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ORIGINAL ARTICLE

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

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Abstract Brassinosteroid (BR)-regulated growth and development in *Arabidopsis* depends on BRASSINOSTEROID INSENSITIVE 1 (BRI1), the BR receptor that is responsible for initiating the events of BR signalling. We analysed the temporal and spatial regulation of BRI1 expression using stable transgenic lines that carried *BRI1* promoter:reporter fusions. In both seedlings and mature plants the tissues undergoing elongation or differentiation showed elevated *BRI1* gene activity, and it could be demonstrated that in the hypocotyl this was accompanied by accumulation of the *BRI1* transcript and its receptor protein product. In seedlings the *BRI1* promoter was also found to be under diurnal regulation, determined primarily by light repression and a superimposed circadian control. To determine the functional importance of transcriptional regulation we complemented the severely BR insensitive *bri1-101* mutant with a *BRI1-luciferase* fusion construct that was driven by promoters with contrasting specificities. Whereas the *BRI1* promoter-driven transgene fully restored the wild phenotype, expression from the photosynthesis-associated *CAB3* and the vasculature-specific *SUC2* and *ATHB8* promoters resulted in plants with varying morphogenic defects. Our results reveal complex differential regulation of *BRI1* expression, and suggest that by influencing the distribution and abundance of the receptor this regulation can enhance or attenuate BR signalling.

Keywords Ectopic expression · Hormone susceptibility · Phytohormone · Reporter gene

Abbreviations

BR	Brassinosteroid
BL	Brassinolide
CS	Castasterone
DD	Continuous dark
LD	Light–dark cycles
LL	Continuous light
DAG	Days after germination
LUC	Firefly luciferase
GUS	β-Glucuronidase

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Introduction

Brassinosteroids (BRs) are polyhydroxylated steroid hormones that control plant development from germination to seed production (Clouse and Sasse 1998; Haubrick et al. 2006). The biologically active BRs castasterone (CS) and brassinolide (BL) are perceived by the plasma membrane-localized leucine-rich repeat receptor-like kinase BRASSINOSTEROID INSENSITIVE 1 (BRI1) (Li and Chory 1997), which then initiates a phosphorylation/dephosphorylation-based signalling cascade that controls the transcriptional activity of BR-responsive genes.

Journal : Large 425 | Dispatch : 27-1-2014 | Pages : 13
Article No : 2031 | ☐ LE | ☐ TYPESET
MS Code : PLAA-D-13-00697 | ☑ CP | ☑ DISK

57 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).

63 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 (BKI1) (Wang and Chory 2006). Once freed from BKI1, 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).

75 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).

87 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.

102 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 (Russinova 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.

125 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.

136 Materials and methods

137 Plant material and growth conditions

138 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 µmol m^{-2} 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).

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Journal : Large 425 | Dispatch : 27-1-2014 | Pages : 13
Article No : 2031 | LE | TYPESET
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Table 1 Gene-specific oligonucleotide primers

Gene	Primer	Sequence (5′ → 3′)
ATHB8	ATHB8pr-F	TTAAAATGGCCTGCAACTGTACGGATAAAC
	ATHB8pr-R	gggTTTGATCCTCTCCGATCTCTC
BRI1	BRI1cs-F	GAGAAATGAAGACTTTTTCAAGCTTCTTTCTCTCTG
	BRI1cs-R	ctcatgggatccCATAATTTTCCTTCAGGAACTTC
	BRI1pr-F	agatcTGCTTGATTATGATGACATTATAG
	BRI1pr-R	ggGTTTGTGAGAGAGAAAAGTGTGGG
	BRI1rt-F	CGCATATCATCCACAGAGAC
	BRI1rt-R	GTATCCATCGCACTCATCAG
TUB2-3	TUBrt-F	CCAGCTTTGGTGATTTGAAC
	TUBrt-R	CAAGCTTTCGGAGGTCAGAG
LUC	LUCrt-F	GGAGCACGGAAAGACGATGACGG
	LUCrt-F	GGAGCACGGAAAGACGATGACGG

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 β-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*II-*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 *Hin*dIII-*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.

207 Detection of reporter gene activity

208 Histochemical localization of *E. coli* β-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 β-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.

