

What Causes Opposing Actions of Brassinosteroids on Stomatal Development?¹

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During the past few years, our understanding of stomatal development and patterning has been advanced by the cloning of a large number of genes in *Arabidopsis* (*Arabidopsis thaliana*). These genes encode peptide ligands (epidermal patterning factor, or EPF), and receptor proteins (TOO MANY MOUTHS [TMM] and the ERECTA family [ERf] of receptor-like kinases [ER, ERL1, and ERL2]; Nadeau and Sack, 2002; Shpak et al., 2005; Torii, 2012). Genes functioning downstream of these receptors include those encoding the mitogen-activated protein kinase (MAPK) module, which consists of the MAPK kinase kinase YODA (YDA); the MAPK kinases MKK4, MKK5, MKK7, and MKK9; and the MAPKs MPK3 and MPK6 (Bergmann et al., 2004; Wang et al., 2007; Lampard et al., 2009). MPK3 and MPK6 phosphorylate and inactivate the basic helix-loop-helix transcriptional factor SPEECHLESS (SPCH), blocking the initiation of stomatal development (MacAlister et al., 2007; Pillitteri et al., 2007; Lampard et al., 2008). It is well known that the interaction of signaling pathways permits the fine tuning of cellular activities needed to carry out complex developmental processes. However, despite the progress made in studies on the stomatal signaling pathway, our understanding of its interaction with other signaling pathway remains very limited.

One of the most well characterized signal transduction pathways in plant biology is the pathway regulated by brassinosteroids (BRs; Kim and Wang, 2010). These plant regulators are perceived by the Leu-rich-repeat receptor-like kinase BRASSINOSTEROID INSENSITIVE1 (BRI1; He et al., 2000). Direct binding of BRs to BRI1 results in the activation of a small family of kinases known as BRASSINOSTEROID SIGNALING KINASEs (Tang et al., 2008). Downstream components of bacterial spore kinases include the GLYCOGEN SYNTHASE KINASE3 (GSK3)-like BRASSINOSTEROID INSENSITIVE2 (BIN2) kinase (Li and Nam, 2002) and two protein phosphatases, BRI1 SUPPRESSOR1 (BSU1; Mora-García et al., 2004) and PROTEIN PHOSPHATASE2A (PP2A; Tang et al., 2011), which control the phosphorylation states of a family of transcription factors including

BRI1-EMS-SUPPRESSOR1 (BES1; Yin et al., 2002) and BRASSINAZOLE RESISTANT1 (BZR1; He et al., 2005). BR signaling leads to the inactivation of BIN2, and PP2A-mediated dephosphorylation and the activation of BZR1 and BES1 (He et al., 2002; Kim and Wang, 2010; Tang et al., 2011).

Two works have revealed that BRs control stomatal production through regulating proteins that function in the stomatal pathway (Gudesblat et al., 2012a; Kim et al., 2012). However, while one of these studies showed that BRs repress stomatal development by alleviating the BIN2-mediated inhibition of YDA (Kim et al., 2012; Fig. 1A), the other study concluded that these plant regulators promote stomatal formation by inhibiting the BIN2-mediated phosphorylation of SPCH (Gudesblat et al., 2012a; Fig. 1B). A number of recent papers (Casson and Hetherington, 2012; Kong et al., 2012), including one published by the authors of one of the original articles (Gudesblat et al., 2012b), highlighted this apparent discrepancy and looked for its possible sources. In this article, I examine data from the cotyledon and hypocotyl separately and propose that BRs have opposite effects on the formation of stomata in these plant organs. I also look for the reasons behind such responses and propose that the TMM action, dampening excessive signal to ERF from the peptide ligands CHALLAH (CHAL) and EPF-LIKE5 (EPFL5) in hypocotyls, may be responsible for the organ-specific effects of BRs.

