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Interactions among gibberellins, brassinosteroids and genes regulate stomatal development in the *Arabidopsis* hypocotyl

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ABSTRACT Stomata are pores on the plant surface that enable gas exchange with the atmosphere. In *Arabidopsis thaliana*, brassinosteroids, which function upstream of the *TTG/bHLHs/MYBs/GL2* transcriptional network, positively regulate stomatal formation in the hypocotyl. Gibberellins also promote stomatal development in the embryonic stem. Here, we investigated the hypothetical interactions between the gibberellin and brassinosteroid signaling pathways during stomatal formation. By combining genetics and gene expression studies, we found that gibberellins promote stomatal development, functioning upstream of both brassinosteroids and the *TTG/bHLHs/MYBs/GL2* network. Gibberellins also regulate an earlier step in stomatal development than that regulated by the receptor-like membrane protein TOO MANY MOUTHS.

KEY WORDS: *Arabidopsis thaliana, brassinosteroid, gibberellin, stomata*

The simplicity of the hypocotyl epidermis of *Arabidopsis Thaliana* makes it an excellent model system for studying cell fate specification and patterning formation. This embryonic organ consists of only two types of epidermal cell files: non-stomata-forming cells and stomata-forming cells. Files devoid of stomata overlie a single cortical cell file, while their neighboring stomatal-forming files are located outside of two cortical cell files (Berger *et al*., 1998; Hung *et al*., 1998). In stomatal-forming files, stomatal development starts with an asymmetric division, which is parallel to the longitudinal axis of the cell (Berger *et al*., 1998). The smaller cell produced by this cell division, the meristemoid, is a self-renewing cell that undergoes a probabilistic sequence of cell divisions before producing the stoma (Berger *et al*., 1998). Beyond providing a framework for the study of a fundamental developmental mechanism, stomata are major components of the terrestrial water and carbon cycles.

Many of the genes controlling stomatal formation in the *Arabidopsis* hypocotyl encode transcription factors that are assembled into a multimeric complex. This complex, which consists of a protein with WD40 repeats named TRANSPARENT TESTA GLABRA (TTG), the R2R3MYB protein WEREWOLF, and two basic helix-loop-helix (bHLH) proteins known as GLABRA3 and ENHANCER OF GLABRA3, represses the formation of stomata in files overlying a single cortical cell file (Larkin *et al*., 2003; Schiefelbein, 2003; Serna, 2004 a; Serna, 2004 b). The function of this complex depends on its ability to induce the transcription of *GLABRA2* (*GL2*), which encodes a homeodomain-leucine zipper protein (Rerie *et al*., 1994; Di Cristina *et al*., 1996), in epidermal cell files overlying a single cortical cell file (Larkin *et al*., 2003; Schiefelbein, 2003; Serna, 2004 a; Serna, 2004 b). Both stomatal development and the repression of *GL2* expression depend on the sequestration of GLABRA3 and ENHANCER OF GLABRA3 by the R3MYB proteins CAPRICE (CPC) and/or TRIPTYCHON (TRY) (Serna, 2008). This event promotes the formation of a complex consisting of CPC (and/or TRY), TTG, GLABRA3, and ENHANCER OF GLABRA3 (Serna, 2008). To form this complex, *CPC* (and probably *TRY*), which is expressed in files characterized by the absence of stomata (Kirik *et al*., 2004; Serna, 2008), moves to the stomatal-forming cell files (Serna, 2008). In addition to this network, *TOO MANY MOUTHS* (*TMM*), which encodes a leucine-rich repeat receptor-like protein without a cytoplasmic domain (Nadeau and Sack, 2002), also controls stomatal formation (Geisler *et al*., 1998). This gene regulates later stages than that regulated by the *TTG/bHLHs/MYBs/GL2* network, promoting stomatal formation from the meristemoids (Bhave *et al*., 2009).

Phytohormones also regulate this process. Both brassinosteroids (Fuentes *et al*., 2012; Gudesblat *et al*., 2012) and gibberellins (Saibo *et al*., 2003) positively regulate stomatal formation, with ethylene and auxin modulating the action of gibberellins (Saibo *et*

Abbreviations used in this paper: CPC, CAPRICE; GFP, GREEN FLUORESCENT PROTEIN; GL2, GLABRA2; TMM, TOO MANY MOUTHS.

