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1 *Research Report*

2

3 **Short title:** BR binding-independent endocytosis of BRI1

4

5 **BRASSINOSTEROID INSENSITIVE1 internalization can occur independent of ligand**
6 **binding**

7

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36

37 **AUTHOR CONTRIBUTIONS**

38

39 L.A.N.C., D.L., N.V., and E.R. conceived, designed, and performed the research. R.P. did
40 modeling. J. L., G.W., and Y.J. contributed unpublished materials. U.H., and M.H. did binding
41 experiments. L.A.N.C., A.S. and S.W. did the xylem phenotype experiments. D.V.D. helped with
42 data analysis and manuscript finalization. L.A.N.C., D.L., and E.R. wrote the manuscript. All
43 authors commented on the results and on the manuscript text.

44

45 **Abstract**

46

47 The brassinosteroid (BR) hormone and its plasma membrane receptor BR INSENSITIVE1 (BRI1)
48 is one of the best-studied receptor-ligand pairs for understanding the interplay between receptor
49 endocytosis and signaling in plants. BR signaling is mainly determined by the plasma membrane
50 pool of BRI1, whereas BRI1 endocytosis ensures signal attenuation. Since BRs are ubiquitously
51 distributed in the plant, the tools available to study BRI1 function without interference from
52 endogenous BRs are limited. Here, we designed a BR-binding-deficient *Arabidopsis*
53 *thaliana*) mutant based on protein sequence-structure analysis and homology modeling of members
54 of BRI1 family. This tool allowed us to re-examine the BRI1 endocytosis and signal attenuation
55 model. We showed that despite impaired phosphorylation and ubiquitination, BR-binding-deficient
56 BRI1 internalizes similarly to the wild-type form. Our data indicate that BRI1 internalization relies
57 on different endocytic machinery. In addition, the BRbinding-deficient mutant provides
58 opportunities to study non-canonical ligand-independent BRI1 functions.

59

60 **Keywords**

61

62 BRI1, brassinosteroids, ligand-binding, endocytosis, non-canonical

63

64 **Introduction**

65
66 Brassinosteroids (BRs) are low abundant and ubiquitously distributed plant steroidal hormones that
67 play essential role in growth, development, immunity and responses to stress (Nolan et al., 2020).
68 BR biosynthetic or signaling mutants display severe phenotypes including dwarfism, darkgreen
69 leaves, photomorphogenesis in the dark, and late flowering (Nolan et al., 2020). BRs are perceived
70 at the cell surface by a leucine-rich repeat (LRR) receptor kinase BR INSENSITIVE1
71 (BRI1) (He et al., 2000; Hothorn et al., 2011; Kinoshita et al., 2005; She et al., 2011; Wang et al.,
72 2001). BR binding triggers the dissociation of the inhibitory proteins BRI1 KINASE INHIBITOR1
73 (BKI1) and BAK1-INTERACTING RECEPTOR-LIKE KINASES3 (BIR3), allowing interaction
74 between BRI1 and its coreceptor BRI1-ASSOCIATED KINASE1 (BAK1), which is required for
75 downstream signaling (Hohmann et al., 2018a; Li et al., 2002; Wang and Chory, 2006). BR signal
76 is conveyed from the cell surface to the nucleus through a sequence of phosphorylation/de-
77 phosphorylation events that activate the transcription factors of the BRASSINAZOLE-
78 RESISTANT1 (BZR1) and BRI1-EMS-SUPPRESSOR1 (BES1)/BZR2 family (Wang et al., 2002;
79 Yin et al., 2002; Chen et al., 2019). BRI1 receptor functions go beyond its canonical BR signaling
80 function as together with the RECEPTOR-LIKE PROTEIN44 (RLP44) and BAK1, BRI1 controls
81 xylem cell fate independently of BRs (Holzwardt et al., 2018, 2020).

82 An important regulatory step in BR signaling is the control of the plasma membrane (PM) pool of
83 BRI1, which is determined by BRI1 endocytosis, recycling, and secretion (Irani et al., 2012; Luo
84 et al., 2015). As a consequence, impaired endocytosis and receptor secretion enhances and reduces
85 BR signaling, respectively (Irani et al., 2012; Luo et al., 2015). Several studies have focused on
86 BRI1 dynamics upon ligand binding. Given that exogenous BRs did not change BRI1
87 internalization dynamics, BRI1 endocytosis was described as ligand-independent (Geldner et al.,
88 2007; Luo et al., 2015; Russinova et al., 2004). Subsequent studies on post-translation
89 modifications (PTM) showed that BRI1 undergoes polyubiquitination that is mediated by the plant
90 U-box (PUB) E3 ubiquitin ligases PUB12 and PUB13 and requires BR binding (Zhou et al., 2018).
91 BRI1 ubiquitination is a signal for BRI1 endocytosis and vacuolar sorting, since disruptions in this
92 process by mutations in either the ubiquitination sites or the E3 ligases translate into accumulation
93 of BRI1 in the PM and consequently BR sensitivity increases (Martins et al., 2015; Zhou et al.,
94 2018).

95 Available tools used to study the dependence of BRI1 trafficking on ligand-binding are
96 limited and rely on the depletion of the endogenous BRs by using BR biosynthetic mutants or the
97 BR biosynthesis inhibitor, brassinazole (BRZ) (Asami et al., 2000). However, the possibility cannot
98 be excluded that treatment with BRZ might not completely deplete bioactive BRs and that the BR
99 biosynthetic mutant might contain biologically active BR precursors. Furthermore, BR biosynthetic
100 mutants display pleiotropic phenotypes that could lead to general changes in membrane trafficking.
101 Therefore, the use of these tools could hamper analysis of ligand-independent BRI1 dynamics.

102 Here, we report the characterization of an Arabidopsis (*Arabidopsis thaliana*) quintuple (Q)
103 BR binding-deficient BRI1 receptor mutant, designated as BRI1^Q, that was generated using
104 homology and structure analysis of BRI1 and its three homologues BRI1-LIKE1 (BRL1), BRL2
105 and BRL3 (Caño-Delgado et al., 2004). This tool revealed that BRI1 endocytosis is largely
106 independent of BRs and despite strongly decreased phosphorylation and ubiquitination the BRI1^Q
107 mutant held normal endocytosis rates. These results reinforce the hypothesis that BRI1 is
108 internalized by means of different endocytic machinery. Moreover, we used the BRI1^Q mutant to
109 investigate BR-independent BRI1 functions, such as xylem cell differentiation. We showed that
110 BRI1^Q can partially complement the xylem cell fate phenotype of the *bril* null mutant, suggesting
111 that BRI1^Q can be used to study BRI1 non-canonical functions.

112

113 **Results and Discussion**

114

115 **BRI1^Q cannot bind BRs**

116 The Arabidopsis genome encodes three BRI1 homologs, designated BRL1, BRL2 and BRL3, of
117 which BRL2 does not bind BRs (Caño-Delgado et al., 2004; Kinoshita et al., 2005). Sequence
118 analyses of BRI1, BRL1, BRL2 and BRL3 ectodomains (Figure 1A) and examination of the crystal
119 structure of the BRI1 ectodomain in a complex with brassinolide (BL), the most active BR (Hothorn
120 et al., 2011; She et al., 2011; Wang et al., 2001) (Figure 1, B-D), revealed five putative residues
121 important for BR binding, three derived from changes in the BRL2 sequence [tyrosine (Y)597,
122 Y599 and methionine (M)657] and two bulky hydrophobic residues located at a 4 Å distance from
123 the BL molecule in BRI1 [Y642 and phenylalanine (F)681] (Figure 1, B and C). Y597, Y599 and
124 Y642 map to the inner surface of the BRI1 island domain, forming the distal part of the BR-binding
125 pocket, whereas M657 and F681 are located in the LRR core and establish hydrophobic interactions
126 with the aliphatic BL moiety (Figure 1, B and C). The identified residues were mutated to the

127 corresponding ones in BRL2 either individually (BRI1^{Y599F} and BRI1^{M657E}) or in a combination
128 (BRI1^{Y597M/Y599F/M657E}) or to alanine (A) (BRI1^{Y597A/Y599A/M657A}).

