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Organ-specific effects of brassinosteroids on stomatal production coordinate with the action of *TOO MANY MOUTHS*

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Abstract In *Arabidopsis*, stomatal development initiates after protodermal cells acquire stomatal lineage cell fate. Stomata or their precursors communicate with their neighbor epidermal cells to ensure the "one cell spacing" rule. The signals from EPF/EPFL peptide ligands received by TOO MANY MOUTHS (TMM) and ERECTA-family receptors are supposed to be transduced by YODA MAPK cascade. A basic helix-loop-helix transcription factor SPEECHLESS (SPCH) is another key regulator of stomatal cell fate determination and asymmetric entry divisions, and SPCH activity is regulated by YODA MAPK cascade. Brassinosteroid (BR) signaling, one of the most well characterized signal transduction pathways in plants, contributes to the control of stomatal production. But opposite organ-specific effects of BR on stomatal production were reported. Here we confirm that stomatal production in hypocotyls is controlled by BR levels. *YODA* and *CYCD4* are not essential for BR stomata-promoting function. Furthermore, we found that BR could confer *tmm* hypocotyls clustered stomatal phenotype, indicating that the BR organ-specific effects on stomatal production might coordinate with the TMM organ-specific actions.

Keywords: *Arabidopsis*; brassinosteroids; development; stomata
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INTRODUCTION

Stomata are pores in the epidermis of the aerial parts of plants, which control carbon dioxide and water vapor exchange between plants and the atmosphere. Proper stomatal density and aperture optimize photosynthesis and transpiration rates. In leaves, the stomatal lineage is initiated from a protodermal cell differentiating into a meristemoid mother cell (MMC). Meristemoid mother cell undergoes an asymmetric division to generate a triangular meristemoid cell (M) and a larger sister cell, named the stomatal lineage ground cell (SLGC). Meristemoid may either directly differentiate into a round guard mother cell (GMC), or undergo asymmetric division one to three times before its differentiation. Guard mother cell divides symmetrically to form two guard cells (GCs) (Nadeau and Sack 2002b; Bergmann and Sack 2007; Lau and Bergmann 2012). In hypocotyls, although protodermal cells could directly differentiate into MMCs, most protodermal cells first differentiate into MMC precursors, which divide symmetrically to form MMCs (Kono et al. 2007). Cyclin CYCD4, which includes CYCD4;1 and CYCD4;2, was reported to be involved in regulating symmetric divisions of MMC precursors (Kono et al. 2007).

Stomata are distributed and spaced across the epidermis. Stomata or their precursors communicate with their neighbor epidermal cells to ensure the "one cell spacing" rule. ERECTA-family (ERf) leucine-rich repeat (LRR) receptor-like kinases ER, ERL1 and ERL2; LRR receptor-like protein TOO MANY MOUTHS (TMM); the EPIDERMAL PATTERNING FACTOR LIKE (EPFL) family peptide ligands, such as EPF1, EPF2, STOMAGEN, and CHALLAH, are identified as the intercellular signals in controlling stomatal production and patterning (Yang and Sack 1995; Nadeau and Sack 2002a; Shpak et al. 2005; Hara et al. 2007, 2009; Sugano et al. 2010; Lee et al. 2012; Shpak 2013). The signals received by TMM-ERf are supposed to be transduced through the mitogen-activated protein kinase (MAPK) cascade, which includes YODA (YDA), MKK4/MKK5, MKK7/MKK9, and MPK3/MPK6 (Bergmann et al. 2004; Wang et al. 2007; Lampard et al. 2009). MPK3 and MPK6 directly phosphorylate a basic helix-loop-helix (bHLH) transcriptional factor SPEECHLESS (SPCH), which is the key regulator of stomatal lineage initiation (MacAlister et al. 2007; Lampard et al. 2008).

Recently, Brassinosteroid (BR) signaling, one of the best characterized signal transduction pathways in plants, has also been shown to control stomatal production (Gudesblat et al. 2012a, 2012b; Kim et al. 2012; Khan et al. 2013). However, BR displays opposite effects on stomatal production in cotyledons and in hypocotyls. Two contradictory signaling pathways, BRASSINOSTEROID-INSENSITIVE 2 (BIN2)-mediated phosphorylation on SPCH, and BIN2-mediated phosphorylation on the MAPK cascade, have been reported (Gudesblat et al. 2012a, 2012b; Kim et al. 2012; Khan et al. 2013). In addition, Qian et al. (2013) proposed that early steps of sterol biosynthesis, which are independent of the final products of sterol biosynthesis and BRs, were involved in regulating stomatal patterning as well as stomatal cell-fate determination after asymmetric division (Qian et al. 2013). Time-lapse analysis revealed that ARGONAUTE1 (AGO1) was another negative regulator in restricting asymmetric spacing divisions in SLGCs. AGO1 may act through the microRNA pathway to regulate SPCH

transcript levels (Yang et al. 2014). Thus, stomatal production and patterning are regulated by the coordination of multiple signaling pathways.

Similar to BR signal, organ-specific responses of stomatal production between cotyledons and hypocotyls were also found in the *tmm* (Yang and Sack 1995). Thus, a model of organ-specific signaling to SPCH was proposed (Casson and Hetherington 2012; Serna 2013). Here we not only confirmed that stomatal production in hypocotyls was closely associated with BR levels, but also found that *YDA* and *CYCD4* were not essential for BR in promoting stomatal production in hypocotyls. In addition, the presence of BR-induced stomatal clusters in *tmm* hypocotyls suggests a crosstalk between BR and TMM-mediated signaling pathways.

RESULTS

BR promotes stomatal formation in hypocotyls

To investigate the relationship between BR signals and stomatal development, we first treated wild-type seedlings with exogenous active epibrassinolide (eBL) and BR biosynthesis inhibitor brassinazole (BRZ). Treatments with eBL promoted stomatal production in hypocotyls in a dose-dependent manner. For example, compared with untreated wild-type hypocotyls, 1 μmol/L eBL increased stomatal number in the hypocotyl by 2.6 times (Figure 1A). Conversely, BRZ treatment reduced the number of stomata in hypocotyls. For example, only about five stomata were found in 10 μmol/L BRZ treated hypocotyls (Figure 1B).

