

## 2 Differential expression of the brassinosteroid receptor-encoding 3 *BRI1* gene in *Arabidopsis*

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6 Received: 7 November 2013 / Accepted: 15 January 2014  
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8 **Abstract** Brassinosteroid (BR)-regulated growth and  
9 development in *Arabidopsis* depends on BRASSINOS-  
10 TEROID INSENSITIVE 1 (*BRI1*), the BR receptor that  
11 is responsible for initiating the events of BR signalling.  
12 We analysed the temporal and spatial regulation of *BRI1*  
13 expression using stable transgenic lines that carried *BRI1*  
14 promoter:reporter fusions. In both seedlings and mature  
15 plants the tissues undergoing elongation or differentia-  
16 tion showed elevated *BRI1* gene activity, and it could be  
17 demonstrated that in the hypocotyl this was accompanied  
18 by accumulation of the *BRI1* transcript and its receptor  
19 protein product. In seedlings the *BRI1* promoter was also  
20 found to be under diurnal regulation, determined primarily  
21 by light repression and a superimposed circadian control.  
22 To determine the functional importance of transcriptional  
23 regulation we complemented the severely BR insensitive  
24 *bri1-101* mutant with a *BRI1-luciferase* fusion construct  
25 that was driven by promoters with contrasting specificities.  
26 Whereas the *BRI1* promoter-driven transgene fully restored

the wild phenotype, expression from the photosynthesis- 27  
associated *CAB3* and the vasculature-specific *SUC2* and 28  
*ATHB8* promoters resulted in plants with varying morpho- 29  
genetic defects. Our results reveal complex differential regu- 30  
lation of *BRI1* expression, and suggest that by influencing 31  
the distribution and abundance of the receptor this regula- 32  
tion can enhance or attenuate BR signalling. 33

**Keywords** Ectopic expression · Hormone susceptibility · 34  
Phytohormone · Reporter gene 35

### Abbreviations 36

BR Brassinosteroid 37

BL Brassinolide 38

CS Castasterone 39

DD Continuous dark 40

LD Light–dark cycles 41

LL Continuous light 42

DAG Days after germination 43

LUC Firefly luciferase 44

GUS β-Glucuronidase 45

### Introduction 46

Brassinosteroids (BRs) are polyhydroxylated steroid hor- 47  
mones that control plant development from germination 48  
to seed production (Clouse and Sasse 1998; Haubrick 49  
et al. 2006). The biologically active BRs castasterone 50  
(CS) and brassinolide (BL) are perceived by the plasma 51  
membrane-localized leucine-rich repeat receptor-like 52  
kinase BRASSINOSTEROID INSENSITIVE 1 (*BRI1*) 53  
(Li and Chory 1997), which then initiates a phosphoryla- 54  
tion/dephosphorylation-based signalling cascade that con- 55  
trols the transcriptional activity of BR-responsive genes. 56

A1 L. Hategan and B. Godza contributed equally to this work.

A2 **Electronic supplementary material** The online version of this  
A3 article (doi:10.1007/s00425-014-2031-4) contains supplementary  
A4 material, which is available to authorized users.

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This intracellular process, which has been well characterized in *Arabidopsis*, is mediated by BRASSINOSTEROID INSENSITIVE 2 (BIN2), a GSK3/SHAGGY-like kinase, and its downstream targets the BRASSINAZOLE RESISTANT transcription factors BZR1 and BZR2/BES1 (for review see: Kim and Wang 2010).

The BRI1 receptor is a key component of BR signalling. Binding of the active hormone by the extracellular domain (Kinoshita et al. 2005) results in activation of the intracellular kinase domain and leads to its dissociation from the BRI1 KINASE INHIBITOR 1 (BKII) (Wang and Chory 2006). Once freed from BKII, BRI1 interacts with its somatic embryogenesis receptor kinase-type co-receptor BRI1-ASSOCIATED KINASE 1 (BAK1/SERK3) (Li et al. 2002; Nam and Li 2002; Russinova et al. 2004) and, following transphosphorylation steps, the activated receptor complex initiates the intracellular events of BR signalling (Wang et al. 2008).

Severe *bri1* mutants lacking functional BR receptors show serious developmental defects, such as severe dwarfness, irregular vascularization, and male sterility (Clouse et al. 1996; Montoya et al. 2002), which have also been observed in BR-deficient plants (Szekeres et al. 1996; Li et al. 1996; Bishop et al. 1996). This indicates the essential role of BRI1 in BR perception. Whereas in *Arabidopsis*, two of its homologues, the closely related BRI1-LIKE 1 and 3 (BRL1, BRL3), are also functional BR receptors (Caño-Delgado et al. 2004; Zhou et al. 2004), inactivation of either or both of these vascularly expressed functions does not lead to visible phenotypic effects (Caño-Delgado et al. 2004).

Unlike other phytohormones, BRs are not subject to active transport (Symons and Reid 2004; Montoya et al. 2005), therefore, the concentration gradient required for eliciting differential responses is formed primarily by regulated local biosynthesis and deactivation of the hormone (Montoya et al. 2005; Nomura et al. 2007; Symons et al. 2012). Variation of BR levels is controlled by homeostatic feedback regulation of the biosynthetic genes (Bancos et al. 2002; Tanaka et al. 2005) and feed-forward regulation of the deactivating genes (Choe et al. 2001; Vert et al. 2008), which limit the concentration range available for hormonal control. This, together with earlier indications of light-regulated BR responsiveness (Turk et al. 2003; Yang et al. 2005; Bancos et al. 2006), suggested that plants may modulate BR signalling via developmental and spatial regulation of their sensitivity to the hormone.

BRI1 directly controls BR responses by interacting with the hormone and initiating the signalling process. Whereas downstream signalling components can influence the output at the transcriptional level, the function and stability of these internal regulators are also modulated by the active receptor complexes (Li and Jin 2007; Wang et al. 2012a). A confocal microscopy-based study revealed considerable cell type-specific differences in the surface density of BRI1, implying

that the intensity of BR signalling correlates with the abundance of the receptor (van Esse et al. 2011). This was shown to depend on the endocytotic internalization of BRI1, which is then followed by its degradation or recycling to the cell surface (Rusinova et al. 2004; Geldner et al. 2007). Receptor availability can also be adjusted via differential expression of BRI1, but earlier results indicated that this is not spatially regulated (Friedrichsen et al. 2000), or that in mature *Arabidopsis* there are only minor differences between organ-specific levels of the *BRI1* transcript (Li and Chory 1997; Shimada et al. 2003). On the other hand, microarray analyses revealed that *BRI1* mRNA accumulation is negatively regulated by BRs (Goda et al. 2002), and our pilot experiments using transgenic seedlings that carried promoter-reporter fusions also indicated differential *BRI1* expression.

Our aim was to find out how *BRI1* expression is regulated at the transcriptional level, and to what extent this control influences BR-dependent development in *Arabidopsis*. We used transgenic lines carrying promoter-reporter fusions to determine the temporal and spatial patterns of *BRI1* gene activity. To clarify the developmental importance of the observed differential regulation, we generated transgenic lines that express *BRI1* ectopically, under the control of well-characterized tissue-specific promoters. Our results reveal that proper morphogenesis requires precise regulation of *BRI1* expression and localization.

## Materials and methods

### Plant material and growth conditions

All experiments were carried out using wild-type *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0) (Nottingham Arabidopsis Stock Centre, UK), and the severely BR insensitive *bri1-101/bin1-1* missense mutant (Li and Chory 1997) of Col-0 background (gift from Jianming Li, University of Michigan, USA). For in vitro cultures, surface-sterilized seeds were spread over Murashige and Skoog medium supplemented with 1 % sucrose and 0.2 % Phytagel (Sigma, St. Louis, MO). Seedlings were germinated and grown at 22 °C in a controlled-environment chamber (SANYO Electronic, Tokyo, Japan) under alternating regimes of 12-h fluorescent white light (photon flux density 50–60  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and 12-h dark (LD). Except illumination, conditions during continuous light (LL) and dark (DD) treatments were identical with those of the corresponding phases of LD. For maintenance and phenotypic characterization, plants were grown in temperature-controlled (20–22 °C) greenhouse. Following 4 to 5 weeks of vegetative growth under short-day conditions (8 h L/16 h D), the plants were brought to flowering and seed production under long-day illumination cycles (16 h L/8 h D).

