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Relationship between brassinosteroids and genes controlling stomatal production in the *Arabidopsis* hypocotyl

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ABSTRACT Stomata are excellent model systems for examining the mechanisms that regulate cell fate determination and pattern formation. It has recently been demonstrated that brassinosteroids control stomatal development by regulating both the MAPK kinase kinase YODA and the basic helix-loop-helix transcriptional factor SPEECHLESS. Here, we show that these plant regulators positively regulate stomatal formation in the hypocotyl and also accelerate their development. Hormone tests, reporter gene studies and mutant analyses revealed that brassinosteroids act upstream of the transcriptional factors *CAPRICE* and *GLABRA2*. These plant regulators control an earlier stage of stomatal production than those regulated by the membrane receptor *TOO MANY MOUTHS*. This work highlights differences in the genetic control of stomatal development between cotyledons or leaves and hypocotyls.

KEY WORDS: *Arabidopsis thaliana, brassinosteroids, CAPRICE, GLABRA2, stomata*

Introduction

The plant epidermis provides an elegant model system that allows exploration of the mechanisms that regulate cell fate specification and patterning formation. In particular, the hypocotyl epidermis of *Arabidopsis thaliana* offers an excellent model system because of its rapid development and simplicity. It reaches maturity one week after germination and consists of two types of files that run parallel to the long axis of the organ (Gendreau *et al*., 1997; Berger *et al*., 1998). These include files consisting of non-protruding cells and located outside two cortical cell files, and files consisting of protruding cells that overlie a single cortical cell file. Stomata develop only in epidermal files located outside two cortical cell files (Berger *et al*., 1998; Hung *et al*., 1998). The first sign of stomatal development is a cell division along its longitudinal axis producing a small and often triangular or rectangular cell named meristemoid (Berger *et al*., 1998), which makes stomatal pathway initiation easy to detect. Meristemoids are self-renewing cells that undergo a probabilistic sequence of cell divisions before stomata formation (Berger *et al*., 1998).

Molecular genetic studies have defined a number of genes that influence stomata formation and patterning in the embryonic stem. The *GLABRA2* (*GL2*) gene, which encodes a homeodomain-leucine zipper protein (Rerie *et al*., 1994; Di Cristina *et al*., 1996), negatively regulates stomata formation in cell files located outside a single cortical cell file (Berger *et al*., 1998; Hung *et al*., 1998). *TRANSPARENT TESTA GLABRA* (*TTG*) encodes a small protein with WD40 repeats (Walker *et al*., 1999), and also represses the formation of ectopic stomata (Rerie *et al*., 1994; Hung *et al*., 1998). The *WEREWOLF* (*WER*) gene negatively regulates stomatal cell fate (Lee and Schiefelbein, 1999). *WER* encodes a R2R3MYB protein that, like *GL2* (Hung *et al*., 1998), is preferentially expressed in epidermal cell files located outside a single cortical cell file (Lee and Schiefelbein, 1999). The *TTG* expression pattern remains unknown. Yeast two-hybrid assays have shown that both TTG and WER proteins physically associate with two BHLH proteins GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) (Payne *et al*., 2000; Bernhardt *et al*., 2003; Zhang *et al*., 2003). These BHLH genes redundantly repress the formation of stomata in files overlying a single cortical cell file (Bernhardt *et al*., 2005). Moreover, *TTG* and *WER* are positive regulators of the *GL2* gene (Hung *et al*., 1998; Lee and Schiefelbein, 1999).

In addition to these repressors of stomata formation, positive regulators have been identified. *CAPRICE* (*CPC*) and *TRIPTYCHON* (*TRY*), both encoding R3MYB proteins (Wada *et al*., 1997; Schellmann *et al*., 2002), redundantly promote stomatal formation (Serna, 2008). Like *WER* and *GL2*, *CPC* (and probably *TRY*) is expressed in files characterized by an absence of stomata (Kirik *et al*., 2004; Serna, 2008). CPC (and probably TRY) moves to

Abbreviations used in this paper: BL, epibrassinolide; CPC, CAPRICE; GL2, GLABRA2; TAF, triadimefon; TMM, TOO MANY MOUTHS.