To further understand the effects of BR on stomatal development, the stomatal phenotypes of BR-related mutants were checked. Both *deetiolated2-1* (*det2-1*), a weak BR biosynthesis mutant (Fujioka et al. 1997), and *brassinosteroid insensitive1-4* (*bri1-4*), a null mutant of BR receptor (Noguchi et al. 1999) displayed a strong reduction in the number of stomata in hypocotyls (Figure 1C, D, F, G). Exogenous application of 1 μmol/L eBL could rescue the production of stomata in *det2-1* to a wild-type level (Figure 1E, J). Conversely, transgenic *BRI1pro:BRI1-GFP* induced excessive stomata in the hypocotyls (Figure 1L), maybe due to its mild *BRI1* gain-of-function. *BRI1pro:BRI1-GFP* could compensate for stomatal deficiency in *bri1-4*, yet eBL failed to compensate for this deficiency (Figure 1I, H, K).

The Glycogen Synthase Kinase 3 (GSK3)-like kinase BIN2 is a negative regulator in BR signaling pathway (Li et al. 2001; Li and Nam 2002); BIN2 directly phosphorylates the downstream transcription factors BRASSINAZOLE RESISTANT1 (BZR1) and bri1-EMS-SUPPRESSOR1 (BES1/BZR2) to inhibit their transcriptional activity (He et al. 2002, 2005; Yin et al. 2002; Ryu et al. 2007, 2010a, 2010b; Sun et al. 2010). In contrast to heterozygous *bin2-1*/+ hypocotyls, dominant *bin2-1* exhibited a significant decrease in stomatal number. However, the dominant mutants *bzr1-1D* and *bes1-D*, which suppressed the dwarf phenotypes of *bin2-1*, *det2*, and *bri1* (Wang et al. 2002; Yin et al. 2002; Kim et al. 2012), showed comparable stomatal number with wild-type in the hypocotyl (Figure 1M).

The epidermis of the *Arabidopsis* hypocotyl consists of protruding cell files and non-protruding cell files, and stomata form in the non-protruding cell files (Serna 2005). Here, we found that although enhanced BR signal increased stomatal number in the hypocotyl and *vice versa*, stomatal cell fate specification was not influenced by BR and stomata still formed exclusively in the non-protruding cell files (Figure 1C–I). Taken together, these results suggest that BR signal promotes stomatal formation in hypocotyls without influencing cell fate specification.

SPCH is critical for BR-induced stomatal initiation in hypocotyls

Stomatal development in hypocotyls initiates from MMC precursors, which divide symmetrically to form MMCs. In the 1 dpg (day post germination), MMC precursors with *TMM* expression were detected in hypocotyls (Kono et al. 2007), indicating that stomatal development in hypocotyls begins after seed germination.

To investigate the mechanism of BR in regulation of stomatal production in hypocotyls, we first checked the effects of BRZ and eBL on the expression of stomatal early-acting genes *ER*, *ERL1*, *ERL2*, *YDA*, and *TMM*. Of the early-acting genes detected, only *TMM* was significantly influenced by BRZ and eBL (Figure 2A). *TMM* is specifically expressed in stomatal lineage cells from MMC precursor stage in hypocotyls (Nadeau and Sack 2002a; Kono et al. 2007). Consistent with the quantitative reverse transcription–polymerase chain reaction (qRT–PCR) results, 10 μmol/L BRZ greatly repressed *TMMpro:GFP* expression in the young hypocotyls (Figure 2B, C), indicating that BR is required for protodermal cells to entry stomatal lineage.

Compared with wild-type, no symmetric division parallel to the apical-basal axis (indicated by a green dashed line in Figure 2E) and no asymmetric division formed in the loss-of-function *spch-4* hypocotyls (Figure 2E, F). Application of 1 μmol/L eBL significantly enhanced the number of cells in non-protruding cell files of *spch-4* hypocotyls (Figure 2D); however, cell divisions related to the initiation of stomatal lineage and the expression of *TMMpro:GFP* were not rescued in *spch-4* hypocotyls treated with 1 μmol/L eBL (Figure 2D, H, I), indicating that the BR-induced stomatal lineage initiation is completely inhibited in *spch-4* hypocotyls.

BR stabilizes SPCH protein

To further explore how SPCH is involved in BR-regulated stomatal production, we determined the effects of BRZ and eBL on the expression of a transcriptional marker *SPCHpro:nucGFP* and a translational marker *SPCHpro:SPCH-GFP* (MacAlister et al. 2007) in the young hypocotyls. *SPCHpro:nucGFP* was observed in both protruding and non-protruding cell files, while *SPCHpro:SPCH-GFP* was only observed in the stomatal lineage cells within the non-protruding cell files, indicating SPCH is post-translationally limited to stomatal stem cells (Figure 3A, C). BRZ treatment had no obvious impacts on *SPCHpro:nucGFP* expression, but greatly suppressed *SPCHpro:SPCH-GFP* expression (Figure 3B, D). Meanwhile qRT–PCR analysis showed that *SPCH* transcriptional levels were not significantly influenced by BRZ and eBL treatment (Figure 3E), indicating that BR signals might post-translationally regulate SPCH.