**Table 1** Gene-specific oligonucleotide primers

Gene	Primer	Sequence (5' → 3')
<i>ATHB8</i>	ATHB8pr-F	TTAAAATGGCCTGCAACTGTACGGATA AAC
	ATHB8pr-R	gggTTTGATCCTCTCCGATCTCTC
<i>BRI1</i>	BRI1cs-F	GAGAAATGAAGACTTTTTCAAGCTTCT TTCTCTCTG
	BRI1cs-R	ctcatgggatccCATAATTTTCCTTCAGGAAC TTC
	BRI1pr-F	agatcTGCTTGATTATGATGACATTATAG
	BRI1pr-R	ggGTTTGTGAGAGAGAAAAGTGTGGG
	BRI1rt-F	CGCATATCATCCACAGAGAC
	BRI1rt-R	GTATCCATCGCACTCATCAG
<i>TUB2-3</i>	TUBrt-F	CCAGCTTTGGTGATTGAAC
	TUBrt-R	CAAGCTTTCGGAGGTCAGAG
<i>LUC</i>	LUCrt-F	GGAGCACGAAAAGACGATGACGG
	LUCrt-F	GGAGCACGAAAAGACGATGACGG

Non-complementary nucleotides are shown in lowercase letters

## 159 Generation and characterization of transgenic plants

160 Reporter fusions and chimeric genes were assembled in  
161 the pPCV-GUS or pPCV-LUC binary reporter vectors, ver-  
162 sions of pPCV812 (Koncz et al. 1994) modified to carry  
163 glufosinate resistance and either  $\beta$ -glucuronidase (*GUS*)  
164 or firefly luciferase (*LUC*) reporter genes. To generate the  
165 *BRI1pro:GUS* and *BRI1pro:LUC* reporter constructs, a  
166 1,899-bp segment of the *BRI1* promoter (At4g39400; -1906  
167 to -8 relative to the translational start) was amplified from  
168 Col-0 genomic DNA by primers BRI1pr-F and BRI1pr-R  
169 (Table 1), which allowed oriented *Bgl*III-*Sma*I insertion in  
170 the respective reporter vectors.

171 For complementation studies, the intronless 3,590 bp  
172 *BRI1* coding sequence, without the termination codon, was  
173 amplified from genomic DNA using the BRI1cs-F and  
174 BRI1cs-R primers (Table 1). To facilitate transgene detec-  
175 tion, the 3' end of the *BRI1* coding sequence was translation-  
176 ally fused to the *LUC* reporter via the hinge region used by  
177 Friedrichsen et al. (2000) in their *BRI1-GFP* fusion. Native  
178 *BRI1*-specific and targeted expression was ensured by fusing  
179 the *BRI1-LUC* coding sequence with promoters of *BRI1*, the  
180 photosynthetic tissue-specific *CAB3* (Mitra et al. 1989), the  
181 vasculature-specific *SUC2* (Truernit and Sauer 1995), and  
182 the procambium-specific *ATHB8* (Baima et al. 1995; Kang  
183 et al. 2003) genes. The promoters of *CAB3* (At1g29910;  
184 -988 to -2) and *SUC2* (At1g22710; -2129 to -2) were avail-  
185 able as *Hind*III-*Bam*HI fragments, the *ATHB8* sequence  
186 (At4g32880; -1721 to -2) was PCR isolated using the ATH-  
187 B8pr-F and ATHB8pr-R primers (Table 1).

188 Stable transgenic *Arabidopsis* lines were generated by  
189 *Agrobacterium*-mediated transformation, as described

190 in Bancos et al. (2006). For each fusion construct at least  
191 10 glufosinate-resistant primary transformants were iso-  
192 lated and T2 progenies were obtained by self-pollination.  
193 Homozygous lines were produced from T2 plants that  
194 showed 3:1 segregation and, when appropriate, the cor-  
195 rect tissue-specificity of the transgene. Representative lines  
196 were chosen from those isolates that shared the most fre-  
197 quently observed expression level and pattern for a par-  
198 ticular transgene. In the case of the *BRI1-LUC* comple-  
199 mentation analyses this selection was based on an initial  
200 phenotype comparison of the parallel homozygous lines  
201 featuring similar phenotypes (Supplementary Table 1).  
202 Subsequent quantitative characterization of inflorescence  
203 and silique development was done with 10, two-month-old  
204 plants of each representative transgenic line, all grown in  
205 parallel, and 50 ripe siliques collected from each batch of  
206 these lines.

## Detection of reporter gene activity

207  
208 Histochemical localization of *E. coli*  $\beta$ -glucuronidase  
209 (*GUS*) reporter activity was carried out according to Jeffer-  
210 son (1987). Seedlings were collected each day after germi-  
211 nation (DAG), whereas organ samples were isolated from  
212 mature, six-week-old plants. All isolates were immediately  
213 fixed by vacuum infiltration with 2 % (w/v) formaldehyde  
214 in 50 mM sodium phosphate (pH 7.0). Following two  
215 washes in the same buffer, samples were stained overnight  
216 at 37 °C in a solution containing 0.5 mg/ml 5-bromo-4-  
217 chloro-3-indolyl  $\beta$ -D-glucuronide (X-Gluc; Biosynth A.G.,  
218 Staad, Switzerland) in 50 mM sodium phosphate (pH 7.0).  
219 Stained samples were soaked in multiple changes of 50 %  
220 (v/v) ethanol to remove plant pigments, and then were pho-  
221 tographed using Nikon SMZ800 microscope with dark  
222 background function.

223 In vivo luminescence of the firefly luciferase (*LUC*)  
224 reporter was detected at constant 22 °C temperature as  
225 described in Kay et al. (1994), using a liquid nitrogen-  
226 cooled digital CCD camera (LN-CCD-512-TKB, Prince-  
227 ton Instruments, Trenton, NJ, USA). For time-course  
228 measurements, patches of 50 one-week-old seedlings  
229 on MS medium were sprayed one day before the first  
230 exposure with sterile 5 mM Tris-phosphate buffer (pH  
231 8.0) containing 2.5 mM D-luciferin (Biosynth A.G.) and  
232 0.01 % (v/v) Triton X-100. For monitoring transgene  
233 activity upon germination, seeds were sown over MS  
234 medium supplemented with 2.5 mM D-luciferin. Ger-  
235 mination in DD was facilitated by a 12-h illumination  
236 period followed by 12-h dark incubation at 4 °C. Lumi-  
237 nescence data were evaluated using Metamorph imaging  
238 software (Meta Series 4.5; Universal Imaging). All meas-  
239 urements were repeated at least three times, with four  
240 replicates.

## 241 Transcript analyses

242 Samples of total RNA were isolated from batches of 50  
243 one-week-old seedlings using RNeasy Plant Mini Kit (Qia-  
244 gen). Traces of genomic DNA were removed by treatment  
245 with RNase-free DNase (Qiagen). RNA was eluted by  
246 RNase-free distilled water and quantitated by OD<sub>260</sub> meas-  
247 urements. Samples were adjusted to 1 µg/µl concentration  
248 and stored at -20 °C until use.

249 For quantitative RT-PCR analyses of relative mRNA lev-  
250 els cDNA was prepared from 1 µg total RNA by RevertAid  
251 First Strand cDNA Synthesis kit (Fermentas) with random  
252 hexanucleotide primers. Measurements, based on SYBR  
253 Green fluorescence, were carried out with 1.5 % amounts of  
254 the cDNA samples, using 7300 Real Time System and soft-  
255 ware (Applied Biosystems). Each assay was performed in  
256 triplicates, with two biological repetitions. Transcript lev-  
257 els were normalized to those of the constitutively expressed  
258 tubulin genes (*TUB2*, At5g62690 and *TUB3*, At5g62700).  
259 The primers for the *BRI1* (*BRI1rt-F* and *BRI1rt-R*), *LUC*  
260 (*LUCrt-F* and *LUCrt-R*) and *TUB* (*TUBrt-F* and *TUBrt-R*)  
261 reactions are shown in Table 1.

262 Quantitation of the *BRI1-LUC* fusion protein

263 Batches of 100 DD-grown seedlings, carrying the  
264 *BRI1pro:BRI1-LUC* transgene in *bri1-101* background,  
265 were harvested at 5 DAG. Following removal of the coty-  
266 ledons and roots the hypocotyls were separated to upper  
267 (apical) and lower (basal) halves and were frozen in liquid  
268 nitrogen. These samples were then used for analyses of the  
269 *BRI1-LUC* mRNA and *BRI1-LUC* protein content.