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stomata-forming cell files where it then acts (Serna, 2008). Like WER, CPC and TRY also associate with both GL3 and EGL3 (Zhang *et al.,* 2003). These findings support the inference that a multimeric complex consisting of WER, TTG, GL3, and/or EGL3 represses stomata formation by promoting *GL2* transcription in epidermal cell files overlying a single cortical cell file (Larkin *et al.*, 2003; Schiefelbein, 2003; Serna, 2004a; Serna, 2004b). Both stomata development and *GL2* expression repression in epidermal cell files located above two cortical cell files depend on the sequestration of GL3 and EGL3 by CPC (and/or TRY), inducing the formation of a complex consisting of CPC (and/or TRY), TTG, GL3, and/or EGL3 (Serna, 2008). *CPC* and *TRY* are not the only positive regulators of stomatal cell fate in the embryonic stem. The *TOO MANY MOUTHS* (*TMM*) gene, which encodes a leucin-rich repeat receptor-like protein without a cytoplasmic domain (Nadeau and Sack, 2002), also promotes stomatal formation (Geisler *et al.*, 1998). This gene controls stomatal progression from meristemoids, but is not essential for entry into the stomatal pathway (Bhave *et al.*, 2009).

It is known that the regulation of stomatal formation in the hypocotyl is controlled by hormones. Stomata development is induced by gibberellins (Saibo *et al.*, 2003). Furthermore, the effect of gibberellins is enhanced by ethylene and auxins (Saibo *et al.*, 2003). In addition, brassinosteroids control stomatal development by regulating both the MAPK kinase kinase YODA and the basic helix-loop-helix transcriptional factor SPEECHLESS (Kim *et al.,* 2012; Gudesblat *et al.*, 2012). Analysis of global transcriptome of *Arabidopsis* seedlings has shown that brassinosteroids induce *WER* expression (Nemhauser *et al.*, 2004), which suggests some relationship between these plant regulators and genes controlling stomatal production. Here, we explore the role of brassinosteroids in stomatal development in the hypocotyl, and their hypothetical interactions with genes regulating this process.

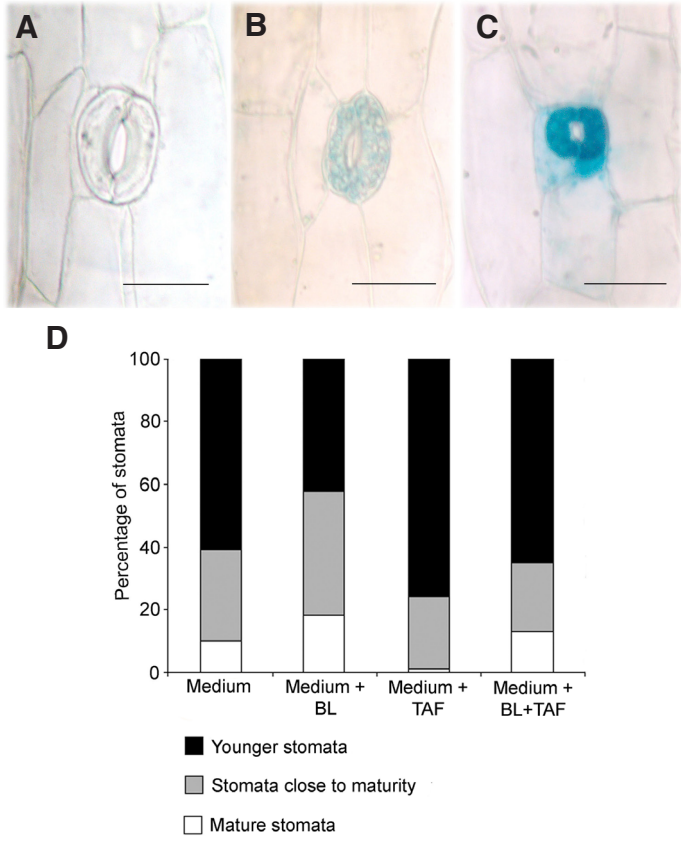

Fig. 1. Stomatal maturation and *TMM (TOO MANY MOUTHS)* expression in response to brassinosteroids. (A-C) *Transgenic plants expressing* GUS *under control of the* TMM *showing* **(A)** *mature stomata,* **(B)** *stomata closer to maturity and* **(C)** *younger stomata.* **(D)** *Effect of brassinosteroids on stomatal maturation. BL, epibrassinolide; TAF, triadimefon. Scale bars: 20 μm in (A-C).*