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al., 2003). In addition, brassinosteroids act upstream of the *TTG/MYBs/bHLHs/GL2* network to promote stomatal production in the *Arabidopsis* hypocotyl (Fuentes *et al.*, 2012).

Many biological processes result from crosstalk among different hormonal signaling pathways. They include, for example, the integration of the primary signaling pathways of auxin and brassinosteroids by auxin response factor 2 (Vert *et al.*, 2008), or the crosstalk between auxin and cytokinins and their antagonistic effects on plant immunity (Naseem *et al.*, 2015). Brassinosteroid-activated BRASSINAZOLE RESISTANT1 (BZR1) and gibberellic acid-inactivated DELLA transcriptional regulators mediate also crosstalk between brassinosteroid and gibberellin pathways to regulate plant growth (Bai *et al.*, 2012; Gallego-Bartolomé *al.*, 2012; Li *et al.*, 2012). Here, we investigate whether brassinosteroids and gibberellins act also in the same signaling pathway during stomatal development in the hypocotyl. We also delve into the interaction between the gibberellin signaling pathway and both the *TTG/bHLHs/MYBs/GL2* transcriptional network and *TMM*.

Results

Brassinosteroids act downstream of gibberellins

Brassinosteroids and gibberellins promote stomatal formation in the hypocotyl (Saibo *et al.*, 2003; Fuentes *et al.*, 2012; Gudesblat *et al.*, 2012; Table 1). In the current study, both epibrassinolide (0.5 μM) and gibberellic acid (10 μM) treatment increased the number of stomata per hypocotyl and the stomatal index in wild-type seedlings. In addition, the reduction in the endogenous production of these regulators in wild-type seedlings through treatment with 0.5 μM paclobutrazol, which inhibits gibberellin biosynthesis (Rademacher, 2000), or 1 μM triadimefon, which induces brassinosteroid deficiency-like phenotypes by binding to DWF4 (which is involved in brassinosteroid biosynthesis) (Asami *et al.*, 2003), reduced stomatal production. These results indicate that the endogenous levels of these regulators are physiologically active.

As expected, mutants affected in gibberellin (*gid1a-2 gid1b-3 gid1c-1*) or brassinosteroid perception (*bri1-1*) did not develop stomata. Treatment of the *gid1a-2 gid1b-3 gid1c-1* triple mutant with epibrassinolide (0.5 μM) partially rescued both the number of stomata per hypocotyl and the stomatal index of this mutant (Table 1). This result suggests that brassinosteroids act downstream of gibberellins. In agreement with this interpretation, application of 10 μM gibberellic acid to *bri1-1* failed to rescue its stomatal phenotype (Table 1). Also as expected, stomatal production in wild-type seedlings grown on medium supplemented with 0.5 μM epibrassinolide and 0.5 μM paclobutrazol was higher than that in plants grown on medium supplemented only with paclobutrazol (0.5 μM) (Table 1). The stomatal production of wild-type seedlings on medium supplemented with 10 μM gibberellic acid and 1 μM triadimefon was also higher than that of plants grown on medium supplemented only with triadimefon (1 μM) (Table 1). Under all growth conditions, stomata were located mainly in epidermal