270 Relative levels of the *BRI1-LUC* fusion protein were  
271 determined by the luminometric method of Viczián and  
272 Kircher (2010). In brief, *LUC* reactions were carried out  
273 in microplates using crude extracts prepared from the  
274 hypocotyl samples, and luminescence values were meas-  
275 ured using a TopCount NXT luminometer (Perkin-Elmer).  
276 Data were normalized to protein content. From the same  
277 sets of samples the levels of the *BRI1-LUC* transcript were  
278 also determined by RT-PCR using *LUC*-specific primers.  
279 The assays were done in triplicate, with two biological  
280 replicates.

## 281 Results

282 *BRI1* expression during germination and early seedling  
283 development

284 To find out how *BRI1* promoter activity is regulated dur-  
285 ing early *Arabidopsis* development we visualized GUS  
286 reporter activity in *BRI1pro:GUS* transgenic seedlings by

287 histochemical staining. Under LD conditions (Fig. 1a),  
288 GUS staining was not detectable on day 1 following the  
289 onset of germination. At days two and three, intense stain-  
290 ing appeared in the straightening hypocotyl and in the  
291 radicle, primarily around its elongation zone. Later on  
292 the GUS activity decreased, but it remained strong near  
293 the root tip and well detectable in the petioles. No stain-  
294 ing was observed in the cotyledons. Upon DD germination  
295 (Fig. 1b), GUS staining was visible from day one in the  
296 emerging radicle. During days two and three this became  
297 more intense, and strong coloration developed also in the  
298 hypocotyl. Subsequently, until day seven, the staining grad-  
299 ually decreased around the joint of the hypocotyl and radi-  
300 cle, and was seen mainly in the distal parts of these organs,  
301 near the root tip and, particularly, the hypocotyl hook. DD  
302 seedlings, just as those grown in LD, lacked visible GUS  
303 staining in their cotyledons.

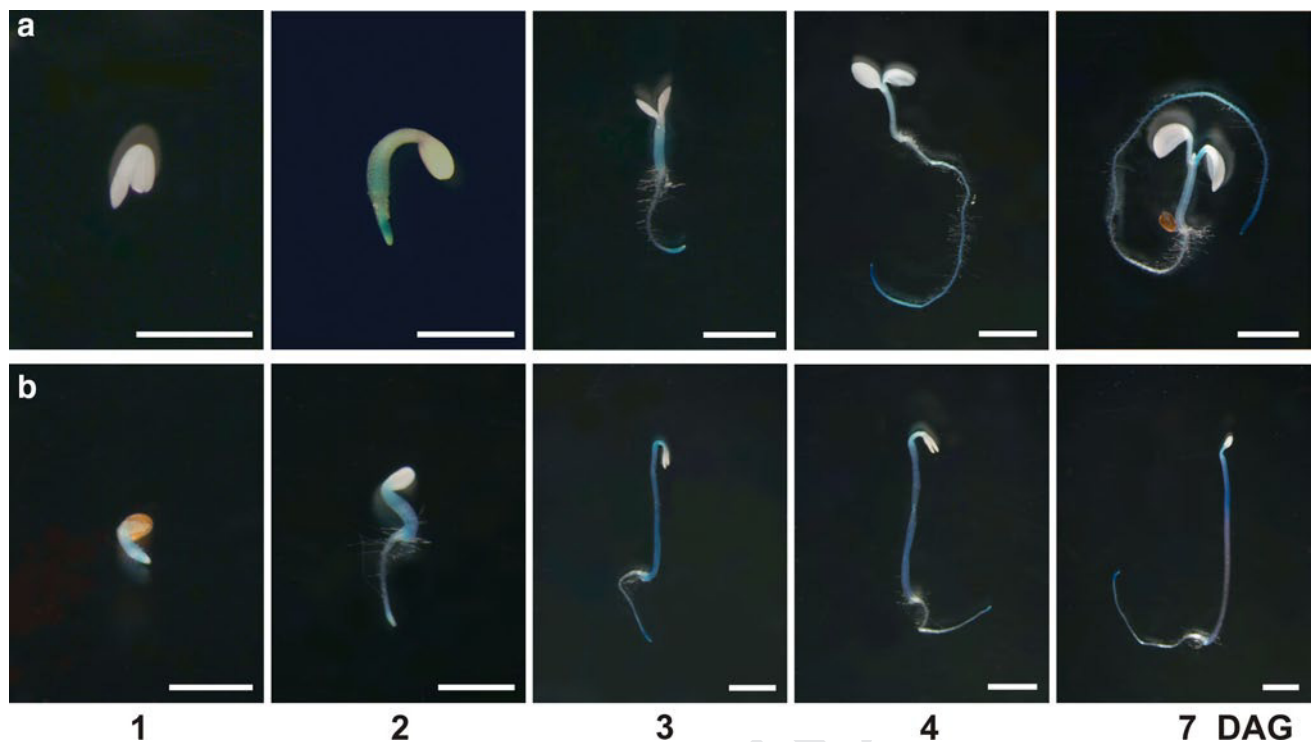
304 Enhanced *BRI1* expression is accompanied  
305 by accumulation of the BR receptor

306 In the hypocotyl of DD seedlings GUS activity decreased  
307 in the basal region, but increased in the apical part after day  
308 four (Fig. 1b). To examine whether the observed unequal  
309 activity of the *BRI1* promoter influences local accumula-  
310 tion of the *BRI1* transcript and the encoded receptor, we  
311 determined the relative levels of the respective mRNA and  
312 protein in the basal and apical halves of five-day-old DD  
313 seedlings.

314 To facilitate detection of the BR receptor, we used a line  
315 carrying the *BRI1pro:BRI1-LUC* transgene in *bri1-101*  
316 background. In this line, BR insensitivity is fully comple-  
317 mented by the *BRI1* coding sequence fused in frame to the  
318 5' end of the *LUC* reporter, and the plants are morphologi-  
319 cally indistinguishable from those of the Col-0 wild type  
320 (Supplementary Fig. S1a). Our quantitative RT-PCR analy-  
321 ses revealed that in the apical segment of the hypocotyls  
322 the abundance of *BRI1:LUC* mRNA was nearly fivefold  
323 higher than the level detected in the basal part (Fig. 2a).  
324 Luminescence-based assays showed similar accumulation  
325 of the *BRI1:LUC* protein in the apical region of the hypoc-  
326 otyls, which contained about 12.5-fold larger amount of the  
327 receptor-reporter fusion than the basal segment (Fig. 2b).  
328 These data indicate good correlation between *BRI1* gene  
329 expression and the accumulation of the *BRI1* receptor.

330 Time course of *BRI1* induction in young seedlings

331 To determine the temporal profile of the observed *BRI1*  
332 induction during early *Arabidopsis* development we fol-  
333 lowed the in vivo luminescence of *BRI1pro:LUC* seed-  
334 lings throughout the first week following germination  
335 (Fig. 3). In these experiments both LD and DD seedlings



**Fig. 1** Histochemical staining of GUS activity in *BR11prom:GUS* transgenic seedlings during the first seven days after germination (DAG). **a** Seedlings germinated and grown in LD cycles. **b** Seedlings germinated and grown in DD. Scale bars correspond to 2 mm

336 showed strong increase of LUC activity between days one  
 337 and three. In LD samples the expression reached its maxi-  
 338 mum on day three, and then decreased to roughly half of  
 339 that level by day four, after which it continued in a periodic  
 340 pattern with maxima at the dark periods (Fig. 3a). In the  
 341 first three days after germination DD induction of the *BR11*  
 342 promoter activity was very similar to that observed in LD.  
 343 Later on, however, the luminescence intensity of DD seed-  
 344 lings remained at an almost stable high level, well above  
 345 the corresponding LD values, until day 6 (Fig. 3b).

#### 346 *BR11* expression in mature plants

347 Our results show organ-specific regulation of *BR11* tran-  
 348 scription in young seedlings, indicating that differential  
 349 expression may be maintained throughout the later stages  
 350 of development. Therefore, we examined the pattern of  
 351 *BR11* promoter activity in rosettes and reproductive organs  
 352 of *BR11-GUS* transgenic plants by GUS histochemical  
 353 analysis.

