the pocket is replaced by tryptophan at position 158 (S5 Fig). Although this tryptophan is not
262 conserved among other legume KAI2b versions used for the alignment (S5 Fig), we predicted
263 that this bulky residue should have a strong impact on ligand binding.

264 To understand the impact of the three divergent candidate amino acids on ligand binding, we
265 generated chimeric receptor proteins (Fig 4B). Exchanging only the two amino acids that are
266 conserved across KAI2a and KAI2b clades comprising the investigated legumes (S5 Fig) was
267 sufficient to influence the melting temperature of the two proteins in response to GR24^{ent-5DS}.
268 *LjKAI2a*^{M160,L190} became less responsive relative to *LjKAI2a* and displayed a slight shift in
269 melting temperature only with 200 μ M GR24^{ent-5DS}, whereas *LjKAI2b*^{L161,S191} gained a weak
270 ability to respond to GR24^{ent-5DS} at 200 μ M. When all three amino acids were swapped, the
271 melting response to GR24^{ent-5DS} was entirely switched between the two receptor proteins:
272 *LjKAI2a*^{M160,L190,W157} did not display any thermal shift in presence of GR24^{ent-5DS}, whereas
273 *LjKAI2b*^{L161,S191,F158} gained a strong response to GR24^{ent-5DS} and displayed a thermal shift with
274 ligand concentrations as low as 25 μ M. Thus, *LjKAI2b*^{L161,S191,F158} seemed to be slightly more
275 prone to ligand-induced destabilisation than wild-type *LjKAI2a*. As W158 appeared to be a
276 critical amino acid for restricting the response to GR24^{ent-5DS}, we also tested whether
277 swapping F157 with W158 alone would suffice to exchange the ability of the receptors to
278 respond to GR24^{ent-5DS}. In effect, *LjKAI2b*^{F158} recapitulated the response of *LjKAI2a* to
279 GR24^{ent-5DS} and likewise *LjKAI2a*^{W157} resembled *LjKAI2b*. Thus, changing this one amino
280 acid in the binding pocket was sufficient to swap ligand specificity. We conclude that the
281 F158/W159 polymorphism predominantly determines the ability of *LjKAI2* proteins to bind
282 GR24^{ent-5DS}, while there is a weaker contribution of L160/M161 and S190/L191.

283 As an alternative means to probe ligand-receptor interactions, intrinsic tryptophan
284 fluorescence assays confirmed the response of wild-type *LjKAI2a* and *LjKAI2b* and all mutant
285 versions to GR24^{ent-5DS} (S6 Fig). Unfortunately, because GR24^{ent-5DS} precipitated above 500
286 μ M, this assay did not allow us to calculate K_d values because saturation of response could

287 not be achieved. Nevertheless, the qualitative results reiterate the strong impact of
288 F158/W159 on the relative affinities of *LjKAI2a* and *LjKAI2b* for GR24^{ent-5DS}.

289 To examine whether the three amino acid residues determine ligand discrimination *in planta*,
290 we transformed Arabidopsis *d14 kai2* double mutants with the mutated *LjKAI2a* and *LjKAI2b*
291 genes driven by the Arabidopsis *KAI2* promoter and performed the hypocotyl growth assay
292 in the presence of GR24^{ent-5DS}. Swapping only the two amino acids conserved in legumes
293 (M160/L161 and S190/L191) was insufficient to exchange the GR24^{ent-5DS} response between
294 lines expressing *LjKAI2a* vs. *LjKAI2b*. However, swapping all three amino acids, negatively
295 affected the capacity of *LjKAI2a*^{M160,L190,W157} to mediate a hypocotyl response to GR24^{ent-5DS},
296 whereas it reconstituted a response via *LjKAI2b*^{L161,S191,F158} in three independent transgenic
297 lines (Fig. 5A and 5B). Although these results do not rule out a contribution of M160/L161
298 and S190/L191 towards ligand preference, they confirm that the phenylalanine to tryptophan
299 substitution at position 157/158 is critical for determining the difference in GR24^{ent-5DS} binding
300 preference between the two *L. japonicus* karrikin receptors KAI2a and KAI2b.

301

302 **The phenylalanine to tryptophan exchange occurred in several unrelated angiosperms**
303 **independently**

304 The phenylalanine-to-tryptophan transition requires two base changes at position two and
305 three of the codon. We asked whether this change also occurred in other species and
306 searched for KAI2 sequences across the plant phylogeny by BLAST-P against the
307 EnsemblPlants, NCBI and 1KP databases [51], and retrieved KAI2 sequences of the parasitic
308 plants *Striga hermonthica*, *Orobanche fasciculata* and *Orobanche minor* from Conn et al.
309 2015 [38]. This analysis showed that the *KAI2c* copy, we detected in the soybean genome

310 (Fig 1) occurred in all analysed genomes of Milletoid legumes, the Genistoid legume *Lupinus*
311 *albus* and the Mimosoid legume *Prosopis alba* (S7 Fig). This suggests that the members of
312 the Hologalegina clade, such as *L. japonicus*, have secondarily lost *KAI2c*. Importantly,
313 among the 156 *KAI2* sequences we analysed, ten in addition to *LjKAI2b* contain a tryptophan
314 at the position corresponding to 157 in *Arabidopsis KAI2* (S7 Fig). One of them was present
315 in another legume (*Prosopis alba*). Five were present in other eudicots, of which three were
316 in the Lamiales (Paulowniaceae: *Paulownia fargesii*; Phrymaceae: *Erythranthe guttata*,
317 Orobanchaceae: *Orobanche fasciculata*) and two in the Ericales (Primulaceae: *Ardisia*
318 *evoluta* and *Ardisia humilis*). Furthermore, we found four in monocots of the Bromeliaceae
319 (*Ananas comosus*), Dioscoreaceae (*Dioscorea rotundata*) and Iridaceae (*Sisyrinchium*
320 *angustifolium*). In these species, W157 does not co-occur with M160 and L190 as in *LjKAI2b*,
321 but mostly in combination with the more widely conserved residues L160 and A190 and also
322 with L160 and L190 in *Prosopis alba* and *Ananas comosus* (S7 Fig). The genomes of all
323 dicotyledon species encoding a *KAI2* version with W157 contained at least one second copy
324 encoding F157. In the monocot *Dioscorea rotundata* two *KAI2* copies encoded the W157,
325 whereas in *Ananas comosus* and *Sisyrinchium angustifolium*, we detected only one *KAI2*
326 copy. However, we cannot exclude the existence of additional copies as several
327 transcriptomes in the 1KP database are likely incomplete.

328 In summary, we demonstrate that the F to W transition has occurred several times
329 independently in the angiosperms without co-dependency on M160 and L190 of *LjKAI2b*, and
330 in most cases it occurred in a duplicate *KAI2* version. Thus, the binding pocket of *KAI2*
331 proteins appears to be subject to diversification, broadening the range of diverse *KAI2*-ligand
332 variants that can be recognized, and at the same time extending the opportunities for binding-

333 and signaling-specificity through KAI2 variants with a less (F157) and/or more (W157)
334 restrictive binding-pocket.

335

336 **Characterization of *L. japonicus* karrikin and strigolactone receptor mutants**

337 To explore the roles of *LjKAI2a* and *LjKAI2b* in *L. japonicus*, we characterized mutants in
338 these genes as well as in *D14* and *MAX2*. We identified *LORE1* retrotransposon insertions
339 in *L. japonicus* *KAI2a*, *KAI2b* and *MAX2* (*kai2a-1*, *kai2b-3*, *max2-1*, *max2-2*, *max2-3*, *max2-*
340 *4*) in available collections [52, 53] and nonsense mutations in *D14* and *KAI2b* (*d14-1*, *kai2b-*
341 *1*, *kai2b-2*) by TILLING [54] (Fig. 6A, S1 Table). Since some of the *max2* and *kai2b* mutants
342 were impaired in seed germination or production (S1 Table) we continued working with *kai2b-*
343 *1*, *kai2b-3*, *max2-3* and *max2-4*. Quantitative RT-PCR analysis revealed that all mutations
344 caused reduced transcript accumulation of the mutated genes in roots of the mutants except
345 for *d14-1* (S8 Fig). Furthermore, the transcript accumulation of *LjKAI2a* and *LjKAI2b* was not
346 affected by mutation of the respective other paralog (S8A Fig).

347 The *LORE1* insertion in the *kai2a-1* mutant is located close (19 bp) to a splice acceptor site.
348 Since some *LjKAI2a* transcript accumulated in the mutant, we sequenced this residual
349 transcript to examine the possibility that a functional protein could still be made through loss
350 of *LORE1* by splicing. We found that indeed a transcript from ATG to stop accumulates in
351 *kai2a-1* but it suffers from mis-splicing leading to a loss of the *LORE1* transposon plus 15 bp
352 (from 369 - 383), corresponding to five amino acids (YLNDV) at position 124-128 of the
353 protein (S9A-9B Fig). This amino-acid stretch reaches from a loop at the surface of the protein
354 into the cavity of the binding pocket (S9C Fig). The artificial splice variant did not rescue the

355 *Arabidopsis kai2-2* hypocotyl phenotype, confirming that it is not functional *in planta* and
356 showing that the amino acids 124-YLNDV-128 are essential for *LjKAI2a* function (S9D Fig).

357

358 **Karrikin and *rac*-GR24 cause reduction in hypocotyl growth of *L. japonicus* in an**
359 ***LjKAI2a*-dependent manner**

360 The *d14-1* and all *max2* mutants displayed increased shoot branching, indicating that the *L.*
361 *japonicus* strigolactone receptor components D14 and MAX2 are involved in shoot branching
362 inhibition (Fig 6B and 6C), as for *Arabidopsis*, pea and rice [4, 43, 55, 56]. In addition, *d14*
363 and *max2* mutants had smaller leaves (Fig 6D), a phenotype that has not yet been associated
364 with strigolactone signalling in other dicotyledon species. Surprisingly, *kai2a* and *kai2b* single
365 mutants as well as *kai2a-1 kai2b-1* double mutants or *max2* mutants did not display the
366 canonical elongated hypocotyl phenotype, which is observed in *Arabidopsis* [4] also in white
367 light conditions (Fig 2, 3 and 5). If anything, the *kai2a-1 kai2b-1* and *max2* mutant hypocotyls
368 were shorter than those of the wild type (Fig. 6E). This indicates that the requirement of KL
369 perception for suppression of hypocotyl elongation under white light is not conserved in *L.*
370 *japonicus* and/or that KL may not be produced under these growth conditions.

371 To examine whether *L. japonicus* hypocotyls are responsive to karrikin treatment, we
372 measured the dose-response of hypocotyl growth in wild-type to KAR₁, KAR₂ and also to *rac*-
373 GR24. Hypocotyl elongation of wild type plants was progressively inhibited with increasing
374 concentrations of all three compounds (S10A Fig). However, it was not suppressed by KAR₁
375 or KAR₂ treatment in the *kai2a-1 kai2b-1* double mutant and the *max2-4* mutant (S10B-10C
376 Fig). This demonstrates that similar to *Arabidopsis*, the hypocotyl response to karrikin of *L.*
377 *japonicus* depends on the KAI2-MAX2 receptor complex. We also examined the KAR₁

378 response of *kai2a* and *kai2b* single mutant hypocotyls and found that *kai2a-1* did not
379 significantly respond to KAR₁ and KAR₂, while the two allelic *kai2b* mutants showed reduced
380 hypocotyl growth in response to both karrikins (S10B Fig). The transcript accumulation
381 pattern of *DLK2* (*Lj2g3v0765370*) – a classical karrikin marker gene in Arabidopsis [4, 45] –
382 was consistent with this observation and *DLK2* was induced in hypocotyls by KAR₁ and KAR₂
383 in a *LjKAI2a*-dependent but *LjKAI2b*-independent manner (S10D Fig). *rac*-GR24 treatment
384 induced an increase of *DLK2* transcripts in a *LjKAI2b*-independent, partially *LjKAI2a*-
385 dependent, and fully *MAX2*-dependent manner, suggesting that this induction is mediated via
386 *LjKAI2a* (*GR24^{ent-5DS}*) and *LjD14* (*GR24^{5DS}*), similar to Arabidopsis [45] (S10C Fig). In
387 summary, *LjKAI2a* appears to be necessary and sufficient to perceive karrikins and *GR24^{ent-}*
388 *5DS* in the *L. japonicus* hypocotyl, possibly because expression of *LjKAI2b* in hypocotyls is too
389 low under short day conditions (S2B Fig).

390

391 ***L. japonicus* root system architecture is modulated by KAR₁ but not by KAR₂ treatment**

392 *rac*-GR24 treatment can trigger root system architecture changes in Arabidopsis and
393 *Medicago truncatula* [57-59], and it has recently become clear for Arabidopsis that lateral and
394 adventitious root formation is co-regulated by karrikin and strigolactone signalling [15, 25].
395 We examined whether *L. japonicus* root systems respond to *rac*-GR24, KAR₁ and KAR₂ (Fig.
396 7A). Surprisingly, in contrast to Arabidopsis and *M. truncatula*, *L. japonicus* root systems
397 responded neither to *rac*-GR24 nor to KAR₂. Only KAR₁ treatment led to a dose-dependent
398 decrease in primary root length and an increase of post-embryonic root (PER) number, and
399 thus to a higher PER density (Fig. 7A). PERs include lateral and adventitious roots that can
400 be difficult to distinguish in *L. japonicus* seedlings grown on Petri dishes. The instability of

401 *rac*-GR24 over time in the medium could potentially prevent a developmental response of the
402 root to this compound in our experiments [60]. However, refreshing the medium with new *rac*-
403 GR24 or karrikins at 5 days post- germination, did not alter the outcome (Fig. 7B).
404 Consistently, we observed *DLK2* induction in roots after KAR₁ but not after KAR₂ treatment
405 (Fig. 7C).

406 Together with the hypocotyl responses to KAR₁, KAR₂ and *rac*-GR24 this indicates organ-
407 specific sensitivity or responsiveness to these three compounds in *L. japonicus* with a more
408 stringent uptake, perception and/or response system in the root.