129 Finally, a quintuple BRI1^{Y597M/Y599F/Y642A/M657E/F681A} version was generated and designated as
130 BRI1^Q. Next, the binding kinetics of BL to the BRI1^Q ectodomain was determined by
131 grating-coupled interferometry (GCI) (Figure 2B). As controls, the ectodomains of Arabidopsis wild
132 type BRI1 and that of the previously characterized Arabidopsis *bri1-6* mutant that carries the
133 glycine (G) 644 to asparagine (D) missense mutation (BRI1^{G644D}) were included (Hohmann et al.,
134 2018a; Hothorn et al., 2011; Kinoshita et al., 2005; Noguchi et al., 1999; Wang et al., 2001).
135 Analytical size-exclusion chromatography and right-angle light scattering experiments confirmed
136 that all BRI1 variants were monodisperse, suggesting that mutations in the BR-binding pocket do
137 not affect the overall shape and oligomeric state of the BRI1 ectodomain (Supplemental Figure S1).
138 The GCI experiments revealed that BRI1 bound BL with a dissociation constant (K_D) of ~10 nM
139 as previously reported (Hohmann et al., 2018a; Wang et al., 2001), BRI1^Q did not bind BL, whereas
140 the BRI1^{G644D} mutant displayed a strongly reduced BL-binding capacity with a K_D of ~11.6 μ M
141 (Figure 2B).

142 After confirmation that BRI1^Q cannot bind BL, mutated full-length BRI1 versions fused to
143 mCitrine (mCit) were expressed in the *bri1* null mutant from the native promoter and plants with
144 similar protein expression levels of the transgenes were selected (Figure 2A; Supplemental Figure
145 S2A). BRI1^{Y599F}-mCit, BRI1^{M657E}-mCit and BRI1^{Y597M/Y599F/M657E}-mCit partially complemented
146 the *bri1* dwarf phenotype and localized in the PM and in intracellular punctate structures, similar
147 to the wild type BRI1 (Geldner et al., 2007; Russinova et al., 2004) (Figure 2A). However,
148 BRI1^{Y597A/Y599A/M657A}-mCit and BRI1^Q-mCit did not complement the *bri1* mutant (Figure 2A).
149 BRI1^{Y597A/Y599A/M657A}-mCit displayed an aberrant accumulation in the vacuole, probably due to
150 misfolding of the LRR domain caused by the mutations. Similar localization was reported for the
151 artificially ubiquitinated BRI1, in which the ubiquitin was recognized before reaching the PM,
152 likely at the *trans*-Golgi network/early endosome (TGN/EE) compartments, and it served as a
153 targeting signal for vacuolar degradation (Martins et al., 2015). In contrast, BRI1^QmCit exhibited
154 correct BRI1 localization in the PM and in intracellular punctate structures. Hence, the absence of
155 BRI1^Q functionality corroborates the *in vitro* ligand-binding deficiency results (Figure 2B). To
156 further characterize the BRI1^Q-mCit line, we tested the BL-induced BRI1 PTMs. It is well
157 established that after ligand binding, BRI1 heterodimerizes with its co-receptor BAK1 and

158 undergoes PTMs such as phosphorylation and ubiquitination (Belkhadir and Jaillais, 2015; Martins
159 et al., 2015; Zhou et al., 2018). Moreover, BL treatment promotes the dephosphorylation of the
160 transcription factor BES1 in a dose-dependent manner, which is frequently used as a BR signaling
161 indicator (Yin et al., 2002). In agreement with the impaired BL binding, BRI1^Q-mCit had no
162 detectable phosphorylation or ubiquitination, did not interact with BAK1 and did not promote
163 BES1 dephosphorylation after BL treatment (Figure 2, C and D).

164 These findings indicate that BRI1^Q is unable to perceive BRs.

165

166 **Endocytosis of BRI1 is independent of BR binding**

167 The lack of BL binding in the BRI1^Q mutant provides a powerful tool to investigate different
168 aspects of BRI1 regulation, including endocytosis, without interference from BRs. Although
169 initially BRI1 endocytosis had been described as ligand independent (Geldner et al., 2007;
170 Russinova et al., 2004), later BR perception has been demonstrated to promote BRI1 ubiquitination
171 (Zhou et al., 2018), which assists BRI1 internalization and vacuolar targeting (Luo et al. , 2022;
172 Martins et al., 2015; Zhou et al., 2018). We revisited the BRI1 ligand-dependent endocytosis model
173 using BRI1^Q by evaluating the internalization of BRI1^Q-mCit in root meristem epidermal cells
174 (Figure 3, A and B). The PM pool of BRI1 is regulated by secretion, recycling, and endocytosis
175 (Irani et al., 2012; Luo et al., 2015). To avoid interference of the newly synthesized and secreted
176 BRI1, we analyzed 5-day-old plants expressing BRI1^Q-mCit treated with 50 μ M of the protein
177 synthesis inhibitor cycloheximide (CHX) for 1.5 h. Interestingly, the PM *vs.* cytoplasm
178 fluorescence intensity, did not significantly differ between BRI1^Q-mCit and the control BRI1-mCit,
179 both in *bril* null background (Figures 3, A and B, upper panel). In addition to the CHX treatment,
180 we applied Brefeldin A (BFA), an inhibitor of endosomal trafficking that is widely used to visualize
181 endocytosis (Geldner et al., 2003). In Arabidopsis roots, BFA treatment promotes the formation of
182 BFA bodies, composed of aggregated TGN/EEs (Geldner et al., 2003; Lam et al., 2009). When
183 combining CHX (50 μ M, 1.5 h) with BFA (50 μ M, 30 min), both BRI1mCit and BRI1^Q-mCit
184 accumulated in similar size BFA bodies (Figure 3, A and B, lower panel). These results were in
185 agreement with the measurements of the PM *vs.* cytoplasm fluorescence intensity in BRI1^Q-mCit
186 (Figure 3, A and B, upper panel). Comparable PM *vs.* cytoplasm fluorescence intensity ratios and
187 BFA body size were also obtained when BRI1^Q-mCit was introduced into the Arabidopsis
188 Columbia-0 (Col-0) wild type (Supplemental Figure S2, B and D) to avoid artefacts in quantitative
189 microscopy due to the strong dwarfism of the *bril* mutant (Figure 3, C and D).

190 We next assessed the recycling dynamics of BRI1^Q-mCit in *bri1*, by performing BFA
191 washout experiments after treatment with CHX (50 μM) for 1 h, followed by a combined
192 application of CHX and BFA (50 μM) for 30 min. Epidermal cells of root meristem were imaged
193 at 0, 30, 60, 90, and 120 min after the BFA washout in the presence of CHX. By quantifying the
194 percentage of epidermal cells with BFA bodies we did not observe differences between the
195 BRI1mCit and BRI1^Q-mCit (Supplemental Figure S3, A and B).

196 Besides recycling, BRI1 is also subjected to vacuole degradation (Martins et al., 2015; Zhou
197 et al., 2018). To evaluate the vacuolar targeting of BRI1^Q we transferred BRI1^Q-mCit/Col-0 and
198 BRI1-mCit/Col-0 plants to dark conditions, which suppress vacuolar lytic activity and allow
199 detection of the vacuolar pH-resistant mCit fluorescent fusion proteins (Tamura et al., 2003). By
200 measuring the fluorescence intensity of BRI1^Q-mCit and BRI1-mCit in the vacuole, we observed
201 that the vacuolar accumulation of BRI1^Q-mCit is slightly reduced when compared to that of BRI1-
202 mCit (Supplemental Figure S3, C and D).