To further confirm the effect of BR on SPCH protein stability, *SPCHpro:SPCH-GFP* seedlings were treated with the protein synthesis inhibitor cycloheximide (CHX) and checked every 30 min. After 2.5-h CHX treatment, SPCH-GFP

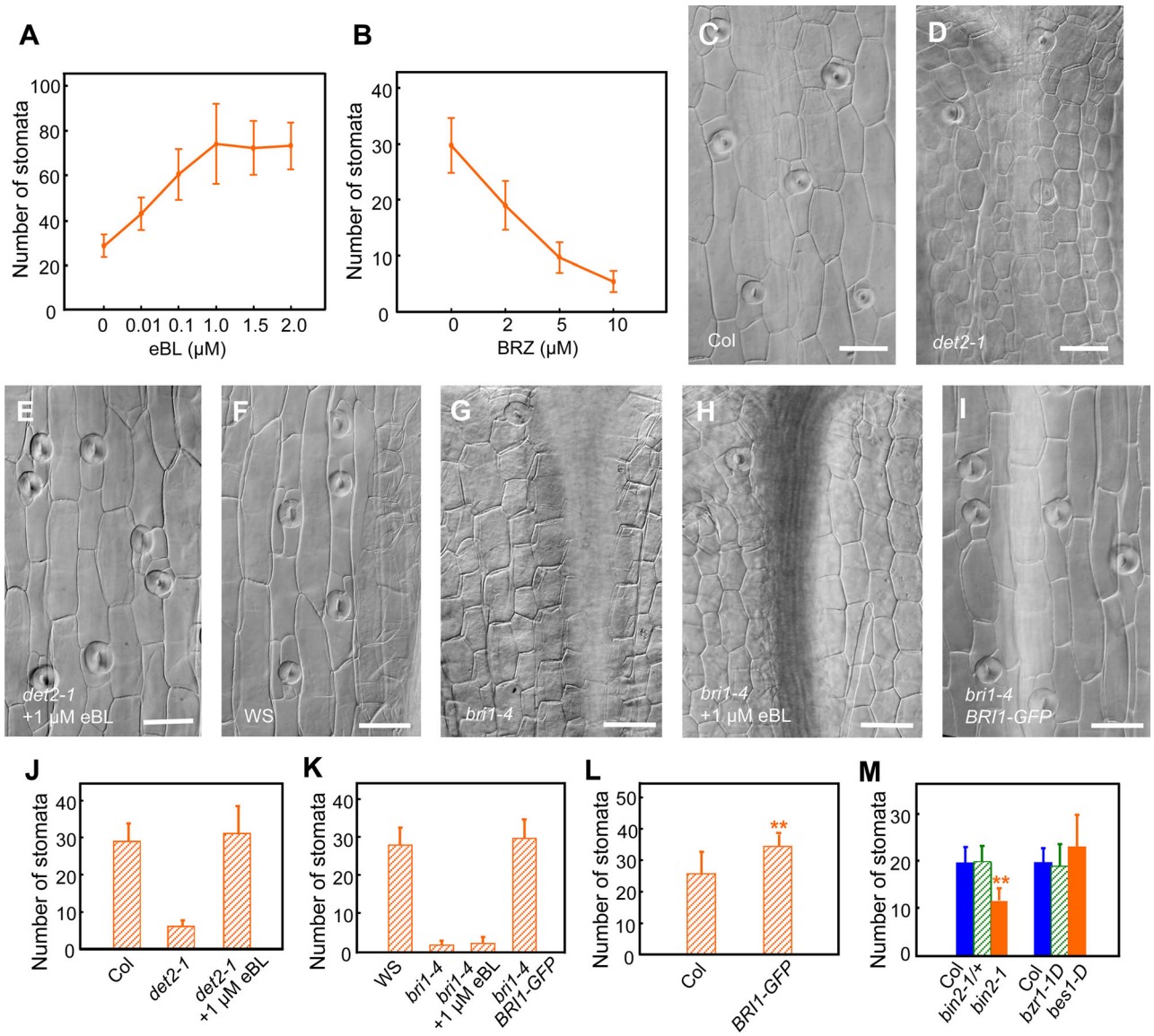

Figure 1. Brassinosteroid (BR) signals promote stomatal production in hypocotyls

(A) Quantification of stomatal number in the whole hypocotyls of 12 days post germination (dpg) wild-type seedlings grown in 1/2 MS medium containing 0, 0.01, 0.1, 1, 1.5, and 2 μmol/L eBL. **(B)** Quantification of stomatal number in the hypocotyl of 12 dpg wild-type seedlings grown in 1/2 MS medium containing 0, 2, 5, and 10 μmol/L BRZ. **(C–E)** Stomata in the hypocotyls of 12 dpg wild-type in Col ecotype **(C)**, *det2-1* **(D)** and 1 μmol/L eBL-treated *det2-1* **(E)**. **(F–H)** Stomata in the hypocotyls of 12 dpg wild-type in WS ecotype **(F)**, *bri1-4* **(G)**, and eBL-treated *bri1-4* **(H)**. **(I)** *bri1-4* mutant harboring *BRI1pro:BRI1-GFP*. **(J)** Quantification of stomatal number in the hypocotyls of 12 dpg Col, *det2-1* and eBL-treated *det2-1*. **(K)** Quantification of stomatal number of in the hypocotyls of 12 dpg WS, *bri1-4*, eBL-treated *bri1-4*, and *bri1-4* harboring *BRI1pro:BRI1-GFP*. **(L)** Quantification of stomatal number in the hypocotyls of 12 dpg Col and *BRI1pro:BRI1-GFP* seedlings. **(M)** Number of stomata in the hypocotyls of 12 dpg Col, *bin2-1*/+, *bin2-1*, *bzr1-1D*, and *bes1-D*. Values are mean ± standard deviation (*SD*), $n \geq 20$. Two asterisks indicate a very significant difference ($P < 0.01$). Bar = 50 μm.

disappeared in the hypocotyl (Figure 3F, G, J). However, after 2.5-h CHX supplemented with 1 μmol/L eBL, GFP-positive cells could still be detected (Figure 3H, I, J), confirming the function of BR stabilizing SPCH protein. Consistently, western blot analysis revealed that SPCH protein accumulation was obviously enhanced by eBL treatment and greatly inhibited by 10 μmol/L BRZ treatment (Figure 3K), indicating that BR promotes stomatal production through regulating SPCH abundance post-translationally.