Results

Brassinosteroids promote stomatal formation in the hypocotyl

Plants treated with 0.5 μM epibrassinolide exhibited a statistically significant increase in number of stomata on the hypocotyl relative to untreated plants (Table 1). Plants treated with 1 μM triadimefon, which inhibits biosynthesis of brassinosteroids and gibberellins (Buchenauer and Rohner, 1981; Asami *et al.*, 2003), showed a small reduction in the number of stomata, with the exception of Col-0 (Table 1). This reduction was counteracted by the co-application of 0.5 μM epibrassinolide, confirming that this plant regulator promotes stomata formation in the hypocotyl (Table 1). The position of stomata relative to cortical tissue did not seem to change with growth conditions, as stomata developed in files overlying two cortical cell files (Table 1).

Two mutants, *brassinosteroid insensitive1* (*bri1*) and *deetiolated2* (*det2*), which were affected in the perception and synthesis of brassinosteroids, respectively (Li *et al.*, 1996; Wang *et al.*, 2001),

TABLE 1

NUMBER OF STOMATA IN THE HYPOCOTYL OF WILD-TYPE AND MUTANT SEEDLINGS GROWN UNDER DIFFERENT CONDITIONS

	Col-0	Ws-2	Col	*bri1-1* (in Col-0)	*bri1-4* (in Ws-2)	*det2-1* (in Col)	*pBRI1:BRI1:GFP* (in *bri1-1*)	*pBRI1:BRI1:GFP* (in *bri1-4*)
Medium	19.0±5.6 (93.3)	30.0±5.4 (93.3)	17.0±3.7 (93.5)	2.0±1.4[a] (92.2)	0.0±0.0[a]	9.0±4.1[a] (93.3)	19.8±4.7 (93.3)	29.4±5.3 (92.2)
Medium + BL (0.5 μM)	41.0±9.3[b] (93.3)	40.0±9.0[b] (100)	25.6±5.7[b] (93.3)	2.0±1.8[a] (93.5)	0.0±0.0[a]	23.0±9.9[ab] (88.3)	39.7±5.9[b] (93.3)	40.5±5.1[b] (93.3)
Medium + TAF (1 μM)	20.0±5.7 (88.3)	23.0±6.2[b] (100)	11.0±4.6[b] (92.2)	2.0±1.6[a] (93.5)	0.0±0.0[a]	8.0±3.2[a] (88.3)	18.3±3.7 (92.2)	25.8±4.6 (93.3)
Medium + BL (0.5 μM) + TAF (1 μM)	38.7±6.7[b] (93.3)	37.0±7.6[b] (93.3)	25.3±5.8[b] (93.5)	3.0±2.3[a] (93.5)	0.0±0.0[a]	27.0±8.9[ab] (88.3)	39.9±3.1[b] (88.3)	37.5±4.1[b] (93.5)

[a]p>0.05 relative to the corresponding wild-type line. [b]p>0.05 relative to untreated-plants.Values indicate mean ± SD of at least 15 seedlings for each line. The percentage of non-ectopic stomata is indicated between brackets. BL, epibrassinolide; TAF, triadimefon.

were chosen for further characterization of brassinosteroid effects on stomatal production in the hypocotyl. Seedlings with null *BR1* mutations did not develop stomata (*bri1-4*) or develop an insignificant number of them (*bri1-1*), and those with *DET2* mutations (*det2-1*) displayed a strong reduction in the number of stomata in comparison to untreated wild-type plants (Table 1). As expected, both *bri1-1* and *bri1-4* mutants showed absolute insensitivity to the different treatments, whereas *det2-1* exhibited an increase in the number of stomata in response to the application of exogenous epibrassinolide (Table 1). The fact that both *bri1-1/bri1-4* mutations block stomatal production but triadimefon only reduces it (Table 1), might suggest that brassinosteroids-regulated determinants of cell fate specification in the hypocotyl epidermis are active during embryogenesis.

In all the mutants, the position of the stomata relative to the cortical tissue was similar to that of wild-type plants (Table 1). As in wild-type seedlings, stomata of these mutants were located in the upper 2/3 of the hypocotyl. Plants with the translation fusion consisting of a *GFP*-tagged *BRI1* construct driven by the 1.7 kb *BRI1* upstream region (*pBRI1:BRI1:GFP*; Friedrichsen *et al.*, 2000) were crossed into the *bri1-1* and *bri1-4* mutants, which rescued the defects induced by the mutations (Table 1). Taken together, these results show that, as expected, both *BRI1* and *DET2* promote stomata formation in the hypocotyl.