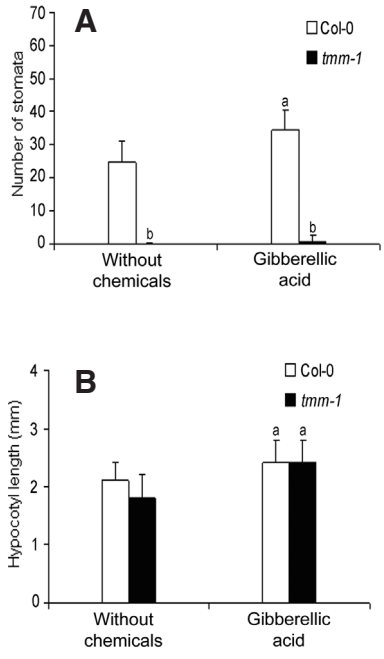

Fig. 1. Effect of gibberellic acid on the hypocotyls of wild-type and *tmm-1* seedlings. (A) *Number of stomata and* **(B)** *hypocotyl length of two-week-old wild-type plants and tmm-1 grown on media supplemented with 10 μM gibberellic acid. Without chemicals denotes growth medium without added gibberellic acid. Values are mean e SD (n ≥ 15).* [a]*Means are significantly different from those of plants grown without added gibberellic acid (p < 0.05).* [b]*The difference between wild-type and* tmm-1 *seedlings is significant (p < 0.05). The Student t-test was used to generate p values.*

TABLE 1

NUMBER OF STOMATA IN THE HYPOCOTYLS OF WILD-TYPE AND MUTANT SEEDLINGS GROWN WITH OR WITHOUT CHEMICAL TREATMENTS

	Col-0		*gid1a-2 gid1b-3 gid1c-1* (in Col-0)		*bri1-1* (in Col-0)	
	Number of stomata per hypocotyl	Stomatal index	Number of stomata per hypocotyl	Stomatal index	Number of stomata per hypocotyl	Stomatal index
Without chemical treatment	20.8±4.9 (93.3)	8.6±2.7	0.0±0.0[a]	0.0±0.0[a]	0.3±0.5[a] (100)	0.6±1.8[a]
Epibrassinolide (0.5 μM)	39±6.6[b] (100.0)	12.2±1.7[b]	6.7±3.1[a,b] (100)	6.3±0.4[a,b]	ND	ND
Gibberellic acid (10 μM)	38.3±6.6[b] (100)	12.8±2.8[b]	ND	ND	0.3±0.5[a] (100)	1.7±2.6[a]
Paclobutrazol (0.5 μM)	7.7±3.7[b] (86.7)	6.5±1.2[b]	ND	ND	ND	ND
Triadimefon (1 μM)	15.2±4.3[b] (93.3)	6.5±2.5[b]	ND	ND	ND	ND
Epibrassinolide (0.5 μM) + Paclobutrazol (0.5 μM)	12.7±3.8[b,c] (90.7)	9.9±1.6[c]	ND	ND	ND	ND
Gibberellic acid (10 μM) + Triadimefon (1 μM)	26.6±7.0[b,d] (93.3)	10.6±2.7[d]	ND	ND	ND	ND

[a]p > 0.05 relative to the corresponding wild-type line. [b]p > 0.05 relative to untreated plants. [c]Difference between plants grown in medium supplemented with paclobutrazol versus epibrassinolide + paclobutrazol (p > 0.05). [d]Difference between plants grown in medium supplemented with triadimefon versus gibberellic acid + triadimefon (p > 0.05). Values indicate mean a SD of at least 14 seedlings per line. The Student t-test was used to generate p values. The percentage of non-ectopic stomata is indicated in parentheses. ND, not determined.

TABLE 2

NUMBER OF STOMATA IN THE HYPOCOTYLS OF WILD-TYPE AND MUTANT SEEDLINGS GROWN WITH OR WITHOUT CHEMICAL TREATMENTS

Number of stomata per hypocotyl	L*er*	Ws-2	*gl2-1* (in L*er*)	*cpc* (in Ws-2)	*try cpc* (in L*er*)
Without chemical treatment	19.0±5.1 (96.7)	30.0±5.4 (93.3)	32.0±8.4[a] (73.3)	14.0±2.3[a] (93.3)	1.0±2.8[a] (90.0)
Gibberellic acid (10 μM)	34.0±7.3[b] (93.3)	40.0±9.0[b] (100)	47.0±10.0[ab] (70.0)	25.0±9.0[ab] (90.0)	2.0±4.1[a] (89.5)
Paclobutrazol (0.5 μM)	13.0±5.5[b] (100)	23.0±6.2[b] (100)	23.0±6.7[ab] (76.7)	14.0±4.0[a] (90.0)	2.0±4.3[a] (90.0)

[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 14 seedlings per line. The Student t-test was used to generate p values. The percentage of non-ectopic stomata is indicated in parentheses.

files overlying two cortical cell files (Table 1), which could favor gas exchange between the atmosphere and the internal tissues.