203 Because BRI1^Q and BRI1 were endocytosed, recycled and degraded with similar dynamics, we
204 hypothesized that they share endomembrane trafficking routes. Indeed, as previously reported for
205 BRI1 (Irani et al., 2012), BRI1^Q co-localized with endomembrane markers labeling TGN/EEs, late
206 endosomes/multivesicular bodies (MVBs) and Golgi compartments but not with the autophagy
207 marker ATG8e (Supplemental Figure S4). Moreover, by taking advantage of the bioactive
208 fluorescently-labeled BR precursor Alexa Fluor 647Castasterone (AFCS), which specifically
209 marks the endocytic route of BRI1 (Irani et al., 2012), we showed that AFCS co-localized with
210 BRI1^Q-mCit in the wild type Arabidopsis (Supplemental Figure S5), suggesting that the two
211 receptors are internalized through similar routes.

212 Despite the widespread use of CHX and BFA treatments to analyze endocytosis in plant
213 cells, it cannot be excluded that the chemical treatments might cause pleiotropic effects (Oksvold
214 et al., 2012; Smith et al., 2014). To circumvent this problem, we expressed BRI1-mCit and BRI1^Q-
215 mCit under the control of the heat shock-inducible promoter (*pHS*) in the Col-0 background and
216 studied the protein internalization during the recovery phase after a heat treatment at 37°C for 1 h,
217 which did not affect the endocytic rates of FM4-64 in wild type seedling (Supplemental Figure S6).
218 Firstly, we selected transgenic lines expressing *pHS::BRI1mCit* and *pHS::BRI1^Q-mCit* with similar
219 expression levels following the heat induction (37°C) for 1 h (Supplemental Figure S2C). Taking
220 advantage of a vertical confocal microscope setup equipped with the TipTracker software (von
221 Wangenheim et al., 2017) that allows the monitoring of growing root tips over time, we observed

222 that BRI1^Q-mCit reached the PM a little later than the BRI1-mCit (Figure 3, E and F). However,
223 after a signal intensity peak in the PM, between 87 and 99 min, the internalization rate of BRI1-
224 mCit and BRI1^Q-mCit was very similar (Figure 3, E and F). Thus, our findings further confirm
225 previous reports (Geldner et al., 2007; Irani et al., 2012; Russinova et al., 2004) that the BRI1
226 internalization is largely independent of BR binding and, consequently, receptor activation. After
227 ligand binding and interaction with BAK1, BRI1 is phosphorylated and ubiquitinated, both
228 essential for receptor internalization (Martins et al., 2015; Zhou et al., 2018). However, although
229 BRI1^Q displayed reduced levels of both PTMs, it still had a normal endocytosis. These results
230 reinforce the hypothesis that BRI1 is internalized using different mechanisms. For instance, the
231 BRI1 internalization has been demonstrated to partially depend on both the classical clathrin
232 Adaptor Protein 2 (AP-2) complex that binds to a canonical
233 YXXΦ endocytic motif in BRI1 (Liu et al., 2020) and on ubiquitin recognition machinery, since
234 endocytosis of the ubiquitin-deficient BRI1^{25KR}-mCit or BRI1-mCit in the *pub12 pub13* double
235 mutant was not completely abolished (Martins et al., 2015; Zhou et al., 2018). Similar observations
236 were reported for the borate exporter BOR1, in which AP-2-dependent and AP-2-independent
237 internalization had been activated by low and high borate concentration, respectively (Yoshinari et
238 al., 2019). Equally in mammals, the well-studied epidermal growth factor receptor (EGFR) is also
239 internalized via different endocytic mechanisms, including canonical ligand-dependent clathrin-
240 mediated endocytosis and clathrin-independent endocytosis, which both depend on the ligand
241 concentration (Zhou and Sakurai, 2022), and a ligand-independent internalization where EGFR
242 endocytosis is induced by stress conditions and does not require kinase activity or ubiquitination
243 (Metz et al., 2021).

244 However, after internalization, BRI1 and BRI1^Q pursued the same trafficking routes.
245 Interestingly and in contrast to BRI1, the immune receptors, FLAGELLIN SENSING2 (FLS2) and
246 PEP RECEPTOR1 (PEPR1) follow distinct trafficking pathways depending on their activation
247 state. Inactive FLS2 and PEPR1 constitutively recycle between PM and TGN/EEs in a BFA-
248 sensitive manner, while after activation, FLS2 and PEPR are internalized in MVBs that are
249 insensitive to BFA (Beck et al., 2012; Ortiz-Morea et al., 2016; Mbengue et al., 2016).

250 Taken together, our results show that BRI1 internalization is not abolished in the BR
251 binding-deficient mutant but it remains to be established which signals or conditions trigger
252 endocytosis of the inactive receptor.

253

254 **BRI1^Q can partially complement the xylem phenotype of *bri1***

255 In addition to its primary role in perceiving BRs, recent studies suggest that BRI1 might also have
256 non-canonical functions in sensing cell wall integrity. After disturbance of the cell wall integrity
257 by the inhibition of the pectin de-methyl esterification, BRI1 is recruited together with the RLP44
258 and BAK1 to activate a BR signaling for a compensatory feedback loop to remodel the cell wall
259 (Wolf et al., 2012). Besides cell wall integrity monitoring, RLP44 is also implicated in controlling
260 xylem cell fate through the phytosulfokine (PSK) signaling (Holzwardt et al., 2018). Interestingly,
261 BRI1 and BAK1 are also necessary to regulate the vasculature cell fate, but independently of BRs,
262 because BR biosynthesis mutants have no ectopic xylem in the procambial position present in the
263 *rlp44* and *bri1* mutants (Holzwardt et al., 2018, 2020). To test whether BRI1^Q retains its non-BRs
264 receptor functions, we examined if BRI1^Q could still interact with RLP44 (Holzwardt et al., 2018).
265 RLP44-RFP was transiently co-expressed with either BRI1-GFP or BRI1^Q-GFP in *Nicotiana*
266 *benthamiana* leaves and co-immunoprecipitation (Co-IP) assay revealed that RLP44-RFP was co-
267 purified with both BRI1-GFP and BRI1^Q-GFP but not with the negative control, indicating that
268 BRI1^Q, like the wild type BRI1, can form a complex with RLP44 (Figure 4A). Furthermore,
269 confocal analysis of *N. benthamiana* leaves transiently expressing BRI1-GFP, BRI1^Q-GFP and
270 RLP44-RFP show that both BRI1 and BRI1^Q co-localize with RLP44 in dynamic punctate
271 structures (Figure 4B). The intracellular punctate structures containing RLP44-RFP and BRI1-GFP
272 or BRI1^Q-GFP are probably endosomes, because BRI1 is a *bona fide* endosomal PM cargo (Geldner
273 et al., 2007; Russinova et al., 2004). Moreover, RLP44 had already been shown to localize in
274 endosomal structures in *Arabidopsis* roots (Wolf et al., 2014). Finally, we tested whether BRI1^Q-
275 mCit could recover the BR-independent xylem cell fate phenotype of the *bri1* null mutants
276 (Holzwardt et al., 2018, 2020). Indeed, BRI1^Q-mCit could partially complement the ectopic number
277 of xylem cells present in the *bri1* mutant (Figure 4). Collectively, our result provides evidence that
278 BRI1^Q could still be active in BR-independent pathways.

279 In conclusion, analyses of the crystal structure of the ectodomain of BR receptors and
280 homology modeling allowed us to create a BR binding-deficient BRI1 mutant, BRI1^Q.
281 Characterization of BRI1^Q showed that it displays a clear *bri1*-like phenotype and is unable to
282 respond to exogenous BRs. By means of BRI1^Q as a tool to study BRI1 endocytosis and in
283 agreement with previous observations (Geldner et al., 2007; Irani et al., 2012; Russinova et al.,
284 2004), we conclude that BRI1 internalization can occur without BR binding. Moreover, the
285 BRbinding-deficient BRI1 mutant might provide opportunities to discover additional non-

286 canonical ligand-independent BRI1 functions in vasculature development and other processes
287 (Graeff et al., 2020; Holzward et al., 2018, 2020).