Brassinosteroids accelerate stomatal formation

TMM is expressed in stomatal precursors and in younger neighbour cells (Nadeau and Sack, 2002). Thus, *TTM* expression can be used to identify the level of maturity of the stomata. Three levels of maturity were established: 1) mature stomata (without *TMM* expression); 2) stomata close to maturity (*TMM* expression in guard cells); and 3) young stomata (*TMM* expression in guard cells and in at least one neighbour cell) (Fig. 1 A-C). When plants were grown in the presence of 0.5 μM epibrassinolide, the number of mature stomata (and those close to maturity) in the developing hypocotyl increased relative to that of untreated plants (Fig. 1D). Plants treated with 1 μM triadimefon exhibited a reduction in numbers of both fully developed stomata and stomata closer to maturity (Fig. 1D). This reduction was counteracted by co-application of 0.5 μM epibrassinolide (Fig. 1D). These results indicate that brassinosteroids, in addition to promoting stomatal formation, accelerate stomatal development.

Brassinosteroids do not reverse the lack of stomata in the tmm-1 *hypocotyl*

The *tmm* hypocotyl has virtually no stomata, but arrested meristemoids (Bhave *et al.*, 2009). To determine whether brassinosteroids suppress this *tmm* dependent defect, *tmm-1* seedlings were grown on media supplemented with 0.5 μM epibrassinolide. The phytohormone, at a concentration of 0.5 μM, was able to induce stomatal production in wild-type plants (Fig. 2A). The *tmm-1* mutant treated in this manner did not develop stomata, but it increased its number of meristemoids (Fig. 2B). Both wild-type and *tmm-1* seedlings treated with 0.5 μM epibrassinolide exhibited elongated hypocotyls (Fig. 2C). These results support that brassinosteroids promote stomatal pathway initiation, and that *TMM* is required for meristemoids progression. Consistent with these results, *bri1-1*, *bri1-4*, *det2-1* mutants and triadimefon-treated wild type plants have not arrested meristemoids like those found in *tmm-1* mutant.

Brassinosteroids control GL2 *and* CPC *expression*

The hypothetical effect of brassinosteroids on expression of genes in the *MYBs-TTG-BHLHs-GL2* network was determined. The expression of two genes in this network with opposite functions was studied: *GL2*, a repressor of stomatal development (Berger *et al.*, 1998; Hung *et al.*, 1998), and *CPC*, a positive regulator of this process (Serna, 2008). In wild-type plants harbouring the *GL2:GUS* construct, GUS activity has been preferentially detected in the upper portion of non-stomata-forming cell files (Hung *et al.*, 1998). The pattern of *GFP* expression under the control of the *GL2* promoter in plants grown in the presence of 0.5 μM epibrassinolide (Fig. 3B) was indistinguishable from that of untreated control plants (Fig. 3A). However, the application of 1 μM triadimefon disrupted the position-dependent pattern of *GFP* expression, leading to a largely random epidermal pattern of expression, with cells in both stomatal forming cell files and non-stomatal forming ones showing *GFP* expression (Fig. 3C). Co-application of 0.5 μM epibrassinolide triggered a *GFP* expression pattern similar to that of untreated plants (Fig. 3D), confirming that the random epidermal pattern of expression is due to inhibition of the synthesis of brassinosteroids and not gibberellins. Taken together, these results indicate that brassinosteroids both prevent *GL2* promoter induction in stomata-forming cell files and promote it in non-stomata forming ones.

