

1 **Integration of Brassinosteroid and Phytosulfokine Signalling Controls Vascular Cell**  
2 **Fate in the *Arabidopsis* Root**

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4 Eleonore Holzwart<sup>a</sup>, Apolonio Ignacio Huerta<sup>a,1</sup>, Nina Glöckner<sup>b</sup>, Borja Garnelo Gómez<sup>a</sup>,  
5 Friederike Ladwig<sup>b</sup>, Sebastian Augustin<sup>a,2</sup>, Jana Christin Askani<sup>a</sup>, Ann-Kathrin Schürholz<sup>a</sup>,  
6 Klaus Harter<sup>b</sup>, and Sebastian Wolf <sup>a,3</sup>

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9 <sup>a</sup>Department of Cell Biology, Centre for Organismal Studies Heidelberg, Heidelberg University,  
10 Im Neuenheimer Feld 230, 69120 Heidelberg, Germany

11 <sup>b</sup>Plant Physiology, ZMBP, Universität Tübingen, Auf der Morgenstelle 32, 72076 Tübingen,  
12 Germany

13 <sup>1</sup>Present address: Department of Biology, ETH Zurich, Universitätstrasse 2, 8092 Zurich,  
14 Switzerland

15 <sup>2</sup>Present address: Department of Plant Molecular Biology, University of Lausanne, 1015  
16 Lausanne, Switzerland

17 <sup>3</sup>To whom correspondence should be addressed. Email: [sebastian.wolf@cos.uni-](mailto:sebastian.wolf@cos.uni-heidelberg.de)  
18 [heidelberg.de](mailto:sebastian.wolf@cos.uni-heidelberg.de)

19

20 Corresponding author: Sebastian Wolf, Im Neuenheimer Feld 230, 69120 Heidelberg,  
21 Germany; Phone: +49 6221545614; email: [sebastian.wolf@cos.uni-heidelberg.de](mailto:sebastian.wolf@cos.uni-heidelberg.de)

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25 **Author contributions:** EH, AIH, NG, BGG, FL, SA, JCA, and A-KS performed experiments  
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33

34 **Abstract**

35 Multicellularity arose independently in plants and animals, but invariably requires robust  
36 determination and maintenance of cell fate. This is exemplified by the highly specialized water-  
37 and nutrient-conducting cells of the plant vasculature, which are specified long before their  
38 commitment to terminal differentiation. Here, we show that the hormone receptor  
39 BRASSINOSTEROID INSENSITIVE 1 (BRI1) is required for root vascular cell fate  
40 maintenance, as BRI1 mutants show ectopic xylem in procambial position. However, this  
41 phenotype is unrelated to classical brassinosteroid signalling outputs. Instead, BRI1 is  
42 required for the expression and function of its interaction partner RECEPTOR-LIKE PROTEIN  
43 44 (RLP44), which, in turn, associates with the receptor for the peptide hormone  
44 phytosulfokine (PSK). We show that PSK signalling is required for the maintenance of  
45 procambial cell identity and is quantitatively controlled by RLP44, which promotes complex  
46 formation between the receptor for PSK and its co-receptor. Mimicking the loss of RLP44,  
47 PSK-related mutants show ectopic xylem in the position of procambium, whereas *rlp44* can  
48 be rescued by exogenous PSK. Based on these findings, we propose that RLP44 controls cell  
49 fate by connecting BRI1 and PSK signalling, providing a mechanistic framework for the  
50 integration of signalling mediated by the plethora of plant receptor-like kinases at the plasma  
51 membrane.

## 52 Introduction

53 A key function of signalling networks in multicellular organisms is to ensure robust  
54 determination and maintenance of cell fate. In plants, extreme specialization is displayed by  
55 the cells of the vascular tissues, vital for the distribution of water, nutrients, and signalling  
56 molecules. Xylem tracheary elements are characterized by lignified secondary cell wall  
57 thickenings that protect against collapse and provide mechanical support for vertical growth.  
58 Positioned between xylem and the nutrient-transporting phloem are the cells of the  
59 procambium, which give rise to the lateral meristems during secondary growth (1). Root  
60 vascular tissue patterning is set up in the embryo and maintained in the post-embryonic root  
61 by mutual antagonism of auxin and cytokinin signalling domains (2-5). After xylem precursor  
62 cells are displaced from the root meristem, an intricate gene-regulatory network connected to  
63 patterning mechanisms by HD-ZIP III transcription factors mediates differentiation into  
64 tracheary elements (6-9). Thus, primary root xylem cell fate can be traced back to early  
65 specification events in the embryo. In contrast, during secondary growth, (pro)cambial cells  
66 adjacent to the existing tracheary elements acquire xylem cell fate dependent on positional  
67 information (10).