288

289 **Materials and Methods**

290

291 **Plant materials, growth conditions and treatments.**

292 The experimental model used in this study was *Arabidopsis* (*Arabidopsis thaliana* (L.) Heynh).

293 The wild type used was accession Columbia-0 (Col-0) . Dual-marker lines were generated by
294 crossing *pBRI1::BRI1-mCit/Col-0* or *pBRI1::BRI1^Q-mCit/Col-0* with plants expressing
295 *pVHAa1::VHAa1-RFP/Col-0* (Dettmer et al., 2006), *pUBQ10::MEMB12-mCherry/Col-0*

296 (Geldner et al., 2009), *pARA7::ARA-RFP/Col-0* (Geldner et al., 2009), and

297 *pUBQ10::mCherryATG8e/Col-0* (Zhao et al., 2022).

298 For phenotypic analysis, plants were grown in soil in a growth chamber at 22°C, 58% relative

299 humidity, and a 16-h light/8-h dark photoperiod for 6 weeks. The *Arabidopsis* seeds were surface-

300 sterilized with chlorine gas, and then placed on plates with half-strength Murashige and Skoog

301 medium (½MS) containing 0.5% (w/v) sucrose, 0.8% (w/v) agar, and 2.5 mM methyl ester

302 sulfonate at pH 5.7. After vernalization for 2 days at 4°C, the plates were moved to the growth

303 chamber under a 16-h/8-h light/dark cycle. *Nicotiana benthamiana* plants were grown in a

304 greenhouse under a normal 14-h light/10-h dark regime at 25°C. For the microsomal protein

305 preparation, plants were grown for 6 days on plates. For the BRI1 internalization assay and the

306 BRI1 transcript analysis, plants were grown for 5 or 7 days on plates. The vacuolar targeting of

307 BRI1 was evaluated in plants grown for 5 days on plates and then transferred to dark for 10 h.

308 MG-132 (10 mM stock in dimethylsulfoxide [DMSO]), BFA (50 mM stock in DMSO), FM4-64 (2

309 mM stock in water), and CHX (50 mM stock in DMSO) were used at the concentrations indicated

310 in the figure legends.

311

312 **Vector construction and plant transformation.** The BRI1-coding region without the stop codon

313 was cloned into *pMD19-T* (simple) (Takara Biotechnology) to generate *pMD19-BRI1* that was used

314 as a template to generate the mutants *BRI1^{Y599F}*, *BRI1^{M657E}*, *BRI1^{Y597M/Y599F/M657E}*,

315 *BRI1^{Y597A/Y599A/M657A}*, and *BRI1^{Y597M/Y599F/Y642A/M657E/F681A}* by overlapping PCR and subcloned into

316 *pDONR221* to generate *pDONRPIP2-BRI1* (with mutations). The primers used to generate the

317 BRI1 mutations are listed in Table S1. The destination vectors were generated by recombining

318 *pK7m34GW*, *pB7m34GW*, *pDONRP4P1r-pBRI1*, *pDONRP4P1r-pHS* (MarquèsBueno et al., 2016)
319 *pDONRP4P1r-pBRI1*, *pDONRP4P1r-pHS*, *pDONR221-BRI1*, and *pDONRP2rP3-mCit* (Martins
320 et al., 2015). The resulting constructs were transformed into the heterozygous *bril* null mutant
321 (GABI_134E10) (Jaillais et al., 2011) or into Col-0 plants by floral dip. For transient expression in
322 *N. benthamiana* leaves, *pDONR221-BRI1* and *pDONR221-BRI1^Q* were recombined in *pK7FWG2*
323 that contained the 35S promoter and C-terminal GFP. The Gateway technology (Invitrogen) was
324 used for cloning.

325

326 **Western blot analysis and immunoprecipitation**

327 For the BRI1 expression assay, 5-day-old seedlings were homogenized in liquid nitrogen. Total
328 proteins were extracted with a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (w/v)
329 sodium dodecyl sulfate (SDS), 100 mM dithiothreitol (DTT), and ethylenediaminetetraacetic acid
330 (EDTA)-free protease inhibitor cocktail cOmplete (Roche). For blocking and antibody dilutions,
331 3% (w/v) bovine serum albumin (BSA) powder in 0.2% (v/v) Tris-buffered saline-containing
332 Tween-20 was used. For the microsomal fraction isolation, 6-dayold seedlings treated with 50 μ M
333 MG-132 for 5 h were ground in liquid nitrogen and resuspended in ice-cold sucrose buffer (100
334 mM Tris [pH 7.5], 810 mM sucrose, 5% [v/v] glycerol, 10 mM EDTA [pH 8.0], 10 mM
335 ethyleneglycoltetraacetic acid [EGTA, pH 8.0], 5 mM KCl, protease inhibitor [Sigma-Aldrich],
336 and phosphatase inhibitor [Sigma-Aldrich]). The homogenate was transferred to polyvinyl
337 polypyrrolidone (PVPP) pellets, mixed, and left to rest for 5 min. Samples were centrifuged for 5
338 min at 600 \times g at 4°C. The supernatant was collected. The extraction was repeated for two more
339 times. The supernatant was filtered through a Miracloth mesh. The same amount of water was
340 added to the clear supernatant and centrifuged at 21,000 \times g for 2 h at 4°C to pellet microsomes
341 (Abas and Luschnig, 2010). The pellet was resuspended in immunoprecipitation buffer (25 mM
342 Tris, pH 7.5, 150 mM NaCl, 0.1% [w/v] SDS, protease inhibitor, and phosphatase inhibitor).
343 Immunoprecipitations were carried out on solubilized microsomal proteins with GFP-Trap-MA
344 (Chromotek) according to the manufacturer's protocol.

345 For protein detection, the following antibodies were used: monoclonal α -GFP horseradish
346 peroxidase-coupled (1/5,000; Miltenyi Biotech), monoclonal α -tubulin (1/10,000; Sigma-
347 Aldrich), α -ubiquitin (Ub) P4D1 (1/2,500; Millipore), α -pThr (1/2,000; Cell Signaling), α -BES1
348 (Yin et al., 2002) (1/4,000), and α -BAK1 (1/5,000; custom-made by Eurogentec). Uncropped blots
349 are shown in Supplemental Figure S7.

350
351 **Xylem staining**
352 Seven-day-old Arabidopsis seedlings were stained with Basic Fuchsin as described (Ursache et al.,
353 2018).

354
355 **AFCS uptake assays**

356 AFCS uptake assay was performed as previously described (Irani et al., 2014) with modifications.
357 6-day-old seedlings grown on solid ½MS were transferred to 200 µl of liquid ½MS on a piece of
358 parafilm placed in a Petri plate, humidified with wet laboratory wipes for 10 min. The medium was
359 replaced with ½MS supplemented with 30 µM AFCS for 40 min (pulse).
360 Seedlings were washed six times and chased for 2 min on ½MS followed by imaging.