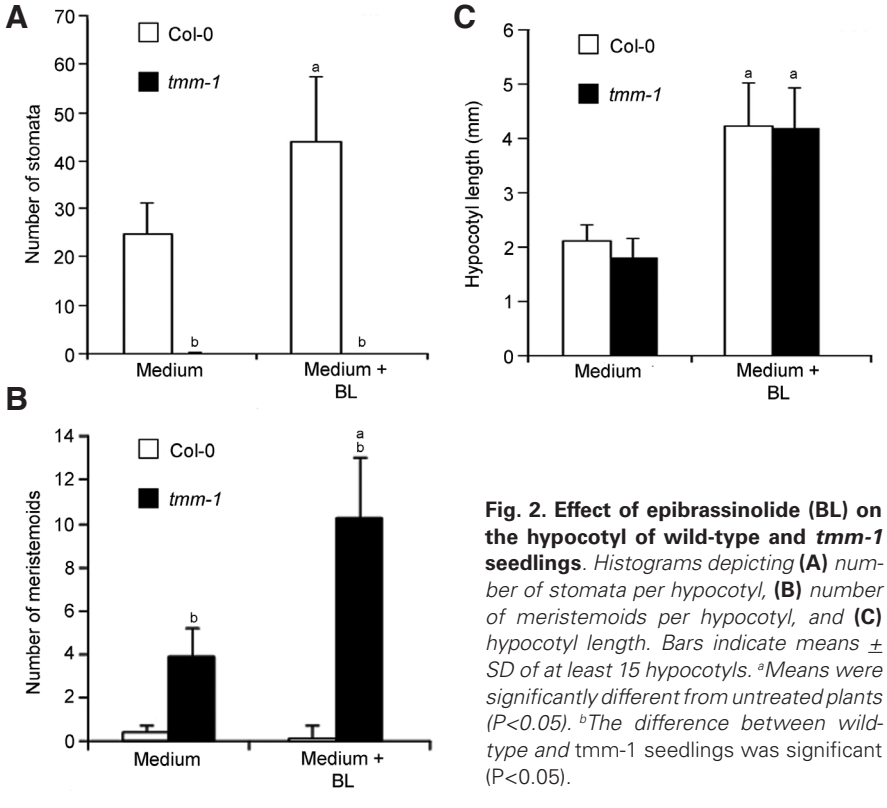

Fig. 2. Effect of epibrassinolide (BL) on the hypocotyl of wild-type and *tmm-1* seedlings. *Histograms depicting* **(A)** *number of stomata per hypocotyl,* **(B)** *number of meristemoids per hypocotyl, and* **(C)** *hypocotyl length. Bars indicate means ± SD of at least 15 hypocotyls.* [a]*Means were significantly different from untreated plants (P<0.05).* [b]*The difference between wild-type and* tmm-1 seedlings was significant (P<0.05).

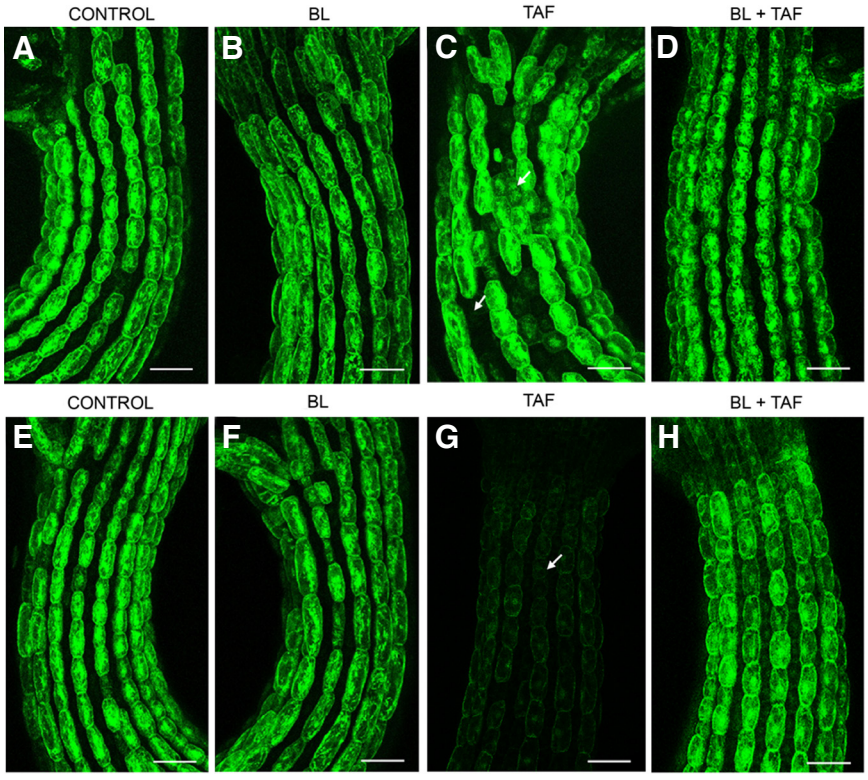

Fig. 3. Brassinosteroid effect on both *GL2* and *CPC* promoter induction in the hypocotyl epidermis. (A-D) *Pattern of* GFP *expression driven by the* GL2 *promoter.* **(E-H)** *Expression of* GFP *under the control of the* CPC *promoter.* **(C)** *Random* GL2:GFP *expression and* **(G)** *strong reduction of* CPC:GFP *expression is indicated by arrows. BL, epibrassinolide; TAF, triadimefon. Scale bars: 150 μm in (A-H).*