BRS REPRESS STOMATAL DEVELOPMENT IN COTYLEDONS

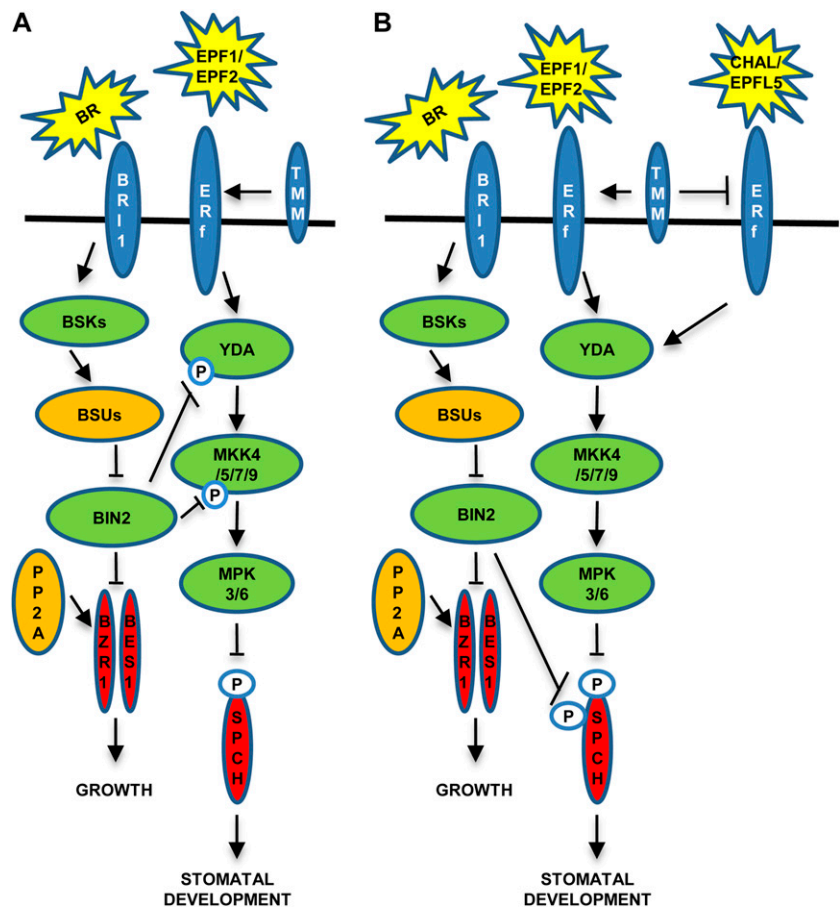
Plants with quadruple loss of function of *BSU*-related phosphatases (*bsu-q*) displayed a striking phenotype, producing a cotyledon surface consisting almost entirely of stomata (Kim et al., 2012). BR-deficient plants (*deetiolated2-1* [*det2-1*]), or those that lack sensitivity to BRs (*bri1-116*, dominant *bin2-1*, and plants overexpressing *BIN2*), also developed an increased number of stomata in their cotyledons (Kim et al., 2012). These mutants also developed stomatal clusters (Kim et al., 2012). By contrast, plants lacking BR-signaling GSK3-like kinases (*bin2-3 bil1 bil2* loss-of-function mutant) and *det2-1* plants overexpressing a member of the *BSU* subfamily (*BSU1-LIKE2*) developed a reduced number of stomata (Kim et al., 2012). These results support the notion that BRs repress stomatal development in cotyledons

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Figure 1. Proposed cross talk between BRs and stomatal development. A, Regulation of stomatal formation in the cotyledon. TMM enhances EPF1 and EPF2 signaling through ERf. At low BR levels, BIN2 phosphorylates and inactivates both YDA and MKK4, switching off the signaling that inactivates SPCH by phosphorylating residues in the MPKTD, which triggers stomatal production. Genetic data also suggest that ERf and TMM regulate BIN2 (or upstream components). B, Stomatal formation in the hypocotyl. TMM enhances EPF1 and EPF2 signaling, but also dampens CHAL and EPFL5 signaling. TMM action reduces EPF1 and EPF2 signaling, leading to a reduction of MAPK activity. Under this scenario, in the absence of BRs, BIN2 inactivates SPCH by phosphorylating residues in the N terminus and in the MPKTD. These phosphorylation events repress stomatal development.