68 Brassinosteroid (BR) hormone signalling (11, 12) has been implicated in xylem differentiation  
69 and vascular patterning (13, 14). BRs are perceived by BRASSINOSTEROID INSENSITIVE  
70 1 (BRI1) (15) which belongs to the large group of plant receptor-like kinases (RLK) with a  
71 leucine-rich repeat (LRR) extracellular domain, a transmembrane domain, and a cytosolic  
72 kinase domain related to animal Irak and Pelle kinases (16). Upon ligand binding, BRI1  
73 heterodimerizes with members of the SOMATIC EMBRYGENESIS RECEPTOR KINASE  
74 (SERK) LRR-RLK family such as BRI1-ASSOCIATED KINASE 1 (BAK1)(17, 18), and  
75 activates a signalling cascade that negatively regulates BRASSINOSTEROID INSENSITIVE  
76 2 (BIN2)(19), a GSK3-like kinase that phosphorylates the BR-responsive transcription factors  
77 BRASSINAZOLE RESISTANT 1 (BZR1)(20) and BRI1 EMS SUPPRESSOR 1 (BES1)/BZR2.  
78 Inhibition of BIN2 activity allows BZR1 and BES1 to translocate to the nucleus, where they  
79 mediate BR-responsive control of transcription (21-23). A so far somewhat enigmatic  
80 relationship exists between BR and PHYTOSULFOKINE (PSK) signalling. PSKs are small  
81 secreted peptide growth factors that have been implicated in a variety of fundamental  
82 processes and are perceived by two close relatives of BRI1, PHYTOSULFOKINE RECEPTOR  
83 1 and 2 (24-27). PSK activity depends on proteolytic processing of the precursor peptides, the  
84 sulfation of two tyrosine residues in the mature pentapeptide (YIYTQ) by TYROSYLPROTEIN  
85 SULFOTRANSFERASE (TPST) (28), and functional BR signalling (29-31). At present, it is not  
86 clear how BR and PSK signalling interact but the receptors for both growth factors share the  
87 requirement for a SERK co-receptor (32, 33).

88 Recently, we demonstrated that feedback information from the cell wall is integrated with BR  
89 signalling at the level of the receptor complex through RECEPTOR-LIKE PROTEIN (RLP) 44  
90 (34). RLP44 is genetically required for the BR-mediated response to impaired cell wall  
91 modification and is sufficient to elevate BR signalling when overexpressed. RLP44 was shown  
92 to be in a complex with BRI1 and BAK1 and to directly interact with BAK1. Thus, we  
93 hypothesized that RLP44 modulates BR signalling strength in response to cues from the cell  
94 wall (34). However, it is not clear whether the RLP44-BR signalling module plays additional  
95 roles in plant physiology, besides a possible mechanism for cell wall homeostasis (35). Here,  
96 we show that RLP44 is required for the maintenance of cell fate in the root vasculature by  
97 connecting components of the BR and PSK signalling pathways. RLP44 directly interacts with  
98 BRI1 and controls xylem differentiation in a BRI1-dependent manner, but independently of BR  
99 signalling outputs. In addition, RLP44 can directly interact with PSKR1, and the *rlp44*  
100 phenotype can be rescued by application of the PSK peptide. Moreover, mutants affected in

101 PSK signalling show an *rlp44*-like xylem phenotype, suggesting that RLP44 has a positive  
102 effect on PSK signalling, which, in turn, promotes procambial identity.

## 103 Results

### 104 RLP44 directly interacts with the brassinosteroid receptor BRI1

105 We previously demonstrated that RLP44 is present in a complex with both BRI1 and its co-  
106 receptor BAK1 and is able to promote BR signalling upon cues from the cell wall or when  
107 overexpressed. In addition, we provided evidence for direct interaction with BAK1 (34). To  
108 assess whether RLP44 can also directly interact with BRI1, we performed a mating-based split  
109 ubiquitin assay in yeast (36). Under selective conditions, interaction of BRI1 and RLP44  
110 enabled yeast growth, similar to what was observed before with BAK1-RLP44 and BAK1-BRI1  
111 (34) (Fig. 1A). In addition, Foerster resonance energy transfer-fluorescence lifetime imaging  
112 microscopy (FRET-FLIM) analysis after transient expression in *Nicotiana benthamiana* leaves  
113 confirmed that BRI1 and RLP44 are able to interact directly (Fig. 1B and Fig. S1A).  
114 Furthermore, endogenous BRI1 and BAK1 were detected in immunoprecipitates of *RLP44*-  
115 *GFP* expressed under the control of its own promoter in the *rlp44<sup>cnu2</sup>* mutant background (Fig.  
116 S1B). In summary, RLP44 and BRI1 form complexes in yeast and *in planta* through a direct  
117 interaction. To assess the potential role of the RLP44-BRI1 signalling modules we sought to  
118 identify the tissues in which it might be active. As BRI1 is ubiquitously expressed, we thus  
119 investigated the expression pattern of RLP44.