GUS activity, in plants harbouring the *CPC:GUS* construct, has also been preferentially detected in non-stomata-forming cell files (Kirik *et al.*, 2004; Serna, 2008). In plants grown in the presence of 0.5 μM epibrassinolide, *CPC:GFP* expression was identical to that of untreated plants (Fig. 3 E,F). A strong reduction of *CPC:GFP* expression was observed in plants treated with 1 μM triadimefon (Fig. 3G), which was restored by co-application of 0.5 μM epibrassinolide (Fig. 3H). These results indicate that brassinosteroids positively regulate *CPC* promoter induction in non-stomata-forming cell files.

Brassinosteroids do not reverse the low number of stomata in the try cpc *hypocotyl*

The *gl2* mutation (*gl2-1*) increases the number of stomata, with many of them being located in epidermal files overlying a single cortical cell file (Berger *et al.*, 1998; Table 2). The *gl2-1* mutant responded to the treatments, increasing stomatal production in response to brassinosteroids and decreasing it in response to triadimefon (Table 2). The number of stomata of *gl2-1* in all growth conditions, with the exception of mediums supplemented with both epibrassinolide and triadimefon, was higher than that of wild-type seedlings given its particular ability to produce ectopic stomata (Table 2).

CPC also controls stomata formation in the embryonic stem, with the *cpc* mutant displaying a reduced number of stomata in comparison to wild-type seedlings (Serna, 2008; Table 2). Interestingly, the *cpc* mutant increased stomatal production in response to epibrassinolide (Table 2). However, *try cpc* had a low number of stomata under all growth conditions showing absolute insensitivity to the different treatments (Table 2). The triple mutant *try cpc det2-1* phenocopied the very low number of stomata of *try cpc* (Table 2). In addition, it also exhibited insensitivity to the phytohormone brassinosteroid, almost blocking stomatal formation under all growth conditions (Table 2).

Discussion

The responses of *bri1-1*, *bri1-4* and *det2-1* mutants to brassinosteroids are consistent with the general effect of these phytohormones to promote stomatal production in the hypocotyl. Both *bri1-1* and *bri1-4* mutants showed absolute insensitivity to the various treatments and *det2-1* had elevated numbers of stomata in response to epibrassinolide. Consistent with these results, the number of stomata in the hypocotyls of *bri1-116*, *cpd* (unable to synthesize brassinosteroids) and of the gain-of function mutant *bin2-1* (*BIN2* represses brassinosteroids signalling) was strongly reduced, and this number was increased in *bin2-3 atsk22 atsk23*, *bin2-3* (*ATSK22*, *ATSK23* and *BIN2* act in a redundant manner) and in plants overexpressing the brassinosteroid biosynthetic gene *DWARF4* (Gudesblat *et al.*, 2012). Interestingly, mutations

TABLE 2

NUMBER OF STOMATA IN THE HYPOCOTYL OF WILD-TYPE AND MUTANT SEEDLINGS GROWN GROWN UNDER DIFFERENT CONDITIONS

	Ws-2	L*er*	*cpc* (in Ws-2)	*try cpc* (in L*er*)	*gl2-1* (in L*er*)	*det2-1 try cpc*
Medium	30.0±5.4 (93.3)	19.0±5.1 (96.7)	14.0±2.3[a] (93.3)	1.0±2.8[a] (90.0)	32.0±8.4[a] (73.3)	1.0±2.9 (89.5)
Medium + BL (0.5 μM)	40.0±9.0[b] (100)	34.0±7.3[b] (93.3)	25.0±9.0[ab] (90.0)	2.0±4.1[a] (89.5)	47.0±10.0[ab] (70.0)	2.0±4.3 (90.0)
Medium + TAF (1 μM)	23.0±6.2[b] (100)	13.0±5.5[b] (100)	14.0±4.0[a] (90.0)	2.0±4.3[a] (90.0)	23.0±6.7[ab] (76.7)	1.0±2.8 (90.0)
Medium + BL (0.5 μM) + TAF (1 μM)	37.0±7.6[b] (93.3)	37.0±6.1[b] (96.7)	29.0±8.7[ab] (90.0)	1.0±2.9[a] (94.1)	32.0±6.1 (63.4)	1.0±2.9 (93.3)