(Kim et al., 2012). In agreement with this interpretation, the pharmacological activation of BR signaling by brassinolide (BL), the most active natural BR, or bixin, an inhibitor of BIN2 activity, reduced stomatal production in cotyledons (Kim et al., 2012). Bixin also fully suppressed the stomatal clustering of *bin2-1* and partially suppressed the severe phenotypes of *bsu-q* (Kim et al., 2012).

Interestingly, gain-of-function mutants of *BZR1* (*bzr1-1D* mutant) exhibited the wild-type phenotype, and *bzr1-1D* also failed to suppress the stomatal phenotypes of *bri1-116*, *bsu-q*, and *bin2-1* (Kim et al., 2012). In light of these results, Kim et al. (2012) proposed that BRs repress stomatal development independent of the BIN2 substrate BZR1.

But does the BR pathway interact with the signaling cascade that controls stomatal development? When CA-YDA, a constitutively active YDA variant that probably acts through activation of the MAPK pathway, which phosphorylates and inactivates SPCH (Wang et al., 2007; Lampard et al., 2008), was expressed in *bri1-116*, *bsu-q*, or *bin2-1* mutants, stomatal development was blocked (Kim et al., 2012). Consistent with these data, the *bsu-q spch-3* mutant exhibited a phenotype similar to that of *spch-3*, that is, lacked stomata (Kim et al., 2012). Therefore, BR signaling components function upstream

of YDA (Kim et al., 2012; Fig. 1A). Bixinin treatment suppressed the weak stomatal clustering of *tmm* and partially rescued the *er erl1 erl2* phenotype, but it had no effect on the *yda* epidermis (Kim et al., 2012). Brassinazole, an inhibitor of BR biosynthesis, enhanced the stomatal phenotype of *tmm*, but it did not further increase the number of stomata in *er erl1 erl2*, probably because almost all of the cells in this mutant were already stomatal cells (Kim et al., 2012). Together, these results suggest that BIN2 functions downstream of ERf and TMM, but upstream of YDA (Kim et al., 2012).

In contrast with the repressive role of BRs in stomatal production, Gudesblat et al. (2012a) found that BR-deficient plants (*constitutive photomorphogenesis and dwarfism [cpd]*) and those lacking BR sensitivity (*bri1-116*) had cotyledons that appeared normal. In addition, cotyledons of plants overexpressing the BR biosynthesis gene *DWARF4* (*DWF4*) also displayed a normal phenotype, while those of plants overexpressing *BRI1* slightly increased in the stomatal production (Gudesblat et al., 2012a). This disparity may have arisen from the use of different experimental growth conditions (Gudesblat et al., 2012b). Thus, in the conditions employed by Gudesblat et al. (2012a), an unknown factor may have masked the effect of BRs on stomatal production in this plant organ.

BRS PROMOTE STOMATAL DEVELOPMENT IN HYPOCOTYLS

Plants that either lacked BRs (*cpd*, *det2-1*) or had reduced sensitivity to BRs (*bri1-1*, *bri1-4*, *bri1-116*, and dominant *bin2-1*) developed a very low number of stomata in the hypocotyl, and those with enhanced BR responses (plants overexpressing either *DWF4* or *BR11*, *bin2-3 bil1 bil2*, and *bin2-3*) displayed the opposite phenotype, that is, a strong increase in stomatal production (Fuentes et al., 2012; Gudesblat et al., 2012a). Indeed, BL increases stomatal production, while brassinazole and triadimefon, two inhibitors of BR biosynthesis, reduce the number of stomata (Fuentes et al., 2012; Gudesblat et al., 2012a). Mutant backgrounds responded to these treatments as expected, with *bri1-1* and *bri1-4* displaying absolute insensitivity to BL, while *det2-1* increased stomatal production in response to this plant regulator (Fuentes et al., 2012). Interestingly, the gain-of-function mutants *bes1-D* and *bzr1-D* had normal numbers of stomata in their hypocotyls (Gudesblat et al., 2012a). Therefore, *BIN2* represses stomatal development in the hypocotyl in a *BZR1*- and *BES1*-independent manner (Gudesblat et al., 2012a).