### 120 RLP44 is expressed in the developing root vasculature

121 To study the function of RLP44, we generated transgenic plants expressing a translational  
122 GFP fusion of RLP44 under control of the RLP44 promoter (*pRLP44:RLP44-GFP*). These  
123 plants displayed elongated, narrow leaf blades and elongated petioles, reminiscent of BRI1  
124 overexpressing plants (Fig. 2A and B) (37), and as previously observed with RLP44  
125 overexpression (34). We crossed a *pRLP44:RLP44-GFP* line with the *RLP44* loss-of-function  
126 mutant *rlp44<sup>cnu2</sup>* derived from *comfortably numb 2* (34), which resulted in plants with a wildtype-  
127 like appearance (Fig. 1C), demonstrating that the fusion protein is functional, and confirming  
128 that the additional transgenic RLP44 expression was causative for the observed  
129 morphological effects (Fig. S2A). In the root apical meristem of *pRLP44:RLP44-GFP* and  
130 *pRLP44:RLP44-GFP (rlp44<sup>cnu2</sup>)*, fluorescence was present in most tissues, but markedly  
131 enriched in epidermis and lateral root cap (Fig. 2D-F and Fig. S2B-E); slightly enhanced  
132 expression was also observed in xylem precursor cells (Fig. S2B). A strong increase of GFP  
133 fluorescence in the stele was observed towards the more mature part of the root (Fig. 2D-G  
134 and Fig. S2B-D), in accordance with previously published transcriptome data (38) and  $\beta$ -  
135 glucuronidase reporter activity under control of the RLP44 promoter (Fig. S2F, G). In the  
136 differentiating part of the root stele, RLP44-GFP fluorescence was relatively weak in the  
137 phloem, intermediate in xylem, and highest in the undifferentiated procambial cells (Fig. 1H, I,  
138 and Fig. S1C and D, compare Fig. 3A for vascular anatomy).

### 139 RLP44 controls xylem cell fate in a BRI1-dependent manner, but independently of BR 140 signalling outputs

141 The prevalence of *pRLP44:RLP44-GFP* fluorescence in the stele prompted us to study the  
142 role of RLP44 in vascular development. To this end, we visualized lignified secondary cell  
143 walls in *rlp44* loss-of-function mutants through basic fuchsin staining and observed  
144 supernumerary metaxylem-like cells, frequently outside the primary xylem axis in the position  
145 of the procambium (Fig. 3A and B), a phenomenon we never observed in wildtype roots.  
146 Quantification of metaxylem cells in seedling roots of both *rlp44<sup>cnu2</sup>* and the T-DNA insertion  
147 line *rlp44-3* six days after germination (dag) showed a significant increase (Fig. 3C),  
148 suggesting that RLP44 controls xylem cell fate. Expression of RLP44 under control of its own

149 promoter complemented this phenotype (Fig. S3). Since we had previously identified RLP44  
150 as an activator of BR signalling, we also included *bri1<sup>enu1</sup>*, a hypomorphic *bri1* allele (39) in the  
151 analysis. Surprisingly, primary root xylem number in *bri1<sup>enu1</sup>* was indistinguishable from  
152 wildtype, suggesting that the ectopic xylem in *rlp44* mutants might be unrelated to the outputs  
153 of BR signalling. We therefore analysed the root xylem of a number of BR-related mutants  
154 spanning a broad range of growth phenotypes. Hypomorphic *bri1* mutants such as *bri1-301*  
155 (19) and *bri1-5* (40), the more severe signalling mutant *bin2-1* (19), and the BR-deficient  
156 biosynthetic mutant *constitutive photomorphogenic dwarf* (*cpd*) (41) did not show a  
157 pronounced increase in xylem cell number (Fig. 3D). In sharp contrast, *bri1* null alleles such  
158 as a previously characterized T-DNA mutant (termed *bri1-null*) (42) and the *bri1 bri1 bri3* triple  
159 mutant (called *bri triple* from hereon) (43) displayed a marked increase in the number of  
160 differentiated xylem cells (Fig. 3D), whereas expression of BRI1 under the control of its own  
161 promoter in *bri1-null* resulted in wildtype-like xylem (Figure 3E). Taken together, our results  
162 show that the xylem differentiation phenotype does not correlate with the severity of BR  
163 deficiency-related growth phenotypes (Fig. 3F). This is best exemplified by the comparison  
164 between the *cpd* and *bri1* null mutants, with *cpd* displaying wildtype-like or even slightly  
165 decreased xylem cell numbers, despite exhibiting a *bri1-null*-like growth phenotype. Thus, the  
166 control of xylem cell number, is largely independent of BR signalling outputs, but requires the  
167 presence of both BRI1 and RLP44. Interestingly, the expression of *RLP44* was reduced in the  
168 *bri1-null* mutant but not in *bri1* hypomorphs or *cpd*, suggesting that reduced RLP44 levels  
169 could partially explain the xylem phenotype of *bri1-null* (Fig. S4A, B). Consistent with this,  
170 uncoupling RLP44 transcription from BRI1 control through the 35S promoter could alleviate  
171 the *bri1-null* xylem phenotype (Fig. S4C), suggesting that BRI1 and RLP44 indeed act in the  
172 same pathway regulating xylem cell fate. In contrast, overexpression of *RLP44* had no effect  
173 on growth BL-insensitivity of *bri1-null* (Fig. S5). Taken together, our findings demonstrate that  
174 the phenotype of *bri1* loss-of-function mutants is partially independent from BR signalling  
175 outputs and suggest that RLP44 exerts its function downstream of BRI1 through other, yet  
176 unidentified signalling components.