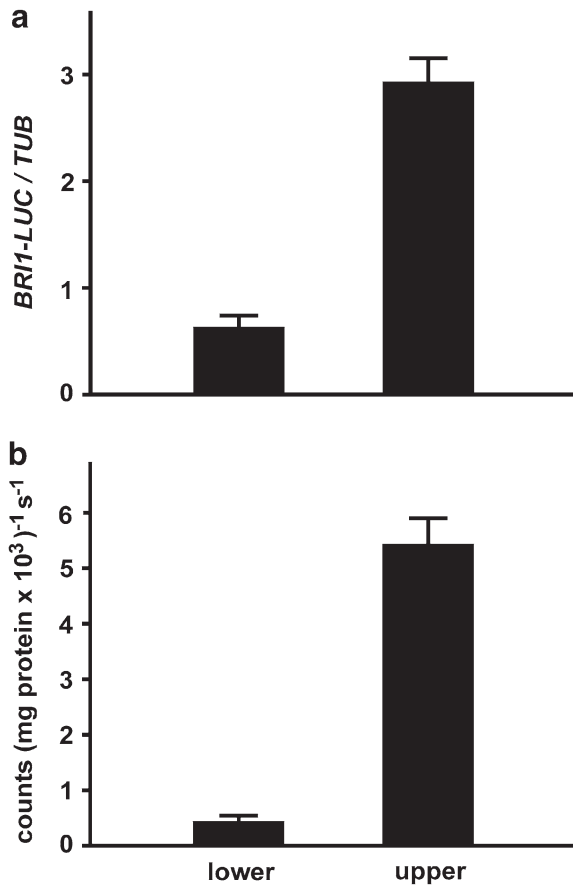
354 In pre-bolting rosettes of five-week-old plants GUS  
 355 staining was observed in young, expanding leaves, mainly  
 356 in the petioles and proximal parts of the central veins. Only  
 357 very weak or no activity could be detected in older leaves  
 358 and in the roots (Fig. 4a). Also low level expression was  
 359 seen in the flowers, where staining occurred only at the

joining of the pedicel and over the stigma (Fig. 4b). By  
 contrast, much stronger GUS activity could be detected in  
 immature siliques, in which staining was most intense in  
 the developing seeds (Fig. 4c, d).

#### Diurnal and light regulation of *BR11* promoter activity

In young LD-grown seedlings we found that following a  
 strong, transient induction *BR11* promoter activity adopted  
 a pattern of daily fluctuation, which became regular by  
 the end of the first week after germination (Fig. 3a). To  
 characterize this periodic regulation we determined the  
 changes of luminescence intensity in seven-day-old LD-  
 grown *BR11pro:LUC* seedlings. In these in vivo time-  
 course measurements we observed daily cycles of weaker  
 transgene activity during the illumination periods and  
 stronger expression in the dark phases (Fig. 5a). Relative  
 to the beginning and end of the photoperiods, a moderate  
 increase of the expression levels could be seen around the  
 middle of both the light and dark stages.

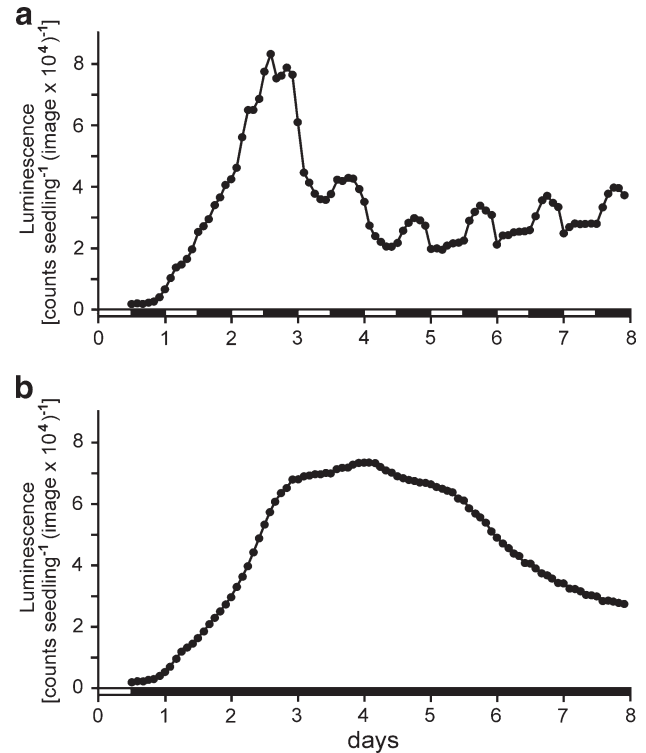
Under LD conditions the abrupt changes of lumines-  
 cence intensity were detected following lights on and lights  
 off, suggesting that *BR11* transcriptional activity is influ-  
 enced by light conditions. Therefore, we also measured  
 the luminescence profiles of seven-day-old *BR11pro:LUC*  
 seedlings upon transfer from LD to LL or DD. In these



**Fig. 2** Differential accumulation of the *BRII-LUC* transcript and *BRII-LUC* fusion protein in the hypocotyls of DD-grown *BRIIprom:BRII-LUC/bri1-101* seedlings. **a** Relative levels of the *BRII-LUC* mRNA in the *lower* and *upper* halves of 5 DAG seedlings. Quantitative RT-PCR measurements were carried out using *LUC*-specific primers. **b** Luminescence generated by the *BRII-LUC* fusion protein in the *lower* and *upper* halves of 5 DAG seedlings. The data represent mean values  $\pm$  SD

384 experiments we found that from the onset of LL the pat-  
 385 tern of expression changed to a low-amplitude oscillation  
 386 with roughly 24-h periodicity, showing maxima toward the  
 387 end of the subjective light periods (Fig. 5b). This circadian  
 388 type regulation of *BRII* activity was maintained for at least  
 389 3 days in LL. By contrast, the shift to DD resulted in a  
 390 more or less steady expression, close to the maximum level  
 391 of the last LD cycle, with only barely recognizable circadian  
 392 changes (Fig. 5c).

393 To further elucidate the regulatory role of light, we  
 394 also measured the time course of *BRII* expression using  
 395 LD conditioned seven-day-old seedlings that were moved  
 396 to DD for 60 h, and then returned to LD cycles (Fig. 6a).  
 397 Compared to the LD control (Fig. 6b), the intensity of  
 398 luminescence remained relatively high and constant during  
 399 the DD phase, just as it has been in extended DD (Fig. 5c).  
 400 Then, upon return to LD, the first 'lights on' decreased the



**Fig. 3** Luminescence intensities of *BRIIprom:LUC* seedlings during germination and early seedling development. **a** LD germinated and raised seedlings. **b** DD germinated and raised seedlings. Zero time is the start of germination following the cold treatment at 4 °C. In the time scale *white* and *black* bars indicate *light* and *dark* phases. Each panel shows the result of a representative measurement

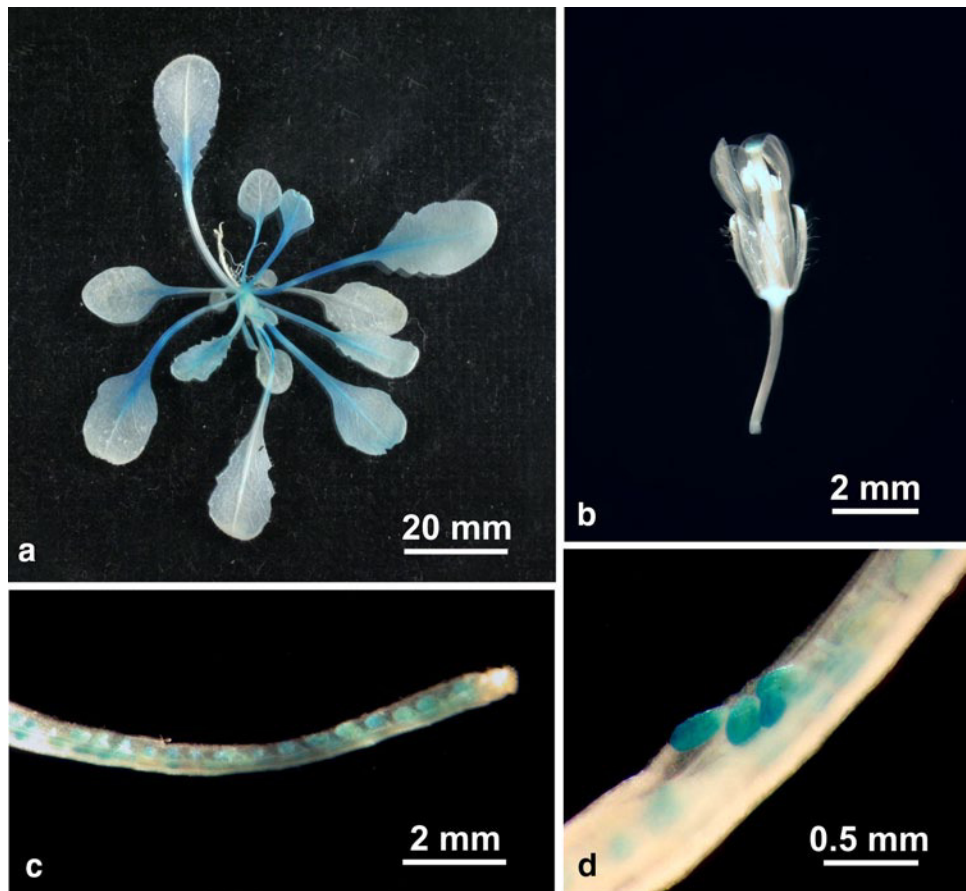
level of expression to approximately half of the preced- 401  
 ing dark values within 4 h. Following this sharp decline 402  
 the luminescence profile resumed the biphasic periodicity 403  
 which is characteristic for the LD seedlings (Fig. 6a, b). 404