The observation that *bsu-q* plants produced large stomatal clusters on hypocotyls is unexpected (Kim et al., 2012). Because BRs promote stomatal development in the embryonic stem, the lack of sensitivity to BRs in *bsu-q* would be expected to block stomatal production. The appearance of these clusters could be explained by the notion that some of the genes mutated in *bsu-q* might play a role in preventing adjacent cells from developing into stomata.

To investigate the possible interaction between BR signaling and genes that function in the stomatal pathway, Gudesblat et al. (2012a) examined mutants with impaired stomatal pathway genes grown in the presence of exogenous BL (Gudesblat et al., 2012a). They found that BL increased stomatal production in the hypocotyls of *epf1*, *epf2*, *er-105*, *erl1-2*, *erl2-1*, and *yda-5* (Gudesblat et al., 2012a). This indicates that BRs act downstream or independent of the genes impaired in these mutants (Gudesblat et al., 2012a). The fact that BL failed to promote stomatal production in *spch-3* is consistent with *SPCH* as a downstream transcriptional factor initiating stomatal cell lineages.

TMM AND BRS FUNCTION DURING DIFFERENT STEPS OF STOMATAL DEVELOPMENT

The hypocotyl epidermis is arranged in files that run parallel to the long axis of this organ, and only some of these files develop stomata (Berger et al., 1998; Hung et al., 1998). The first step of stomatal development in the hypocotyl is a cell division along its longitudinal axis, which is parallel to the longitudinal axis of the organ (Berger et al., 1998). This cell division produces a small and often triangular or rectangular cell named the meristemoid (Berger et al., 1998). Meristemoids are

self-renewing cells that undergo a sequence of asymmetric cell divisions before stomata formation (Berger et al., 1998). The *tmm* mutant hypocotyl lacked stomata, but it exhibited arrested meristemoids (Bhave et al., 2009). Interestingly, BL not only failed to reverse the lack of stomata in the *tmm-1* hypocotyl (Fuentes et al., 2012; Gudesblat et al., 2012a) but also increased the production of meristemoids (Fuentes et al., 2012). In light of these results, Fuentes et al. (2012) proposed that BRs promote the initiation of the stomatal pathway, and *TMM* is required in later stages of stomatal development for the progression of meristemoids toward stomata. Consistent with these results, *bri1-1*, *bri1-4*, and *det2-1* mutants and wild-type plants treated with the inhibitor of BR biosynthesis triadimefon did not display arrested meristemoids (Fuentes et al., 2012).

Stomata-forming cell files have more cells than their neighboring nonstomatal cell files (Hung et al., 1998). Gudesblat et al. (2012a) found that BL increased the number of cells in stomata-forming cell files, with *tmm* mutants displaying a response similar to that found in wild-type plants. Then, BL increases the number of nonstomatal cells and promotes the initiation of the stomatal pathway in a *TMM*-independent manner.

BIN2 PHOSPHORYLATES SEVERAL PROTEINS IN STOMATAL SIGNALING CASCADE

In vitro and in vivo experiments have demonstrated that *YDA* binds to *BIN2* (Kim et al., 2012). In addition, *CA-YDA*, which lacks 23 putative *GSK3* phosphorylation sites, binds to *BIN2* in yeast two-hybrid assays (Kim et al., 2012). As might be expected, *BIN2* phosphorylates the region deleted in *CA-YDA*, which inhibits *YDA* activity (Kim et al., 2012; Fig. 1A). In consonance with these data, the kinase activities of *MPK3* and *MPK6* were reduced when *BIN2* was activated (*det2* mutants) but were increased when *BIN2* was repressed (bikinin or BL treatments in wild-type plants; Kim et al., 2012). Because BRs increase *MAPK* activity by repressing *BIN2*, and *MAPKs* repress *SPCH* activity, it is expected that BL or bikinin treatments would decrease *SPCH* activity, repressing stomatal development in cotyledons.