409 Surprisingly, we found that the roots responded to *rac*-GR24 treatment with increased *DLK2*
410 transcript accumulation (Fig. 7D) although no change in root architecture was observed in
411 response to this treatment (Fig. 7A). This suggests that different ligands may be transported
412 to different tissues or may have a divergent impact on receptor conformation, thereby
413 mediating different downstream responses. To confirm the contrasting responses of *L.*
414 *japonicus* root systems to KAR₁ and *rac*-GR24, and to test whether they result from divergent
415 molecular outputs, we examined transcriptional changes after one, two and six hours
416 treatment of *L. japonicus* wild-type roots with KAR₁ and *rac*-GR24 using microarrays.
417 Statistical analysis revealed a total number of 629 differentially expressed (DE) genes for
418 KAR₁-treated and 232 genes for *rac*-GR24-treated roots (Table S2). In agreement with
419 previous reports from Arabidopsis and tomato [44, 61, 62] the magnitude of differential
420 expression was low. Most of the DE genes upon KAR₁ and *rac*-GR24 treatment responded
421 solely after 2h (S11 Fig). Interestingly, only a minority of 48 genes responded in the same
422 direction in response to both KAR₁ and *rac*-GR24, while the majority of genes responded
423 specifically to KAR₁ (580 DEGs) or *rac*-GR24 (169 DEGs). If *rac*-GR24 were to simply mimic

424 the effect of KAR (GR24^{ent-5DS}) and SL (GR24^{5DS}) on roots, one would have expected a large
425 overlap with KAR₁ responses, and in addition a number of non-overlapping DEGs, regulated
426 through D14. In summary, the microarray experiment confirmed largely non-overlapping
427 responses of *L. japonicus* root response to KAR₁ and *rac*-GR24.

428

429 **Both *LjKAI2a* and *LjKAI2b* mediate root architecture-responses to KAR₁ but only**
430 ***LjKAI2a* mediates *DLK2* expression in response to GR24^{ent-5DS}**

431 To inspect which α/β -hydrolase receptor mediates the changes in *L. japonicus* root system
432 architecture in response to KAR₁ treatment, we examined PER density in the karrikin receptor
433 mutants. The *Ljkai2a-1 kai2b-1* double mutant and the *max2-4* mutant did not respond to
434 KAR₁ treatment with changes in root system architecture (Fig. 8A, S12 Fig). With 1 μ M KAR₁,
435 we obtained contradictory results for the single *kai2a* and *kai2b* mutants in independent
436 experiments (S12A and S12C Fig). However, *kai2a* and *kai2b* single mutants but not the
437 *kai2a kai2b* double mutant responded to a slightly higher concentration of 3 μ M KAR₁ (Fig.
438 8A), indicating that *LjKAI2a* and *LjKAI2b* redundantly perceive KAR₁ (or a metabolite thereof)
439 in *L. japonicus* roots. This pattern was mirrored by *DLK2* expression in roots: both *kai2a* and
440 *kai2b* single mutants responded to KAR₁ with increased *DLK2* expression, while the *kai2a-1*
441 *ka2b-1* double mutant and the *max2-4* mutant did not respond (Fig. 8B). Since *LjKAI2b* did
442 not respond to GR24^{ent-5DS} *in vitro* as well as in the heterologous Arabidopsis background
443 (Fig 4 and 5, S6 Fig), we examined its ability to mediate *DLK2* induction by GR24^{ent-5DS} in *L.*
444 *japonicus* roots. Wild-type and *kai2b* mutant roots responded to GR24^{ent-5DS} with increased
445 *DLK2* expression, but this was not the case for *kai2a* roots confirming that *LjKAI2b* cannot
446 bind and mediate responses to GR24^{ent-5DS} (Fig 8C). In summary, *LjKAI2a* and *LjKAI2b* act

447 redundantly in roots in mediating responses to KAR₁ but only KAI2a can perceive GR24^{ent-}
448 ^{5DS}.

449

450 **Discussion**

451 Gene duplication followed by sub- or neofunctionalization is an important driver in the
452 evolution of complex signalling networks and signalling specificities during the adaptation to
453 new or diverse environments. In the legumes, the karrikin receptor gene *KAI2* multiplied
454 possibly during the whole genome duplication that occurred in the Papilionoidaea before the
455 diversification of legumes 59 million years ago [63]. While the Mimosoids, Genistoids and
456 Millettioids, contain three different *KAI2* versions, the Hologalegina clade appears to have lost
457 one of them, retaining the more closely related *KAI2a* and *KAI2b* versions. Here, we provide
458 evidence that *L. japonicus* *KAI2a* and *KAI2b* diversified in their ligand-binding specificity as
459 well as organ-specific requirement (Fig 9).

460 *LjKAI2a* and *LjKAI2b* differ in their quantitative sensitivity to KAR₁ and KAR₂, which vary only
461 by the presence of one methyl group in KAR₁ (Fig. 3B). An increased hydrophobicity of the
462 *LjKAI2b* binding pocket as compared to *LjKAI2a* may mediate the preference towards the
463 more hydrophobic KAR₁, similar to the fire-following plant *Brassica tournefortii* [40]. The
464 difference in ligand preference of *LjKAI2a* vs. *LjKAI2b* is more dramatic for GR24^{ent-5DS}, an
465 enantiomer of the synthetic strigolactone analogue *rac*-GR24, which acts through Arabidopsis
466 *KAI2* when applied to plants, promotes interaction of *KAI2* with *SMAX1* in yeast and binds to
467 *AtKAI2 in vitro* [8, 15, 34, 45, 46]. We show that *LjKAI2a* mediates strong hypocotyl growth
468 responses to GR24^{ent-5DS} in Arabidopsis as well as transcriptional activation of *DLK2* in *Lotus*
469 *japonicus* roots. *LjKAI2b* is incapable of triggering these responses to the compound, while

470 being able to induce the same responses upon KAR₁ treatment. The dramatic difference in
471 the ability of *LjKAI2a* and *LjKAI2b* to bind GR24^{ent-5DS} is confirmed *in vitro* by DSF and intrinsic
472 tryptophan fluorescence assays. Together, these results demonstrate that the individual α/β -
473 fold hydrolase receptor is sufficient to explain ligand sensitivity in planta.

474 Identifying the determinants of ligand-binding specificity of D14 and different KAI2 and KAI2-
475 like proteins is an area of active research. Binding specificity of D14 and KAI2 to SLs and
476 KARs respectively has been associated with the geometry and size of the binding pocket [6,
477 64]. Changes in amino acid residues located in the pocket of divergent KAI2 versions in
478 parasitic weeds have enabled alterations in pocket architecture and evolution of a chimeric
479 receptor that perceives strigolactones like D14 but mediates germination like KAI2 [38, 39].
480 The rigidity of lid helices forming the tunnel of the binding pocket have been proposed to
481 determine specificity of KAI2-like proteins for strigolactone-like molecules vs. KAR₁ in
482 *Physcomitrella patens* [37].

483 We identified three amino acids at the ligand-binding pocket that differ between *LjKAI2a* and
484 *LjKAI2b*. Two of these are conserved across the legume KAI2a and KAI2b clades, namely
485 L160 and S190 in KAI2a and M161 and L191 in KAI2b. This pattern of conservation suggests
486 functional relevance in maintaining flexibility for different KAI2 ligands in legumes. Indeed,
487 exchanging these two amino acids slightly changes the thermal instability of the two KAI2
488 versions in the DSF assay. Neither amino acid change is predicted to substantially impact
489 the pocket volume or geometry but the amino acids of *LjKAI2b* are more hydrophobic, which
490 may explain the preference for the more hydrophobic KAR₁ over KAR₂. A similar
491 phenomenon was observed in *Brassica tournefortii*, a fire-following weed that has two
492 functional *KAI2* genes [40]. Similar to the situation in *L. japonicus*, *BtKAI2b* mediated a

493 greater sensitivity to KAR₁ over KAR₂ in the Arabidopsis background, while it was the reverse
494 for *BtKAI2a*. This was explained by one amino acid polymorphism in the binding pocket
495 towards a more hydrophobic amino acid (V98L) in *BtKAI2b*. Notably, this residue (V98L) is in
496 a very different position than the polymorphics residues in *L. japonicus* KAI2a/KAI2b,
497 suggesting that the receptors are highly plastic and that similar binding-specificities may be
498 achieved by changing hydrophobicity in different positions of the pocket.

499 Exchanging L160/M161 and S190/L191 between *L. japonicus* KAI2a and KAI2b was
500 sufficient to change their sensitivity to GR24^{ent-5DS} in the DSF *in vitro* assay. However, the
501 developmental response of Arabidopsis hypocotyls was hardly changed, possibly because *in*
502 *vivo*, suboptimal ligand binding to the receptor can be stabilized by interacting proteins. A
503 third amino acid difference (F157/W158) between the two KAI2 proteins occurs in *L.*
504 *japonicus*. This residue critically determines sensitivity to GR24^{ent-5DS} *in vitro* as well as in
505 Arabidopsis likely because the bulky tryptophan may sterically hinder GR24^{ent-5DS} binding,
506 while still allowing binding of the smaller karrikins. In fact, swapping F157 with W158 alone
507 was sufficient to swap the ability to respond to GR24^{ent-5DS} in DSF as well as intrinsic
508 fluorescence assays.

509 *B. tournefortii* is a fire-following plant, the seeds of which respond to karrikins with dormancy
510 breaking and germination [40]. Therefore, to maintain two copies of KAI2, one of which is
511 specialized for KAR₁, the most abundant KAR in smoke, and the other of which may be
512 specialized for the endogenously produced ligand makes adaptive sense for *B. tournefortii*.
513 For *L. japonicus*, which is not a fire-follower, KAR₁, KAR₂ and GR24^{ent-5DS} are likely not natural
514 KAI2 ligands. Nevertheless, the maintenance of two *KAI2* genes in the Hologalegina
515 legumes, each with amino acid polymorphisms conferring differences in binding preferences

516 to artificial ligands, requires an adaptive basis. One possibility is that *L. japonicus* KAI2a and
517 KAI2b have specialized to bind different ligands *in planta*, indicative of legumes producing at
518 least two different versions of the as-yet-unknown KAI2 ligand. The distinct expression
519 patterns and developmental roles of *LjKAI2a* and *LjKAI2b* might also be consistent with a
520 tissue-specific diversity of ligands, or even an endogenous ligand versus an exogenous
521 ligand derived from the rhizosphere. From our assays with artificial ligands we extrapolate
522 that *LjKAI2b* has a higher ligand selectivity than *LjKAI2a*. The additional amino acid change
523 that occurred in *L. japonicus* but not in the other examined legumes may indicate that the KL
524 bouquet of *L. japonicus* has further diversified. Alternatively, the F to W substitution in *LjKAI2b*
525 may confer resistance to (a) toxic allelochemical(s) that may be released into the rhizosphere
526 by competing neighbouring plants or by microorganisms. This speculative hypothesis is
527 consistent with the role of *LjKAI2b* in roots (but not in hypocotyls) and with the observation
528 that the F157W exchange occurred in several unrelated plant species independently that may
529 all encounter compounds capable of blocking KAI2a in their natural habitat. It will be exciting
530 to investigate the biological significance of this receptor sub-functionalization and the putative
531 diversity of their ligands, once the molecule class of KL and its variants have been identified.

532 In addition to ligand-binding specificity at the level of the receptor, we identified a surprising
533 organ-specific responsiveness to synthetic KAI2 ligands in *L. japonicus*. While hypocotyl
534 growth is inhibited in response to KAR₁, KAR₂ and *rac*-GR24, root systems only respond to
535 KAR₁ with architectural changes (Fig. 9A). To our knowledge such an organ-specific
536 discrimination of different but very similar KAR molecules has not previously so clearly been
537 observed. However, a similar scenario could be at play in rice, in which transcriptome analysis
538 of KAR₂-treated rice roots identified no differentially expressed gene [16], whereas rice

539 mesocotyls respond with growth inhibition to the same treatment [7, 16]. Although KAI2 can
540 be shown to bind KAR₁ *in vitro* by isothermal titration calorimetry or fluorescent microdialysis
541 [5, 6, 65], there is evidence suggesting that KARs are not directly bound by KAI2 *in vivo*, but
542 may be metabolized first to yield the correct KAI2-ligand, which may bind with higher affinity
543 [34, 46]. It is possible that substrate specificities differ among enzymes involved in KAR
544 metabolism in hypocotyls vs. roots. This would imply that the single methyl group, which
545 distinguishes KAR₁ from KAR₂, is sufficient to impact specialized metabolism of karrikins.
546 Alternatively, the transport of KAR₂ or the KAR₂-derived metabolic product could be limited in
547 the root system, or KAR₂-derivatives may be rapidly catabolised in roots, thus limiting their
548 effect. While KAR₂ fails to induce increased PER density as well as *DLK2* expression in *L.*
549 *japonicus* roots, GR24^{ent-5DS} triggers *DLK2* transcript accumulation albeit being unable to
550 increase PER density. *DLK2* induction by GR24^{ent-5DS} requires KAI2a, thus involvement of
551 D14 can be excluded. Furthermore, *LjKAI2a* and *LjKAI2b* act redundantly in mediating KAR₁-
552 induced root system changes, excluding the possibility that they are regulated exclusively by
553 *LjKAI2b*, which cannot bind GR24^{ent-5DS}. It is tempting to speculate that conformational
554 changes of KAI2 proteins may differ depending on the ligand and that the extend of the
555 change may influence the interaction strength with the karrikin signaling repressor SMAX1,
556 MAX2 and/or additional proteins [8, 66]. Perhaps *DLK2* expression is more sensitive to
557 quantitative SMAX1 removal than genes required to be induced for root system changes,
558 such as the ethylene biosynthesis gene *ACS7* [66]. Alternatively, SMAX1 proteins inhibiting
559 *DLK2* expression are more accessible to the receptor complex, thereby allowing interaction
560 even when the receptor binds a suboptimal ligand, as compared to SMAX1 individuals
561 supressing transcriptional acitivity of genes involved in root system changes; or GR24^{ent-5DS}

562 is only taken up into a subset cells, in which SMAX1 removal does not mediate root system
563 changes.