177 It has been previously reported that root vascular development is responsive to environmental  
178 conditions (7). We compared xylem cell numbers of plants grown on standard medium (0.9%  
179 agar) and those that experienced increased mechanical force by growth on reclined hard agar  
180 plates (2% agar) (44). Interestingly, the number of xylem cells in the wildtype increased  
181 substantially under those conditions, whereas it did not further increase in *rlp44-3* (Fig. S6).

### 182 **Vascular cell fate determination by RLP44 and BRI1 is independent of BR signalling-** 183 **mediated control of cell proliferation**

184 We next asked whether the increase in xylem cell number observed in the *rlp44* mutant is  
185 reflected in enhanced cell proliferation in the root and therefore quantified vascular cell number  
186 of the seedling roots 6 dag. In *rlp44-3*, vascular cell number was indistinguishable from  
187 wildtype in the differentiation zone, suggesting normal cell proliferation in the root meristem  
188 (Fig. 4A). The *bri1<sup>enu1</sup>* mutant, which did not display ectopic xylem cells, showed a significant  
189 increase in total vascular cell number (Fig. 4A), consistent with the recently described role of  
190 BR signalling in controlling formative cell divisions (45). These results suggest that increased  
191 xylem and increased proliferation in the vasculature are independent phenomena, and confirm  
192 a role for BR signalling in controlling formative cell divisions in the root meristem (Fig. 4A). In  
193 line with this, depletion of BRs in wildtype roots by application of the BR biosynthesis inhibitor  
194 PPZ (46) resulted in pronounced increase of vascular cell number (Fig. 4B, C). When PPZ-  
195 treated roots were supplemented with a low dose (0.5 nM) of BL, both root growth and vascular  
196 cell number were fully recovered (Fig. 4B, C). A higher-than-optimal dose of BL (5 nM)  
197 suppressed root growth and led to a strongly decreased vascular cell number (Fig. 4C). The  
198 *rlp44<sup>enu2</sup>* mutant displayed a wildtype-like response to the manipulation of BR levels in terms

199 of cell number (Fig. 4C), further supporting the independence of the xylem cell fate phenotype  
200 from the BR-signalling mediated control of cell proliferation (45).

### 201 **RLP44 controls xylem cell fate by promoting phyto­sulfokine signalling**

202 The results described so far suggested that the maintenance of procambial cell identity in the  
203 root requires the presence of both BRI1 and RLP44. In addition, RLP44 seems to act  
204 downstream of BRI1 in controlling vascular cell fate. As it could be ruled out that RLP44 exerts  
205 its effect on xylem differentiation through BR signalling, we reasoned that in the absence of a  
206 kinase domain, RLP44 is required to interact with and influence the activity of another  
207 signalling component which, in turn, controls xylem cell fate. Interestingly, besides BRI1 and  
208 its close homologues BRL1, BRL2, and BRL3, the LRR X clade of RLKs harbours the  
209 receptors for the peptide growth factor PSK, PSKR1 and 2 (16). As PSK signalling has also  
210 been implicated in promoting the trans-differentiation of *Zinnia elegans* mesophyll cells into  
211 tracheary elements (31, 47) and depends on functional BR signalling (29), we tested the  
212 association of RLP44 with PSKR1. Co-immunoprecipitation experiments in *N. benthamiana*  
213 showed that *PSKR1-GFP* (33) was present in RLP44-RFP immunoprecipitates (Fig. 5A). In  
214 addition, FRET-FLIM analysis showed a pronounced reduction in fluorescence lifetime when  
215 *PSKR1-GFP* was co-expressed with RLP44-RFP, suggesting a direct interaction (Fig. 5B) that  
216 was not affected by exogenous application of PSK (Fig. S7A and B). Moreover, exogenous  
217 application of PSK peptide reverted *rlp44* xylem back to the stereotypical wildtype pattern of  
218 mostly two protoxylem and three metaxylem cells in one axis (Fig. 5C). Further supporting a  
219 role of PSK signalling in the control of xylem cell fate, the *pskr1-3 pskr2-1* double mutant (48)  
220 showed an increased number of xylem cells reminiscent of the *rlp44* mutant (Fig. 6D and E).  
221 A similar phenotype was observed in the double mutant of *pskr1-3* and the related RLK *psy1r1*,  
222 as well as in the *tpst-1* mutant, impaired in the biosynthesis of PSK and other sulfated peptides  
223 (Fig. 5D and E) (28, 49). We then asked how RLP44 might promote PSK signalling. As RLP44  
224 is a direct interaction partner of both PSKR1 and its co-receptor BAK1, we assessed whether  
225 presence of RLP44 could increase association of receptor and co-receptor. Indeed, more  
226 BAK1 was detected in immunoprecipitates of PSKR1-GFP expressed in *N. benthamiana* when  
227 RLP44-RFP was co-expressed (Fig. 5F), suggesting that RLP44 might act as a scaffold in the  
228 complex. Supporting these results, BAK1 levels in immunoprecipitates of PSKR1-GFP were  
229 reduced in the *rlp44<sup>cnu2</sup>* mutant (Fig. 5G). Notably, presence of PSKR1-GFP had a negative  
230 effect on the amount of RLP44 in immunoprecipitates of BRI1-RFP (Fig. S7C), suggesting that  
231 the two pathways might compete for RLP44. Taken together, our data suggest that RLP44  
232 interacts with, and stabilizes a complex between, PSKR1 and BAK1 to promote PSK  
233 signalling, which, in turn, suppresses the progression from procambial to xylem identity (Fig.  
234 5H).