In vitro kinase assays have shown that *BIN2* also phosphorylates both *MKK4* and *MKK5* (Khan et al., 2013). In addition, *BIN2* phosphorylation of *MKK4* represses its activity against *MPK6* in vitro (Khan et al., 2013; Fig. 1A). *BIN2* phosphorylates two residues of *MKK4* (Khan et al., 2013): Ser-230 and Thr-234. Since mutations in Thr-234 abolished the activity of *MKK4* against *MPK6* in vitro, Khan et al. (2013) proposed that Thr-234 may be the main phosphorylation site of *MKK4* by *BIN2*. Moreover, the overexpression of a mutant version of *MKK4* in Thr-234, by replacing Thr with Ala to prevent phosphorylation, conferred a cotyledon epidermis composed almost entirely of stomata (Khan et al., 2013). This phenotype mirrors those in which the activities of either *MKK4* and *MKK5* or *MPK3* and *MPK6* are lost (Wang et al., 2007), which suggests that the *MKK4*

mutant version favorably competes with the endogenous one in binding MPK3 and MPK6, interfering with the activation of MPK3 and MPK6 (Khan et al., 2013). This would prevent SPCH repression, triggering stomatal development. It will be interesting to determine the significance of BIN2-mediated Ser-230 phosphorylation.

Gudesblat et al. (2012a) investigated whether BIN2 phosphorylates SPCH. They found that BIN2 phosphorylates SPCH *in vitro*, and such phosphorylation is abolished after incubation with bikinin (Gudesblat et al., 2012a). BIN2 phosphorylates five residues of SPCH located within the MAPK target domain (MPKTD; Gudesblat et al., 2012a). BIN2 also phosphorylates five residues of SPCH located at the N terminus of the protein (Gudesblat et al., 2012a). Two of the five *in vitro* phosphorylated MPKTD residues, and one of the five *in vitro* phosphorylated N-terminal residues, were also found to be phosphorylated *in vivo* (Gudesblat et al., 2012a). The differences in results obtained in *in vitro* and *in vivo* phosphorylation experiments can easily be explained by the fact that kinases have broader substrate specificity *in vitro* than *in vivo*. BL reduced the phosphorylation of two of the three SPCH residues that were phosphorylated by BIN2 *in vitro* and *in vivo* (Gudesblat et al., 2012a; Fig. 1B): Ser 65 at the N-terminal domain and Ser 171 at the MPKTD. BRs may therefore promote SPCH activity, and thus stomatal development in the hypocotyl, by inhibiting the phosphorylation of these two BIN2 target residues. The expression of mutant versions of SPCH in these residues, by replacing them with Ala, rescued stomatal production in the *spch-3* cotyledon (Gudesblat et al., 2012a). The expression of this mutant protein also increased the proliferation of nonstomatal epidermal cells, suggesting a role of BIN2 in regulating cell division through SPCH (Gudesblat et al., 2012a).

BL increased the amount of SPCH protein but not the transcript level, and brassinazole reduced the concentration of SPCH (Gudesblat et al., 2012a). In addition, the proteasome inhibitor MG132 also increased the level of SPCH (Gudesblat et al., 2012a). These data are consistent with a model in which BRs control stomatal production by regulating SPCH levels. Given that the expression of mutant versions of SPCH protein with mutations in its BIN2-specific phosphorylation sites (Ser 65 and others at the N-terminal domain) increased the level of SPCH in *spch-3*, it is likely that SPCH degradation depends on the phosphorylation of these sites (Gudesblat et al., 2012a).