#### Developmental consequences of ectopic *BRII* expression 405

Our results revealed complex regulation of *BRII* gene 406  
 activity, suggesting that these expressional control mecha- 407  
 nisms can influence plant development by modulating the 408  
 availability of the BR receptor. Therefore, we assumed that 409  
 altering the expression pattern would have well-recogniza- 410  
 ble developmental consequences. We tested this possibility 411  
 in transgenic *Arabidopsis* lines expressing *BRII* under the 412  
 control of well-characterized tissue-specific promoters. 413

We analysed the developmental effects of targeted *BRII* 414  
 misexpression by complementing the *bri1-101* mutant 415  
 with the *BRII-LUC* gene fusion driven by the photosyn- 416  
 thesis-associated *CAB3*, the vascular tissue-specific *SUC2*, 417  
 or the procambium-specific *ATHB8* promoters. When 418  
 comparing the transcript level of seven-day-old seed- 419  
 lings to that of *BRIIprom:BRII-LUC/bri1*, it was roughly 420  
 double in *CAB3pro:BRII-LUC/bri1*, approximately the 421

**Fig. 4** GUS activity in mature *BRI1prom:GUS* plants. **a** Five-week-old rosette. **b** A flower. **c**, **d** Segments of opened immature siliques



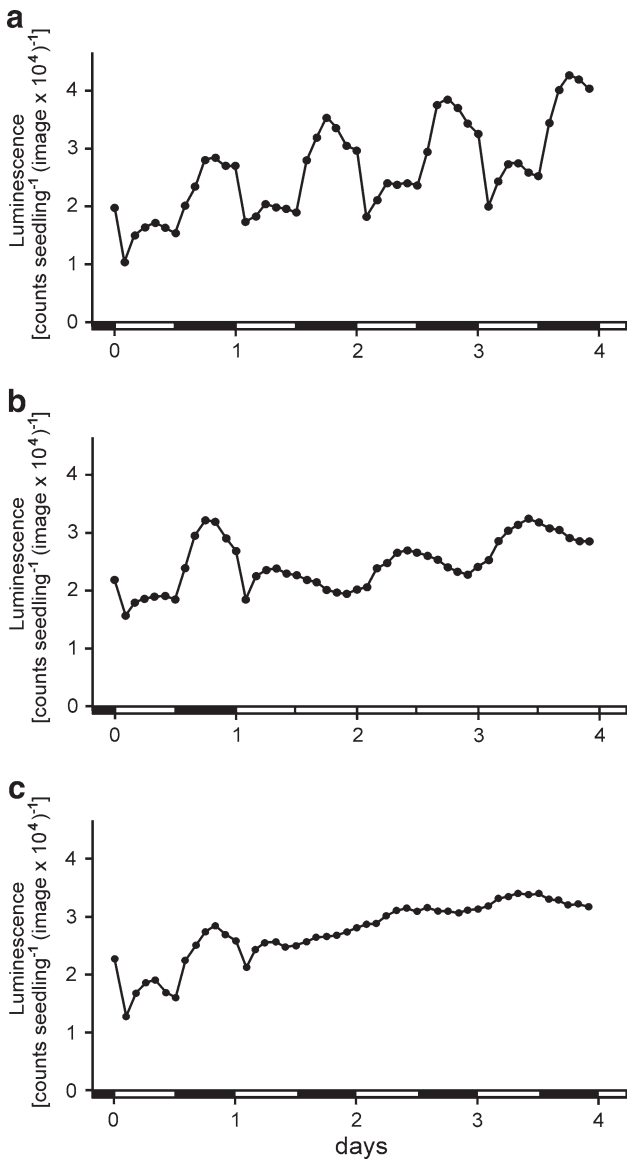
422 same in *SUC2pro:BRI1-LUC/bri1*, and less than 15 % in  
 423 *ATHB8pro:BRI1-LUC/bri1* plants (Fig. 7a). The leaves  
 424 of five-week-old *CAB3pro:BRI1-LUC/bri1* rosettes had  
 425 hyponastic blades and longer petioles than those of the  
 426 *BRI1pro:BRI1-LUC/bri1* control. The expression of the  
 427 transgene, as revealed by LUC activity, was observed over  
 428 the entire area of the leaves, but was not visible in the root  
 429 system (Fig. 7b, c). *SUC2pro:BRI1-LUC/bri1* plants of  
 430 the same age had severe dwarf phenotype, similar to that  
 431 of the non-complemented *bri1-101* mutant. Their lumi-  
 432 nescence was much weaker in the mature leaves, and only  
 433 moderately stronger in the expanding leaves and the root  
 434 (Fig. 7d). The *ATHB8pro:BRI1-LUC/bri1* transgenic lines  
 435 had semidwarf rosettes with flat, rounded leaves. In these  
 436 plants most of the luminescence was observed in the veins  
 437 of the leaves and in the roots (Fig. 7e).

438 The developmental consequences of ectopic *BRI1*  
 439 expression were clearly visible in two-month-old mature  
 440 plants. When compared to Col-0, the *CAB3pro:BRI1-*  
 441 *LUC/bri1* line showed close resemblance, with an inflo-  
 442 rescence of comparable height, leaves of similar size, and  
 443 only slightly shorter siliques with nearly the same number  
 444 of seeds (Fig. 8a, b; Table 2). By contrast, size propor-  
 445 tions between the organs of *SUC2pro:BRI1-LUC/bri1* were

severely distorted. While the leaves were short and epinas-  
 tic as those of the *bri1-101*, the inflorescence stem became  
 much more elongated, reaching more than half the height  
 of Col-0 (Fig. 8a, b; Table 2). The siliques were only about  
 half as long as those of the wild type and contained much  
 fewer seeds (Table 2). The *ATHB8pro:BRI1-LUC/bri1*  
 plants were more or less proportionately dwarfed, featur-  
 ing rounded but flat leaves and inflorescence stems approx-  
 imately twice higher than those of *bri1-101* (Fig. 8a, b;  
 Table 2). The average length of the siliques was only about  
 two-thirds compared to that of Col-0, but they produced  
 nearly the same number of seeds (Table 2).