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Journal : Large 425 | Dispatch : 27-1-2014 | Pages : 13
Article No : 2031 | LE | TYPESET
MS Code : PLAA-D-13-00697 | CP | DISK

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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_{260} 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 hyopcotyl 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

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Journal : Large 425 | Dispatch : 27-1-2014 | Pages : 13
Article No : 2031 | LE | TYPESET
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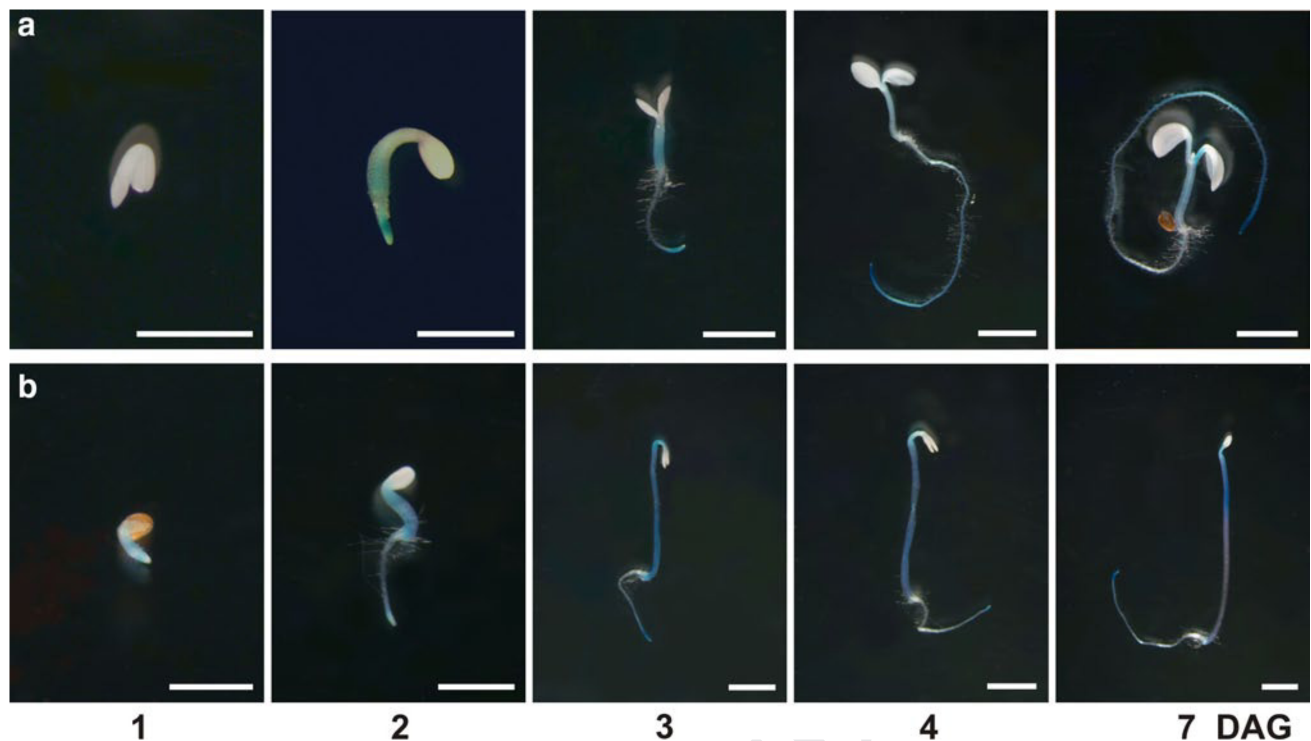

Fig. 1 Histochemical staining of GUS activity in *BRI1prom:GUS* transgenic seedlings during the first seven days after germination (DAG). **a** Seedlings germinated and grown in LD cycles. **b** Seedlings germinated and grown in DD. Scale bars correspond to 2 mm

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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 *BRI1* 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 *BRI1* expression in mature plants

347 Our results show organ-specific regulation of *BRI1* 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 *BRI1* promoter activity in rosettes and reproductive organs 352 of *BRI1-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 360 contrast, much stronger GUS activity could be detected in 361 immature siliques, in which staining was most intense in 362 the developing seeds (Fig. 4c, d). 363

Diurnal and light regulation of *BRI1* promoter activity 364

In young LD-grown seedlings we found that following a 365 strong, transient induction *BRI1* promoter activity adopted 366 a pattern of daily fluctuation, which became regular by 367 the end of the first week after germination (Fig. 3a). To 368 characterize this periodic regulation we determined the 369 changes of luminescence intensity in seven-day-old LD- 370 grown *BRI1pro:LUC* seedlings. In these in vivo time- 371 course measurements we observed daily cycles of weaker 372 transgene activity during the illumination periods and 373 stronger expression in the dark phases (Fig. 5a). Relative 374 to the beginning and end of the photoperiods, a moderate 375 increase of the expression levels could be seen around the 376 middle of both the light and dark stages. 377

Under LD conditions the abrupt changes of lumines- 378 cence intensity were detected following lights on and lights 379 off, suggesting that *BRI1* transcriptional activity is influ- 380 enced by light conditions. Therefore, we also measured 381 the luminescence profiles of seven-day-old *BRI1pro:LUC* 382 seedlings upon transfer from LD to LL or DD. In these 383

Journal : Large 425 | Dispatch : 27-1-2014 | Pages : 13
Article No : 2031 | ☐ LE | ☐ TYPESET
MS Code : PLAA-D-13-00697 | ☑ CP | ☑ DISK