But, how can BIN2 regulate different proteins depending on the organ where it is acting (YDA and MKK4 in cotyledons and SPCH in hypocotyls)? The simplest explanation is that organ type affects the levels of the components of the MAPK module, with high levels in cotyledon epidermal cells and low ones in hypocotyl cells. High levels of the MAPK components in cotyledon cells could allow that BIN2-mediated repression of both YDA and MKK4 was the dominant effect, promoting stomatal formation. In contrast, low MAPK levels in hypocotyl cells could allow BIN2

regulation of SPCH to repress stomata development. Under this scenario, the repression of stomatal cluster development in the leaves of brassinazole-treated MKK4 or MKK5 overexpressing plants (Khan et al., 2013), suggests the existence of an excess of MKK4 and MKK5 that cannot be phosphorylated by BIN2, repressing SPCH activity.

It is known that BIN2 controls the output of BR signaling through the control of the subcellular localization of BES1 (Ryu et al., 2010a, 2010b) and BZR1 (Ryu et al., 2007). In a similar way to the BIN2-mediated regulation of BES1 or BZR1, this hypothetical scenario, with different MAPK activities depending on the organ, does not exclude that BIN2 may regulate the output of BR signaling also through the control of the subcellular localization of YDA and SPCH. This does not seem to be the case of MKK4, whose nucleocytoplasmic localization unchanged after treatment with BR or brassinazole (Khan et al., 2013).

TMM ACTION AND LEVELS OF MAPKS

Mutations in stomatal regulators confer patterning defects that are uniform across all organs that produce stomata. Unlike most stomatal mutants, *tmm* exhibits contrasting phenotypes in different organs (Geisler et al., 1998; Bhave et al., 2009); for example, *tmm* hypocotyls produce no stomata, but *tmm* cotyledons develop stomatal clusters. How can *TMM* play opposite roles depending of the organ where it acts? Mutations in the *CHAL* putative peptide ligand gene, which is expressed in internal tissues of the hypocotyl and stem (Abrash and Bergmann, 2010; Abrash et al., 2011), restored stomata to *tmm* hypocotyl without significantly affecting cotyledon stomata development (Abrash and Bergmann, 2010). Interestingly, overexpression experiments reveal that *CHAL* needs *ERf* to inhibit stomatal development but that, in striking contrast to the putative secretory peptide genes *EPF1* and *EPF2*, whose repression of stomatal development depends on both *ERf* members and *TMM*, the effects of *CHAL* overexpression are dramatically enhanced in absence of *TMM* (Hara et al., 2007, 2009; Hunt and Gray, 2009; Abrash and Bergmann, 2010). In contrast to *CHAL*, *EPF1* and *EPF2* are expressed across all aerial organs examined, in overlapping but also in different stomatal precursor cells (Hara et al., 2007, 2009; Hunt and Gray, 2009). This allows proposing that *TMM* permits or enhances *EPF1* and *EPF2* signaling through *ERf* receptors and across all organs that produce stomata, but dampens *CHAL* signaling, also through *ERf* receptors, in the hypocotyl and stem (Abrash and Bergmann, 2010; Torii, 2012; Fig. 1B). Then, in the *tmm* mutant, *CHAL* signaling would prevent stomatal formation in the hypocotyl and stem, and the lack of the *EPF1* and *EPF2* signaling would trigger stomatal cluster formation in cotyledons. *TMM* also dampens the signaling mediated by the *CHAL* paralog *EPFL5*, whose promoter also is induced in the hypocotyl, although in a

different pattern from that of *CHAL* (Abrash et al., 2011). Consistent with this model, *tmm erl1* resumed stomatal formation in stems (Shpak et al., 2005).