564 We observed that KAR₁ treatment triggers increased PER density in *L. japonicus*. This is
565 somewhat contradictory to *kai2* and *max2* mutants in Arabidopsis, which display an increased
566 lateral root density [15]. The discrepancy may result from different physiological optima
567 between the two species or from nutrient conditions in the two experimental systems. We
568 observed the KAR₁ response of *L. japonicus* root systems in half-Hoagland solution with low
569 phosphate levels (2.5µM PO₄³⁻) and without sucrose, whereas the root assay in Arabidopsis
570 was conducted in ATS medium (*Arabidopsis thaliana* salts) with 1% sucrose [15]. Phosphate
571 and sucrose levels have previously been described to influence the effect of strigolactone
572 and *rac*-GR24 on Arabidopsis root architecture [57, 67, 68].

573 In Arabidopsis and rice, KAI2/D14L is required to inhibit hypocotyl and mesocotyl elongation,
574 respectively [3, 4, 16]. Since these two species are evolutionarily distant from each other, but
575 have both retained a function of KL signalling in inhibiting the growth of similar organs, it
576 seemed likely that this function would be conserved among a large number of plant species.
577 Surprisingly, in *L. japonicus*, we observed no elongated hypocotyl phenotype for the *kai2a-1*
578 *kai2b-1* double and two allelic *max2* mutants (Fig. 5). However, we could trigger a reduction
579 of hypocotyl elongation by treatment with KAR₁, KAR₂ and *rac*-GR24 in the wild type and in
580 a *LjKAI2a* and *LjMAX2*-dependent manner. Perhaps the endogenous KL levels in *L.*
581 *japonicus* hypocotyls are insufficient to cause inhibition of hypocotyl elongation, at least under
582 our growth conditions.

583

584 In summary, we have demonstrated sub-functionalization of two KAI2 copies in *L. japonicus*
585 with regard to their ligand-binding specificity and organ-specific relevance. Furthermore, we
586 find organ-specific responsiveness of *L. japonicus* to two artificial KAI2 ligands. A
587 phenylalanine to tryptophan transition independently occurred in the KAI2-binding pocket in
588 several angiosperms, while a leucine-to-methionine and a serine-to-leucine exchange are
589 conserved in KAI2a and KAI2b across legumes. This conservation and independent multiple
590 occurrence of specific amino acid polymorphisms suggests that they bear functional relevance
591 for discriminating diverse KAI2 ligands. Our findings open novel research avenues towards
592 understanding the diversity in KL ligand-receptor relationships and in developmental
593 responses to, as yet, unknown natural as well as synthetic butenolides that influence diverse
594 aspects of plant development.

595

596 **Materials and methods**

597 **Plant material and seed germination**

598 The *A. thaliana kai2-2* (Ler background) and *d14-1* (Col-0 background) mutants are from [4],
599 the *d14-1 kai2-2* double mutant from [45], the *htl-2* mutant was provided by Min Ni [69] and
600 the cross with K02821 is from [16]. Seeds were surface sterilized with 70% EtOH. For
601 synchronizing the germination, seeds were placed on ½ MS 1% agar medium and maintained
602 at 4°C in the dark for 72 hours.

603 The *L. japonicus* Gifu *max2-1*, *max2-2*, *max2-3*, *max2-4*, *kai2a-1* and *kai2b-3* mutations are
604 caused by a LORE1 insertion. Segregating seed stocks for each insertion were obtained from
605 the Lotus Base (<https://lotus.au.dk>, [70]) or Makoto Hayashi (NIAS, Tsukuba, Japan, [53] for
606 *max2-2*). The *d14-1*, *kai2b-1* and *kai2b-2* mutants were obtained by TILLING [54] at

607 RevGenUK (<https://www.jic.ac.uk/technologies/genomic-services/revgenuk-tilling-reverse->
608 genetics/). Homozygous mutants were identified by PCR using primers indicated in S3 Table.
609 For germination, *L. japonicus* seeds were manually scarified with sand-paper and surface
610 sterilized with 1% NaClO. Imbibed seeds were germinated on 1/2 Hoagland medium
611 containing 2.5µM PO₄³⁻ and 0.4% Gelrite (www.duchefa-biochemie.com), at 24°C for 3 days
612 in the dark, or on ½ MS 0.8% agar at 4°C for 3 days in dark (only for the experiment in Fig.
613 6E).

614

615 **Phylogenetic, synteny and protein sequence analysis**

616 *Lotus japonicus* KAI2, D14 and MAX2 sequences were retrieved using tBLASTn with AtKAI2,
617 AtD14 and AtMAX2, against the NCBI database, the plantGDB database and the *L. japonicus*
618 genome V2.5 (<http://www.kazusa.or.jp/lotus>). The presence of MAX2-like was identified by
619 tBLASTn in an in-house genome generated by next generation sequencing using CLC Main
620 Workbench [71]. Pea sequences were found by BLASTn on “pisum sativum v2” database
621 with AtKAI2 as query (<https://www.coolseasonfoodlegume.org>). For Fig 1, the MUSCLE
622 alignment of the protein sequences was used to generate Maximum-likelihood tree with 1000
623 bootstrap replicates in MEGAX [72]. For the synteny analysis of MAX2 and MAX2-like,
624 flanking sequences were retrieved from the same in-house genome [71]. For S7 Fig, KAI2
625 sequences across the plant phylogeny were retrieved by BLAST-P search against the
626 EnsemblPlants, NCBI and 1KP databases [51], in addition, KAI2 sequences of the parasitic
627 plants *Striga hermonthica* and *Orobanche cumana* were retrieved from Conn et al. 2015 [38].
628 The MUSCLE alignment, generated in MEGAX [72], was used to produce a tree with 1000
629 bootstrap replicates with IQTREE [73].

630

631 **Structural homology modelling of proteins**

632 Proteins were modelled using SWISS-MODEL tool (<https://swissmodel.expasy.org>) with the
633 *A. thaliana* KAI2 (4JYM) templates [5].

634

635 **Bacterial protein expression and purification**

636 Full-length *L. japonicus* coding sequences were cloned into pE-SUMO Amp using primers in
637 S3 Table. Clones were sequence-verified and transformed into Rosetta DE3 pLysS cells
638 (Novagen). Subsequent protein expression and purification were performed as described
639 previously [34], with the following modifications: the lysis and column wash buffers contained
640 10 mM imidazole, and a cobalt-charged affinity resin was used (TALON, Takara Bio).

641

642 **Differential scanning fluorimetry**

643 DSF assays were performed as described previously [34]. Assays were performed in 384-
644 well format on a Roche LightCycler 480 II with excitation 498 nm and emission 640 nm
645 (SYPRO Tangerine dye peak excitation at 490 nm). Raw fluorescence values were
646 transformed by calculating the first derivative of fluorescence over temperature. These data
647 were then imported into GraphPad Prism 8.0 software for plotting. Data presented are the
648 mean of three super-replicates from the same protein batch; each super-replicate comprised
649 four technical replicates at each ligand concentration. Experiments were performed at least
650 twice.

651

652 **Intrinsic tryptophan fluorescence (ITF) assay**

653 The ITF assay was performed in 384-well format on a BMG Labtech CLARIOstar multimode
654 plate reader, using black FLUOtrac microplates (Greiner 781076). Reactions (20 μ L) were
655 set up in quadruplicate and contained 10 μ M protein, 20 mM HEPES pH 7.5, 150 mM NaCl,
656 1.25% (v/v) glycerol, and 0-500 μ M ligand. Ligands were initially prepared in DMSO at 20x
657 concentration, and therefore reactions also contained 5% (v/v) DMSO. Ligands were
658 dissolved in buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 1.25% (v/v) glycerol) at 2x
659 concentration immediately before use, of which 10 μ L per well was dispensed with a
660 multichannel pipette. An equivalent volume of 2x solution of protein in buffer was prepared
661 and then dispensed onto the plate using an Eppendorf Multipipette with a 0.1 mL tip. The plate
662 was mixed at 120 rpm for 2 min, centrifuged at 500x *g* for 2 min, and then incubated in the
663 dark for 20 min at room temperature. Fluorescence measurements were taken first with fixed
664 wavelength filters (excitation 295/10 nm; longpass dichroic 325 nm; emission 360/20 nm),
665 followed by the linear variable filter monochromator for emission wavelength scans (excitation
666 295/10 nm, emission 334-400 nm, step width 2 nm, emission bandwidth 8 nm).
667 Measurements were performed at 25 °C using 17 flashes per well for fixed filters or 20 flashes
668 per well for wavelength scans. Gain and focus settings were set empirically for each
669 experimental run. Data were blank-corrected by subtraction of fluorescence values from an
670 identical set of wells containing ligand and buffer but no protein. Data analysis was performed
671 in Graphpad Prism v8.4. Best fit curves were generated from untransformed fluorescence
672 readings using nonlinear regression and the in-built “One site - Total” model, with least
673 squares regression as a fitting method and an asymmetrical (profile-likelihood) 95%
674 confidence interval. As saturation was not reached, only ambiguous values for K_d were
675 returned. For emission wavelength scans, fluorescence values at each wavelength were

676 normalised by expressing as a percentage of the corresponding value from samples lacking
677 ligand.

678

679 **Plasmid generation**

680 Genes and promoter regions were amplified using Phusion PCR according to standard
681 protocols and using primers indicated in S3 Table. Plasmids were constructed by Golden
682 Gate cloning [74] as indicated in S4 Table.

683

684 **Plant transformation**

685 *kai2-2* and *d14-1* mutants were transformed by floral dip in *Agrobacterium tumefaciens* AGL1
686 suspension. Transgenic seedlings were selected by mCherry fluorescence and resistance to
687 20 µg/mL hygromycin B in growth medium. Experiments were performed using T2 or T3
688 generations, with transformed plants validated by mCherry fluorescence.

689

690 **Shoot branching assay**

691 *A. thaliana* and *L. japonicus* were grown for 4 and 7 weeks, respectively in soil in the
692 greenhouse at 16h/8h light/dark cycles. Branches with length >1cm were counted, and the
693 height of each plant was measured.

694

695 **Hypocotyl elongation assay**

696 *A. thaliana* seedlings were grown for 5 days on half-strength Murashige and Skoog (MS)
697 medium containing 1% agar (BD). *L. japonicus* seedlings were grown for 6 days on half-
698 strength Hoagland medium containing 2.5µM PO₄³⁻ and 0.4% Gelrite (www.duchefa-

699 biochemie.com), or on half-strength MS containing 0.8% agar (only for experiment in Fig.
700 6D). Long-day conditions with 16h/8h light/dark cycles were used to test restoration of *A.*
701 *thaliana* hypocotyl growth suppression by cross-species complementation (Fig. 2A). For
702 Karrikin, *rac*-GR24, GR24^{5DS} and GR24^{ent-5DS} treatments the medium was supplied with KAR₁
703 (www.olchemim.cz), KAR₂ (www.olchemim.cz), *rac*-GR24 (www.chiralix.com) GR24^{5DS} and
704 GR24^{ent-5DS} (www.strigolab.eu) or equal amounts of the corresponding solvent as a control.
705 Karrikins were solubilized in 75% methanol and *rac*-GR24 and the GR24 stereoisomers in
706 100% acetone, at 10mM stock solution. Short-day conditions at 8h/16h light/dark cycles were
707 used to test hormone responsiveness of *A. thaliana* as well as *L. japonicus* hypocotyls. After
708 high-resolution scanning, the hypocotyl length was measured with Fiji (<http://fiji.sc/>).

709

710 **Root system architecture assay**

711 *L. japonicus* germinated seeds were transferred onto new plates containing KAR₁
712 (www.olchemim.cz), KAR₂ (www.olchemim.cz), *rac*-GR24 (www.chiralix.com) or the
713 corresponding solvent. Karrikins were solubilized in 75% methanol and *rac*-GR24 in 100%
714 acetone, at 10 mM stock solution. Plates were partially covered with black paper to keep the
715 roots in the dark, and placed at 24°C with 16-h-light/8-h-dark cycles for 2 weeks. After high-
716 resolution scanning, post-embryonic root number was counted and primary root length
717 measured with Fiji (<http://fiji.sc/>).

718

719 **Treatment for analysis of transcript accumulation**

720 Seedling roots were placed in 1/2 Hoagland solution with 2.5 μM PO₄³⁻ containing 1 or 3 μM
721 Karrikin₁ (www.olchemim.cz for qPCR analysis, synthesized according to [75] for microarray

722 analysis), Karrikin₂ (www.olchemim.cz), *rac*-GR24 (www.chiralix.com) or equal amounts of
723 the corresponding solvents for the time indicated in Figure legends and the roots were
724 covered with black paper to keep them in the dark.

725

726 **Microarray analysis**

727 Three biological replicates were performed for each treatment. Root tissues were harvested,
728 rapidly blotted dry and shock frozen in liquid nitrogen. RNA was extracted using the Spectrum
729 Plant Total RNA Kit (www.sigmaaldrich.com). RNA was quantified and evaluated for purity
730 using a Nanodrop Spectrophotometer ND-100 (NanoDrop Technologies, Willington, DE) and
731 Bioanalyzer 2100 (Agilent, Santa Clara, CA). For each sample, 500 ng of total RNA was used
732 for the expression analysis of each sample using the Affymetrix GeneChip® Lotus1a520343
733 (Affymetrix, Santa Clara, CA). Probe labeling, chip hybridization and scanning were
734 performed according to the manufacturer's instructions for IVT Express Labeling Kit
735 (Affymetrix). The Microarray raw data was normalized with the Robust Multiarray Averaging
736 method (RMA) [76] using the Bioconductor [77] package "Methods for Affymetrix
737 Oligonucleotide Arrays" (affy version 1.48.0) [78]. Control and rhizobial probesets were
738 removed before statistical analysis. Differential gene expression was analyzed with the
739 Bioconductor package "Linear Models for Microarray Data" (LIMMA version 3.26.8) [79]. The
740 package uses linear models for parameter estimation and an empirical Bayes method for
741 differential gene expression assessment [80]. P-values were adjusted due to multiple
742 comparisons with the Benjamini-Hochberg correction (implemented in the LIMMA package).
743 Probesets were termed as significantly differentially expressed, if their adjusted p-value was
744 smaller than or equal to 0.01 and the fold change for at least one contrast showed a difference

745 of at least 50%. To identify the corresponding gene models, the probeset sequences were
746 used in a BLAST search against *L. japonicus* version 2.5 CDS and version 3.0 cDNA
747 sequences (<http://www.kazusa.or.jp/lotus/>). If, based on the bitscore, multiple identical hits
748 were found, we took the top hit in version 2.5 CDS as gene corresponding to the probe. For
749 version 3.0 cDNA search we used the best hit, that was not located on chromosome 0, if
750 possible. For probesets known to target chloroplast genes (probeset ID starting with Lj_), we
751 preferred the best hit located on the chloroplast chromosome, if possible. Probeset
752 descriptions are based on the info file of the *L. japonicus* Microarray chip provided by the
753 manufacturer (Affymetrix).