### Discussion

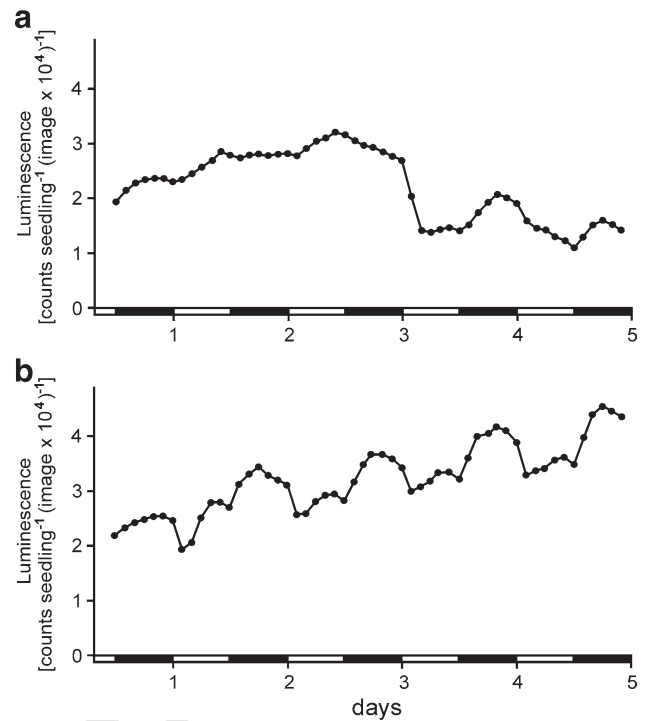
An earlier analysis of *BRI1* expression and localization,  
 which used a *BRI1prom:BRI1-GFP* transgenic line, indi-  
 cated that in *Arabidopsis* seedlings the distribution of the  
 receptor is not spatially regulated (Friedrichsen et al. 2000).  
 This result was in line with RNA gel blot and mRNA  
 microarray hybridisation data (Li and Chory 1997; Goda  
 et al. 2002), which showed only moderate variation of *BRI1*  
 transcript levels between mature organs. These studies



**Fig. 5** Luminescence profiles of one-week-old LD conditioned *BRI1prom:LUC* seedlings. **a** In LD. **b** Upon shift from LD to LL. **c** Upon shift from LD to DD. Zero time is the onset of the last common light period. In the time scale *white* and *black* bars indicate *light* and *dark* phases, whereas *grey* bars correspond to subjective dark (**b**) or subjective light (**c**) periods. Each *panel* shows the result of a representative measurement

467 implied that *BRI1* abundance may not have an important  
 468 role in influencing differential BR responses, which could  
 469 depend primarily on local levels of the active hormone.

470 Other results, however, suggested that physiological  
 471 responses to BRs are also influenced by differential hor-  
 472 mone susceptibility. Müssig et al. (2003) reported that  
 473 while 24-epiBL stimulated root growth at sub-nanomolar  
 474 concentrations, the nanomolar concentrations that pro-  
 475 moted hypocotyl elongation were already inhibitory to root  
 476 development. Dark-grown seedlings were found to be more



**Fig. 6** Luminescence responses of one-week-old LD conditioned *BRI1prom:LUC* seedlings to changing light regimes. **a** Shift from LD to DD from lights off on day 1, and then back to LD from lights on day 3. **b** LD control. Zero time is the onset of the last common light period. *White* and *black* bars indicate *light* and *dark* phases; *grey* bars correspond to subjective light periods. Each *panel* shows the result of a representative measurement

477 responsive to treatments with exogenous BL or 24-epiBL  
 478 than those raised under light–dark conditions (Turk et al.  
 479 2003; Yang et al. 2005). It was also observed that in seed-  
 480 lings exposed to prolonged darkness the BR-repressible  
 481 *CPD* gene became strongly downregulated, despite the  
 482 unchanged level of active BRs (Bancos et al. 2006). These  
 483 results seemed to indicate that the regulation of BR sensi-  
 484 tivity could have a role in enhancing or attenuating physi-  
 485 ological responses to the hormone.

486 Whereas the extent of BR effects can be influenced by  
 487 the availability and/or phosphorylation state of down-  
 488 stream signalling components (Kim and Wang 2010), the abun-  
 489 dance of the *BRI1* receptor, which directly interacts with  
 490 the hormone and initiates the signalling process, is crucial  
 491 in regulating the responses. Accordingly, a receptor-over-  
 492 expressing line shows phenotypic features consistent with  
 493 enhanced BR exposure (Wang et al. 2001), similar to those  
 494 seen in plants that overproduce the hormone (Choe et al.  
 495 2001). As *de novo* synthesis is assumed to be an important  
 496 factor in determining the availability of the receptor, we  
 497 wanted to find out how the expression of *BRI1* is regulated  
 498 in *Arabidopsis*. To this end we generated transgenic plants