## 235 **Discussion**

### 236 **RLP44 controls vascular cell fate in a BRI1-dependent manner**

237 Cell fate determination in plants mainly relies on positional information provided by perception  
238 of hormone gradients and non-cell autonomous factors (50-52). The expanded family of plant  
239 RLK proteins and their ligands play central roles in intercellular communication, cell identity  
240 maintenance, and the regulation of cell expansion and proliferation (53). Currently, our view  
241 of these pathways is evolving to appreciate the extensive cross-talk and interdependence of  
242 diverse signalling pathways (54). The response to BRs, mediated by what is probably the best-  
243 characterized plant signalling cascade (11), is found to be integrated with a growing number  
244 of other pathways at two cross-talk “hot spots”, namely the GSK3-like kinase BIN2 (55-57)  
245 and the BR-responsive transcription factors BZR1 and BES1 (58-61). Here, we report that BR  
246 and PSK signalling are coupled at the level of the plasma membrane receptors through RLP44  
247 and that this signalling module is required to control xylem cell fate. Our genetic and

248 biochemical analyses support a scenario where PSK signalling strength is quantitatively  
249 controlled by RLP44, which itself is dependent on the presence of BRI1. While we do not rule  
250 out post-translational control of RLP44 by BRI1, for example through phosphorylation of the  
251 cytoplasmic domain or because the presence of BRI1 is required for correct receptor complex  
252 assembly, this dependency is at least partially based on transcriptional regulation. The *bri1-*  
253 *null* loss-of-function mutant, but not hypomorphs or the BR biosynthetic mutant *cpd*, showed  
254 reduced RLP44 expression. Consistent with this, uncoupling RLP44 from its native  
255 transcriptional regulation by constitutive expression could rescue the *bri1-null* xylem  
256 phenotype. Conversely, the novel *bri1* loss-of-function phenotype of ectopic xylem in  
257 procambial position is unrelated to BR-signalling. In line with this, only limited overlap of  
258 differentially expressed genes in *bri1-116* and the morphologically similar BR biosynthetic  
259 mutant *dwf4* was observed, potentially indicating additional brassinosteroid-independent  
260 functions of BRI1 (21).

261 More work will be needed to mechanistically understand the interaction between RLP44 and  
262 RLKs. External application of PSK (this study) or BR (34) ligands had no influence on the  
263 association between RLP44 and PSKR1 or BRI1, respectively, in line with ligand-  
264 independence of many, but not all, RLP-RLK interactions (62, 63). BRI1 and PSKR1/2 belong  
265 to the LRR X subfamily of LRR-RLKs (16) and share the requirement for interaction with SERK  
266 co-receptors to form an active, heteromeric signalling complex (32, 33, 64). With the exception  
267 of the ligand binding domain, the SERK-bound BRI1 and PSKR1 complex structures are very  
268 similar (32, 64), therefore it is conceivable that RLP44 binds both receptors through the same  
269 mechanism. These results are in line with the emerging theme of dynamic, promiscuous, and  
270 flexible interactions of plasma membrane proteins to integrate signalling information and fine-  
271 tune cellular responses to external cues (65-67). Interestingly, the mechanism by which RLPs  
272 influence signalling seems to differ widely, ranging from direct participation in ligand binding  
273 (62, 68), to the control of signalling specificity through blocking access of RLK ligands (68), to  
274 the guarding of extracellular proteins targeted by pathogens (69). Here, we propose a  
275 scaffolding function of RLP44 for the interaction between PSKR1 and its co-receptor BAK1,  
276 expanding the mechanistic diversity of RLPs. We have previously reported that RLP44 is  
277 involved in the response to cell wall state; along those lines, it will be interesting to see whether  
278 cues from the cell wall are able to influence the balance between the two known roles of  
279 RLP44 by shifting its interaction with BRI1 or PSKR1.