754

755 **qPCR analysis**

756 Tissue harvest, RNA extraction, cDNA synthesis and qPCR were performed as described
757 previously [71]. qPCR reactions were run on an iCycler (Bio-Rad, www.bio-rad.com) or on
758 QuantStudio5 (Applied Biosystems, www.thermofisher.com). Expression values were
759 calculated according to the $\Delta\Delta C_t$ method [81]. Expression values were normalized to the
760 expression level of the housekeeping gene *Ubiquitin*. For each condition three to four
761 biological replicates were performed. Primers are indicated in Table S4.

762

763 **Statistics**

764 Statistical analyses were performed using Rstudio (www.rstudio.com) after log
765 transformation for qPCR analysis. F- and p-values for all figures are provided in S5 Table.

766

767

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778

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1033

1034 **Figure legends**

1035 **Figure 1. The KAI2 gene underwent duplication prior to diversification of the legumes.**

1036 Phylogenetic tree of KAI2 and D14 rooted with bacterial RbsQ from indicated species (*Lotus*
1037 *japonicus*; *Glycine max*; *Pisum sativum*; *Medicago truncatula*; *Arabidopsis thaliana*; *Populus*
1038 *trichocarpa*; *Oryza sativa*; *Zea mays*; *Sorghum bicolor*; *Marchantia polymorpha*). MEGAX
1039 was used to align the protein sequences with MUSCLE and generate a tree inferred by
1040 Maximum Likelihood method [72]. The tree with the highest log likelihood (-7359.19) is
1041 shown. The percentage of trees in which the associated taxa clustered together is shown
1042 next to the branches. Values below 50 were ignored. *KAI2* duplication in the legumes is
1043 highlighted by red and blue branches.

1044

1045 **Figure 2. *Lotus japonicus* D14, KAI2a and KAI2b can replace D14 and KAI2 in** 1046 ***Arabidopsis*, respectively.**

1047 (A) Hypocotyl length of *A. thaliana* wild-type (Ler), *kai2-2* and *kai2-2* lines complemented by
1048 *AtD14*, *AtKAI2*, *LjD14*, *LjKAI2a* and *LjKAI2b*, driven by the *AtKAI2* promoter at 6 days post
1049 germination (dpg). Seedlings were grown in 8h light / 16h dark periods (n=37-122). (B)
1050 Shoots of *A. thaliana* *d14-1*, with an empty vector (EV) or complemented with *AtD14*, *AtKAI2*,
1051 *LjD14*, *LjKAI2a* and *LjKAI2b*, driven by the *AtD14* promoter at 26 dpg. Scale bar = 10 cm. (C)
1052 Rosette branch number at 26 dpg of *A. thaliana* wild-type (Col-0), *d14-1* and *d14-1* lines

1053 carrying an empty vector (EV) or plasmids containing *AtD14*, *AtKAI2*, *LjD14*, *LjKAI2a* and
1054 *LjKAI2b*, driven by the *AtD14* promoter (n=24). Letters indicate different statistical groups
1055 (ANOVA, post-hoc Tukey test).

1056

1057 **Figure 3. *Lotus japonicus* KAI2a, KAI2b and rice D14L confer divergent hypocotyl**
1058 **growth responses to KAR₁ and KAR₂ in Arabidopsis.**

1059 (A) Structures of KAR₁, KAR₂, GR24^{5DS} and GR24^{ent-5DS}. (B-C) Hypocotyl length of *A.*
1060 *thaliana kai2* mutants complemented with *KAI2* from *A. thaliana*, *L. japonicus* and rice, after
1061 treatment with solvent (Mock), 1 μM KAR₁ or KAR₂ at 6 dpv. (B) *Ler* wild-type, *kai2-2* and
1062 *kai2-2* lines complemented with *AtKAI2*, *LjKAI2a* and *LjKAI2b*, driven by the *AtKAI2* promoter
1063 (n= 33-128). (C) *Ler* and Col-0 wild-type, *htl-2* (*Ler*), K02821-line transgenic for *p35S:OsD14L*
1064 (Col-0), and two homozygous F₃ lines from the *htl-2* x K02821 cross [16] (n= 80-138). (D)
1065 Hypocotyl length of *A. thaliana* Col-0 wild-type, *d14-1 kai2-2* double mutants, and *d14-1 kai2-*
1066 *2* lines complemented with *LjKAI2a* and *LjKAI2b*, driven by the *AtKAI2* promoter after
1067 treatment with solvent (Mock), 1 μM GR24^{5DS} or GR24^{ent-5DS} (n= 59-134). (B-D) Seedlings
1068 were grown in 8h light / 16h dark periods. Letters indicate different statistical groups (ANOVA,
1069 post-hoc Tukey test).

1070

1071 **Figure 4. Binding of GR24^{ent-5DS} to LjKAI2a is determined by three amino acids.**

1072 (A) The ligand-binding cavity regions of *LjKAI2a* and *LjKAI2b* proteins after structural
1073 homology modelling on the KAI2 crystal structure of *A. thaliana* [5]. Conserved residues in
1074 the cavity that differ between the KAI2a and KAI2b clades, and that are also different between
1075 *LjKAI2b* and *AtKAI2*, are shown in green. The phenylalanine residue in *LjKAI2a*, which is

1076 changed to tryptophan in *LjKAI2b*, is shown in violet. The catalytic triad is coloured in red. **(B)**
1077 DSF curves of purified SUMO fusion proteins of wild-type *LjKAI2a* and *LjKAI2b*, and versions
1078 with swapped amino acids *LjKAI2a*^{M160,L190}, *LjKAI2b*^{L161,S191}, *LjKAI2a*^{W157,M160,L190},
1079 *LjKAI2b*^{F158,L161,S191}, *LjKAI2a*^{W157}, *LjKAI2b*^{F158} at the indicated concentrations of of GR24^{ent-}
1080 ^{5DS}. The first derivative of the change of fluorescence was plotted against the temperature.
1081 Each curve is the arithmetic mean of three sets of reactions, each comprising four technical
1082 replicates. Peaks indicate the protein melting temperature. The shift of the peak in *LjKAI2a*
1083 indicates ligand-induced thermal destabilisation consistent with a protein-ligand interaction.
1084 Insets plot the minimum value of (-dF/dT) at the melting point of the protein as determined in
1085 the absence of ligand (means ± SE, n = 3). Asterisks indicate significant differences to the
1086 solvent control (ANOVA, post-hoc Dunnett test, N.S.>0.05, *≤0.05, **≤0.01, ***≤0.001,
1087 ****≤0.0001).

1088

1089 **Figure 5. Amino acid swaps reverse sensitivity of *LjKAI2a* and *LjKAI2b* to GR24^{ent-5DS}**
1090 **in *Arabidopsis hypocotyls*.**

1091 Hypocotyl length of *A. thaliana* Col-0 wild-type, *d14-1 kai2-2* double mutants, and *d14-1 kai2-*
1092 *2* lines complemented with *LjKAI2a* and *LjKAI2b* variants driven by the *AtKAI2* promoter and
1093 after treatment with solvent (Mock), 1 μM GR24^{5DS} or GR24^{ent-5DS}. **(A)** *LjKAI2a*^{M160,L190} and
1094 *LjKAI2a*^{W157,M160,L190} (n = 46-84). **(B)** *LjKAI2b*^{L161,S191} and *LjKAI2b*^{F158,L161,S191} (n= 49-102). **(A-**
1095 **B)** Seedlings were grown in 8h light / 16h dark periods Asterisks indicate significant
1096 differences versus mock treatment (Welch t.test, *≤0.05, **≤0.01, ***≤0.001, ****≤0.0001).

1097

1098 **Figure 6. Role of *D14*, *KAI2a*, *KAI2b* and *MAX2* in shoot and hypocotyl development of**
1099 ***Lotus japonicus*.**

1100 (A) Schematic representation of the *L. japonicus* *D14*, *KAI2a*, *KAI2b* and *MAX2* genes. Black
1101 boxes and lines show exons and introns, respectively. *LORE1* insertions are indicated by red
1102 triangles and EMS mutations by red stars. (B) Shoot phenotype of *L. japonicus* wild-type and
1103 karrikin and strigolactone perception mutants at 8 weeks post germination (wpg). Scale bars:
1104 7 cm. (C) Number of branches and of *L. japonicus* wild-type, karrikin and strigolactone
1105 perception mutants at 7 wpg (n = 12-21). (D) Leaf size of the indicated genotypes at 9 wpg
1106 (n = 12-15 plants with an average of 3 leaves). (E) Hypocotyl length of the indicated
1107 genotypes of *L. japonicus* under short day conditions (8h light/ 16h dark) at 1 wpg (n = 79-
1108 97). (C-E) Letters indicate different statistical groups (ANOVA, post-hoc Tukey test).

1109

1110 **Figure 7. *Lotus japonicus* root system architecture is affected specifically by treatment**
1111 **with *KAR*₁ but not *KAR*₂.**

1112 (A) Primary root length (PRL), post-embryonic root (PER) number and PER density of wild-
1113 type plants 2 wpg after treatment with solvent (M) or three different concentrations of *KAR*₁,
1114 *KAR*₂ or *rac*-GR24 (GR24) (n = 32-57). (B) PER density of wild-type plants at 2 wpg and
1115 treated with solvent (Mock) 1 μM *KAR*₁, 1 μM *KAR*₂, or 1 μM *rac*-GR24 (n = 43-51). Plants
1116 were transferred onto fresh hormone-containing medium after 5 days. (C-D) qRT-PCR-based
1117 expression of *DLK2* normalized to *Ubiquitin* expression in roots at 2 wpg after 2 hours
1118 treatment with solvent (Mock), (C) 1 μM *KAR*₁ and 1 μM *KAR*₂, (D) 1 μM *rac*-GR24 (n = 4).
1119 (A and C) Letters indicate different statistical groups (ANOVA, post-hoc Tukey test). (B)
1120 Asterisks indicate significant differences (ANOVA, Dunnett test, N.S.>0.05, *≤0.05). (D)

1121 Asterisk indicate significant differences versus mock treatment (Welch t.test, * \leq 0.05, ** \leq 0.01,
1122 *** \leq 0.001).

1123

1124 **Figure 8. *LjKAI2a* and *LjKAI2b* operate redundantly in the response of roots to KAR₁**

1125 (A) Post-embryonic-root (PER) density of *L. japonicus* plants, 2 wpg after treatment with
1126 solvent (M) or 3 μ M KAR₁ (n=34-72). (B-C) qRT-PCR-based expression of *DLK2* in roots of
1127 *L. japonicus* plants at 2 wpg after 2 hours treatment with solvent (Mock) or (B) 3 μ M KAR₁ or
1128 (C) 1 μ M GR24^{ent-5DS}. Expression values were normalized to those of the housekeeping gene
1129 *Ubiquitin* (n= 3-4). (A-C) Asterisks indicate significant differences versus mock treatment
1130 (Welch t.test, * \leq 0.05, ** \leq 0.01, *** \leq 0.001).

1131

1132 **Figure 9. *L. japonicus* KAI2a and KAI2b display organ-specific redundancy and differ**
1133 **in their ligand-binding specificity.**

1134 (A) *LjKAI2a* is required to mediate inhibition of hypocotyl growth in response to KAR₁ and
1135 KAR₂. In roots *LjKAI2a* and *LjKAI2b* redundantly promote lateral root density, but only in
1136 response to KAR₁ treatment. (B) Upper panel: In the Arabidopsis *kai2-2* background *LjKAI2a*
1137 mediates hypocotyl growth inhibition in response to KAR₁, KAR₂ and GR24^{ent-5DS}. In the same
1138 background, *LjKAI2b* mediates a stronger response to KAR₁ than to KAR₂ and no response
1139 to GR24^{ent-5DS} (indicated by a red cross). Three divergent amino acids at the binding pocket
1140 are indicated in white. Lower panel: Swapping the three divergent amino acids in the binding
1141 pocket reconstitutes GR24^{ent-5DS} activity through *LjKAI2b* and abolishes GR24^{ent-5DS} activity
1142 through *LjKAI2a*. Among the three amino acids F157/W158 are decisive for GR24^{ent-5DS}
1143 binding (strong colors), while L160/M161 and S190/L191 play a weaker role (pale colors).

1144 Amino acids from *LjKAI2a* have a red/pale red and amino acids from *LjKAI2b* a violet/pale
1145 background.

1146

1147 **Supplementary figure legends**

1148 **S1 Fig. MAX2-like underwent pseudogenization.**

1149 **(A)** Schematic representation of the syntenic regions containing the *MAX2* and *MAX2-like*
1150 loci in *L. japonicus*. Coloured arrows and black lines show exons and introns respectively.

1151 **(B)** Protein alignment of *LjMAX2*, *LjMAX2-like* and an artificial *LjMAX2-like* with a deletion of
1152 the thymine at the position 453 in the coding sequence (*LjMAX2-like* Δ T453). Position of the
1153 nucleotide deletion is indicated in the translated sequence by a red triangle. Amino-acid
1154 conservation between *MAX2* and *MAX2-like* is indicated by a dark background.

1155

1156 **S2 Fig. Organ-specific accumulation of *D14*, *KAI2a*, *KAI2b* and *MAX2* transcripts.**

1157 **(A-C)** Transcript accumulation in wild-type of *D14*, *KAI2a*, *KAI2b* and *MAX2* normalized to
1158 expression of *Ubiquitin*, in **(A)** leaf, stem, flower and root of plants grown in pots, and in **(B)**
1159 hypocotyl and roots of 1 wpg plants grown on Petri dishes in 8h light / 16h dark cycles, and
1160 in **(c)** roots of 2 wpg plants grown on Petri dishes in 16h light / 8h dark cycles (n = 3).