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

The *tmm-1* hypocotyl develops meristemoids that are unable to develop into stomata (Bhave *et al*., 2009). To determine whether gibberellins reverse this *tmm-1*-dependent defect, we grew *tmm-1* seedlings on medium supplemented with gibberellic acid. Treatment with 10 μM gibberellic acid increased stomatal production in wild-type plants but failed to induce stomatal formation in *tmm-1* (Fig. 1 A). The length of the hypocotyl of both wild-type plants and *tmm-1* mutant increased significantly in response to gibberellic acid (Fig. 1 B). These results support the notion that gibberellins promote stomatal pathway initiation, whereas *TMM* is required for meristemoid progression.

Gibberellins control GL2 *and* CPC *expression*

To determine the effects of gibberellins on the expression of genes in the *MYBs-TTG-bHLHs-GL2* network, we investigated the expression of *GL2*, a negative regulator of stomatal development (Berger *et al*., 1998; Hung *et al*., 1998), and *CPC*, a promoter of this process (Serna, 2008). In wild-type plants, the *GL2* promoter is preferentially induced in the upper portion of non-stomata-forming cell files (Hung *et al*., 1998). When plants were grown in the presence of 10 μM gibberellic acid, the pattern of *GFP* expression, driven by the *GL2* promoter, was indistinguishable from that of untreated plants: GFP activity detected in non-stomata-forming cell files but not in stomata-forming cells files (Fig. 2 A and B). However, plants treated with 0.5 μM paclobutrazol exhibited *GL2:GFP* expression also in some cells of stomata-forming cell files (Fig. 2C). This result indicates that gibberellins repress *GL2* promoter activity in stomata-forming cell files.

The *CPC* promoter is also preferentially induced in the upper portion of non-stomata-forming cell files (Kirik *et al*., 2004; Serna, 2008). When plants were grown in medium supplemented with 10 μM gibberellic acid, the pattern of *CPC:GFP* expression was identical to that of untreated plants (Fig. 2 D and E). However, *CPC:GFP* expression extended to all files in plants treated with 0.5 μM paclobutrazol (Fig. 2 F). These data indicate that gibberellins repress *CPC* promoter activity in stomata-forming cell files.

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

Treatment with 10 μM gibberellic acid increased the number of stomata in the hypocotyl of both L*er* and Ws-2 ecotypes (Table 2). In addition, the reduction of gibberellin biosynthesis with 0.5 μM paclobutrazol reduced stomatal formation in these ecotypes (Table 2). Under all growth media, stomata of both L*er* and Ws-2 ecotypes were mainly placed in epidermal cell files that overly two cortical cell files (Table 2). The *gl2* mutant (*gl2-1*) develops an increased number of stomata compared to wild-type seedlings,