YDA and CDYD4 might be not essential for BR promoting stomatal production in hypocotyls

It is well known that SPCH stabilization is regulated by the YDA-MAPK pathway (Lampard et al. 2008). Next we investigated

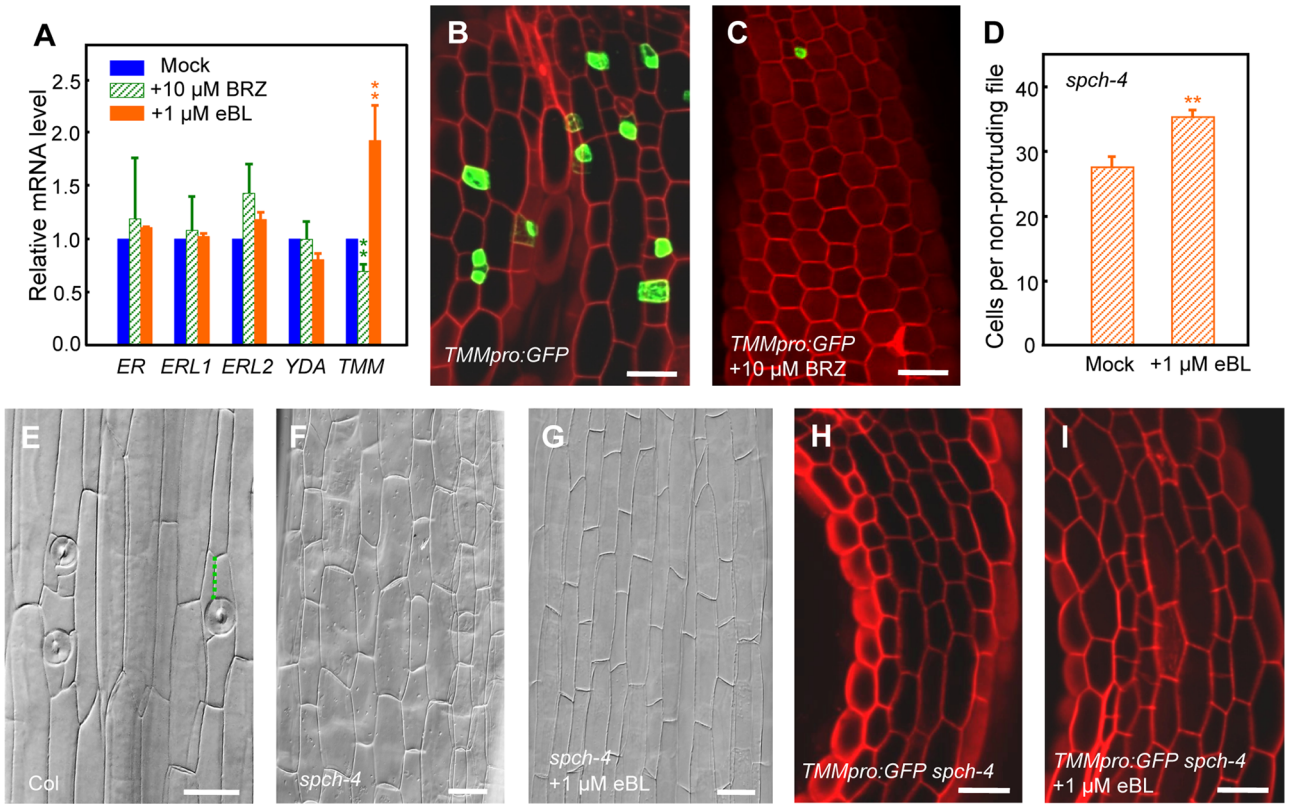

Figure 2. Brassinosteroid (BR) fails to restore stomatal initiation in *spch-4* hypocotyls
(A) Quantitative reverse transcription–polymerase chain reaction (qRT–PCR) analysis of *ER*, *ERL1*, *ERL2*, *TMM*, and *YDA* transcriptional levels in the 1.5 dpg Col seedlings grown in medium containing 10 μmol/L BRZ, or 1 μM eBL. **(B, C)** *TMMpro:GFP* expression in 1.5 dpg hypocotyls (**B**) was suppressed by BRZ treatment (**C**). **(D)** Quantification of the number of cells within non-protruding cell files from the hypocotyls of 12 dpg *spch-4* and eBL-treated *spch-4*. Cell number from at least three cell files in each hypocotyl was counted. **(E–G)** Stomatal phenotype in 12 dpg hypocotyls of wild-type **(E)**, *spch-4* **(F)** and eBL-treated *spch-4* **(G)**. **(H, I)** *TMMpro:GFP* expression in 1.5 dpg hypocotyls of *spch-4* **(H)** and eBL-treated *spch-4* **(I)**. Green dashed line in **(E)** indicates the symmetric division parallel to the apical-basal axis that is absent in *spch-4* hypocotyls (**F**). Values in **(A)** and **(D)** are mean ± *SD* of three individual experiments. Two asterisks indicate a very significant difference ($P < 0.01$). Bars = 50 μm.

whether YDA was essential for BR promotion of stomatal production. We generated *yda-10 bri1-4* double mutant by crossing. *yda-10* produces strong stomatal clusters in leaves just as *yda-1* (Kang et al. 2009). In the hypocotyl, *yda-10* formed stomatal clusters (Figure 4A), and *bri1-4* only formed several stomata (Figure 4B). The introduction of *bri1-4* weakened cluster formation in the *yda-10* hypocotyl; both the number and the size of clusters were decreased in double mutants (Figure 4C, E, F). In contrast, when *yda-10* was treated with 1 μmol/L eBL, both the number and the size of clusters were greatly increased in the hypocotyl (Figure 4D–F), suggesting the YDA-MAPK signaling pathway might be not essential for BR promotion of stomatal production in the hypocotyl.

Cyclin CYCD4, including CYCD4;1 and CYCD4;2, has been reported to promote the symmetric division of MMC precursors during the initiation of the stomatal lineage in hypocotyls (Kono et al. 2007). To investigate whether promotion of stomatal initiation in the hypocotyl by BR signal is dependent on CYCD4, null mutant *cycd4;1-2 cycd4;2-3* was treated with exogenous eBL. The results showed that 1 μmol/L eBL could still enhance stomatal formation in the *cycd4;1-2 cycd4;2-3* hypocotyl, and the increase in stomatal number in double mutant (by 2.7 times) was comparable with that in wild-type (by 2.5 times) (Figure 4G), indicating that BR promotion of stomatal initiation in the hypocotyl is independent of CYCD4.