1161

1162 **S3 Fig. Subcellular localisation of *LjD14*, *LjKAI2a*, *LjKAI2b* and *LjMAX2* in *Nicotiana* 1163 *benthamiana* leaves.**

1164 **(A)** Subcellular localization of *LjD14*, *LjKAI2a*, *LjKAI2b* and *LjMAX2* in *N. benthamiana* leaf
1165 epidermal cells. *LjD14*, *LjKAI2a* and *LjKAI2b* are N-terminally fused with mOrange. *LjMAX2*
1166 is N-terminally fused with T-Sapphire. Scale bars: 25 μ m. **(B)** Western blot of protein extracts

1167 from *N. benthamiana*, showing that the mOrange tag fused with *LjD14*, *LjKAI2a* and *LjKAI2b*
1168 was not cleaved at detectable amounts.

1169

1170 **S4 Fig. SDS-PAGE of purified SUMO fusion proteins and DSF assay with GR24^{5DS}.**

1171 (A) 200 pmol (approx. 8 µg) of purified proteins were separated by 12% SDS-PAGE
1172 containing 2,2,2-trichlorethanol as a visualization agent. Below each lane is the calculated
1173 protein size in kiloDaltons. S, protein size standards (Precision Plus Dual Color Standards,
1174 Bio-Rad #1610394) with corresponding sizes in kDa shown on the left. Optimal exposures of
1175 recombinant proteins and size standards were taken separately under UV transillumination
1176 and red epi-illumination, respectively. The two images were merged in post-processing, and
1177 the junction between them is indicated by a vertical line. (B) DSF curves of purified SUMO
1178 fusion proteins of wild-type *LjKAI2a* and *LjKAI2b*, and versions with swapped amino acids
1179 *LjKAI2a*^{W157,M160,L190}, *LjKAI2b*^{F158,L161,S191}, *LjKAI2a*^{W157}, *LjKAI2b*^{F158}, at the indicated
1180 concentrations of GR24^{5DS}. The first derivative of the change of fluorescence was plotted
1181 against the temperature. Each curve is the arithmetic mean of four technical replicates. Peaks
1182 indicate the protein melting temperature. There is no ligand-induced thermal destabilisation
1183 consistent with no protein-ligand interaction.

1184

1185 **S5 Fig. Amino acid differences between the legume KAI2a and KAI2b clades.**

1186 Protein sequence alignment of KAI2a and KAI2b homologs from the legumes *Lotus*
1187 *japonicus*, *Pisum sativum*, *Medicago truncatula* and *Glycine max*, in comparison with
1188 *Arabidopsis* KAI2 and rice D14L. Residues conserved within the KAI2a and KAI2b clades but
1189 different between these clades are coloured in green and blue. Residues of the catalytic triad

1190 are coloured in red. A non-conserved tryptophan in *LjKAI2b* located in the protein cavity is
1191 coloured in violet. Yellow triangles indicated amino acid residues located in the ligand-binding
1192 cavity of the proteins. Orange triangles indicate the three amino acids responsible for
1193 differences in GR24^{ent-5DS}-binding between *LjKAI2a* and *LjKAI2b*.

1194

1195 **S6 Fig. Intrinsic tryptophan fluorescence assay confirms inability of *LjKAI2b* to**
1196 **interact with GR24^{ent-5DS}**

1197 Intrinsic tryptophane fluorescence of wild-type *LjKAI2a* and *LjKAI2b*, and protein versions
1198 with swapped amino acids *LjKAI2a*^{M160,L190}, *LjKAI2b*^{L161,S191}, *LjKAI2a*^{W157,M160,L190},
1199 *LjKAI2b*^{F158,L161,S191}, *LjKAI2a*^{W157}, *LjKAI2b*^{F158} measured with (A) fixed wavelength filters
1200 (excitation 295/10 nm; longpass dichroic 325 nm; emission 360/20 nm) and (B) with a linear
1201 variable filter monochromator for emission wavelength scans (excitation 295/10 nm, emission
1202 334-400 nm, step width 2 nm, emission bandwidth 8 nm) at the indicated GR24^{ent-5DS}
1203 concentrations.

1204

1205 **S7 Fig. The F157 to W replacement occurred multiple times in angiosperm KAI2**
1206 **proteins.**

1207 Phylogenetic tree of KAI2 proteins rooted with *A. thaliana* DLK2. The KAI2a and KAI2b clades
1208 in legumes are highlighted by red and blue branches. Monophyletic groups corresponding to
1209 a same order or clade are highlighted by colored rectangular boxes. Amino-acids at the
1210 positions corresponding to AtKAI2 157, 160 and 190 are indicated with single-letter code. A
1211 black background indicates the presence of the most common residues in KAI2 proteins:
1212 F157, L160 and A190. A blue background indicates residues M160 and L190, conserved in

1213 legume KAI2b. A red background indicates S190, conserved in legume KAI2a. A green
1214 background indicates a W at position 157. A brown background indicates a different residue.

1215

1216 **S8 Fig. Transcript accumulation in the *L. japonicus* KAR and SL receptor mutants.**

1217 (A) qRT-PCR based transcript accumulation of *LjKAI2a* and *LjKAI2b*, in roots of wild type and
1218 *kai2a-1*, *kai2b-1*, *kai2b-3*, *kai2a-1 kai2b-1* and *max2-4* as well as *LjMAX2* and *LjD14* in *max2-*
1219 *4* and *d14-1*, respectively (n=4). Expression values were normalized to those of the
1220 housekeeping gene *Ubiquitin*. (B) *LjKAI2b* transcript accumulation in wild-type, *kai2b-1* (stop
1221 codon) and *kai2b-3* (LORE1 insertion) mutants by semi-quantitative RT-PCR using primer
1222 pairs located 5' and 3' of the mutations, as well as flanking (ML) the mutations. Transcript
1223 accumulation of the housekeeping gene *Ubiquitin* is also shown.

1224

1225 **S9 Fig. Characterisation of the *kai2a-1* allele.**

1226 (A) Schematic representation of mis-splicing caused by the LORE1 insertion in the *kai2a-1*
1227 mutant. (B) cDNA alignment showing the absence of nucleotides 369 to 383 in the *kai2a-1*
1228 transcript, causing a deletion of amino acids 124 to 128 (orange). (C) Protein model of
1229 *LjKAI2a* based on the *AtKAI2-KAR₁* complex 4JYM [5] showing *KAR₁* in green, residues of
1230 the catalytic triad in red and the amino acids missing in a hypothetical *LjKAI2a-1* protein in
1231 orange. (D) Hypocotyl elongation at 6 dpg in Arabidopsis *kai2-2* mutants transgenically
1232 complemented with genomic and the cDNA of wild-type *LjKAI2a* and *Ljkai2a-1* driven by the
1233 *AtKAI2* promoter (n = 75-106). Plants were grown in 8h light / 16h dark cycles. Letters
1234 indicate different statistical groups (ANOVA, post-hoc Tukey test).

1235

1236 **S10 Fig. *Lotus japonicus* hypocotyls respond to KAR₁ and KAR₂ in a *LjKAI2a*- and**
1237 ***LjMAX2*-dependent manner.**

1238 (A) Hypocotyls and (B) hypocotyl length of *L. japonicus* seedling at 1 wpg after treatment with
1239 solvent (M) or three different concentrations of KAR₁, KAR₂ or *rac*-GR24 (GR24) (n= 95-105).
1240 Letters indicate different statistical groups (ANOVA, post-hoc Tukey test). (C) Hypocotyl
1241 length of the indicated genotypes at 1 wpg after treatment with solvent (Mock), 1 μM KAR₁
1242 or 1 μM KAR₂ (n = 73-107). (D) Hypocotyl length of wild-type and *max2-4* seedlings 1 wpg
1243 after treatment with solvent (Mock), 1 μM KAR₁, 1 μM KAR₂ (n = 66-96). (E) RT-qPCR-based
1244 expression of *DLK2* in hypocotyls at 1 wpg after 2 hours treatment with solvent (Mock), 1 μM
1245 KAR₁, 1 μM KAR₂, or 1 μM *rac*-GR24 (GR24) (n = 3). Expression values were normalized to
1246 those of the housekeeping gene *Ubiquitin*. (A-E) Seedlings were grown in 8h light / 16h dark
1247 cycles. (C-E) Asterisks indicate significant differences of the compounds versus mock
1248 treatment (ANOVA, post-hoc Dunnett test, N.S.>0.05, *≤0.05, **≤0.01, ***≤0.001).

1249

1250 **S11 Fig. Small overlap between transcriptional responses of *Lotus japonicus* roots to**
1251 **KAR₁ and *rac*-GR24.**

1252 Number of differentially expressed genes (DEGs, adjusted p-value < 0.01) as assessed by
1253 microarray analysis. Left panel: DEGs responding to 1 μM KAR₁ after 1h, 2h and 6h
1254 incubation. Middle panel: DE genes responding to 1 μM *rac*-GR24 1h, 2, 6h incubation. Right
1255 panel: comparison of DE genes responding to 2 h treatment with KAR₁ and *rac*-GR24.

1256

1257 **S12 Fig. KAR perception mutants are less responsive to KAR₁ treatment.**

1258 (A-C) Post-embryonic-root (PER) density of *L. japonicus* plants, 2 wpg after treatment with
1259 solvent (Mock) or 1 μ M KAR₁, of wild-type, (A) *kai2a-1*, *kai2b-1* and *kai2a-1 kai2b-1* (n= 32-
1260 50); (B) *max2-4* (n= 34-43); (c) *kai2a-1*, *kai2b-3* and *kai2a-1 kai2b-1* (n= 37-72). (A-C)
1261 Asterisks indicate significant differences versus mock treatment (Welch t.test, * \leq 0.05,
1262 ** \leq 0.01, *** \leq 0.001).

1263

1264 **S13 Fig. KAR₁ response in roots requires *LjKAI2a* or *LjKAI2b* and *LjMAX2*.**

1265 Primary-root length (PRL) and post-embryonic-root (PER) number of *L. japonicus* plants, 2
1266 wpg after treatment with solvent (Mock) or 3 μ M KAR₁ (n=34-72) displayed in Fig 9A.
1267 Asterisks indicate significant differences versus mock treatment (Welch t.test, * \leq 0.05,
1268 ** \leq 0.01, *** \leq 0.001).

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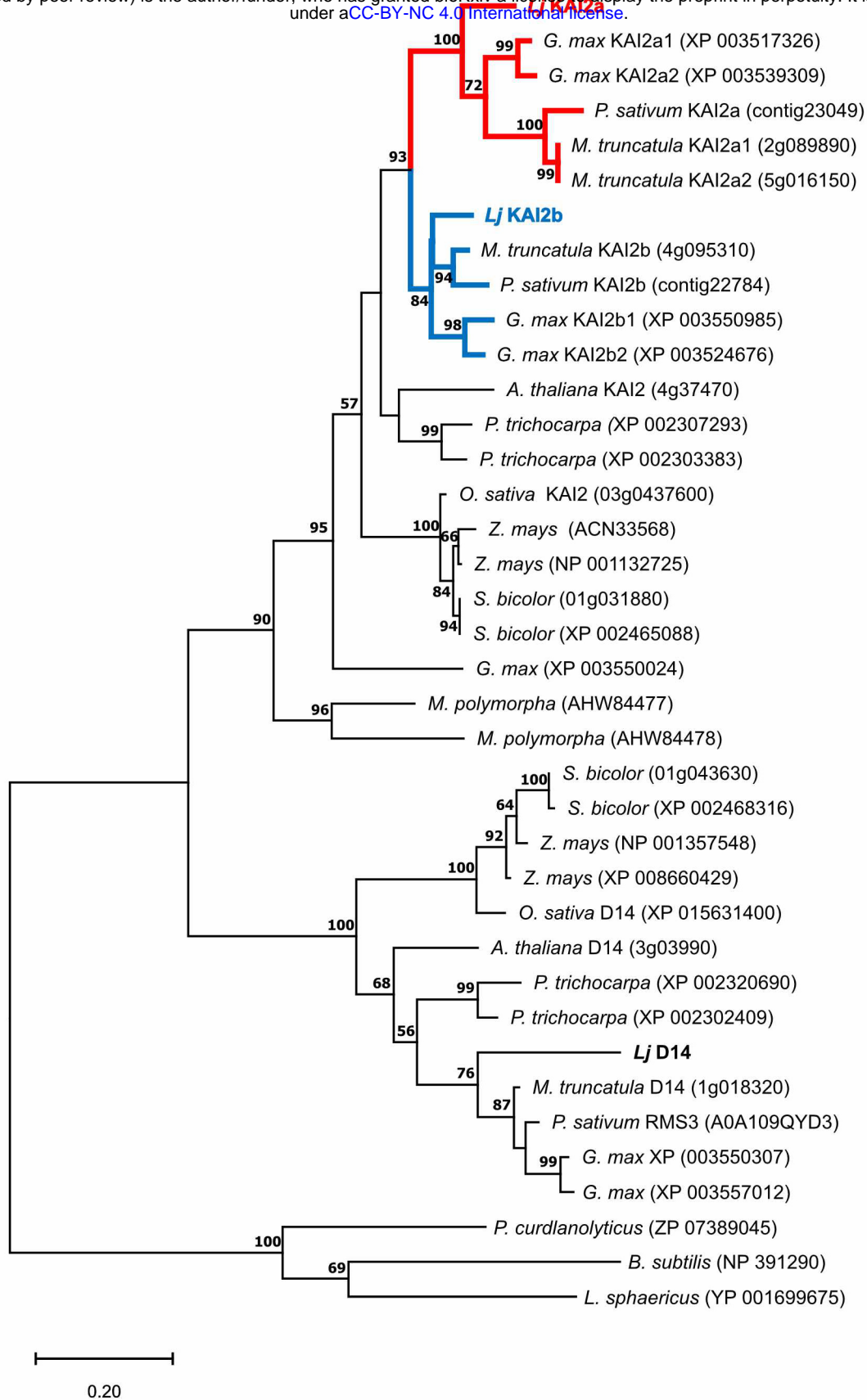


Figure 1. The KAI2 gene underwent duplication prior to diversification of the legumes.