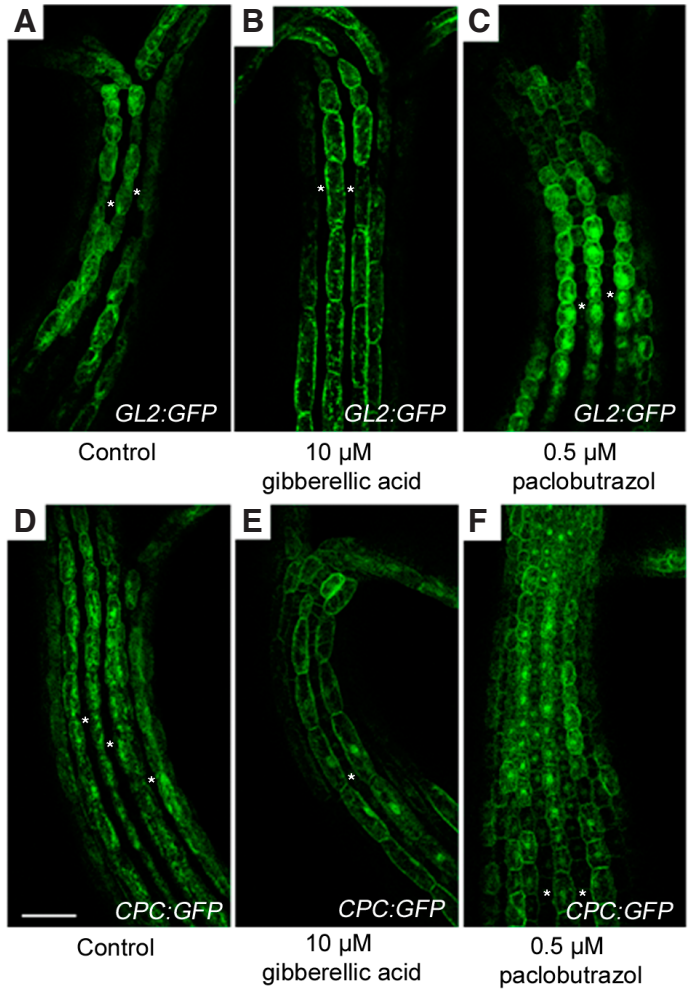

Fig. 2. Effect of gibberellic acid on both (A–C) *GL2:GFP* and (D–F) *CPC:GFP* expression. *Asterisks indicate stomatal-forming cell files. Scale bars, 50 μm; all images were photographed at the same magnification. GFP was excited with an argon laser at 488 nm. GFP emission was detected by setting the spectral detector between 500 and 525 nm.*

with many of the stomata located in files placed outside of a single cortical cell file (Berger *et al.*, 1998; Table 2). This mutant exhibited increased stomatal production when 10 μM gibberellic acid was added to the growth medium but reduced stomatal production on growth medium supplemented with 0.5 μM paclobutrazol (Table 2). On all growth media, stomatal production was higher in the *gl2-1* mutant than in wild-type seedlings, with many of its stomata found in ectopic positions (Table 2).

The *cpc* mutant exhibits a reduced number of stomata compared with wild-type plants (Serna, 2008; Table 2). Treatment with 10 μM gibberellic acid caused an increase in the number of stomata in the *cpc* mutant (Table 2). However, *try cpc*, which develops a very low number of stomata (Serna, 2008; Table 2), was insensitive to gibberellic acid (Table 2). Under all growth conditions, stomata of both *cpc* and *try cpc* were mainly located in epidermal cell files that overly two cortical cell files (Table 2). These results suggest that *CPC* and *TRY* redundantly regulate stomatal production downstream of gibberellic acid. Treatment with 0.5 μM paclobutrazol did not reduce the low stomatal production in the *cpc* mutant (Table 2).

Discussion

Both brassinosteroids and gibberellins control stomatal formation in the embryonic stem (Saibo *et al.*, 2003; Fuentes *et al.*, 2012; Gudesblat *et al.*, 2012), suggesting that their signaling pathways may interact. Crosstalk occurs between these plant regulators through a direct interaction between the brassinosteroids-activated BZR1 and gibberellic acid-inactivated DELLA transcriptional regulators to regulate plant growth (Bai *et al.*, 2012; Gallego-Bartolomé *et al.*, 2012; Li *et al.*, 2012). However, the finding that brassinosteroids regulate stomatal development independent of BZR1 and BRASSINOSTEROID INSENSITIVE1-EMS-SUPPRESSOR1 (BES1; Gudesblat *et al.*, 2012) suggests the existence of an alternative and upstream point of interaction between these two signaling pathways. Epibrassinolide partially rescued stomatal formation in the *gid1a-2 gid1b-3 gid1c-1* triple mutant, suggesting that gibberellins function upstream of brassinosteroids (Fig. 3). The absence of a significant effect of gibberellins on the *bri1-1* mutant strongly supports this suggestion, and suggests that gibberellin signaling pathway interacts with the brassinosteroid signaling pathway upstream of, and/or directly with, BRI1. Consistent with this interpretation, epibrassinolide- and paclobutrazol-treated plants developed more stomata than paclobutrazol-treated plants. Strikingly, treatment with gibberellic acid and triadimefon rescued the stomatal production of triadimefon-treated plants. One possible explanation for this finding is that, as suggested by Asami and co-workers (2003), triadimefon may affect both brassinosteroid and gibberellin biosynthesis. Low mitogen-activated protein kinase levels could allow brassinosteroids, through their regulation by gibberellins, promote stomatal development by inhibiting the GLYCOGEN SYNTHASE KINASE-like BRASSINOSTEROID INSENSITIVE2 (BIN2) repression of SPEECHLESS (Serna, 2013; Fig. 3).