BR confers *tmm* hypocotyls the "too many mouths" stomatal phenotype

In the hypocotyl of *tmm-1*, stomatal initiation was greatly inhibited with few *TMMpro:GFP* (Nadeau and Sack 2002a) expressing stomatal stem cells observed. In addition, stomatal differentiation was also inhibited in the hypocotyl of *tmm-1*; these cells could not differentiate into stomata, instead forming arrested-meristemoids (Figure 5A, C) (Bhave et al. 2009). Exogenous 1 μmol/L eBL promoted *TMMpro:GFP* expression in the hypocotyl of *tmm-1*, stomata production

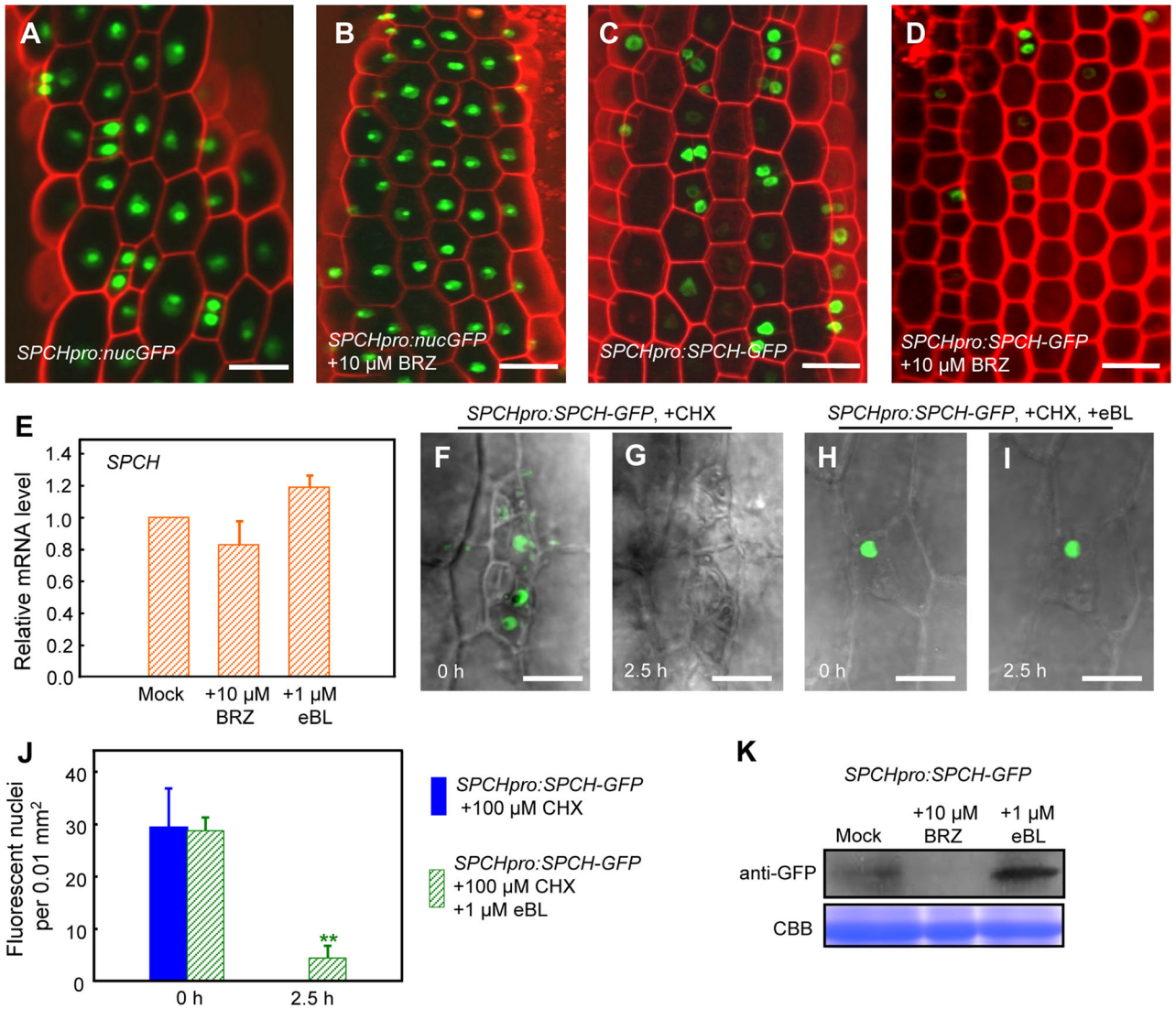

Figure 3. Brassinosteroid (BR) is required for SPEECHLESS (SPCH) protein stabilization
(A) *SPCHpro:nucGFP* promoter marker expresses in all epidermal cells of 1 day post germination (dpg) hypocotyls. (**B**) No obvious changes are found after 10 μmol/L BRZ treatment. **(C)** *SPCHpro:SPCH-GFP* expression is limited to cells related to stomatal lineage in 1 dpg hypocotyls. (**D**) The number of SPCH-GFP positive cells is reduced after BRZ treatment. **(E)** Quantitative reverse transcription–polymerase chain reaction (qRT–PCR) analysis of *SPCH* transcript levels in 1.5 dpg Col seedlings grown in medium containing 10 μmol/L BRZ, 1 μmol/L eBL. **(F–I)** *SPCHpro:SPCH-GFP* expression in the hypocotyl of 2 dpg seedlings before cycloheximide (CHX) treatment **(F and H)**. GFP disappeared after 2.5 h of 100 μmol/L CHX treatment **(G)**, but GFP could still be detected after 2.5 h 100 μmol/L CHX together with 1 μmol/L eBL treatment **(I)**. **(J)** Quantification of GFP-expressing nuclei in the hypocotyls of 2 dpg seedlings before and after CHX treatment, or CHX together with eBL treatment. Values are mean ± *SD* of triple experiments. Two asterisks indicate a very significant difference ($P < 0.01$). **(K)** Western blotting using 1.5 dpg *SPCHpro:SPCH-GFP* seedlings. SPCH-GFP proteins are not detected in seedlings grown in medium containing 10 μmol/L BRZ but accumulate in seedlings grown in medium containing 1 μmol/L eBL. Bars = 50 μm.

was also restored, especially in terms of stomata cluster formation (Figure 5B, D). However, when introducing *bri1-4* into *tmm-1*, arrested-meristemoid formation in the hypocotyl was completely inhibited (Figure 5E), and eBL could not rescue the generation of arrested-meristemoids in the hypocotyls of *tmm-1 bri1-4* (Figure 5F), suggesting that BR-induced stomatal initiation and further differentiation in *tmm-1* hypocotyls depends on a functional BRI1-mediated signaling pathway.