280

### 281 **PSK signalling likely promotes procambial identity**

282 Alongside classical plant hormones, signalling peptides have been revealed to play major  
283 roles in plant development and stress responses (70, 71). The sulfated pentapeptide PSK has  
284 been implicated in a number of diverse processes (24, 71). Here, we propose that PSK  
285 signalling controls xylem cell fate through promoting the maintenance of procambial identity.  
286 A number of observations support this hypothesis. First, PSK treatment rescued the ectopic  
287 xylem phenotype in *rlp44* mutants. Second, PSK-related mutants showed increased xylem  
288 differentiation in procambial position and PSK genes are co-expressed with RLP44 in  
289 procambial cells (Fig. S7D)(38). Third, PSK expression is transiently increased prior to the  
290 acquisition of a procambial intermediate state by cells trans-differentiating into tracheary  
291 elements (31, 72), which could explain why PSK promotes tracheary element formation in *Z.*  
292 *elegans* only when applied early to the cell culture (31, 47). Finally, PSK signalling promotes  
293 callus growth and longevity, in line with a role in the maintenance of cell identity (27).  
294 Accordingly, it has been proposed that PSK signalling maintains the responsiveness to  
295 intrinsic and extrinsic cues to tune proliferation or differentiation (27). However, it is unclear  
296 how PSK signalling affects cellular behaviour, in part due to a lack of knowledge about  
297 potential downstream targets. It will be interesting to see how the BRI1-RLP44-PSK signalling

298 module described here integrates with the fundamental patterning processes and the gene  
299 regulatory networks controlling xylem differentiation (2, 7).

### 300 **The role of BR signalling in vascular development**

301 It has long been described that BR signalling plays an important role in the development of  
302 vascular tissue (13, 14). In addition, it has been reported that BR signalling is kept at low levels  
303 in procambial cells of leaf and hypocotyl to prevent their differentiation into xylem cells (57).  
304 Our results suggest that in the primary xylem of the root, BR signalling only plays a minor role  
305 in controlling differentiation, in marked contrast to the strong patterning defects of BR  
306 signalling and biosynthetic mutants in the shoot (14). Conversely, at least in the root, presence  
307 of BRI1 has a negative effect on xylem cell fate through RLP44 and PSK signalling-mediated  
308 maintenance of procambial identity. Therefore, our results identify a novel role of BRI1 in root  
309 development which is independent of its role as BR receptor.

310

### 311 **Materials and Methods**

312 Details of materials and methods are provided in the Supplemental Information file. Mutants  
313 and transgenic lines used in this study are listed in Table S1.

314

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498

## 499 Figure Legends

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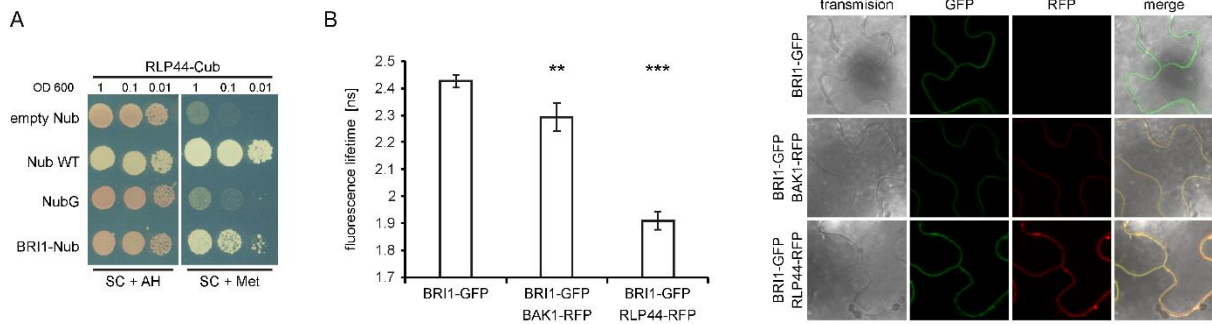
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571