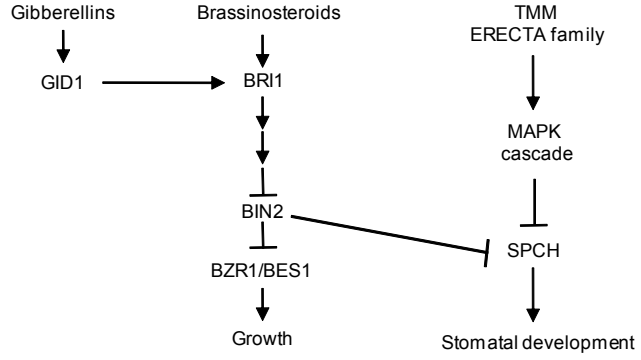

Fig. 3. Model of the genetic and hormonal control of stomatal development in the hypocotyl. *The gibberellin signaling pathway interacts, through GID1 or downstream components, with the brassinosteroid signaling pathway directly with (and/or upstream of) BRI1, which negatively regulates BIN2. Low mitogen-activated protein kinase (MAPK) levels in the hypocotyl allow gibberellins promote stomatal development by negatively regulating the BIN2 repression of SPEECHLESS (SPCH). Arrows indicate positive regulation; blunted lines indicate negative action.*

A number of findings have also increased our understanding of the potential interactions between gibberellins and the *MYBs-bHLHs-TTG-GL2* network. Although the *cpc* mutant increased stomatal production in response to gibberellic acid treatment, the *try cpc* mutant showed absolute insensitivity to this phytohormone. It is therefore likely that gibberellic acid promotes stomatal development upstream of *CPC* and *TRY* and that *cpc* responds to this hormone because of genetic redundancy. The response of the *gl2-1* mutant to changes in both gibberellin and brassinosteroid levels is striking. One possible explanation is that unidentified genes might perform the functions of *GL2* in the *gl2-1* mutant background. In agreement with this suggestion, paclobutrazol treatment promoted *GL2* expression also in some cells of stomatal-forming cell files. In these cells, the absence (or low levels) of gibberellins may block stomatal development. Unexpectedly, paclobutrazol treatment promoted *CPC* promoter induction also in cells of stomatal-forming cell files. This finding suggests that CPC controls unidentified processes in these cells, and that *CPC* expression does not necessarily trigger stomatal formation. Post-transcriptional modification of CPC in non-stomatal-forming cell files may be required to induce stomatal development. Gibberellins then most probably function upstream of the *TTG/bHLHs/MYBs/GL2* network to regulate *GL2* and *CPC* expression. Brassinosteroids also act upstream of *CPC* and *GL2* (Fuentes *et al.,* 2012), but their effects are not identical to those mediated by gibberellins. This indicates that gibberellins do not regulate the expression of these genes through brassinosteroid signaling.

TMM regulates stomatal development, with *tmm-1* developing arrested meristemoids and lacking stomata (Bhave *et al.*, 2009). Gibberellins, like brassinosteroids (Fuentes *et al.*, 2012; Gudesblat *et al.*, 2012), cannot induce stomatal formation in *tmm-1*. These two phytohormones control the initiation of stomatal development, and *TMM* functions in later stages, inducing stomatal formation from meristemoids. In agreement with this interpretation, *gid1a-2 gid1b-3 gid1c-*, as well as paclobutrazol- and triadimefon-treated wild-type plants, do not have arrested meristemoids. The number of meristemoids in *tmm-1* is smaller than the number of stomata in wild-type plants (Fuentes *et al.*, 2012), suggesting that *TMM* also promotes entrance into the stomatal pathway. TMM may reduce mitogen-activated protein kinase activity in the embryonic stem, which would prevent the regulation of SPEECHLESS by BIN2 to promote stomatal development (Serna, 2013).