These results indicated that BR signal not only promoted stomatal initiation (and/or differentiation), but also lead to defects in stomatal pattern, conferring *tmm* hypocotyls the "too many mouths" stomatal phenotype that was found in other organs, such as cotyledons and leaves.

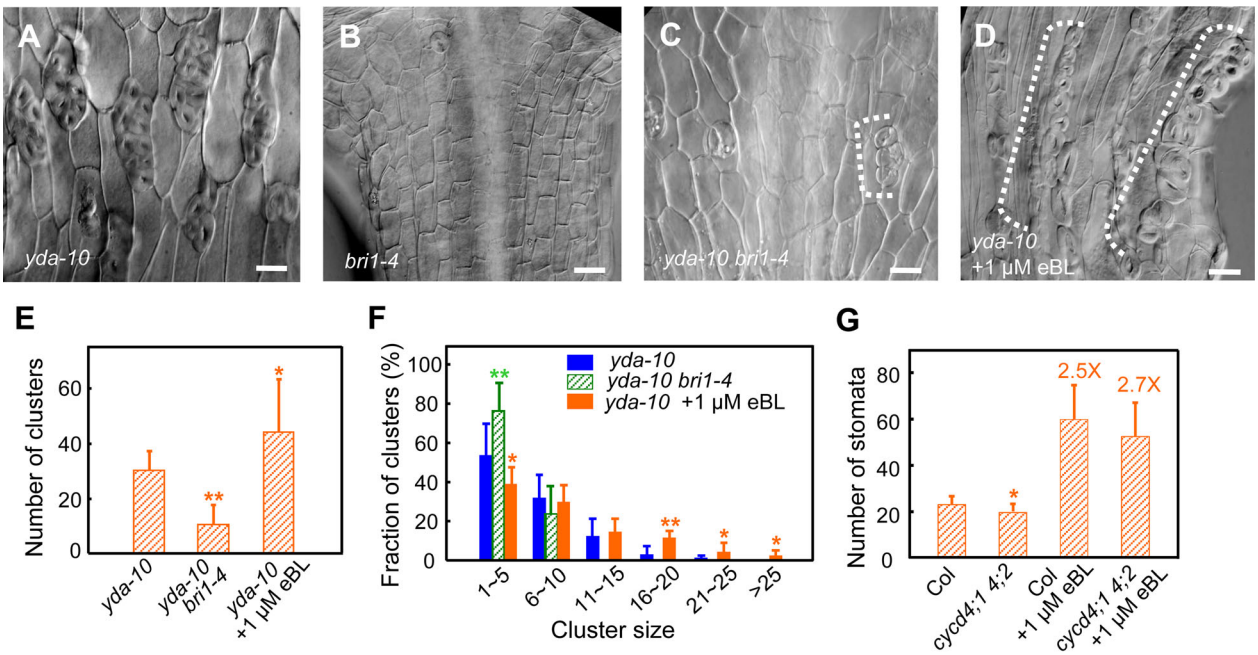

Figure 4. YODA (YDA) and CDYD4 are not essential for brassinosteroid (BR) in promoting hypocotyl stomatal production
(A–D) Stomatal phenotype in the hypocotyls of *yda-10* **(A)**, *bri1-4* **(B)**, double mutant *yda-10 bri1-4* **(C)**, and *yda-10* grown in medium containing 1 μmol/L eBL **(D)**. Brackets in **(C)** and **(D)** indicate the stomatal clusters. **(E)** Quantification of the total number of clusters in the whole hypocotyls of *yda-10*, *yda-10 bri1-4*, and *yda-10* grown in medium containing 1 μmol/L eBL. **(F)** According to the stomatal number in each cluster, cluster size is divided into six categories, 1–5, 6–10, 11–15, 16–20, 21–25, and >25. The statistic data show the percentage of clusters in each category in *yda-10*, *yda-10 bri1-4*, and *yda-10* grown in medium containing 1 μmol/L eBL. **(G)** Quantification of stomatal number in 12 dpg hypocotyls of Col and *cycd4;1-2 cycd4;2-3* double mutants. Stomatal production in Col and *cycd4;1-2 cycd4;2-3* mutant hypocotyls response to 1 μmol/L eBL treatment. Values are mean ± *SD* $n \geq 15$. One asterisk indicates a significant difference ($P < 0.05$), and two asterisks indicate a very significant difference ($P < 0.01$). Bars = 50 μm.

DISCUSSION

BR promotes stomatal formation in hypocotyls by stabilizing SPCH

Previous work indicated that both the BR-deficient mutant, that is, *det2-1*, and the BR-insensitive mutants, that is, *bri1-116* and *bin2-1*, produced stomatal clusters in their cotyledons; and the cotyledon of *bsu-q*, the loss-of-function mutant of *BSU*-related phosphatases, produced a striking phenotype consisting of almost entirely stomata. In contrast, seedlings treated with the GSK3-like kinases inhibitor bikinin and the loss-of-function mutant *bin2-3 bil1 bil2* developed a reduced number of stomata in the cotyledon (Kim et al. 2012). The gain-of-function mutant *bzr1-1D*, which could restore the dwarf phenotype of *bri1-116*, *bsu-q*, and *bin2-1*, failed to suppress their stomatal phenotype. Interestingly, BR regulates stomatal formation through BIN2-mediated phosphorylation in a BZR1-independent manner (Kim et al. 2012). Khan et al. (2013) also found that BR signal deficiency induced stomatal patterning defects in *Arabidopsis* cotyledons, in agreement with the BR-repressed stomatal formation in leaves by BIN2-mediated phosphorylation. However, the presence of stomatal patterning defects found in BR mutants might depend on growth conditions. For example, sucrose or light conditions might be beneficial for cluster formation (Gudesblat et al. 2012a).