[a]p>0.05 relative to the corresponding wild-type line. [b]p>0.05 relative to untreated plants. Values indicate mean ± SD of at least 15 seedlings for each line. The percentage of non-ectopic stomata is indicated between brackets. BL, epibrassinolide; TAF, triadimefon.

in the downstream targets of *BIN2*, *BZR* and *BES1*, do not disrupt stomatal formation in the hypocotyl, suggesting that *BIN2* control stomatal development by regulation of other targets (Gudesblat *et al*., 2012). In addition, the brassinosteroid-insensitive quadruple mutant *bsu-q* exhibits stomatal clusters (Kim *et al*., 2012).

Epibrassinolide did not reverse the lack of stomata in the *tmm-1* hypocotyl, but it increased the number of meristemoids, which supports that brassinosteroids promote stomata pathway initiation, and *TMM* is required in later stages for meristemoids progression. TMM signalling cascade leads to SPCH inactivation, which is required for early stages of stomatal development (Pillitteri and Torii, 2012). The fact that epibrassinolide increases meristemoids production in *tmm-1*, indicates that these plant regulators do not play upstream of *TMM* regulating stomatal initiation.

Hormone tests, reporter gene studies, and mutant analyses have increased our understanding of the potential interactions between brassinosteroids and the *MYBs-BHLHs-TTG-GL2* network during stomatal formation in the hypocotyl. Supplementing medium with epibrassinolide did not affect the *GL2* promoter induction pattern (in which the *GL2* promoter is preferentially induced in non-stomata forming cell files) or the stomatal pattern (in which most stomata are located in files overlying two cortical cell files). However, growth of plants in the presence of triadimefon resulted in a random *GL2* promoter induction and in a reduction in the number of stomata. Similar to the *GL2* promoter, the *CPC* promoter pattern did not change when plants were grown in the presence of epibrassinolide. However, triadimefon reduced *CPC* promoter induction, which agrees with the insensitivity to the inhibitor displayed by the *cpc* mutant. These results suggest that inhibition of brassinosteroids synthesis represses stomata formation in part by repressing *CPC* expression. Genetic redundancy between *TRY* and *CPC*, which show high sequence similarity, might explain the sensitivity of the *cpc* mutant to epibrassinolide. Consistent with this inference, *try cpc* did not respond to the phytohormone. Interestingly, *gl2-1* responded to the treatments, suggesting that other genes might be masking this absence of *gl2-1* response. The fact that the triple mutant *try cpc det2-1* phenocopied the near absence of stomata of *try cpc,* exhibiting also insensitivity to the phytohormone, supports that brassinosteroids act upstream of *CPC* and *TRY*. These results suggest that brassinosteroids control stomatal cell fate through regulation of both *GL2* and *CPC* expression. In contrast to our interpretation, and based on the number of cells per file, Gudesblat *et al.,* (2012) have just proposed that brassinosteroids do not affect cell fate in the hypocotyl epidermis. It is then likely that brassinosteroids act a two different levels on stomatal development in the hypocotyl. On one hand, brassinosteroids regulate the repressive cascade that inactivates the transcriptional factor SPCH necessary for stomata formation (Gudesblat *et al*., 2012). On the other, they control cell patterning specification through the regulation of the expression of genes playing in the MYBs-TTG-BHLHs-GL2 network. How these two networks might interact is an open question.