361
362 **Confocal microscopy and image analysis.** For BRI1 localization and BFA washout Arabidopsis
363 seedlings were imaged with an Olympus FluoView1000 confocal laser scanning microscope
364 equipped with UPLSAPO 60×/1.2 n.a. water-corrected immersion objective at digital zoom 2 and
365 UPLSAPO 40×/1.3 n.a. oil-corrected immersion objective at digital zoom 1, respectively. The
366 excitation/emission wavelengths were 514 nm/530-600 nm for BRI1-mCit, with laser intensity of
367 40 % and gain 667. For BRI1 internalization, BFA treatment, and FM4-64 uptake, a Leica SP8X
368 confocal microscope was used with a HC PL 584 APO CS2 40x/1.1 n.a. water-corrected immersion
369 objective at digital zoom 5, 3, and 5, respectively. The excitation/emission wavelengths were 514
370 nm/530-600 nm for BRI1-mCit, and 514 nm/600-700 nm for FM4-64 with white light laser (WLL)
371 laser intensity of 70 % and gain 150. For BRI1 co-localization, Leica SP8X confocal microscope
372 was used with a HC PL APO CS2 63x/1.20 water-corrected immersion objective at digital zoom
373 2.5. The excitation/emission wavelengths were 514 nm/530600 nm for BRI1-mCit, and 594
374 nm/600-650 nm for RFP and mCherry with WLL laser intensity of 70 % and gain 110. For the
375 BRI1 internalization and FM4-64 uptake, the membrane of individual cells was selected using the
376 brush tool of ImageJ with a size of 5 pixels as well as using the polygon selection tool to mark the
377 intracellular space. The average intensity of the top 5 % highest pixels for both the PM and the
378 intracellular space was used to obtain a ratio between PM and intracellular fluorescence. The BFA
379 body size and percentage of epidermal cells with BFA bodies was calculated as previously
380 described (Luo et al., 2015).

381 For AFCS uptake, epidermal cells of the root meristematic zone were imaged using laser scanning
382 confocal microscope Leica SP8X equipped with an HC PL APO CS2 63×/1.20 watercorrected
383 objective with 4x digital zoom. The excitation wavelength was 633 nm by white light laser.
384 Emission was detected at 650-700 nm by Leica hybrid detectors with WLL laser intensity of 70 %
385 and gain 180.

386 Xylem was imaged with Leica SP8X confocal microscope equipped with a HC PL APO CS2
387 63x/1.20 n.a. water-corrected immersion objective. The excitation/emission wavelengths used were
388 of 561 nm/600-650 nm for Basic Fuchsin staining with WLL laser intensity of 70 % and gain 180.
389 The BRI1 heat shock lines were analyzed under a vertical ZEISS LSM900 microscope equipped
390 with a Plan-Apochromat M27 20x/ 0.8 n.a. objective. The excitation/emission wavelengths were
391 514 nm/530-600 nm for BRI1-mCit with laser intensity of 10 % and gain 660. The root tip was
392 tracked over time with the TipTracker software (von Wangenheim et al., 2017). Quantification was
393 obtained by measuring the PM signal of the same cell over time and normalized by the time point
394 with the highest PM signal. Because the heat shock promoter generated a patchy expression pattern,
395 for quantification cells with similar fluorescence intensity were selected. Image processing and
396 quantification were performed with the Fiji software package.

397

398 **Grating-coupled interferometry (GCI)**

399 A Creoptix WAVE system (Creoptix AG, Switzerland) was used for the GCI binding assays.
400 Experiments were done on a 4PCH WAVE GCI chip (long polycarboxylate surface; Creoptix AG).
401 After a borate buffer conditioning (100 mM sodium borate, pH 9.0, 1 M NaCl; XanTec
402 Bioanalytics, Düsseldorf, Germany), streptavidin was immobilized through a standard amine
403 coupling protocol, followed by passivation of the surface (0.5% BSA [Roche] in 10 mM sodium
404 acetate, pH 5.0), and final quenching with 1 M ethanolamine, pH 8.0 for 7 min (XanTec
405 Bioanalytics). The LRR ectodomains of wild type BRI1 and the respective mutants were
406 biotinylated and coupled to the streptavidin-coated chip. For the BL binding experiments, BL was
407 injected in a 1:2 dilution series, starting from 3 μ M, in 20 mM citrate, pH 5.0, 250 mM NaCl at
408 25°C. Blank injections were used for double referencing and a DMSO calibration curve for bulk
409 correction. All analyses and corrections were done with the Creoptix WAVE control software, with
410 a one-to-one binding model with bulk correction used to fit all experiments.

411

412 **Analytical size-exclusion chromatography (SEC)**

413 Analytical SEC experiments were carried out on a Superdex 200 increase 10/300 GL column (GE
414 Healthcare), preequilibrated in 20 mM sodium citrate, pH 5.0, 250 mM NaCl. 200 µg of protein,
415 injected in a 100-µl volume, was loaded onto the column and elution at 0.75 ml/min was monitored
416 by ultraviolet absorbance at $\lambda = 280$ nm. Peak fractions were analyzed by SDS-PAGE.

417

418 **Right-angle light scattering (RALS)**

419 BRI1 ectodomains (residues 1-788 with a C-terminal Avi-tag as well as a TEV protease cleavable
420 TwinStrep – 9x His tag) were expressed and purified as described previously (Hohmann et al
421 2018b) and analyzed by size-exclusion chromatography (SEC) paired with a RALS and a refractive
422 index (RI) detector, using an OMNISEC RESOLVE / REVEAL system. The calibration of the
423 instrument was carried out with a BSA standard (Thermo Scientific). In a 50 µl volume, 100 µg of
424 protein was separated on a Superdex 200 increase column (GE Healthcare) in 20 mM sodium
425 citrate, pH 5.0, 250 mM NaCl at a column temperature of 35°C and a 0.7 ml/min.

426 Data were analyzed using the OMNISEC software (v10.41).

427

428 **Homology modeling and structure visualization**

429 BRI^Q ectodomain structure was modeled using the Modeller 9.18 program (Šali and Blundell,
430 1993) with the BRI1 ectodomain (PDB ID 3RJ0) as a template. The structures of wild type BRI1
431 and BRI1^Q ectodomains (Figure 1) were visualized by UCSF Chimera (Pettersen et al., 2021).

432 Multiple sequence alignment was prepared using the Jalview program (Waterhouse et al., 2009)

433

434 **Reverse transcription quantitative PCR (RT-qPCR)**

435 Seven-day-old seedlings in liquid half-strength Murashige and Skoog medium were transferred to
436 37°C for 1 h and let to recover at room temperature for another 1 h. Total RNA was extracted with
437 the RNeasy kit (Qiagen). cDNA was synthesized from RNA with the qScript cDNA Supermix
438 (Quantabio). RT-qPCR was conducted with SYBR green I Master kit (Roche) on a LightCycler
439 480 (Roche). The mCitrine expression was normalized to that of *ACTIN2* and *GAPDH*. The cycling
440 conditions were as follows: preincubation at 95°C for 10 min; 45 amplification cycles at 95°C for
441 10 s, 60°C for 15 s, and 72°C for 15 s; melting curve at 95°C for 1 s, and 65°C for 1 s, followed by
442 cooling at 40°C for 10 s.

443

444 **Quantification and statistical analysis**

445
446 The data were subjected to statistical analysis using GraphPad Prism
447 (<https://www.graphpad.com/scientific-software/prism/>) and Excel software. Comparisons between
448 two groups were made with *t*-tests. Comparisons between three or more groups were made with
449 Mann Whitney tests or two-way ANOVAs with subsequent post hoc Sidak's multiple comparisons
450 tests. Comparisons between discrete groups were made using Chi-square tests. The measurements
451 are shown as box plots displaying the first and third quartiles and split by medians (center lines),
452 with whiskers extending to 1.5-fold the interquartile range from the 25th and 75th percentiles.

453
454 **Accession Numbers**

455
456 Sequence data from this article can be found in the GenBank/EMBL data libraries under
457 accession numbers: At4g39400 (BR11), At1g55610 (BRL1), At2g01950 (BRL2), At3g13380
458 (BRL3), At4g33430 (BAK1), At3g49750 (RLP44), At1g19350 (BES1), At1g50010 (TUBULIN),
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477

478 **DECLARATION OF INTERESTS**

479

480 The authors declare no competing interests.

481

482 **FIGURE LEGENDS**

483

484 **Figure 1. Selection of the essential residues for brassinosteroid (BR) binding in the BRI1** 485 **ectodomain.**

486 (A) Sequence alignment of the wild type BRI1 ectodomain (only the region corresponding to
487 BRI1 550-700 is shown) with that of BRL1, BRL2 and BRL3 and BRI1^Q with the mutated residues
488 highlighted in red. The mutated residues are marked with an asterisk.