Here, we focus on hypocotyls in which the total stomatal number and pattern are easier to score compared to cotyledons and leaves. According to our observation, stomatal production in hypocotyls is closely associated with BR levels. Our genetic analysis indicates that the YDA-MAPK pathway, which directly controls SPCH stabilization, might not be essential for BR-promoted stomatal production in hypocotyls. TMM dampens excessive signaling to ER family receptors from EPFL6/CHALLAH (CHAL) family ligands in hypocotyls. Serna (2013) proposed that CHAL family ligands might be responsible for BR organ-specific regulation of stomata development, but no data are currently available to verify this concept. Thus, the mechanism of BR in regulating stomatal formation is more complicated than the current proposed models. Further investigation on organ, cellular and substrate levels will bring new insights of how BR regulates stomatal production (Le et al. 2014).

Cross-talk between BR and TMM-mediated signaling pathways

Brassinosteroid represses stomatal formation in cotyledons and promotes stomatal production in hypocotyls. TMM displays similar organ-specific regulation of stomatal development, with loss-of-function of *TMM* resulting in stomatal cluster formation in leaves and no stomatal generation in

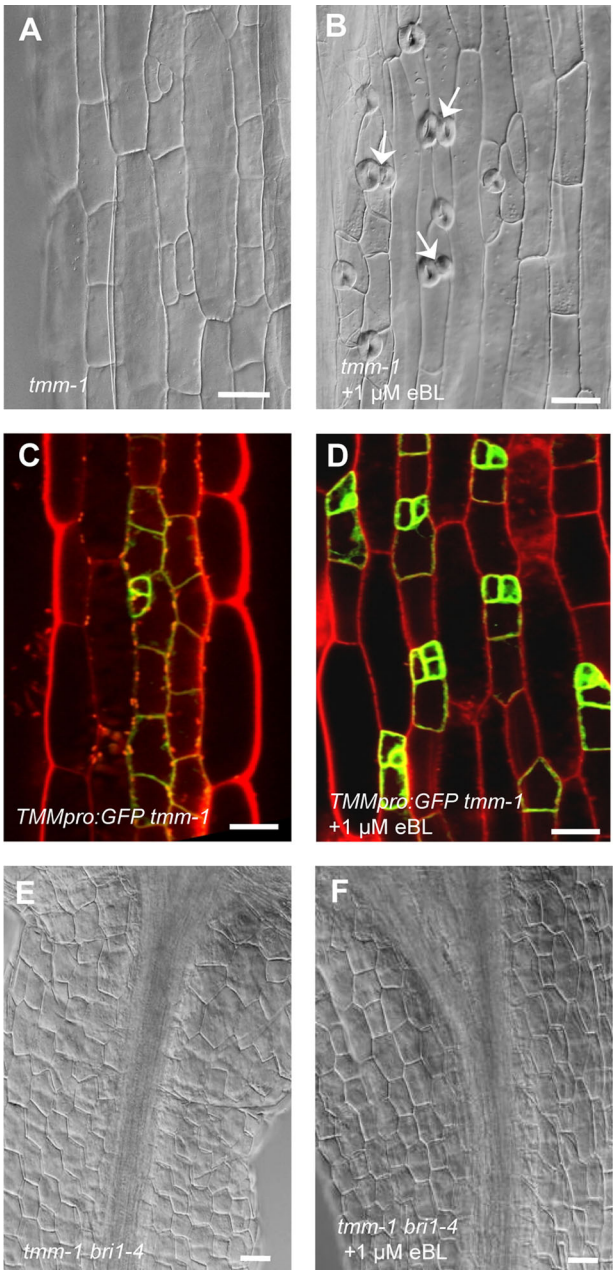

Figure 5. Brassinosteroid (BR) promotes stomatal production in *tmm-1* hypocotyl

(A) No stoma is produced except a couple of arrested meristemoids in a 12 dpg *tmm-1* hypocotyl. **(B)** Stomatal clusters (arrows) are presented in the hypocotyls of 12 dpg *tmm-1* grown in medium containing 1 μmol/L eBL. **(C and D)** *TMMpro:GFP* expression in the hypocotyls of 1.5 day post germination (dpg) *tmm-1* **(C)** and 1 μmol/L eBL treated *tmm-1*, eBL induces the formation of cells expressing *TMMpro:GFP* **(D)**. **(E and F)** No asymmetric division forms in the hypocotyls of 12 dpg *tmm-1 bri1-4* **(E)** and *tmm-1 bri1-4* grown in medium containing 1 μmol/L eBL **(F)**. Bars = 50 μm.

hypocotyls (Yang and Sack 1995; Geisler et al. 1998; Bhave et al. 2009). Interestingly, here we found that 1 μmol/L eBL restored stomata, especially stomatal cluster production in *tmm* hypocotyls. This observation is different from the previously published results that exogenous brassinolide (BL) could enhance cell divisions and meristemoids formation but could not restore stomatal production in the *tmm* hypocotyl (Fuentes et al. 2012; Gudesblat et al. 2012b). Different chemical activities and concentrations of exogenous active BRs, as well as different growth conditions, might be the reason for this difference.

That BR confers the "too many mouths" stomatal phenotype in *tmm* hypocotyls reminds us of the *CHAL* family genes, including *CHAL*, *EPFL5/CHALLAH-LIKE1* (*CLL1*) and *EPFL4/CHALLAH-LIKE2* (*CLL2*). *CHAL* was identified as a suppressor of the *tmm* stoma-deficient phenotype in the hypocotyl; knock-down or knock-out of *CHAL* could rescue stomatal generation in *tmm* hypocotyls (Abrash and Bergmann 2010). The triple mutants *tmm chal cll1*, *tmm chal cll2*, *tmm cll1 cll2* and the quadruple mutant *tmm chal cll1 cll2* all form stomatal clusters in the hypocotyls (Abrash et al. 2011), which is similar to the cluster phenotype in the hypocotyls of eBL- treated *tmm*. On the one hand, the effects of BR on stomatal formation in *tmm* hypocotyls might be an indirect function. The eBL-induced increase in cell size may reduce the relative concentration of CHAL family ligands in the epidermis cells resulting in stomatal generation in *tmm* hypocotyls. On the other hand, a direct cross-talk between TMM-CHAL and BR signaling pathways might exist in regulating stomatal formation, and further investigation will be required to explore this.