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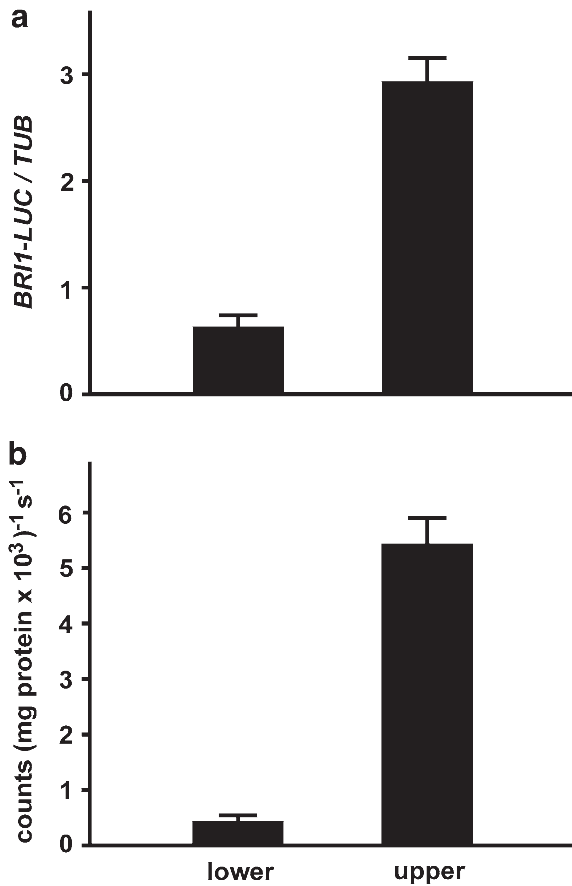

Fig. 2 Differential accumulation of the *BRI1-LUC* transcript and BRI1-LUC fusion protein in the hypocotyls of DD-grown *BRI1pro m:BRI1:LUC/bri1-101* seedlings. **a** Relative levels of the *BRI1-LUC* mRNA in the *lower* and *upper halves* of 5 DAG seedlings. Quantitative RT-PCR measurements were carried out using *LUC*-specific primers. **b** Luminescence generated by the BRI1-LUC fusion protein in the *lower* and *upper halves* of 5 DAG seedlings. The *data* represent mean values ± SD

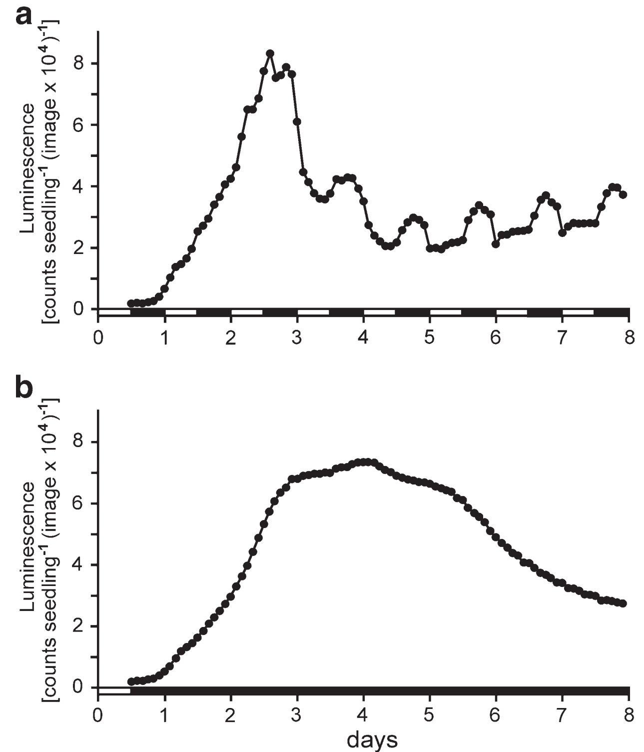

Fig. 3 Luminescence intensities of *BRI1prom:LUC* seedlings during germination and early seedling development. **a** LD germinated and raised seedlings. **b** DD germinated and raised seedlings. Zero time is the start of germination following the cold treatment at 4 °C. In the time scale *white* and *black bars* indicate *light* and *dark phases*. Each *panel* shows the result of a representative measurement

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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 *BRI1* 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 circa-
392 dian changes (Fig. 5c).
393 To further elucidate the regulatory role of light, we
394 also measured the time course of *BRI1* 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
401 level of expression to approximately half of the preced-
402 ing dark values within 4 h. Following this sharp decline
403 the luminescence profile resumed the biphasic periodicity
404 which is characteristic for the LD seedlings (Fig. 6a, b).