Phylogenetic tree of KAI2 and D14 rooted with bacterial RbsQ from indicated species (*Lotus japonicus*; *Glycine max*; *Pisum sativum*; *Medicago truncatula*; *Arabidopsis thaliana*; *Populus trichocarpa*; *Oryza sativa*; *Zea mays*; *Sorghum bicolor*; *Marchantia polymorpha*). MEGAX was used to align the protein sequences with MUSCLE and generate a tree inferred by Maximum Likelihood method [72]. The tree with the highest log likelihood (-7359.19) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Values below 50 were ignored. KAI2 duplication in the legumes is highlighted by red and blue branches.

A

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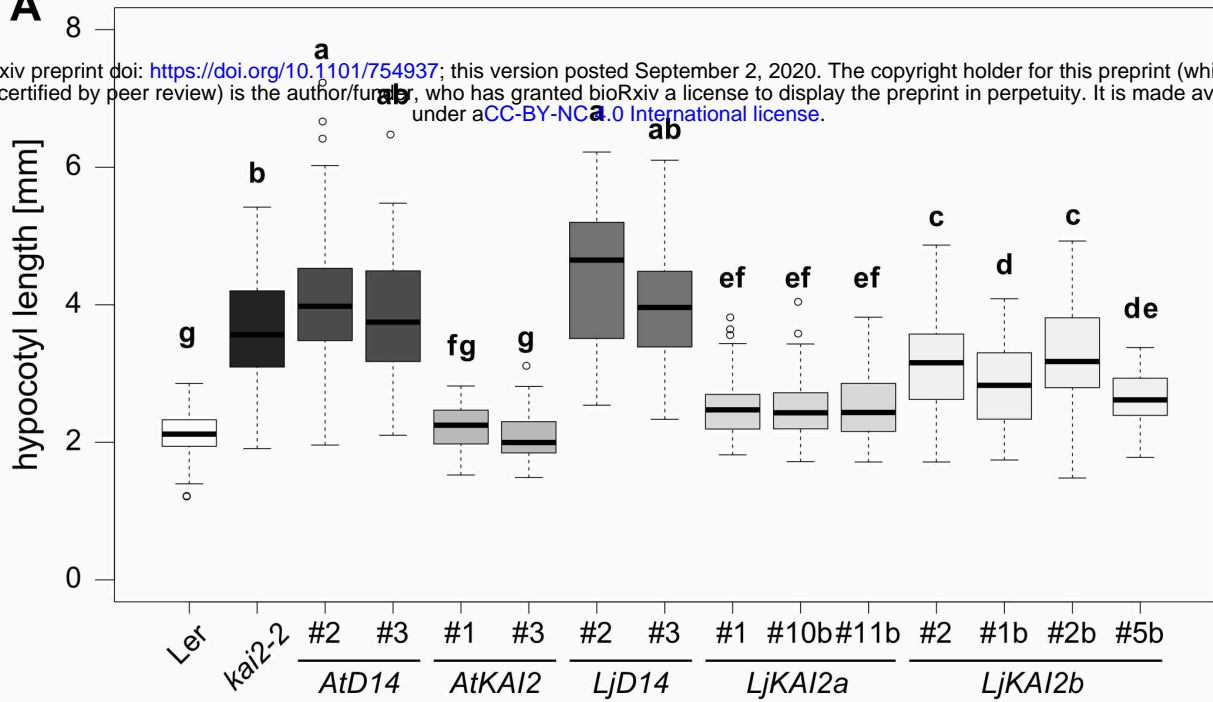
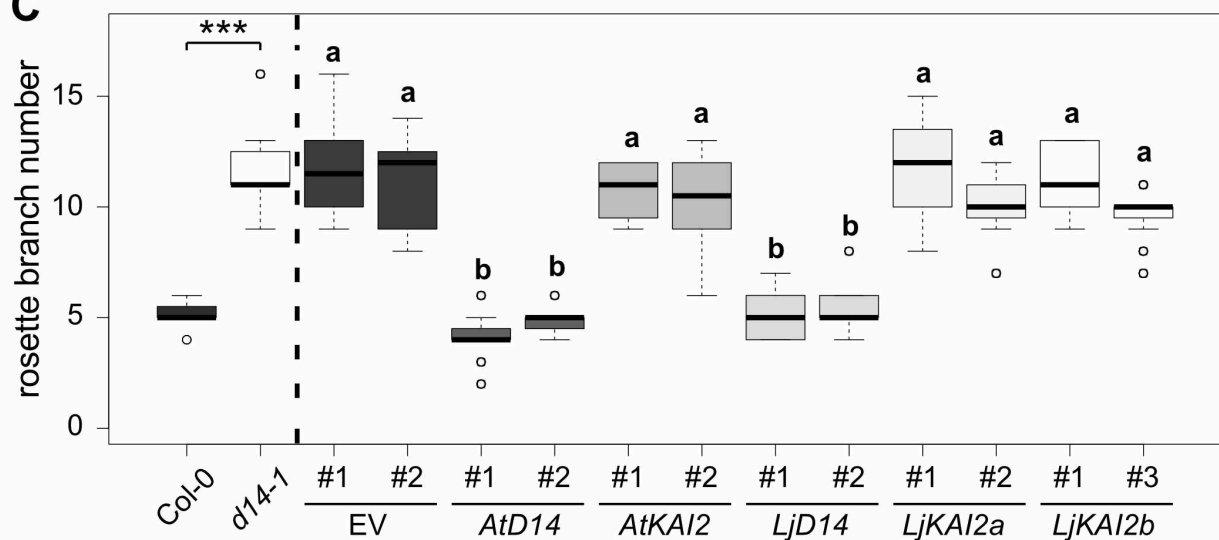
**B****C**

Figure 2. *Lotus japonicus* D14, KAI2a and KAI2b can replace D14 and KAI2 in Arabidopsis, respectively.

(A) Hypocotyl length of *A. thaliana* wild-type (Ler), *kai2-2* and *kai2-2* lines complemented by *AtD14*, *AtKAI2*, *LjD14*, *LjKAI2a* and *LjKAI2b*, driven by the *AtKAI2* promoter at 6 days post germination (dpg). Seedlings were grown in 8h light / 16h dark periods (n=37-122). (B) Shoots of *A. thaliana* *d14-1*, with an empty vector (EV) or complemented with *AtD14*, *AtKAI2*, *LjD14*, *LjKAI2a* and *LjKAI2b*, driven by the *AtD14* promoter at 26 dpg. Scale bar = 10 cm. (C) Rosette branch number at 26 dpg of *A. thaliana* wild-type (Col-0), *d14-1* and *d14-1* lines carrying an empty vector (EV) or plasmids containing *AtD14*, *AtKAI2*, *LjD14*, *LjKAI2a* and *LjKAI2b*, driven by the *AtD14* promoter (n=24). Letters indicate different statistical groups (ANOVA, post-hoc Tukey test).

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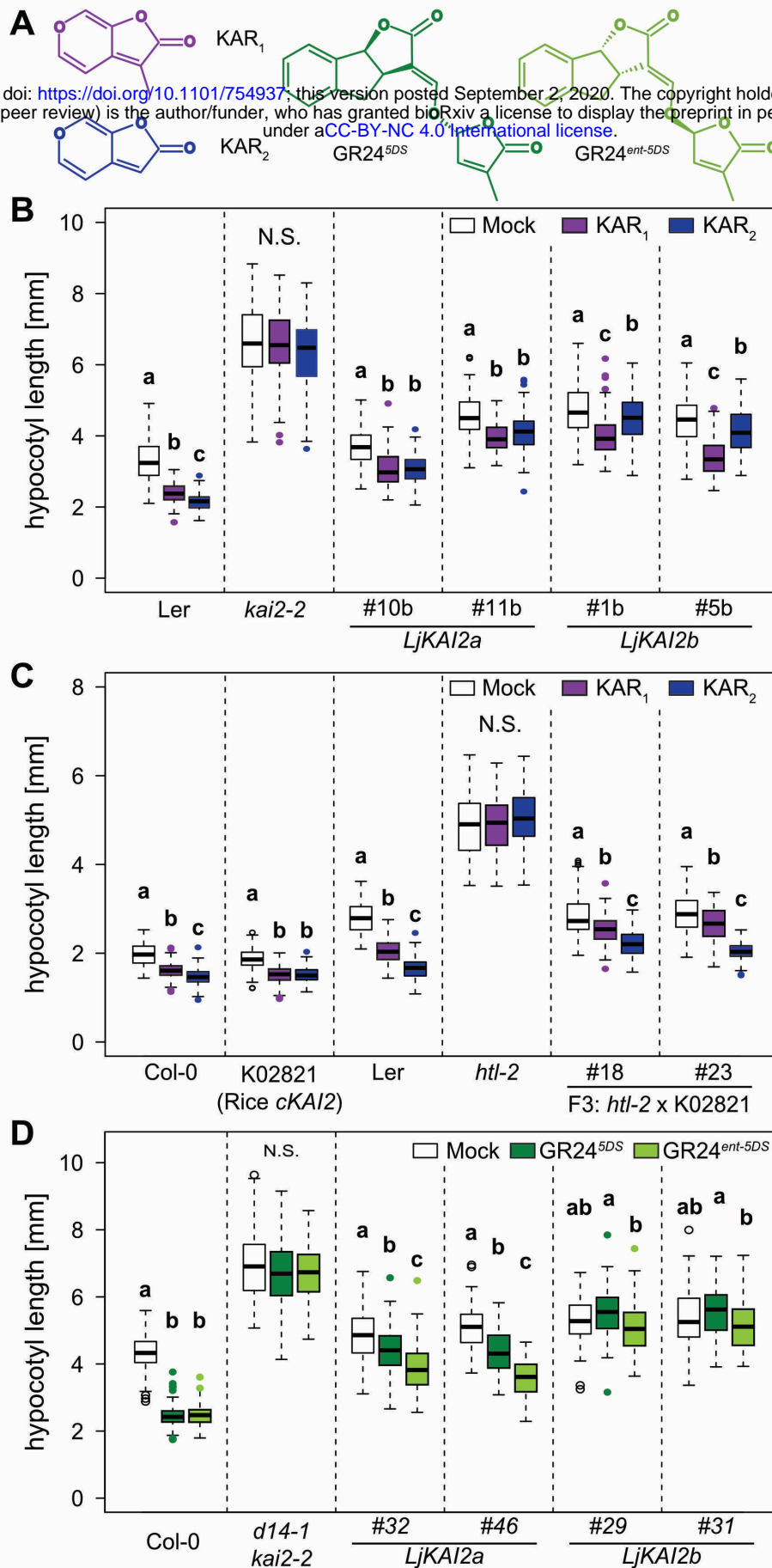


Figure 3. *Lotus japonicus* KAI2a, KAI2b and rice D14L confer divergent hypocotyl growth responses to KAR₁ and KAR₂ in *Arabidopsis*.

(A) Structures of KAR₁, KAR₂, GR24^{5DS} and GR24^{ent-5DS}. **(B-C)** Hypocotyl length of *A. thaliana* *kai2* mutants complemented with *KAI2* from *A. thaliana*, *L. japonicus* and rice, after treatment with solvent (Mock), 1 μ M KAR₁ or KAR₂ at 6 dpg. **(B)** Ler wild-type, *kai2-2* and *kai2-2* lines complemented with *AtKAI2*, *LjKAI2a* and *LjKAI2b*, driven by the *AtKAI2* promoter (n= 33-128). **(C)** Ler and Col-0 wild-type, *htl-2* (Ler), K02821-line transgenic for *p35S:OsD14L* (Col-0), and two homozygous F₃ lines from the *htl-2* x K02821 cross [16] (n= 80-138). **(D)** Hypocotyl length of *A. thaliana* Col-0 wild-type, *d14-1 kai2-2* double mutants, and *d14-1 kai2-2* lines complemented with *LjKAI2a* and *LjKAI2b*, driven by the *AtKAI2* promoter after treatment with solvent (Mock), 1 μ M GR24^{5DS} or GR24^{ent-5DS} (n= 59-134). **(B-D)** Seedlings were grown in 8h light / 16h dark periods. Letters indicate different statistical groups (ANOVA, post-hoc Tukey test).