BR and other signaling pathways in hypocotyl stomatal development

Similarly to BR, the plant hormone gibberellin (GA) also promotes stomatal formation in hypocotyls, but has no effects on stomatal development in leaves (Saibo et al. 2003). In the hypocotyls of the GA-deficient mutant *ga1-3* or GA biosynthesis inhibitor (PAC) treated wild-type seedlings, stomatal formation is eliminated (Saibo et al. 2003). Although PAC severely inhibited stomatal production in the hypocotyl, it did not affect *TMMpro:GUS* expression in MMC precursors, indicating that GA is mainly required for stomatal differentiation (Kono et al. 2007). Here, we show that the SPCH protein level, the key early regulator of stomatal initiation, was dramatically reduced by BRZ treatment. Thus the BRZ-suppressed expression of *TMMpro:GFP* in hypocotyls can be caused by the reduced number of stomatal lineage cells that is controlled by SPCH transcription factor. However, we can not exclude the existence of BR-mediated transcriptional regulation of the *TMM* gene. Thus both BR and GA promote stomatal development in hypocotyls, but they might function in different pathways and developmental stages.

Loss-of-function of *SPCH* results in the failure of stomatal initiation as no asymmetric division occurs and no stoma forms in the hypocotyl. Application of 1 μmol/L eBL could not rescue asymmetric division and stomatal production in *spch*. At the same time, symmetric divisions parallel to the apical-basal axis were neither generated in *spch* nor 1 μmol/L eBL treated *spch* (Figure 2F, G). However, 1 μmol/L eBL enhanced the number of cells in the non-protruding cell files of *spch* (Figure 2D),

suggesting that these cells are produced from symmetric division perpendicular to the apical-basal axis of hypocotyls. These cells are not stomatal lineage cells with no *TMM* expression (Figure 2I). Thus, we conclude that, although the postembryonic cell divisions in the hypocotyl are considered to be related with stomatal formation (Saibo et al. 2003), the longitudinal divisions rather than the transverse divisions are stomata-specific.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thalina mutants *det2-1* (Fujioka et al. 1997), *bin2-1* (Li et al. 2001), *bzr1-1D* (Wang et al. 2002), *tmm-1* (Yang and Sack 1995), *cycd4;1-2 cycd4;2-3* (Kono et al. 2007), *spch-4* (Yang et al. 2014), and *yda-10* (Kang et al. 2009) are in the Columbia ecotype backgrounds except *bri1-4* (Noguchi et al. 1999) in the Wassilewskija (Ws-2). *spch-4* and *bri1-4* were obtained from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA) and confirmed by PCR analysis. All seedlings were grown at 22 °C in half-strength Murashige and Skoog (MS) medium or in soil under 16 h/8 h day/night light cycles.

Chemical treatments

For epibrassinolide (eBL, Sigma-Aldrich, St Louis, MO, USA, E1641) or brassinazole (BRZ, TCI, B2829) treatments, seeds were germinated in half-strength MS medium containing eBL or BRZ. eBL was dissolved in 80% ethanol, BRZ was dissolved in acetone to make stock solution. eBL was added to the medium at final concentration of 0.01, 0.1, and 1 μmol/L; BRZ at 2, 5, and 10 μmol/L.

For time-lapse, 2 dpg hypocotyls were used for the observation of *SPCHpro:SPCH-GFP* using a confocal laser scanning microscope (Olympus, Tokyo, Japan, FV1000-MPE), then seedlings were transplanted into half-strength MS medium containing 100 μmol/L cycloheximide (CHX) or 100 μmol/L CHX together with 1 μmol/L eBL, and expression of *SPCHpro:SPCH-GFP* in hypocotyls was observed every half an hour.

Microscopy

Twelve-day-old hypocotyls were fixed and cleared as described by Malamy and Benfey (1997). Stomatal images were taken using Nomarski optics on an Olympus BX51 microscope. For fluorescence observation, hypocotyls were first stained with 0.5% (w/v) propidium iodide (PI) and imaged using a confocal laser scanning microscope (Olympus, Tokyo, Japan, FV1000-MPE). For stomatal number quantification, stomata in each whole hypocotyl from 12 dpg seedlings were counted.

Total RNA isolation and quantitative qRT-PCR analysis

Total RNA was extracted from 1.5 dpg seedlings using the TRNzol reagent (Tiangen, Beijing, China). First-strand cDNA was synthesized with Moloney Murine Leukemia Virus Reverse Transcriptase (Promega). cDNAs were amplified using SYBR Premix ExTaqTM (TaKaRa, Dalian, China) with Corbett RG3000. ACTIN2 was used as the internal control. RNA isolation and qRT-PCR experiments were repeated three times. The primers used in qRT-PCR experiments for testing *ER*, *ERL1*, *ERL2*, *YDA*, and *TMM* were designed according to Yamamuro et al. (2014); and primers used for testing *SPCH* expression were designed as Yang et al. (2014).

Western blotting

Total proteins were extracted from 2 g (fresh weight) of 1.5 dpg seedlings of *SPCHpro:SPCH-GFP* and *SPCHpro:SPCH-GFP* grown in medium containing 1 μmol/L eBL or 10 μmol/L BRZ. The extraction buffer contained 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP-40, and protease inhibitor cocktail (Roche Diagnostics), pH7.5. Extracts were centrifuged at 14,000 *g* at 4 °C for 40 min. Total proteins were separated using 12% SDS–PAGE and analyzed with an anti-GFP antibody (MBL, Nagoya, Japan) at 1:10,000 dilution.

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