405 Developmental consequences of ectopic *BRI1* expression

406 Our results revealed complex regulation of *BRI1* gene
407 activity, suggesting that these expressional control mecha-
408 nisms can influence plant development by modulating the
409 availability of the BR receptor. Therefore, we assumed that
410 altering the expression pattern would have well-recogniza-
411 ble developmental consequences. We tested this possibility
412 in transgenic *Arabidopsis* lines expressing *BRI1* under the
413 control of well-characterized tissue-specific promoters.
414 We analysed the developmental effects of targeted *BRI1*
415 misexpression by complementing the *bri1-101* mutant
416 with the *BRI1-LUC* gene fusion driven by the photosyn-
417 thesis-associated *CAB3*, the vascular tissue-specific *SUC2*,
418 or the procambium-specific *ATHB8* promoters. When
419 comparing the transcript level of seven-day-old seed-
420 lings to that of *BRI1pro:BRI1-LUC/bri1*, it was roughly
421 double in *CAB3pro:BRI1-LUC/bri1*, approximately the

Journal : Large 425 | Dispatch : 27-1-2014 | Pages : 13
Article No : 2031 | ☐ LE | ☐ TYPESET
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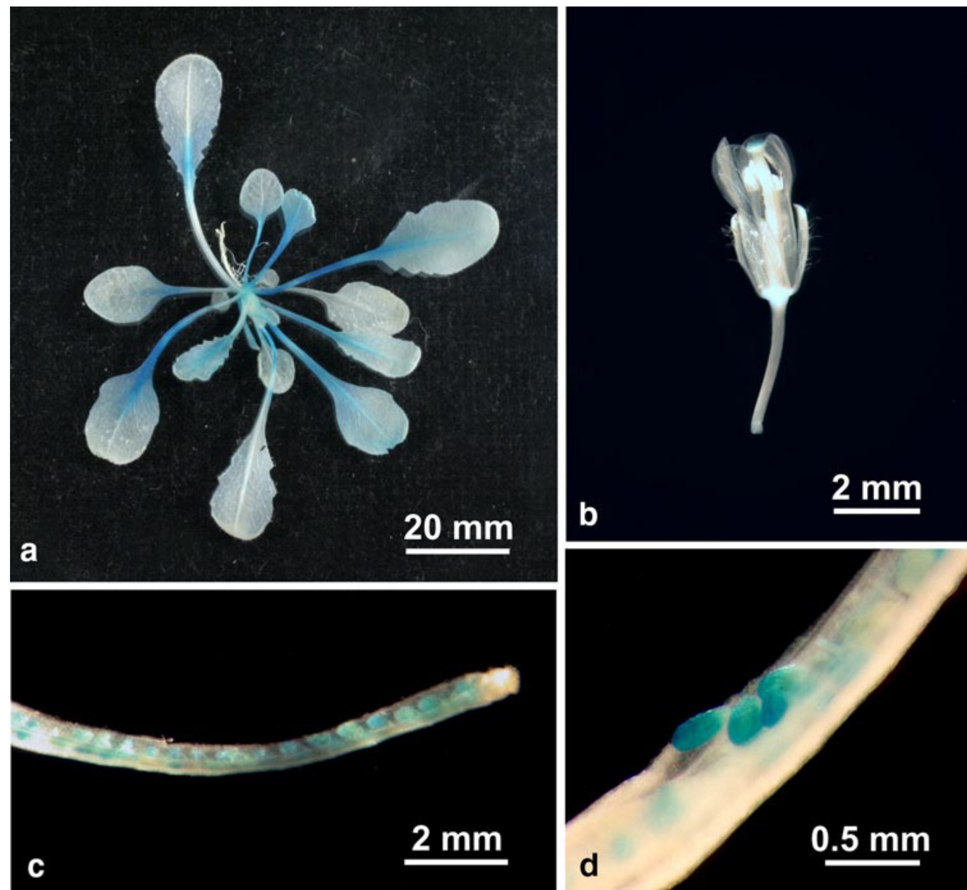

Fig. 4 GUS activity in mature *BRI1prom:GUS* plants. **a** Five-week-old rosette. **b** A flower. **c**, **d** Segments of opened immature siliques

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- 446 tic as those of the *bri1-101*, the inflorescence stem became 447 much more elongated, reaching more than half the height 448 of Col-0 (Fig. 8a, b; Table 2). The siliques were only about 449 half as long as those of the wild type and contained much 450 fewer seeds (Table 2). The *ATHB8pro:BRI1-LUC/bri1* 451 plants were more or less proportionately dwarfed, featur- 452 ing rounded but flat leaves and inflorescence stems approx- 453 imately twice higher than those of *bri1-101* (Fig. 8a, b; 454 Table 2). The average length of the siliques was only about 455 two-thirds compared to that of Col-0, but they produced 456 nearly the same number of seeds (Table 2). 457

Discussion 458

An earlier analysis of BRI1 expression and localization, 459 which used a *BRI1prom:BRI1-GFP* transgenic line, indi- 460 cated that in *Arabidopsis* seedlings the distribution of the 461 receptor is not spatially regulated (Friedrichsen et al. 2000). 462 This result was in line with RNA gel blot and mRNA 463 microarray hybridisation data (Li and Chory 1997; Goda 464 et al. 2002), which showed only moderate variation of *BRI1* 465 transcript levels between mature organs. These studies 466