489 (B) The BRI1 ectodomain structure in a complex with brassinolide (BL) (Protein Data Bank ID
490 3RJ0).

491 (C and D) The five residues selected are indicated as single-letter abbreviation with their locations,
492 either colored in green for wild type BRI1 or in red for BRI1^Q. The figures (B-D) were generated
493 with UCSF Chimera where the BL molecule is shown in a balls-and-sticks representation and
494 colored in purple.

495

496 **Figure 2. BRI1^Q cannot bind brassinolide (BL).**

497 (A) Phenotypes (upper panel) and subcellular localizations (lower panel) of homozygous *bri1*
498 mutant transgenic plants expressing the indicated BRI1 isoform mutations grown in short-day
499 cycle for 6 weeks. The quintuple BRI1^{Y597M/Y599F/Y642A/M657E/F681A} mutation is designated
500 BRI1^Q. Epidermal root meristem cells of 5-day-old seedlings were imaged. Scale bars, 2 cm
501 (upper panel) and 10 μ m (lower panel).

502 (B) Binding kinetics for BL vs wild type BRI1, BRI1^{G644D} and BRI1^Q as obtained from
503 gratingcoupled interferometry (GCI). Sensograms with recorded data are shown in red with
504 the respective fits in black (when applicable) and include the corresponding association rate
505 constant (k_a), dissociation rate constant (k_d) and dissociation constant (K_D).

506 (C) BRI1-mCitrine (mCit) and BRI1^Q-mCit phosphorylation and ubiquitination state and
507 interaction with BAK1 were tested by isolation of microsomal fractions of 5-day-old seedlings
508 followed by immunoprecipitation (IP) and Western blot (WB) analysis with α -ubiquitin (α -
509 Ub), α -pThreonine (α -pThr) and α -BAK1 antibodies.

510 (D) BES1 phosphorylation state assessed in 5-day-old seedlings treated with DMSO (mock),
511 1 nM or 10 nM BL for 1 h were subjected to WB analysis with the α -BES1 antibody; α -tubulin was
512 used as loading control. All experiments were repeated twice with similar results (C and D).

513

514 **Figure 3. BRI1^Q endocytosis is independent of ligand binding.**

515 (A and C) Representative confocal images of epidermal root meristem cells of 5-day-old
516 seedlings expressing BRI1-mCitrine (mCit) or BRI1^Q-mCit treated with cycloheximide (CHX) (50
517 μ M) for 1.5 h or pretreated with CHX for 1 h, followed by treatment for 30 min with CHX and
518 Brefeldin A (BFA) (50 μ M). Scale bar, 10 μ m.

519 (B and D) Plasma membrane (PM) vs intracellular BRI1-mCit fluorescence intensity and BFA
520 body size. For each line, 15 cells from at least 5 seedlings were measured. Box plots show the first
521 and third quartiles, split by the medians (lines), with whiskers extending 1.5-fold interquartile range
522 beyond the box, and dots as outliers. Statistical analysis was performed using Mann Whitney test.

523 (E and F) Time series analysis of meristem epidermal cells of 5-day-old seedling expressing BRI1-
524 mCit or BRI1^Q-mCit after heat induction (1 h at 37°C). Images were taken with a vertical confocal
525 microscope with a 12 min interval between the frames. Scale bar, 10 μ m. PM fluorescence intensity
526 of the same cells was quantified for all time points and the signal peak was set as 1. Box plots show
527 the first and third quartiles, split by the medians (lines), with whiskers extending 1.5-fold
528 interquartile range beyond the box, and dots as outliers. Asterisks indicate the cells that were
529 quantified. Four roots and 3 to 5 cells were measured per genotype. Asterisks indicate statistically
530 significant differences, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, based on twoway ANOVA and post
531 hoc Sidak's multiple comparisons test. All experiments were repeated twice with similar results (A-
532 F).

533

534 **Figure 4. BRI1^Q partially recovers *bri1* xylem cell fate phenotype.**

535 (A) Coimmunoprecipitation of RLP44-RFP transiently co-expressed with BRI1-GFP,
536 BRI1^QGFP, or free GFP (negative control) in *Nicotiana benthamiana* leaf epidermal cells. Proteins

537 were extracted (Input) and immunoprecipitated (IP) by means of magnetic GFP beads. The
538 immunoblots were done with α -GFP and α -RFP antibodies.

539 (B) Co-localization of RLP44-RFP with BRI1-GFP or BRI1^Q-GFP in subcortical discrete
540 punctate structures in *Nicotiana benthamiana* leaf epidermal cells. Insets show enlarged images.

541 Scale bars, 10 μ m.

542 (C) 7-day-old roots stained with Basic Fuchsin for the visualization of xylem cells. Scale bars,
543 10 μ m.

544 (D) Frequency quantification of roots with the indicated number of metaxylem cells. Asterisks
545 indicate statistically significant differences, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Chi-square test.
546 $n = 20-57$ as indicated. All experiments were repeated twice with similar results (C-D).

547

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|-------------------|-----|--------|--------|--------|-------|-------|--------|--------|--------|-------|------|------|-----|--------|------|-----|-----|-------|------|------|-----|---|---|---|---|---|-------|------|------|----|---|---|---|---|---|---|----|-----|----|---|---|---|---|---|---|---|---|---|---|---|---|---|-----|
| BRI1 | 550 | GNIPAE | LGDCRS | LIWLDL | NLNTN | LFNGT | TPAAMF | KQSGK- | IAANFI | AGKRV | VYIK | NDGM | KKK | CHGAGN | LLEF | QGI | RSE | 624 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| BRI1 ^Q | 550 | GNIPAE | LGDCRS | LIWLDL | NLNTN | LFNGT | TPAAMF | KQSGK- | IAANFI | AGKRM | VFIK | NDGM | KKK | CHGAGN | LLEF | QGI | RSE | 624 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| BRL1 | 537 | GNVPR | QLGNC | SLIWLD | LNSN | LTGDL | PGE | LASO | AGL- | VM | PGSV | SGK | Q | F | F | V | R | NEGG- | TDCR | GAGL | VE | F | E | G | I | R | A | E | 610 | | | | | | | | | | | | | | | | | | | | | | | | |
| BRL2 | 509 | GEI | PP | ELGK | TL | VWLD | LNTN | HLT | GEI | PP | RL | G | R | O | P | G | S | K | A | L | S | G | L | L | S | G | N | T | M | A | F | V | R | N | V | G | -- | NS | CK | G | V | G | L | V | E | F | S | G | I | R | P | E | 582 |
| BRL3 | 537 | GNIP | SELG | NCL | IWLD | LNSN | LT | GNL | P | G | E | L | A | S | O | A | G | L- | VM | PGSV | SGK | Q | F | F | V | R | NEGG- | TDCR | GAGL | VE | F | E | G | I | R | A | E | 610 | | | | | | | | | | | | | | | |