Similar to stomata, root hairs develop in files located outside two cortical cell files (Dolan *et al*., 1993; Dolan *et al*., 1994; Galway *et al*., 1994). In addition, a number of experimental studies have shown that the same MYBs-BHLHs-TTG-GL2 network controls epidermal cell fate in both organs (Larkin *et al*., 2003; Schiefelbein *et al*., 2003; Serna 2004a; Serna, 2004b). In roots, brassinosteroids also promote root hair formation, acting upstream of the MYBs-BHLHs-TTG-GL2 network (Kuppusamy *et al*., 2009). Similar to what occurs in the hypocotyl epidermis, the loss of brassinosteroid signalling in the root results in random patterns of *GL2* expression and in a strong reduction of *CPC* expression (Kuppusamy *et al*., 2009). The MYBs-BHLHs-TTG-GL2 network does not seem to play a role in stomata formation in the cotyledon (and leaf) as suggested by the fact that mutations in *TTG* or *GL2* do not induce an apparent phenotype (Berger *et al*., 1998). However, brassinosteroids control stomatal production in cotyledons and leaves (Gudesblat *et al.,* 2012; Kim *et al*., 2012), although their specific role need clarification. Genetic and biochemical analyses have demonstrated that these plant regulators inhibit stomatal development in the cotyledons through the GSK3-like BIN2 kinase, which phosphorylates and inactivates the stomatal MAPK kinase kinase YDA, acting downstream of the *ERECTA* family of receptor-like kinases and of the receptor protein *TMM* (Kim *et al*., 2012). In contrast to these results, Gudesblat *et al.,* (2012) have shown that brassinosteroids promote stomatal formation in the leaves and cotyledons by inhibition of BIN2-mediated phosphorilation of SPCH. What seems certain is that while the genetic control of the production of stomata in the leaves (or cotyledons) differs from that of the hypocotyl, brassinosteroids control the stomatal formation in both organs.

Materials and Methods

Plant material and growth conditions

Mutants and transgenic plants used in this work included *bri1-1* (N3723), *bri1-4* (N3953), *det2-1* (N6159), *tmm-1* (N6140), *gl2-1* (N65), *cpc*, *try cpc*, *GL2:GFP* (N8851), *CPC:GFP*, *TMM:GUS:GFP*, and *pBRI1:BRI1:GFP*. The wild-type strains included Wassilewskija (Ws-2), Columbia (Col), Columbia (Col-0) and Landsberg *erecta* (L*er*).

Seeds were vernalized at 4ºC for several days and surface-sterilized in 5% sodium hypochlorite. They were sown on 1% agar-solidified medium containing Murashige and Skoog salts (Sigma) and 1% sucrose with or without epibrassinolide and/or triadimefon. Seedlings were germinated and grown on horizontally oriented dishes at 22º in light (16-hours-light/8-hours-dark cycle).

Characterization of epidermal features

To determine the total number of stomata (or meristemoids) per hypocotyl, 2-week-old seedlings were mounted on slides, examined, and digitized under Nomarsky optics. Localization of stomatal cells relative to underlying cortical cells was determined from two randomly selected stomata per hypocotyl (15 seedlings) by focusing alternatively on both epidermal and cortical focal planes. Stomata in epidermal files making contact with a single cortical cell file were defined as ectopics and those in files that contact two cortical cell files as non-ectopics (Berger *et al*., 1998; Hung *et al*., 1998).

GUS staining

For histochemical analysis of *GUS* expression, 3-day-old seedlings harbouring the transgenes were assayed for GUS activity as previously described (Jefferson *et al*., 1987). After histochemical GUS assays, seedlings were washed in graded ethanol to remove chlorophyll and enhance contrast. All samples were examined by light microscopy with a Leica DC 300F camera attached to a Leica MZ6 stereomicroscope and to a Leica DMIRB inverted microscope. Images were acquired with the Leica Application Suite and processed with Adobe Photoshop CS3 (Adobe).

GFP imaging

GFP fluorescence from 3-day-old seedlings was monitorized with a DMIRB inverted Leica TCS SP2 confocal microscope using a 488 nm laser line of an Argon laser. For visualization of GFP, the emission window was set at 500-525 nm. GFP fluorescence was performed using a 40x/NA

1.25-0.75 PL APO (oil immersion) objective. Images were recorded with picture size of 1024 x 1024 pixels. They were arranged and labelled using Adobe Photoshop 6.0.

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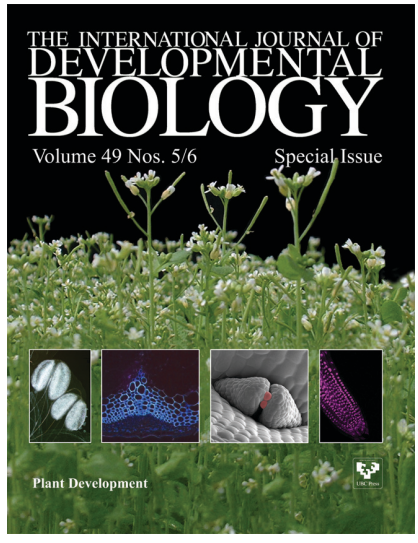

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