572 **Figures**



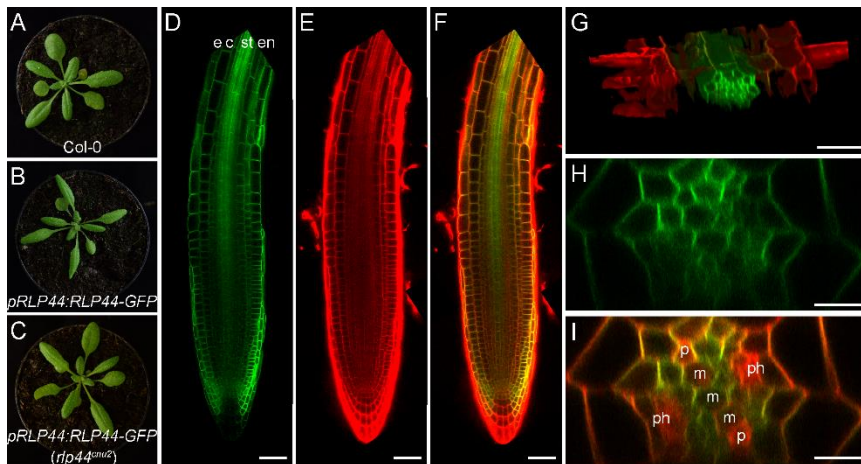
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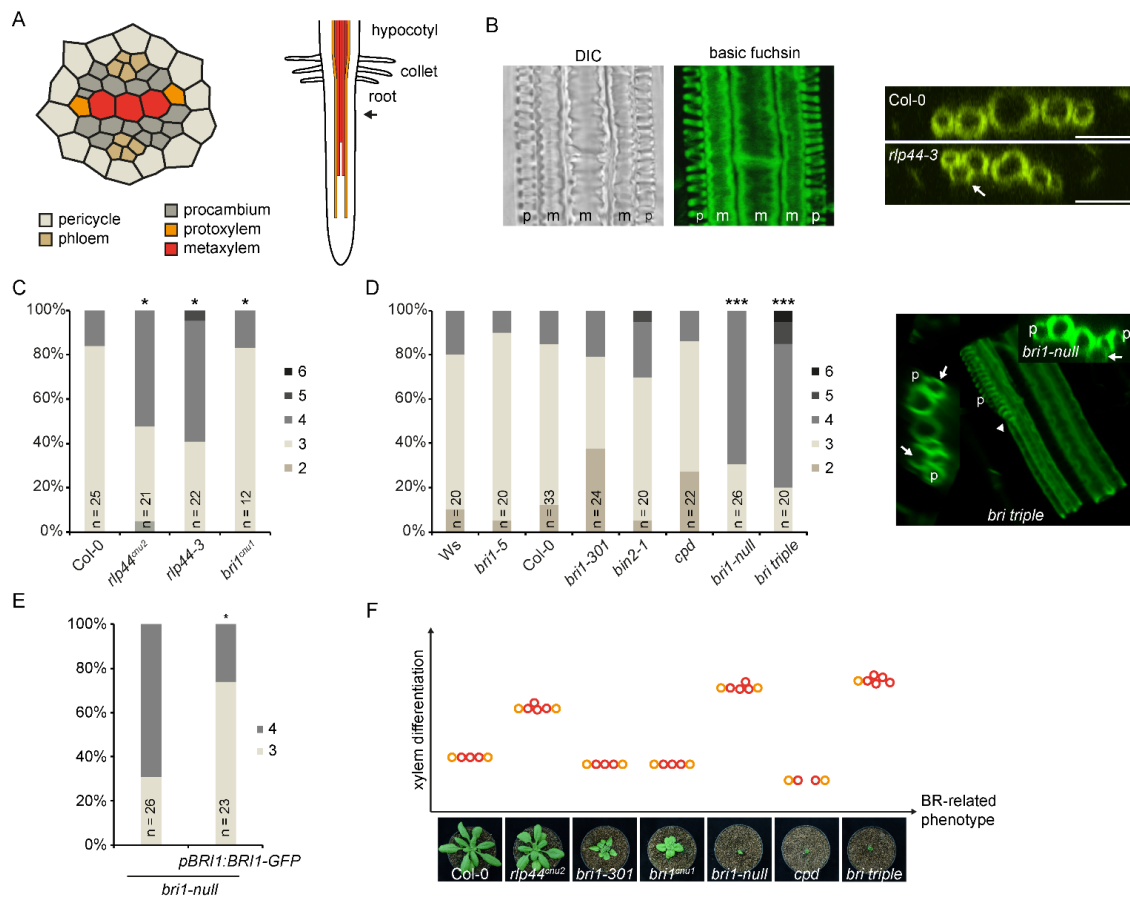
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