Disruption of BR biosynthesis, perception, or signaling displays phenotypes that mirror those found in the *tmm* mutant. Does the TMM action explain the fact that BRs play opposite roles in cotyledons and hypocotyls? In the embryonic stem, TMM not only potentiates the transduction of EPF1 and EPF2 but also dampens the excessive signal from CHAL and EPFL5 to the ERf. Consequently, one could speculate that this mechanism limits the availability of TMM and/of ERf for EPF1 and EPF2, reducing EPF1 and EPF2 signaling. This would lead to a reduction of MAPK activity, triggering an accumulation of SPCH. Under this scenario, BIN2 would predominantly regulate SPCH, negatively regulating stomatal formation. In the cotyledon, the absence of CHAL and EPFL5 signaling would increase EPF1 and EPF2 signaling, leading to a robust activation of MAPK and, consequently, allowing the repression of both YDA and MKK4 by BIN2 to be the dominant effect. This would promote stomata formation. Thus, it is likely that the TMM action, dampening excessive signal to ERf from CHAL and EPFL5 in hypocotyls, is responsible for the organ-specific effects of BRs.

Loss of *CHAL* increased meristemoids in *tmm* hypocotyl (Abrash and Bergmann, 2010), in the same way as BL (Fuentes et al., 2012), most likely because of the repression of both YDA and MKK4 by BIN2 in this double mutant. Some of these meristemoids developed into stomata in *tmm chal* (Abrash and Bergmann, 2010), indicating that *CHAL* is required for a full repression of meristemoid progression in the *tmm* background.

BRS AND CELL-FATE SPECIFICATION IN THE HYPOCOTYL

The *MYBs-BHLHs-TTG-GL2* network controls stomatal cell-fate specification in the hypocotyl (Serna, 2004). Although BL did not affect the expression pattern of either *GLABRA2* (*GL2*) or *CAPRICE* (*CPC*), whose promoters are induced in nonstomata-forming cell files, the treatment with the BR biosynthesis inhibitor triadimefon resulted in a random induction of the *GL2* promoter and a loss of induction of the *CPC* promoter in the hypocotyl epidermis (Fuentes et al., 2012). This suggests that BRs control stomatal cell fate by regulating the expression of at least two members of this network (Fuentes et al., 2012). Supporting this hypothesis, the *cpc* mutant failed to respond to triadimefon, while *try cpc* (*TRIPTYCHON* [*TRY*] shares high sequence similarity with *CPC*) exhibited insensitivity to BL. In addition, *try cpc det2-1* phenocopied the stomatal phenotype of *try cpc*, also exhibiting insensitivity to the phytohormone (Fuentes et al., 2012). Unexpectedly, *gl2-1* responded to both BL and triadimefon, suggesting that other genes might be masking this absence of *gl2-1* response. In contrast to this interpretation, and based on the number of cells per file, Gudesblat

et al. (2012a) proposed that BRs do not affect cell fate in the hypocotyl epidermis. However, because no study has been reported that demonstrates a causal relationship between the number of cells per file and stomatal cell fate, an alternative interpretation would suggest that BRs control separately two aspects of the hypocotyl epidermis: stomatal cell fate and cell division.

CONCLUSION

In line with Casson and Hetherington (2012), this paper supports the hypothesis that cotyledons and hypocotyls respond differently to BRs, resulting in a decrease in stomatal production in cotyledons and an increase in hypocotyls. I propose that the TMM action, dampening excessive signal to ERf from CHAL and EPFL5 in hypocotyls, may be responsible for a reduction of MAPK activity in this plant organ, triggering, under low BR levels, BIN2 regulation of SPCH to repress stomatal development. In contrast, in cotyledons, high MAPK levels could allow the inhibition of both YDA and MKK4 to be the dominant effect, promoting stomatal formation. Interestingly, BR-deficient and BR-insensitive Arabidopsis mutants not only produced more stomata in their cotyledons, but they were also more sensitive to abscisic acid than wild-type plants (Xue et al., 2009), suggesting that plants coordinate both development and physiology to optimize photosynthesis and water use.

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