A**LjKAI2a****LjKAI2b**

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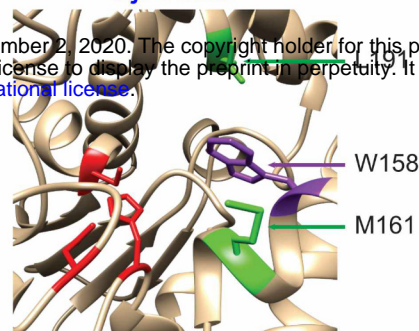
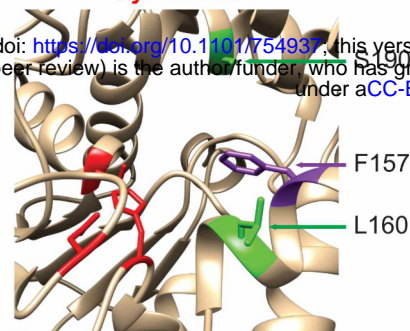
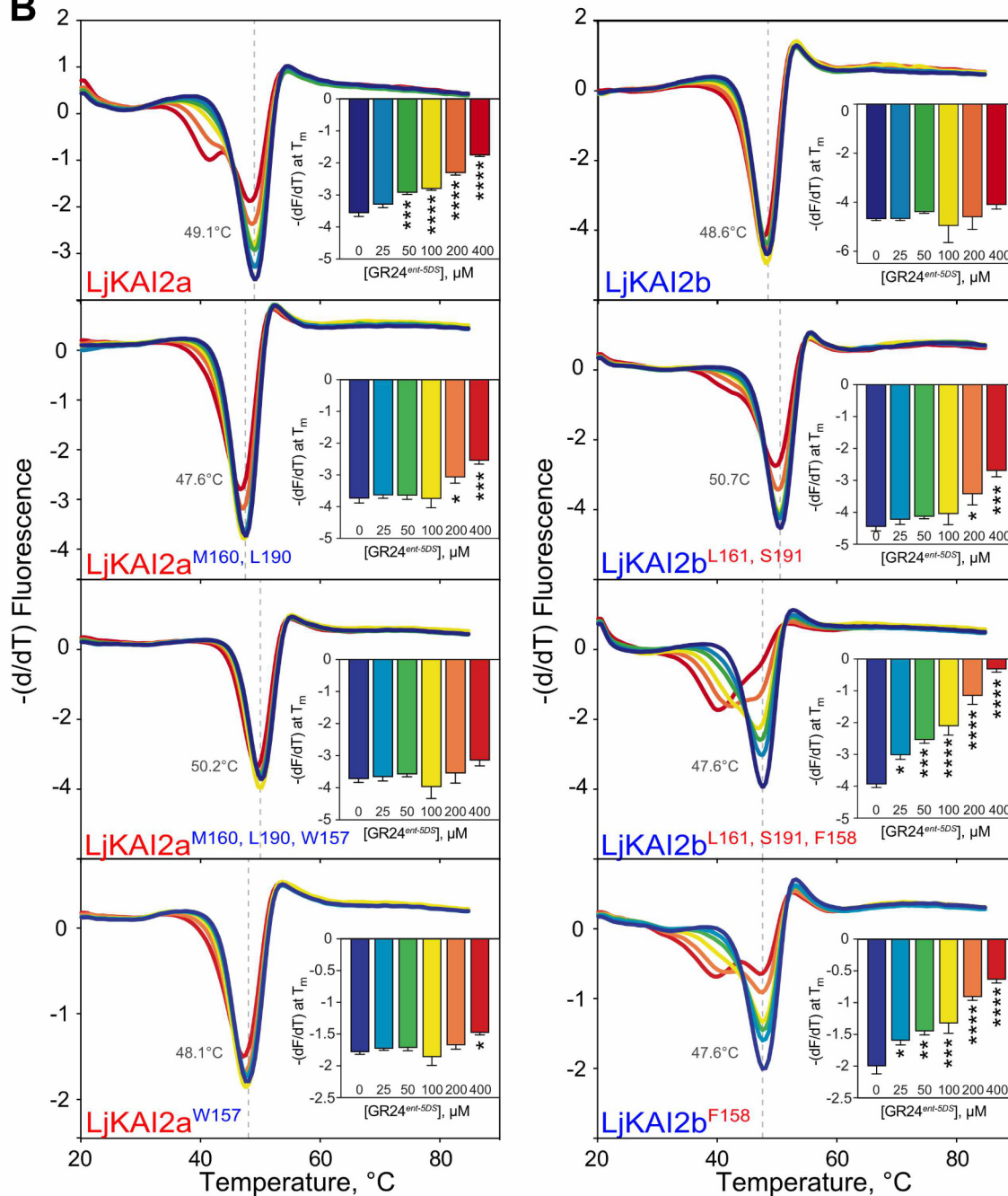
**B**

Figure 4. Binding of GR24^{ent-5DS} to LjKAI2a is determined by three amino acids.

(A) The ligand-binding cavity regions of LjKAI2a and LjKAI2b proteins after structural homology modelling on the KAI2 crystal structure of *A. thaliana* [5]. Conserved residues in the cavity that differ between the KAI2a and KAI2b clades, and that are also different between LjKAI2b and AtKAI2, are shown in green. The phenylalanine residue in LjKAI2a, which is changed to tryptophan in LjKAI2b, is shown in violet. The catalytic triad is coloured in red. (B) DSF curves of purified SUMO fusion proteins of wild-type LjKAI2a and LjKAI2b, and versions with swapped amino acids LjKAI2a^{M160, L190}, LjKAI2b^{L161, S191}, LjKAI2a^{W157, M160, L190}, LjKAI2b^{F158, L161, S191}, LjKAI2a^{W157}, LjKAI2b^{F158} at the indicated concentrations of GR24^{ent-5DS}. The first derivative of the change of fluorescence was plotted against the temperature. Each curve is the arithmetic mean of three sets of reactions, each comprising four technical replicates. Peaks indicate the protein melting temperature. The shift of the peak in LjKAI2a indicates ligand-induced thermal destabilisation consistent with a protein-ligand interaction. Insets plot the minimum value of $-(dF/dT)$ at the melting point of the protein as determined in the absence of ligand (means \pm SE, $n = 3$). Asterisks indicate significant differences to the solvent control (ANOVA, post-hoc Dunnett test, N.S. > 0.05, * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , **** ≤ 0.0001).

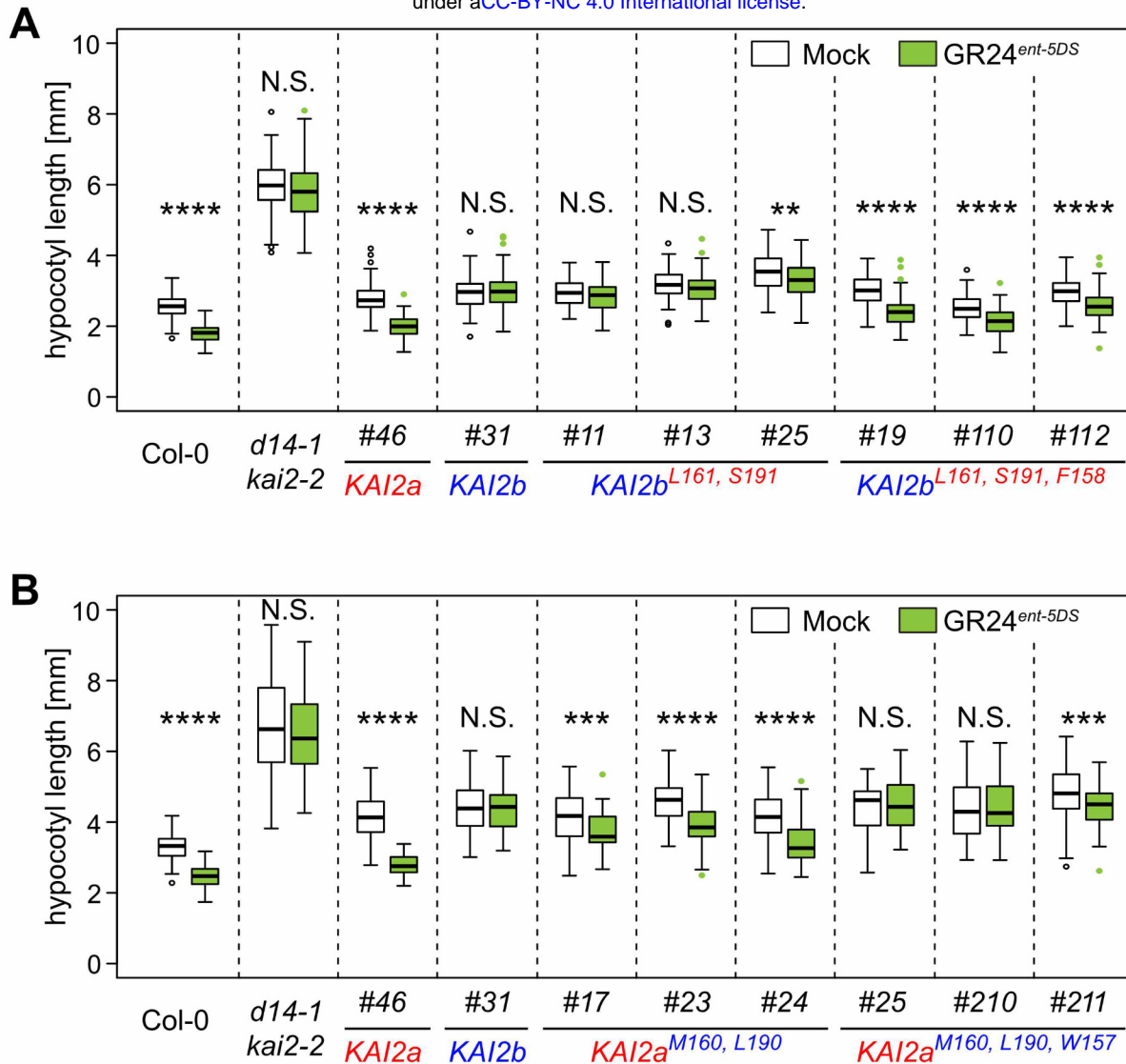


Figure 5. Amino acid swaps reverse sensitivity of *LjKAI2a* and *LjKAI2b* to GR24^{ent-5DS} in Arabidopsis hypocotyls.

Hypocotyl length of *A. thaliana* Col-0 wild-type, *d14-1 kai2-2* double mutants, and *d14-1 kai2-2* lines complemented with *LjKAI2a* and *LjKAI2b* variants driven by the *AtKAI2* promoter and after treatment with solvent (Mock), 1 μ M GR24^{5DS} or GR24^{ent-5DS}. (A) *LjKAI2a^{M160, L190}* and *LjKAI2a^{M160, L190, W157}* (n = 46-84). (B) *LjKAI2b^{L161, S191}* and *LjKAI2b^{L161, S191, F158}* (n = 49-102). (A-B) Seedlings were grown in 8h light / 16h dark periods Asterisks indicate significant differences versus mock treatment (Welch t.test, * \leq 0.05, ** \leq 0.01, *** \leq 0.001, **** \leq 0.0001).

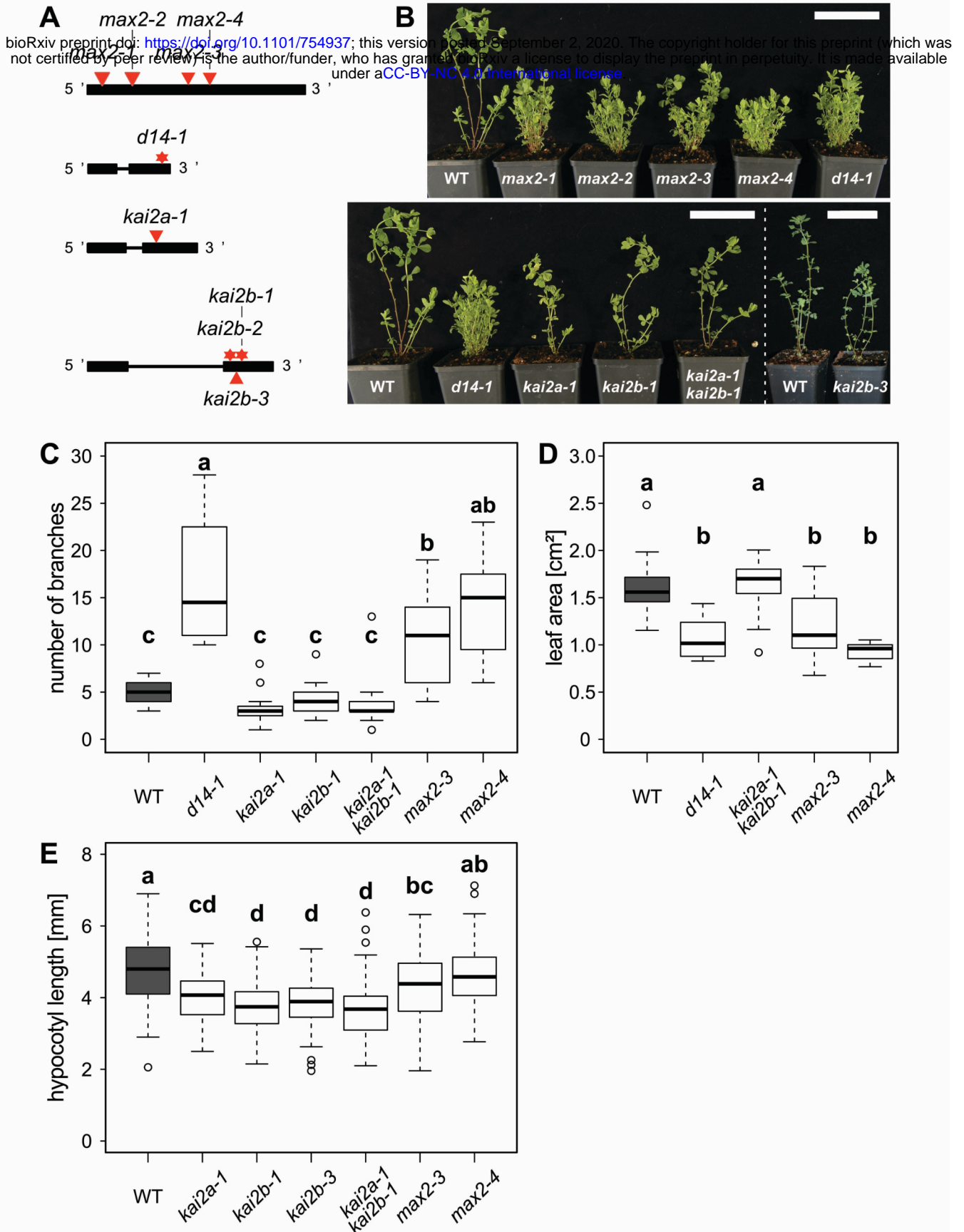


Figure 6. Role of *D14*, *KAI2a*, *KAI2b* and *MAX2* in shoot and hypocotyl development of *Lotus japonicus*.

(A) Schematic representation of the *L. japonicus* *D14*, *KAI2a*, *KAI2b* and *MAX2* genes. Black boxes and lines show exons and introns, respectively. *LORE1* insertions are indicated by red triangles and EMS mutations by red stars. **(B)** Shoot phenotype of *L. japonicus* wild-type and karrikin and strigolactone perception mutants at 8 weeks post germination (wpg). Scale bars: 7 cm. **(C)** Number of branches and of *L. japonicus* wild-type, karrikin and strigolactone perception mutants at 7 wpg (n = 12-21). **(D)** Leaf size of the indicated genotypes at 9 wpg (n = 12-15 plants with an average of 3 leaves). **(E)** Hypocotyl length of the indicated genotypes of *L. japonicus* under short day conditions (8h light/ 16h dark) at 1 wpg (n = 79-97). **(C-E)** Letters indicate different statistical groups (ANOVA, post-hoc Tukey test).