Materials and Methods

Plant materials and growth conditions

Mutant plants used in this work include *bri1-1* (N3723), *gid1a-2 gid1b-3 gid1c-1* (N16297), *tmm-1* (N6140), *gl2-1* (N65), *cpc*, and *try cpc*. Transgenic plants carrying either the *GL2:GFP* (Lin and Schiefelbein, 2001; Fuentes *et al*., 2012) or *CPC:GFP* (Fuentes *et al*., 2012) construct were also used. The wild-type strains included Wassilewskija (Ws-2), Columbia (Col-0), and Landsberg *erecta* (L*er*).

Vernalized seeds were sterilized using 5% sodium hypochlorite and sown on plates containing 1% agar, 1% sucrose, and Murashige and Skoog salts. Some media were supplemented with epibrassinolide, triadimefon, gibberellic acid, and/or paclobutrazol. Seedlings were grown for 2 weeks on horizontally oriented dishes in the light (16 hour light/8 hour dark cycle) at 22 °C.

Sterile seeds from *gid1a-2 gid1b-3 gid1c-1* were sown on plates containing Murashige and Skoog salts medium. The seed coats of non-germinating *gid1a-2 gid1b-3 gid1c-1* seeds were manually removed. Dissected embryos were plated on Murashige and Skoog medium containing 1% sucrose with (or without) epibrassinolide.

Characterization of epidermal features

Two-week-old seedlings were examined and digitized under Nomarski optics to determine the total number of stomata per hypocotyl. Stomata placed in epidermal files that contacted a single cortical cell file were defined as ectopics, and those in files making contact with two cortical cell files were defined as non-ectopics (Berger *et al*., 1998; Hung *et al*., 1998). To determine the percentage of ectopic (or non-ectopic) stomata, two randomly selected stomata per hypocotyl (at least 14 seedlings) were examined by focusing alternatively on the epidermal and cortical focal planes.

The ratio of the number of stomata in a given file divided by the total number of stomata and other epidermal cells in that same file (stomatal index) was also determined. Two cell files per plant, and at least 14 plants, were analyzed. All samples were examined by light microscopy with a Leica DC 300F camera attached to a Leica DMIRB inverted microscope. Images were acquired with the Leica Application Suite and processed with Adobe Photoshop.

GFP imaging

Three-day-old seedlings were used to visualize GFP fluorescence. GFP was excited at 488 nm using an argon laser, and fluorescence was monitored with an emission window setting of 500–525 nm. GFP fluorescence acquisition was performed using the 40x/1.15 Oil CS 0.17/E,0.27 ACS APO objective of a DMIRB inverted Leica TCS SP-II confocal microscope. Images, recorded with a picture size of 1024 × 1024 pixels, were arranged and labeled using Adobe Photoshop.

Author Contributions

L.S. conceived and supervised this study. D.G. and S.F. performed all experiments and related analyses.

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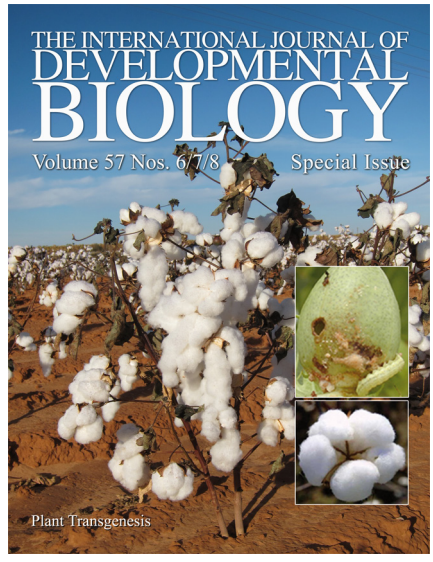

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