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|-------------------|-----|-------|---|---|---|---|---|---|---|---|---|---|---|---|----|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|-----|---|---|-----|-----|
| BRI1 | 625 | QLNRL | S | T | R | N | P | C | N | I | T | S | R | V | Y | G | G | H | T | S | P | T | F | D | N | G | S | M | M | F | L | D | M | S | Y | N | M | L | S | G | Y | I | P | K | E | I | G | S | M | P | Y | L | F | I | L | N | L | G | H | N | D | I | S | G | S | I | P | D | E | V | G | D | 700 | | | | |
| BRI1 ^Q | 625 | QLNRL | S | T | R | N | P | C | N | I | T | S | R | V | A | G | G | H | T | S | P | T | F | D | N | G | S | M | E | F | L | D | M | S | Y | N | M | L | S | G | Y | I | P | K | E | I | G | S | M | P | Y | L | A | I | L | N | L | G | H | N | D | I | S | G | S | I | P | D | E | V | G | D | 700 | | | | |
| BRL1 | 611 | R | L | E | R | L | P | M | V | H | S | C | P | A | T- | R | I | Y | S | G | M | I | M | Y | T | F | S | A | N | G | S | M | I | Y | F | D | I | S | Y | N | A | V | S | G | F | I | P | P | G | Y | G | N | M | G | Y | L | O | V | L | N | L | G | H | N | R | I | T | G | T | I | P | D | S | F | G | 685 | |
| BRL2 | 583 | R | L | L | Q | L | P | S | L | K | S | C | D | E | T- | R | M | Y | S | G | E | L | L | S | L | F | T | R | Y | Q | T | I | E | Y | L | D | L | S | Y | N | Q | L | R | G | K | I | P | D | E | I | G | E | M | I | A | L | Q | V | L | E | L | S | H | N | Q | L | S | G | E | I | P | F | T | I | G | Q | 657 |
| BRL3 | 611 | R | L | E | H | F | P | M | V | H | S | C | P | K | T- | R | I | Y | S | G | M | I | M | Y | T | F | S | A | N | G | S | M | I | Y | L | D | L | S | Y | N | A | V | S | G | S | I | P | L | G | Y | G | A | M | G | Y | L | O | V | L | N | L | G | H | N | L | L | T | G | T | I | P | D | S | F | G | 685 | |

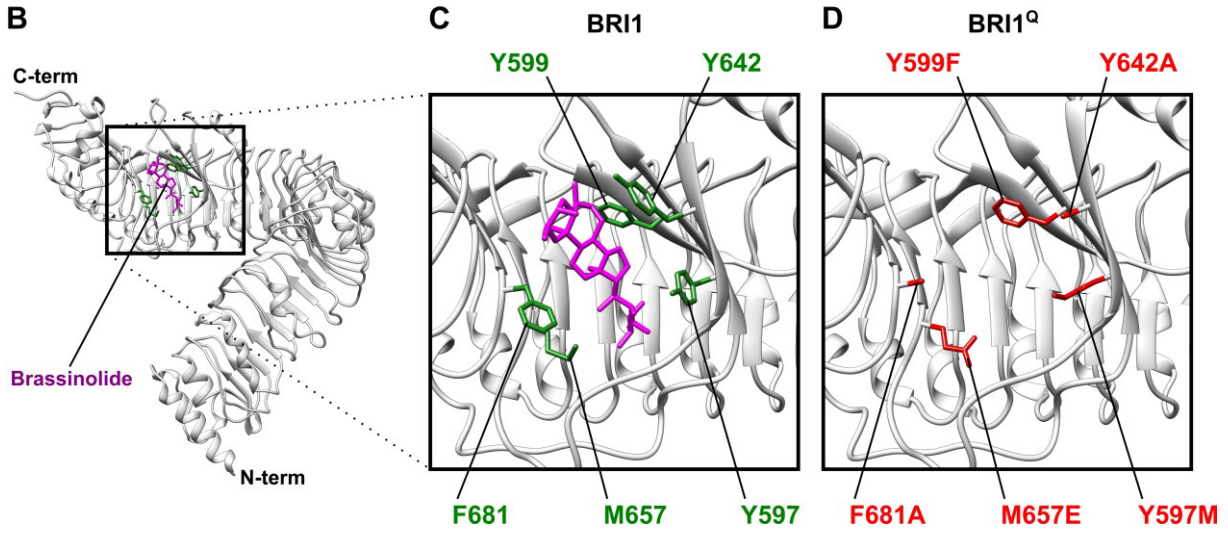


Figure 1
160x115 mm (1.8 x DPI)

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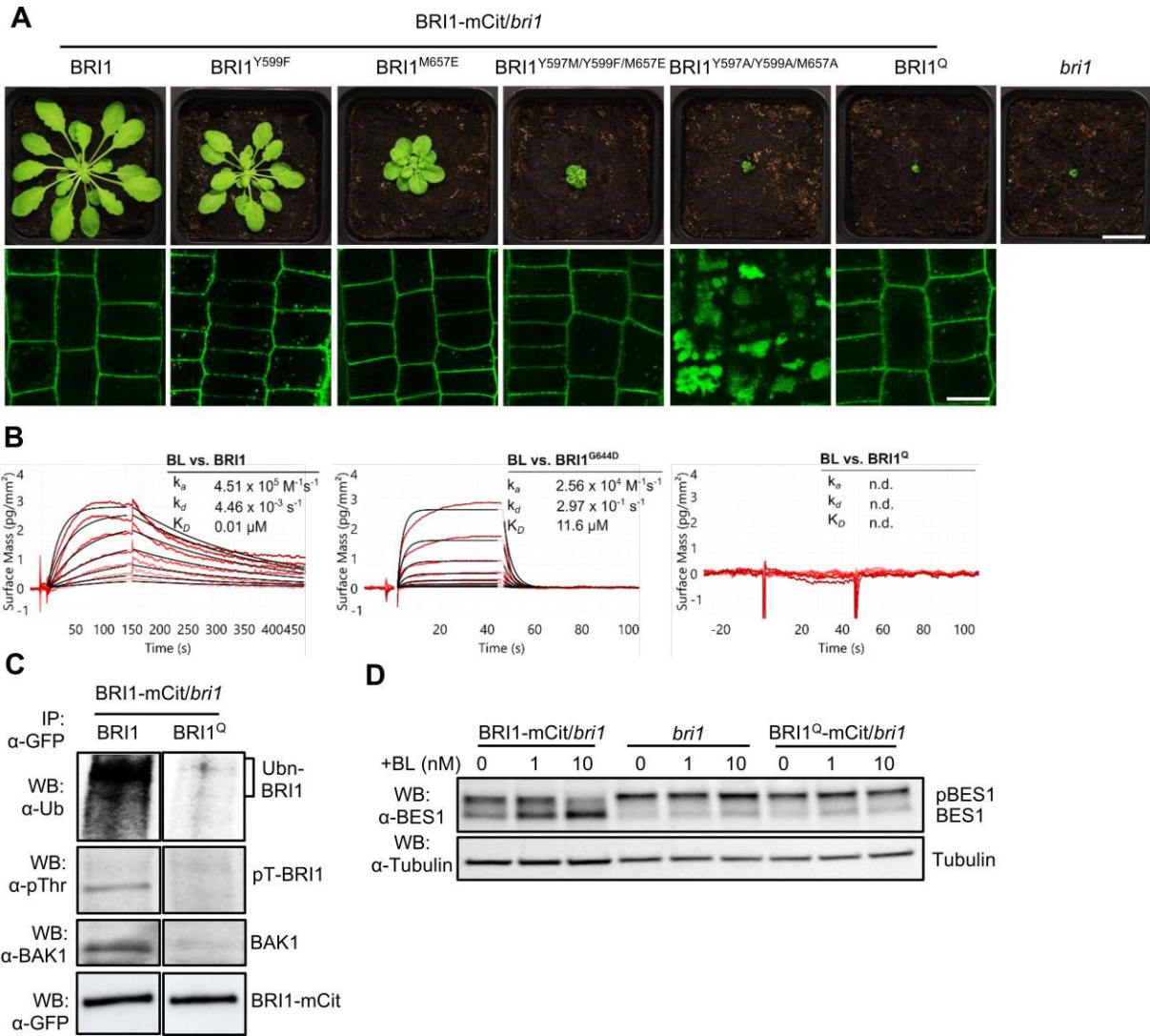


Figure 2
160x143 mm (1.8 x DPI)