Journal : Large 425 | Dispatch : 27-1-2014 | Pages : 13
Article No : 2031 | ☐ LE | ☐ TYPESET
MS Code : PLAA-D-13-00697 | ☑ CP | ☑ DISK

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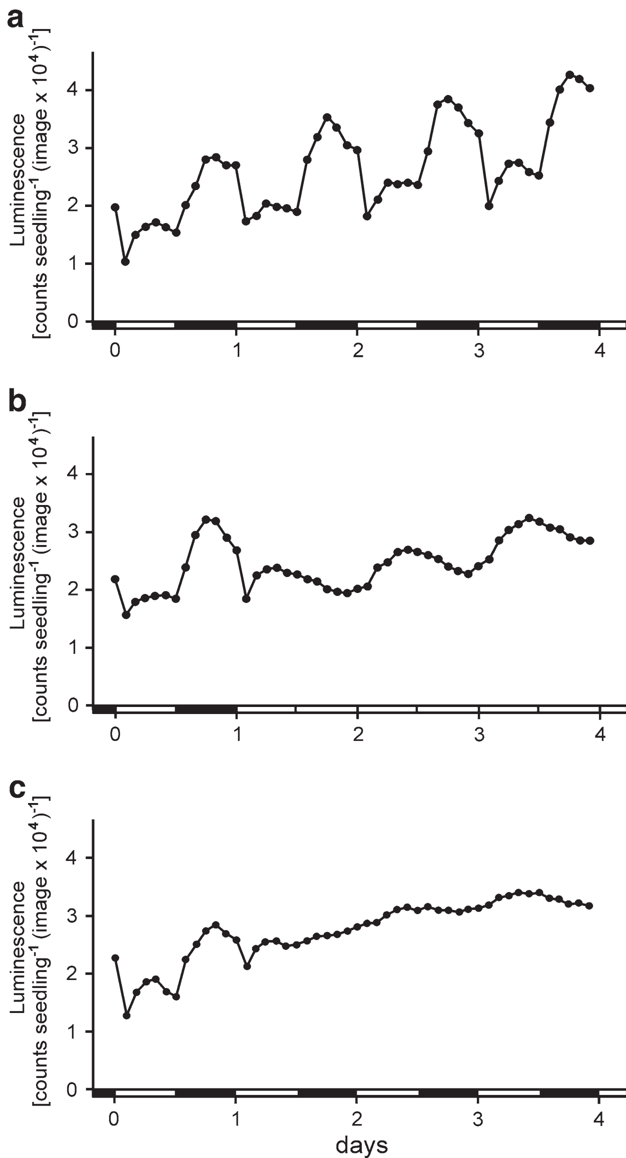

Fig. 5 Luminescence profiles of one-week-old LD conditioned *BRI1prom:LUC* seedlings. **a** In LD. **b** Upon shift from LD to LL. **c** Upon shift from LD to DD. Zero time is the onset of the last common light period. In the time scale *white* and *black bars* indicate *light* and *dark phases*, whereas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

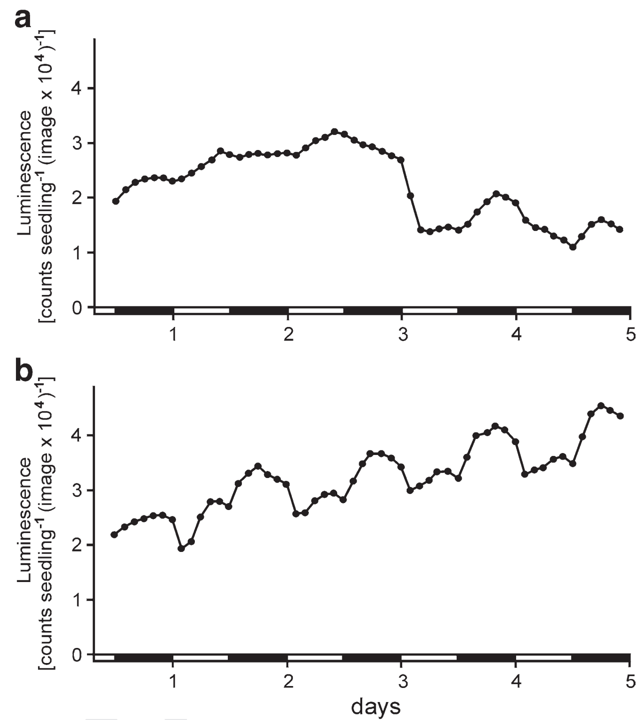

Fig. 6 Luminescence responses of one-week-old LD conditioned *BRI1prom:LUC* seedlings to changing light regimes. **a** Shift from LD to DD from lights off on day 1, and then back to LD from lights on of day 3. **b** LD control. Zero time is the onset of the last common light period. *White* and *black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