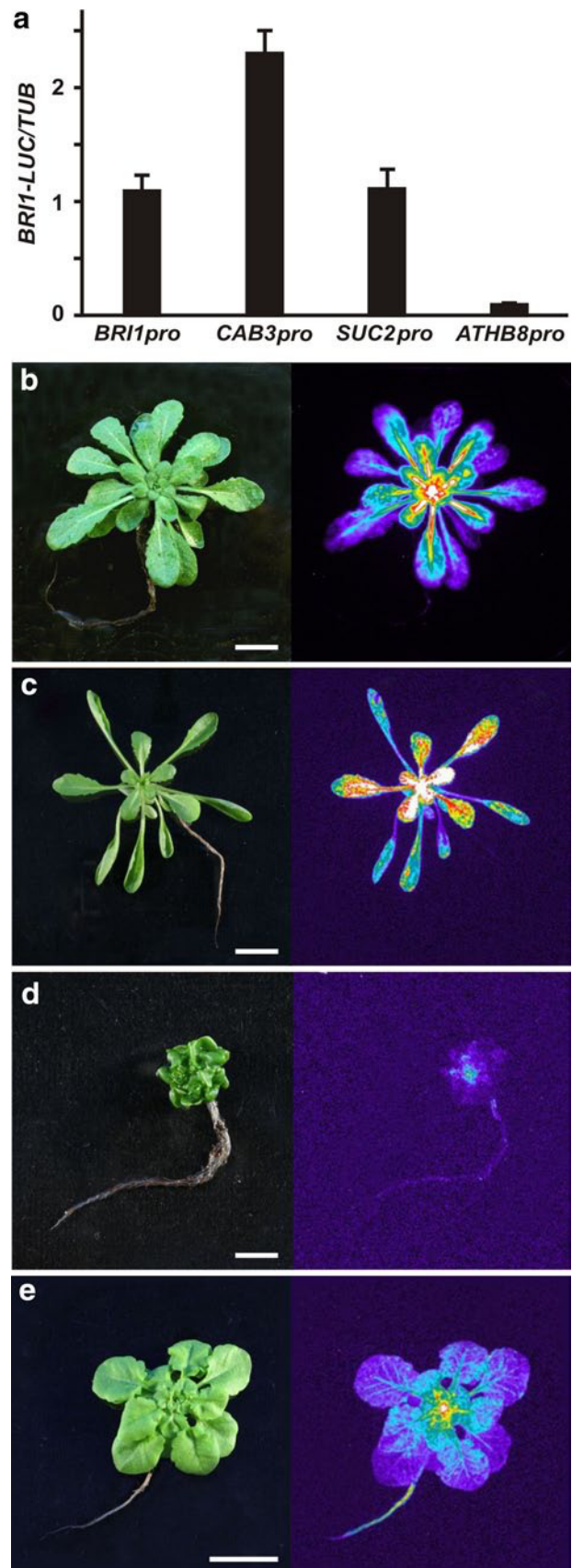


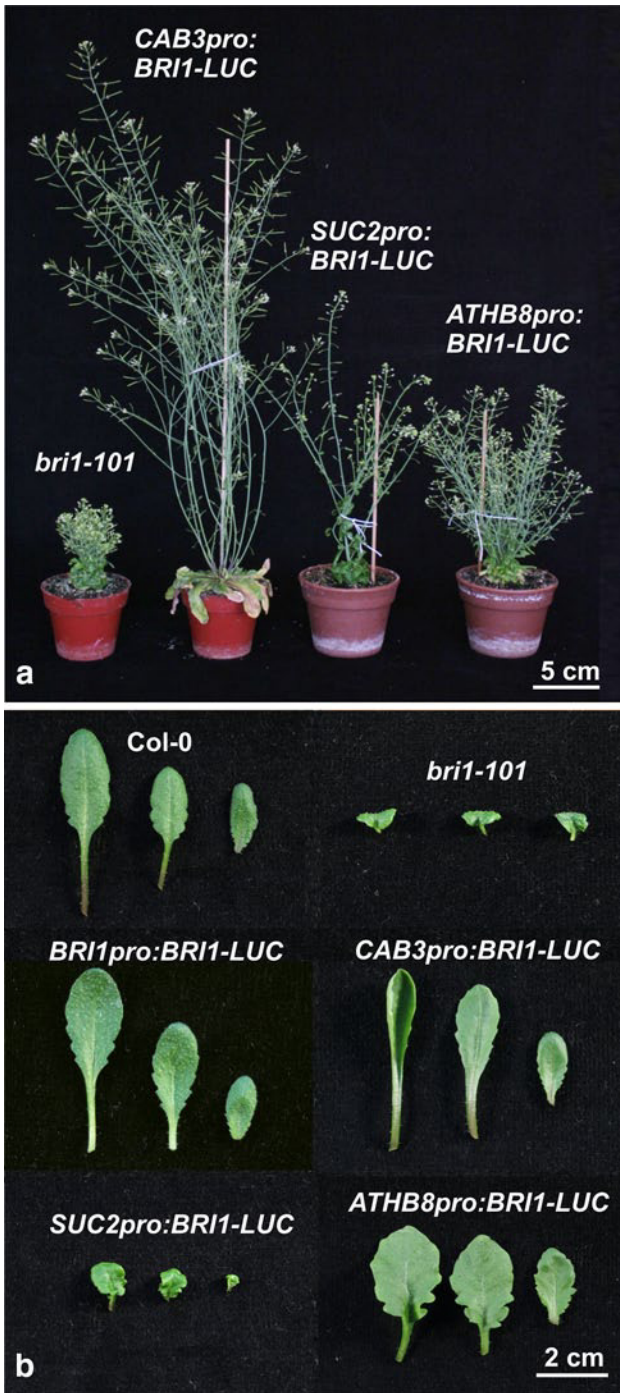
**Fig. 7** Ectopic expression of *BRI1-LUC* in *bri1-101* mutant background. **a** Relative levels of the *BRI1-LUC* transcript expressed under the control of the *BRI1*, *CAB3*, *SUC2* and *ATHB8* promoters in one-week-old transgenic seedlings with *bri1-101* background. Quantitative RT-PCR measurements were carried out using *LUC*-specific primers. The data represent mean values  $\pm$  SD. **b–e** Morphology (left) and luminescence (right) images of one-month-old *BRI1prom:BRI1-LUC* (**b**), *CAB3prom:BRI1-LUC* (**c**), *SUC2prom:BRI1-LUC* (**d**) and *ATHB8prom:BRI1-LUC* (**e**) transgenic rosettes. Scale bars correspond to 1 cm

499 that express the readily detectable GUS or LUC reporters  
500 under the control of the *BRI1* promoter.

501 Our histochemical analyses of *BRI1pro:GUS* seedlings  
502 revealed characteristic developmental and organ-specific  
503 patterns of *BRI1* promoter activity. Following germination  
504 GUS staining was seen primarily in the hypocotyls  
505 and the distal part of the radicle, but could not be observed  
506 in the cotyledons. Although GUS activity appeared earlier  
507 and became more intense in DD- than in LD-grown  
508 seedlings, its localization was similar under both types of  
509 light regimes (Fig. 1). Following day fourth the staining  
510 of DD hypocotyls became stronger toward the cotyledons  
511 (Fig. 1b).

512 To test whether our GUS histochemical assays reliably  
513 reflected differences in *BRI1* expression, we determined  
514 the relative levels of mRNA and receptor accumulation  
515 in the upper and lower halves of DD hypocotyls isolated  
516 from 5 DAG *BRI1pro:BRI1-LUC/bri1-101* seedlings.  
517 In this transgenic line *BRI1* was replaced by the easily  
518 detectable *BRI1-LUC* chimeric receptor that could fully  
519 restore wild-type BR sensitivity in the mutant background  
520 (Supplementary Fig. S1). The quantitative analyses also  
521 showed elevated amounts of the *BRI1-LUC* transcript and  
522 the receptor–reporter fusion protein in the upper hypocotyl  
523 region (Fig. 2a, b). At this stage of DD development  
524 the elongation of epidermal cells is restricted to the apical  
525 region of the hypocotyl (Gendreau et al. 1997), and  
526 MDP40, a BR-controlled regulator of the elongation pro-  
527 cess, is preferentially expressed in the upper half of the  
528 hypocotyl (Wang et al. 2012b). Correlation between *BRI1*  
529 transcriptional activity and the receptor level could also be  
530 observed when comparing two-week-old *BRI1prom:LUC/*  
531 *Col-0* and *BRI1prom:BRI1-LUC/bri1-101* plantlets, which  
532 both showed luminescence primarily in the expanding  
533 leaves and near the root tips (Supplementary Fig. S1b,  
534 c). Whereas the receptor activities of *BRI1* and its LUC-  
535 tagged version may slightly differ, these data suggest that  
536 the upregulation of *BRI1* gene activity contributes to the  
537 accumulation of the receptor and, at least during DD elon-  
538 gation of the hypocotyl, also to the enhancement of BR  
539 signalling.





**Fig. 8** Morphology of transgenic plants with ectopic expression of *BRI1*. **a** Mature, two-month-old plants. **b** Leaves isolated from six-week-old rosettes

540 *BRI1pro:GUS* plants provided information on the  
 541 localization of *BRI1* expression, however, the ~50 h half-  
 542 life of GUS enzyme (Jefferson et al. 1987) did not allow  
 543 detailed temporal monitoring of the changes in *BRI1* pro-  
 544 moter activity. For this purpose we used a *BRI1pro:LUC*  
 545 transgenic line, in which the short (2–3 h) half-life of

the reporter (Millar et al. 1992) permits quasi real-time  
 546 expression analyses. Our data showed a strong induction  
 547 of *BRI1* during the first three days following germination,  
 548 and that this was largely independent of the light condi-  
 549 tions (Fig. 3a, b). Subsequently, *BRI1* activity was quickly  
 550 repressed in LD, falling back to about half of the day three  
 551 maximum value within 24 h (Fig. 3a). By contrast, the level  
 552 of expression in DD remained high for further 2 to 3 days,  
 553 and then decreased gradually (Fig. 3b), reaching a value  
 554 similar to the LD control only by day seven.  
 555

The expression analyses using promoter–reporter fusion  
 556 transgenic lines revealed apparent coincidence between BR-  
 557 dependent morphogenic events and the levels of *BRI1* gene  
 558 activity. In seedlings, the intense GUS staining of LL root  
 559 tips and DD hypocotyls was in good agreement with prefer-  
 560 ential elongation of these regions under the mentioned light  
 561 regimes. Earlier studies found similar expression patterns  
 562 of *Arabidopsis CYP85A2* and tomato (*Solanum lycopersi-*  
 563 *cum*) *CYP85A1* (Castle et al. 2005; Montoya et al. 2005),  
 564 the genes encoding the main C-6 oxidase enzymes required  
 565 for the synthesis of bioactive BRs in these species (Bishop  
 566 et al. 1999; Shimada et al. 2003). Furthermore, the strong  
 567 *BRI1* activity of developing *Arabidopsis* seeds (Fig. 4c, d)  
 568 is accompanied by enhanced *CYP85A2* expression (Castle  
 569 et al. 2005), and concomitant induction of the genes that  
 570 encode the *BRI1* and *CYP85* orthologues has also been  
 571 observed in germinating pea (*Pisum sativum*) (Nomura  
 572 et al. 2007). BRs have an important role in seed and fruit  
 573 development (Huang et al. 2013), and combined mRNA and  
 574 BR analyses in various dicot species revealed that induc-  
 575 tion of the *CYP85* genes during these processes results in  
 576 transient accumulation of bioactive BRs (Montoya et al.  
 577 2005; Nomura et al. 2005, 2007; Symons et al. 2006). Taken  
 578 together, these results support the notion that local induction  
 579 of BR biosynthesis, and the resulting accumulation of the  
 580 hormone, tends to coincide with enhanced *BRI1* expression.  
 581

In LD-grown seedlings *BRI1* promoter activity exhib-  
 582 its a recognizable diurnal periodicity, with expression  
 583 levels higher in the dark and lower during the light peri-  
 584 ods (Fig. 5a). The observed biphasic pattern results from a  
 585 weak circadian fluctuation showing maxima at the middle  
 586 of the subjective light periods, and a superimposed nega-  
 587 tive light regulation that allows strongest activity during  
 588 the dark phases (Fig. 5a–c). The circadian minima and  
 589 maxima are clearly recognizable on the diurnal pattern,  
 590 which, however, is determined primarily by the light regu-  
 591 lation. Whereas the circadian oscillation is well recogniz-  
 592 able in LL, its amplitude is strongly dampened in DD. This  
 593 is probably due to the elevated, near-maximum expression  
 594 of *BRI1* in the dark (Fig. 5c). The functional significance  
 595 of the diurnal regulation of *BRI1* is not clear, but it seems  
 596 likely that it can cause periodic daily changes in receptor  
 597 availability and, hence, BR responsiveness.  
 598