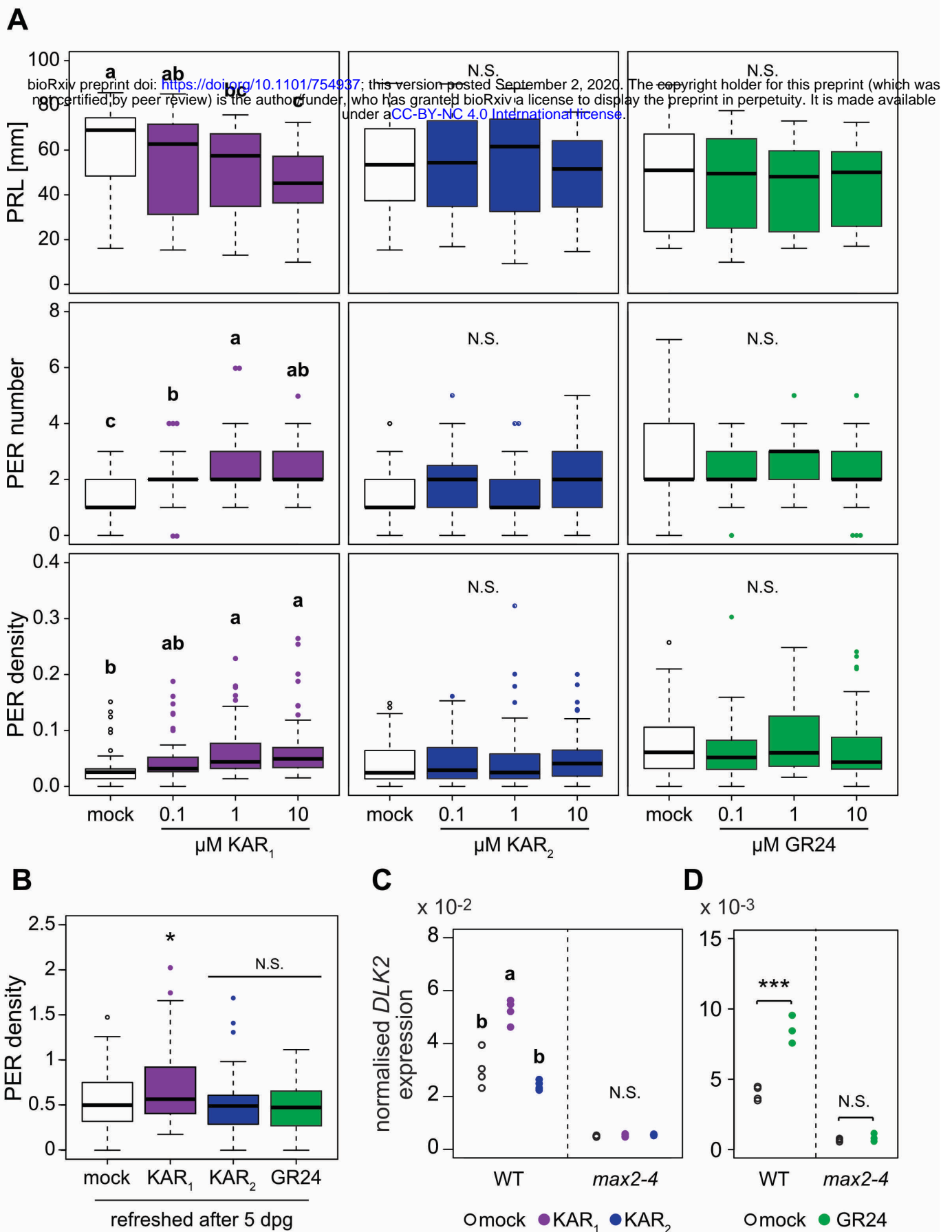


Figure 7. *Lotus japonicus* root system architecture is affected specifically by treatment with KAR₁ but not KAR₂. (A) Primary root length (PRL), post-embryonic root (PER) number and PER density of wild-type plants 2 wpg after treatment with solvent (M) or three different concentrations of KAR₁, KAR₂ or *rac*-GR24 (GR24) (n = 32-57). (B) PER density of wild-type plants at 2 wpg and treated with solvent (Mock) 1 μM KAR₁, 1 μM KAR₂, or 1 μM *rac*-GR24 (n = 43-51). Plants were transferred onto fresh hormone-containing medium after 5 days. (C-D) qRT-PCR-based expression of *DLK2* normalized to *Ubiquitin* expression in roots at 2 wpg after 2 hours treatment with solvent (Mock), (C) 1 μM KAR₁ and 1 μM KAR₂, (D) 1 μM *rac*-GR24 (n = 4). (A and C) Letters indicate different statistical groups (ANOVA, post-hoc Tukey test). (B) Asterisks indicate significant differences (ANOVA, Dunnett test, N.S.>0.05, *≤0.05). (D) Asterisk indicate significant differences versus mock treatment (Welch t.test, *≤0.05, **≤0.01, ***≤0.001).

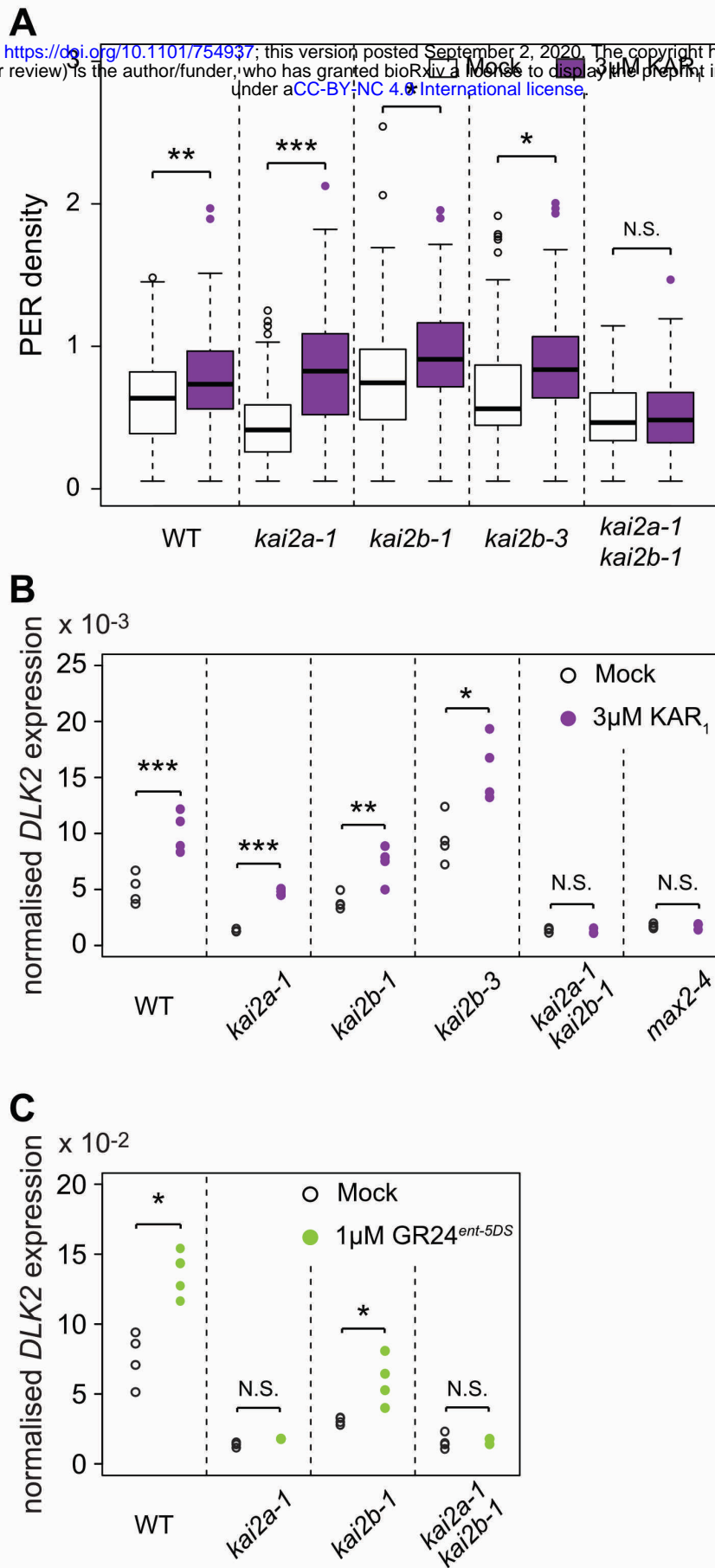


Figure 8. *LjKAI2a* and *LjKAI2b* operate redundantly in the response of roots to KAR_1
(A) Post-embryonic-root (PER) density of *L. japonicus* plants, 2 wpg after treatment with solvent (M) or 3 μM KAR_1 (n=34-72). **(B-C)** qRT-PCR-based expression of *DLK2* in roots of *L. japonicus* plants at 2 wpg after 2 hours treatment with solvent (Mock) or **(B)** 3 μM KAR_1 or **(C)** 1 μM $GR24^{ent-5DS}$. Expression values were normalized to those of the housekeeping gene *Ubiquitin* (n= 3-4). **(A-C)** Asterisks indicate significant differences versus mock treatment (Welch t.test, * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001).

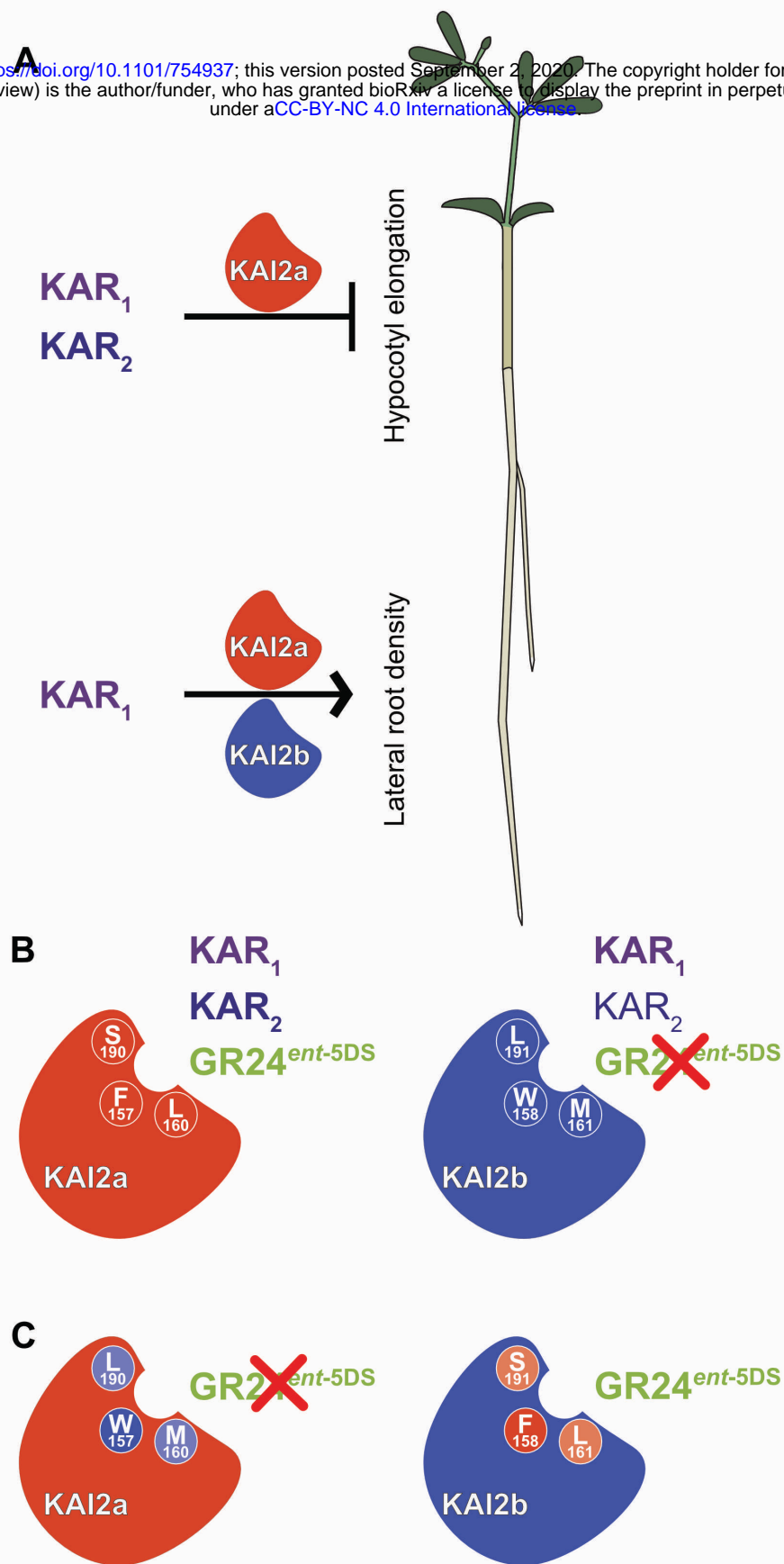


Figure 9. *L. japonicus* KAI2a and KAI2b display organ-specific redundancy and differ in their ligand-binding specificity. (A) *LjKAI2a* is required to mediate inhibition of hypocotyl growth in response to KAR_1 and KAR_2 . In roots *LjKAI2a* and *LjKAI2b* redundantly promote lateral root density, but only in response to KAR_1 treatment. (B) Upper panel: In the Arabidopsis *kai2-2* background *LjKAI2a* mediates hypocotyl growth inhibition in response to KAR_1 , KAR_2 and $GR24^{ent-5DS}$. In the same background, *LjKAI2b* mediates a stronger response to KAR_1 than to KAR_2 and no response to $GR24^{ent-5DS}$ (indicated by a red cross). Three divergent amino acids at the binding pocket are indicated in white. Lower panel: Swapping the three divergent amino acids in the binding pocket reconstitutes $GR24^{ent-5DS}$ activity through *LjKAI2b* and abolishes $GR24^{ent-5DS}$ activity through *LjKAI2a*. Among the three amino acids F157/W158 are decisive for $GR24^{ent-5DS}$ binding (strong colors), while L160/M161 and S190/L191 play a weaker role (pale colors). Amino acids from *LjKAI2a* have a red/pale red and amino acids from *LjKAI2b* a violet/pale background.