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

Other results, however, suggested that physiological responses to BRs are also influenced by differential hormone susceptibility. Müssig et al. (2003) reported that while 24-epiBL stimulated root growth at sub-nanomolar concentrations, the nanomolar concentrations that promoted hypocotyl elongation were already inhibitory to root development. Dark-grown seedlings were found to be more responsive to treatments with exogenous BL or 24-epiBL than those raised under light–dark conditions (Turk et al. 2003; Yang et al. 2005). It was also observed that in seedlings exposed to prolonged darkness the BR-repressible *CPD* gene became strongly downregulated, despite the unchanged level of active BRs (Bancos et al. 2006). These results seemed to indicate that the regulation of BR sensitivity could have a role in enhancing or attenuating physiological responses to the hormone.

Whereas the extent of BR effects can be influenced by the availability and/or phosphorylation state of downstream signalling components (Kim and Wang 2010), the abundance of the BRI1 receptor, which directly interacts with the hormone and initiates the signalling process, is crucial in regulating the responses. Accordingly, a receptor-overexpressing line shows phenotypic features consistent with enhanced BR exposure (Wang et al. 2001), similar to those seen in plants that overproduce the hormone (Choe et al. 2001). As de novo synthesis is assumed to be an important factor in determining the availability of the receptor, we wanted to find out how the expression of *BRI1* is regulated in *Arabidopsis*. To this end we generated transgenic plants

Journal : Large 425 | Dispatch : 27-1-2014 | Pages : 13
Article No : 2031 | LE | TYPESET
MS Code : PLAA-D-13-00697 | CP | DISK

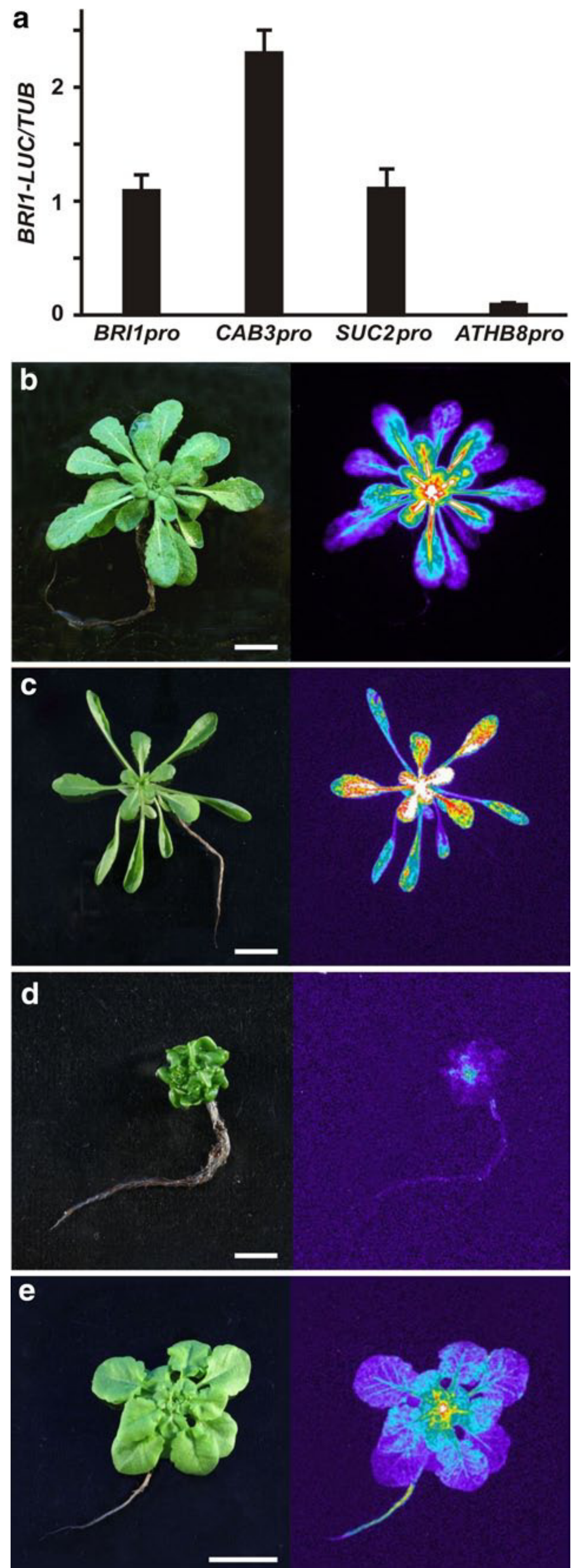

Fig. 7 Ectopic expression of *BRI1-LUC* in *bri1-101* mutant background. **a** Relative levels of the *BRI1-LUC* transcript expressed under the control of the *BRI1*, *CAB3*, *SUC2* and *ATHB8* promoters in one-week-old transgenic seedlings with *bri1-101* background. Quantitative RT-PCR measurements were carried out using *LUC*-specific primers. The data represent mean values ± 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