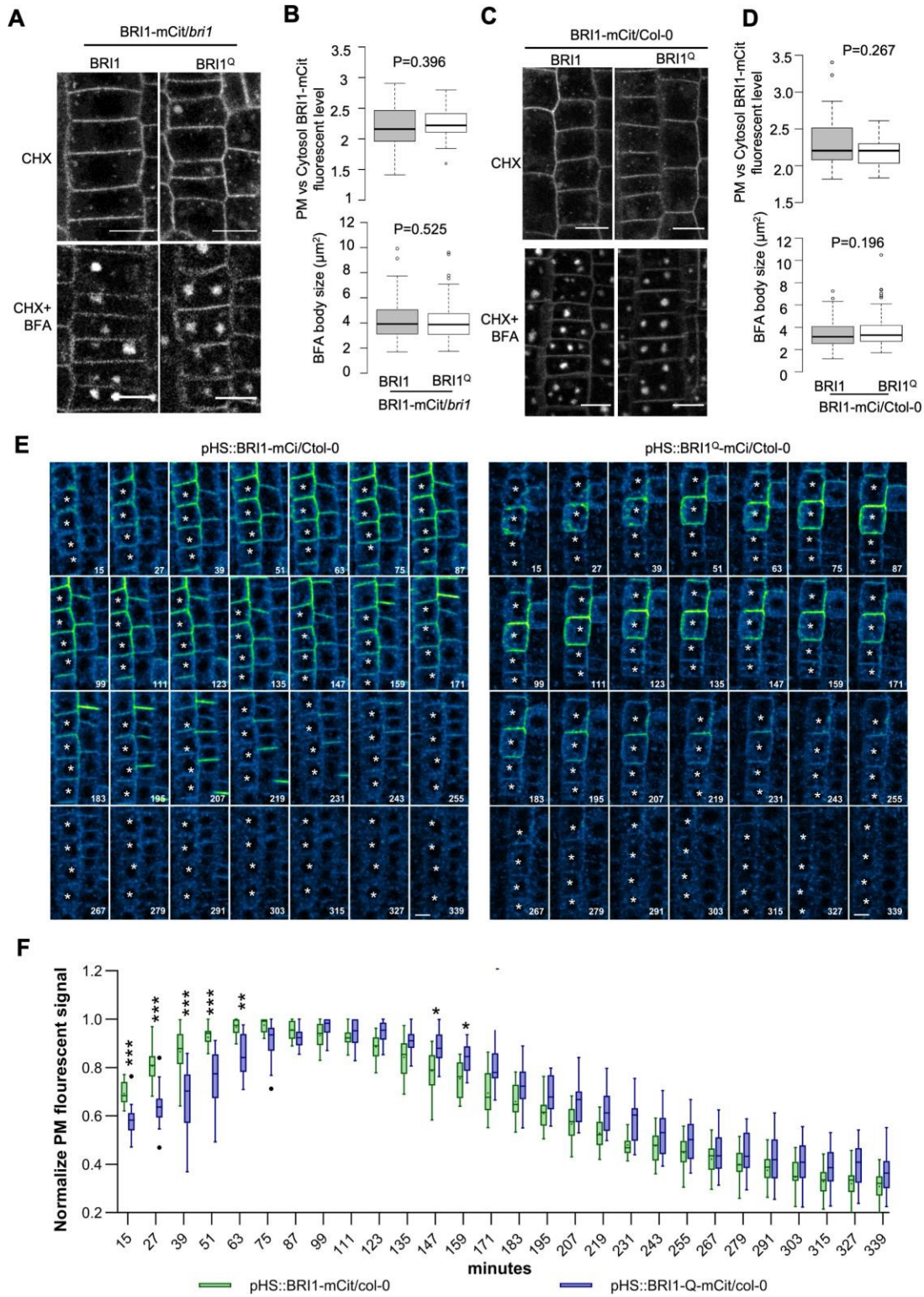


Figure 3
160x222 mm (1.8 x DPI)

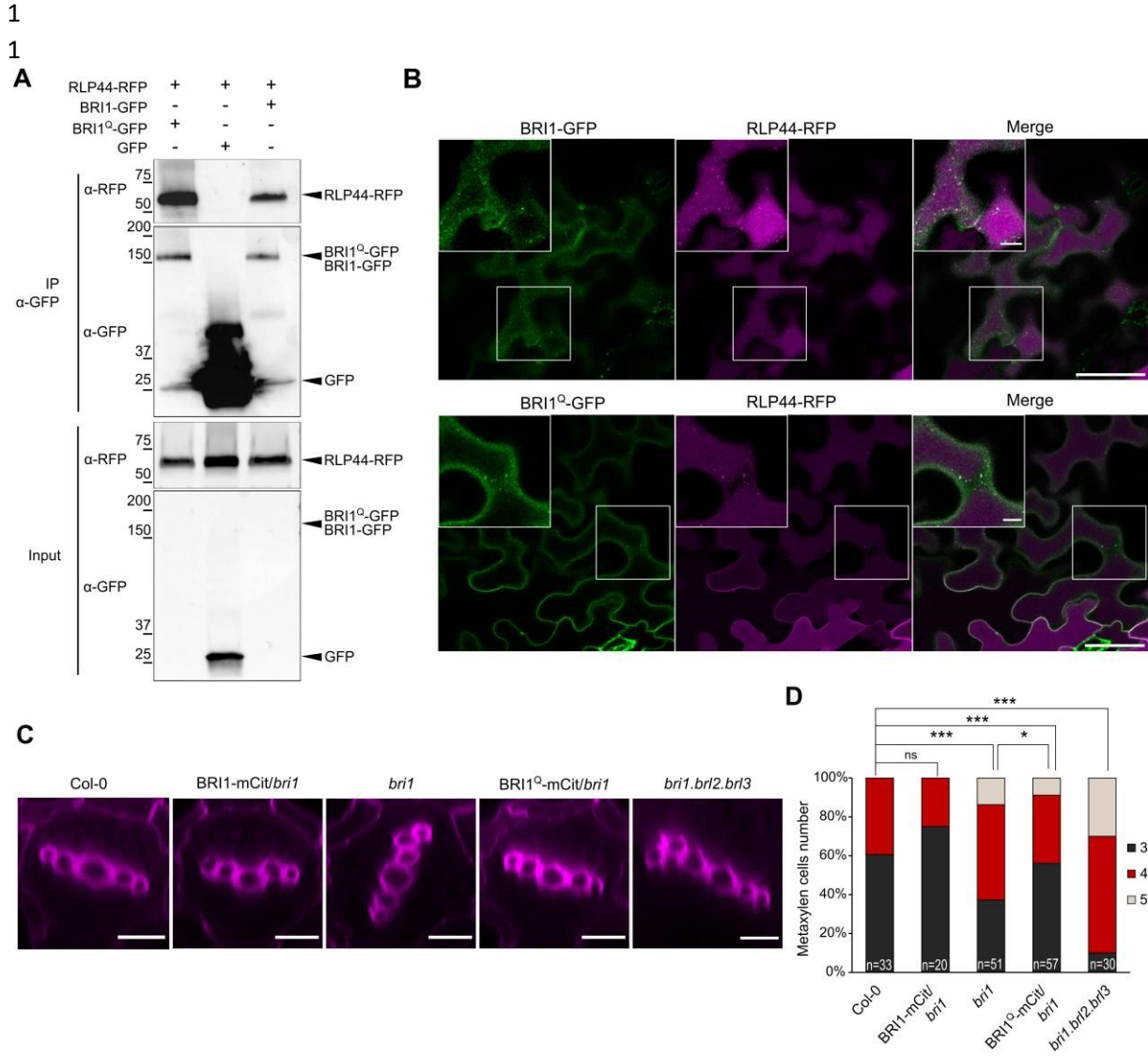


Figure 4
160x138 mm (1.8 x DPI)