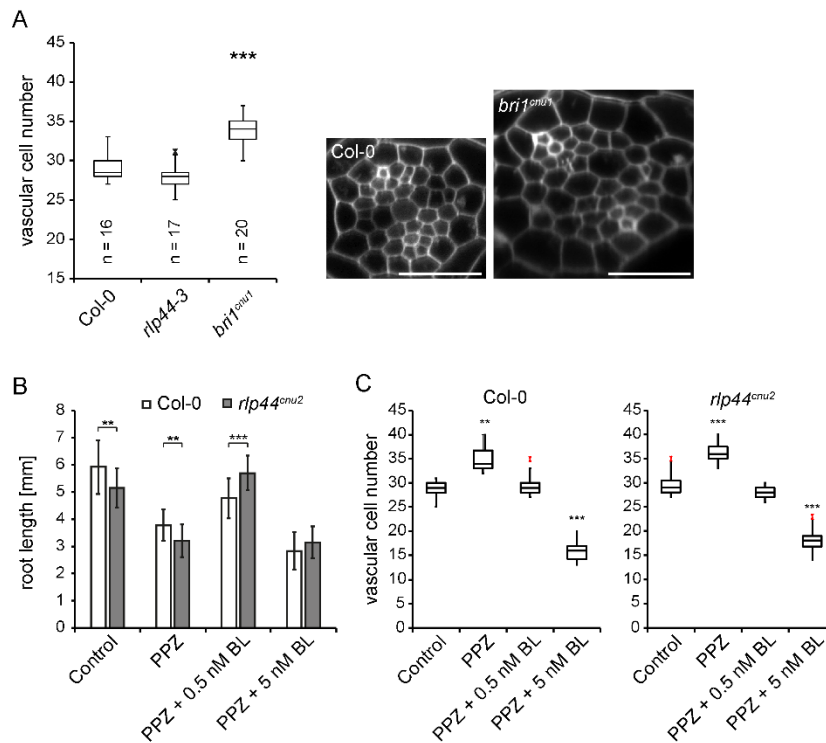
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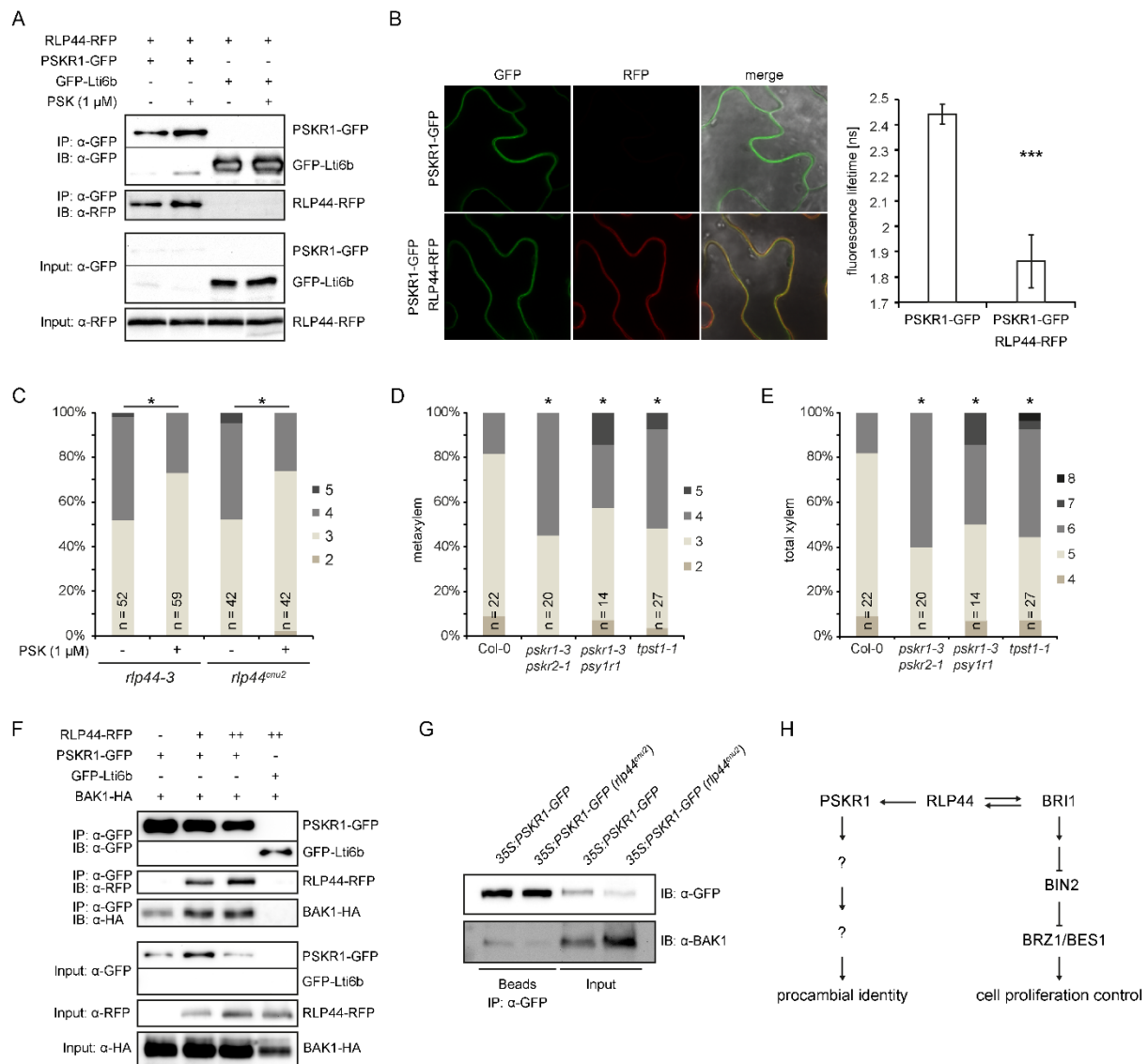




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