**Table 2** Inflorescence and silique development in *BRII-LUC*-complemented lines

<i>Arabidopsis</i> line	Inflorescence height (mm)	Silique length (mm)	Seeds per silique
Col-0	396 ± 52	13.6 ± 0.9	40 ± 5
<i>bri1-101</i>	63 ± 9	6.5 ± 0.6	8 ± 3
<i>BRIIpro:BRII-LUC</i>	388 ± 68	14.4 ± 0.8	41 ± 7
<i>CAB3pro:BRII-LUC</i>	412 ± 75	12.9 ± 1.0	37 ± 7
<i>SUC2pro:BRII-LUC</i>	227 ± 40	6.7 ± 0.8	27 ± 4
<i>ATHB8pro:BRII-LUC</i>	149 ± 14	10.9 ± 0.8	38 ± 3

Data are mean values ± SD

599 Earlier studies revealed that in *Arabidopsis* seedlings BR  
600 responses depend on light conditions and the time of the  
601 day. Yang et al. (2005) observed that 1 μM 24-epibrassi-  
602 nolide promoted hypocotyl elongation in LD seedlings,  
603 whereas in the DD control the concentrations above 10 nM  
604 were already inhibitory. Upon prolonged DD exposure of  
605 LD-grown seedlings the BR-repressible *CPD* expression  
606 decreased dramatically, though the level of active endog-  
607 enous BRs remained unchanged (Bancos et al. 2006). It  
608 has also been described that under short-day conditions  
609 shifting the peak of *BRII* transcript accumulation from the  
610 end to the beginning of the dark period can alter the rescue  
611 effect in the strong *bri1-116* mutant background (Michael  
612 et al. 2008). Considering that the half-life of the *BRII*  
613 protein is approximately 5 h (Geldner et al. 2007), these  
614 results suggest that light regulation of *BRII* transcription  
615 can be a means of modulating receptor abundance and BR  
616 susceptibility.

617 The complex regulation of *BRII* expression implies that  
618 differential expression is important for ensuring the proper  
619 BR control of developmental processes. Therefore, to ver-  
620 ify that inappropriate regulation of *BRII* gene activity inter-  
621 feres with normal morphogenesis, we generated transgenic  
622 lines expressing the receptor ectopically. We used *BRII-LUC*  
623 fusion, which allowed easy localization of the recep-  
624 tor. The fusion protein contained the same linker peptide as  
625 that of the chimeric *BRII-GFP* (Friedrichsen et al. 2000),  
626 in which the receptor function was not compromised. As  
627 expected, the *BRIIpro:BRII-LUC* fusion restored the wild  
628 phenotype in the strong BR insensitive *bri1-101* mutant  
629 (Supplementary Fig. S1a).

630 When compared to wild-type Col-0 and the  
631 *BRIIpro:BRII-LUC*-complemented line, we found very  
632 similar phenotype in the *CAB3pro:BRII-LUC* line.  
633 Although these plants had more elongate, hyponas-  
634 tic leaves, their inflorescence height, silique length and  
635 seed number were very close to those of the wild type  
636 (Fig. 8a, b; Table 2). This is consistent with the strong,  
637 photosynthetic tissue-specific activity of the *CAB3* pro-  
638 moter, and the observation that the rosette versus root  
639 distribution of the *BRII-LUC* fusion product is compar-  
640 able in the *CAB3pro:BRII-LUC* and *BRIIpro:BRII-LUC*  
641 plants (Fig. 7b, c). The longer, hyponastic leaves of the

*CAB3pro:BRII-LUC* line can be attributed to an enhanced  
642 receptor production in the mesophyll cells, which seems to  
643 result in a stronger BR-dependent elongation and expan-  
644 sion at the abaxial side of the leaf blade. In contrast to the  
645 *CAB3pro:BRII-LUC* plants, only partial and disproportional  
646 complementation could be seen in the transgenic  
647 lines that expressed *BRII* under the control of vascular  
648 tissue-specific promoters. Whereas *SUC2pro:BRII-LUC*  
649 plants developed about threefold higher inflorescence than  
650 *bri1-101*, the shape and size of its rosette leaves and the  
651 length of its siliques did not appreciably differ from those  
652 of the non-complemented mutant. Despite their similar  
653 appearance, the seed production of the *SUC2pro:BRII-LUC*  
654 siliques was substantially higher than those of the  
655 mutant (Fig. 8a, b; Table 2). The *ATHB8pro:BRII-LUC* line  
656 also showed reduced inflorescence height and leaf expan-  
657 sion, but in this case the complementation was stronger in  
658 the rosette leaves and weaker in the inflorescence stems.  
659 Despite the shorter siliques, their seed number was roughly  
660 equal to those of the wild type (Fig. 8a, b; Table 2). These  
661 results, in agreement with the findings of Savaldi-Goldstein  
662 et al. (2007), show that primarily vascular expression of  
663 *BRII* can only partially restore BR sensitivity in severe *bri1*  
664 mutants. The observed phenotypic differences between  
665 the *SUC2pro:BRII-LUC* and *ATHB8pro:BRII-LUC* plants  
666 likely result from the differing activity and developmen-  
667 tal regulation of the phloem-specific *SUC2* (Truernit and  
668 Sauer 1995) and the strictly procambium-specific *ATHB8*  
669 (Kang et al. 2003) promoters. Our results show that ectopic  
670 expression of *BRII* can severely disturb the development  
671 of *Arabidopsis* plants, and that correct spatial and temporal  
672 transcriptional control of the receptor gene is required for  
673 the proper coordination of organ morphogenesis.

674 In addition to BR levels, the initiation of signalling by  
675 this hormone also depends on the availability of *BRII* and  
676 its SERK-type co-receptor. A transgenic line overexpress-  
677 ing the *BRII-GFP* fusion showed excess leaf elongation,  
678 similar to that observed in BR overproducing plants, and  
679 the BR-binding capacity of its microsomal fractions was  
680 found higher than that of the wild type (Wang et al. 2001).  
681 Similar, but less pronounced enhancement of BR effects  
682 could be observed when the co-receptor *BAK1/SERK3*  
683 was overexpressed (Nam and Li 2002). This weaker effect  
684

685 and the semidwarf phenotype of the *bak1-1* null mutant  
 686 (Li et al. 2002) can be attributed to the functional redun-  
 687 dancy between BAK1/SERK3 and three other members of  
 688 the SERK family (Gou et al. 2012) which, in addition to  
 689 their role in the BR receptor complexes, also function as  
 690 co-receptors in pathogen-induced defence signalling path-  
 691 ways (Roux et al. 2011). Although SERK co-receptors are  
 692 indispensable for the initiation of BR signalling (Gou et al.  
 693 2012), the formation of active receptor complexes seems  
 694 to be limited by the less abundant BRI1 component. Our  
 695 results indicate that in *Arabidopsis* *BRI1* gene activity is  
 696 under complex regulation, and that this transcriptional con-  
 697 trol has a role in determining the distribution of the recep-  
 698 tor. The data of the complementation analyses support the  
 699 notion that in BR insensitive background proper restoration  
 700 of the wild phenotype requires *BRI1* promoter-specific dif-  
 701 ferential regulation of the receptor gene.

702 We demonstrated that *BRI1* expression is under devel-  
 703 opmental, organ-specific and diurnal regulation. In addi-  
 704 tion, it is also controlled by phytohormones, as BRs can  
 705 downregulate (Goda et al. 2002), whereas auxin can  
 706 enhance the level of transcription (Nemhauser et al. 2004;  
 707 Sakamoto et al. 2013). Thus, the activity of *BRI1* is deter-  
 708 mined in a complex way, similar to that of the key *BR* bio-  
 709 synthetic genes (Hategan et al. 2011; Zhao and Li 2012).  
 710 This can allow optimal coordination of BR accumula-  
 711 tion and susceptibility, and suggests that the differential  
 712 regulation of receptor abundance is an important means  
 713 of enhancing or attenuating physiological effects of the  
 714 hormone.

715 **Acknowledgments** This work was supported by the Hungarian Sci-  
 716 entific Research Fund (Grant T 68201) and the ‘BRAVISSIMO’ Marie  
 717 Curie Initial Training Grant of the European Union. The authors thank  
 718 Márta Börcsök S. and Mária Tóth S. for their help with preparing the  
 719 photographic material.

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