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

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

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

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Journal : Large 425 | Dispatch : 27-1-2014 | Pages : 13
Article No : 2031 | LE | TYPESET
MS Code : PLAA-D-13-00697 | CP | DISK

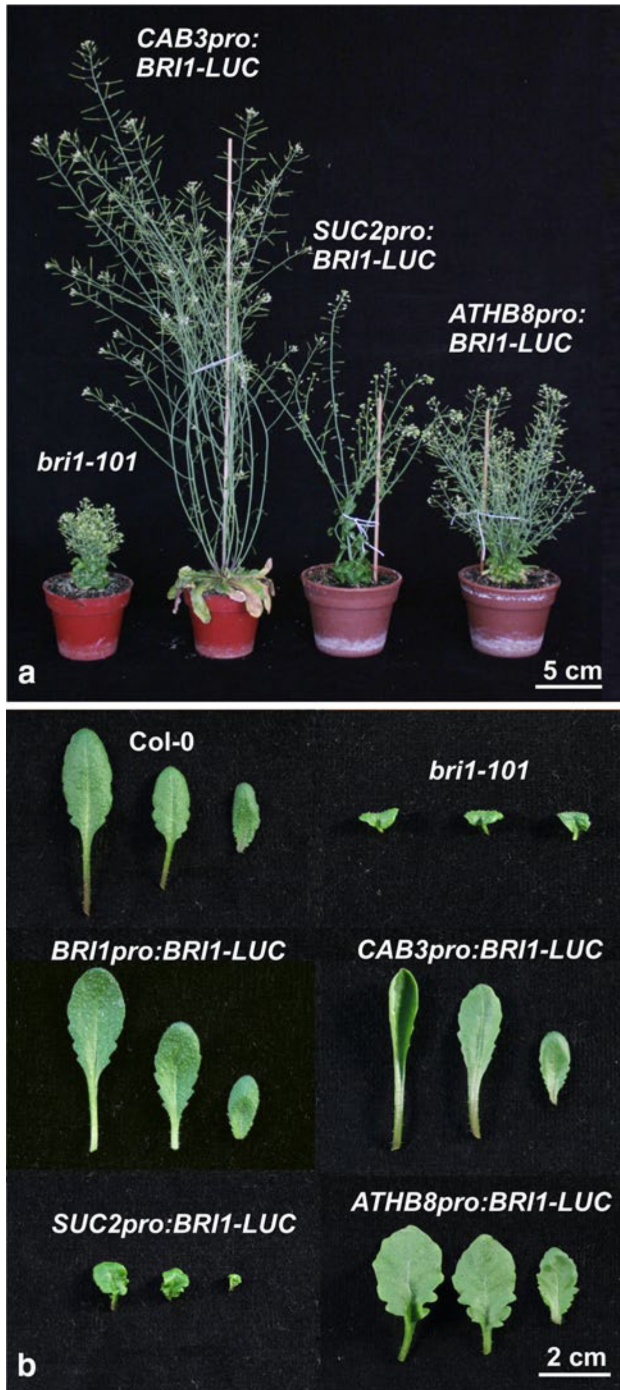

Fig. 8 Morphology of transgenic plants with ectopic expression of *BRI1*. **a** Mature, two-month-old plants. **b** Leaves isolated from six-week-old rosettes

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

Journal : Large 425 | Dispatch : 27-1-2014 | Pages : 13
Article No : 2031 | ☐ LE | ☐ TYPESET
MS Code : PLAA-D-13-00697 | ☑ CP | ☑ DISK

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Table 2 Inflorescence and silique development in *BRI1-LUC*-complemented lines

Arabidopsis line	Inflorescence height (mm)	Silique length (mm)	Seeds per silique
Col-0	396 ± 52	13.6 ± 0.9	40 ± 5
bri1-101	63 ± 9	6.5 ± 0.6	8 ± 3
BRI1pro:BRI1-LUC	388 ± 68	14.4 ± 0.8	41 ± 7
CAB3pro:BRI1-LUC	412 ± 75	12.9 ± 1.0	37 ± 7
SUC2pro:BRI1-LUC	227 ± 40	6.7 ± 0.8	27 ± 4
ATHB8pro:BRI1-LUC	149 ± 14	10.9 ± 0.8	38 ± 3

Data are mean values ± SD

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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 *BRI1* 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 BRI1 613 protein is approximately 5 h (Geldner et al. 2007), these 614 results suggest that light regulation of *BRI1* transcription 615 can be a means of modulating receptor abundance and BR 616 susceptibility.

617 The complex regulation of *BRI1* 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 *BRI1* gene activity inter- 621 feres with normal morphogenesis, we generated transgenic 622 lines expressing the receptor ectopically. We used *BRI1-* 623 *LUC* fusion, which allowed easy localization of the recep- 624 tor. The fusion protein contained the same linker peptide as 625 that of the chimeric BRI1-GFP (Friedrichsen et al. 2000), 626 in which the receptor function was not compromised. As 627 expected, the *BRI1pro:BRI1-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 *BRI1pro:BRI1-LUC*-complemented line, we found very 632 similar phenotype in the *CAB3pro:BRI1-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 BRI1-LUC fusion product is compara- 640 ble in the *CAB3pro:BRI1-LUC* and *BRI1pro:BRI1-LUC* 641 plants (Fig. 7b, c). The longer, hyponastic leaves of the 642 *CAB3pro:BRI1-LUC* line can be attributed to an enhanced 643 receptor production in the mesophyll cells, which seems to 644 result in a stronger BR-dependent elongation and expan- 645 sion at the abaxial side of the leaf blade. In contrast to the 646 *CAB3pro:BRI1-LUC* plants, only partial and dispropor- 647 tional complementation could be seen in the transgenic 648 lines that expressed BRI1 under the control of vascular 649 tissue-specific promoters. Whereas *SUC2pro:BRI1-LUC* 650 plants developed about threefold higher inflorescence than 651 *bri1-101*, the shape and size of its rosette leaves and the 652 length of its siliques did not appreciably differ from those 653 of the non-complemented mutant. Despite their similar 654 appearance, the seed production of the *SUC2pro:BRI1-* 655 *LUC* siliques was substantially higher than those of the 656 mutant (Fig. 8a, b; Table 2). The *ATHB8pro:BRI1-LUC* line 657 also showed reduced inflorescence height and leaf expan- 658 sion, but in this case the complementation was stronger in 659 the rosette leaves and weaker in the inflorescence stems. 660 Despite the shorter siliques, their seed number was roughly 661 equal to those of the wild type (Fig. 8a, b; Table 2). These 662 results, in agreement with the findings of Savaldi-Goldstein 663 et al. (2007), show that primarily vascular expression of 664 *BRI1* can only partially restore BR sensitivity in severe *bri1* 665 mutants. The observed phenotypic differences between 666 the *SUC2pro:BRI1-LUC* and *ATHB8pro:BRI1-LUC* plants 667 likely result from the differing activity and developmen- 668 tal regulation of the phloem-specific *SUC2* (Truernit and 669 Sauer 1995) and the strictly procambium-specific *ATHB8* 670 (Kang et al. 2003) promoters. Our results show that ectopic 671 expression of *BRI1* can severely disturb the development 672 of *Arabidopsis* plants, and that correct spatial and temporal 673 transcriptional control of the receptor gene is required for 674 the proper coordination of organ morphogenesis.

675 In addition to BR levels, the initiation of signalling by 676 this hormone also depends on the availability of BRI1 and 677 its SERK-type co-receptor. A transgenic line overexpress- 678 ing the BRI1-GFP fusion showed excess leaf elongation, 679 similar to that observed in BR overproducing plants, and 680 the BR-binding capacity of its microsome fractions was 681 found higher than that of the wild type (Wang et al. 2001). 682 Similar, but less pronounced enhancement of BR effects 683 could be observed when the co-receptor BAK1/SERK3 684 was overexpressed (Nam and Li 2002). This weaker effect

Journal : Large 425 | Dispatch : 27-1-2014 | Pages : 13
Article No : 2031 | ☐ LE | ☐ TYPESET
MS Code : PLAA-D-13-00697 | ☑ CP | ☑ DISK

685 and the semidwarf phenotype of the *bak1-1* null mutant (Li et al. 2002) can be attributed to the functional redundancy between BAK1/SERK3 and three other members of the SERK family (Gou et al. 2012) which, in addition to their role in the BR receptor complexes, also function as co-receptors in pathogen-induced defence signalling pathways (Roux et al. 2011). Although SERK co-receptors are indispensable for the initiation of BR signalling (Gou et al. 2012), the formation of active receptor complexes seems to be limited by the less abundant BRI1 component. Our results indicate that in *Arabidopsis BRI1* gene activity is under complex regulation, and that this transcriptional control has a role in determining the distribution of the receptor. The data of the complementation analyses support the notion that in BR insensitive background proper restoration of the wild phenotype requires *BRI1* promoter-specific differential regulation of the receptor gene.

We demonstrated that *BRI1* expression is under developmental, organ-specific and diurnal regulation. In addition, it is also controlled by phytohormones, as BRs can downregulate (Goda et al. 2002), whereas auxin can enhance the level of transcription (Nemhauser et al. 2004; Sakamoto et al. 2013). Thus, the activity of *BRI1* is determined in a complex way, similar to that of the key *BR* biosynthetic genes (Hategan et al. 2011; Zhao and Li 2012). This can allow optimal coordination of BR accumulation and susceptibility, and suggests that the differential regulation of receptor abundance is an important means of enhancing or attenuating physiological effects of the hormone.

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