Spotlight

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Brassinosteroids tailor stomatal production to different environments

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Two recent reports show that brassinosteroids control stomata production by regulating the GSK3-like kinase BIN2-mediated phosphorylation of two different stomatal signalling components resulting in opposite stomatal phenotypes. We discuss how these two mechanisms might differentially control stomatal generation under diverse growth conditions.

A new role for brassinosteroids uncovered

Stomata are microscopic pores on the leaf surface that control carbon dioxide and water vapour exchange between the plants and their environment. Regulation of stomatal aperture and density ensures optimal photosynthesis and transpiration rates. The stomatal lineage is initiated from an undifferentiated protodermal cell through multiple asymmetric cell divisions and cell fate decisions (Figure 1). Within this cell lineage, the stomatal fate is limited by positional signalling events that ensure that stomata are separated from each other by at least one non-stomatal epidermal cell. Development of stomata is regulated by a mitogen-activated protein kinase (MAPK) signalling cascade including the MAPK kinase kinase (MAPKKK) YODA (YDA) that eventually phosphorylates and inactivates the basic helix–loop–helix (bHLH) transcription factor SPEECHLESS (SPCH) required for stomata lineage initiation and progression. The MAPK module is activated by receptor–ligand complexes, including the ERECTA (ER) family of leucine-rich repeat receptor-like kinases (LRR RLKs), the LRR receptor protein TOO MANY MOUTHS (TMM) and the peptide ligands belonging to the EPIDERMAL PATTERNING FACTOR (EPF) class [1].

The plant brassinosteroid (BR) hormones control diverse aspects of plant development and are recognised by the LRR RLK BR-INSENSITIVE 1 (BRI1). Upon BR recognition, BRI1 is activated and a signal is relayed through a phosphorylation cascade that inhibits BR-INSENSITIVE 2 (BIN2), a GSK3-like kinase whose function is to phosphorylate and inactivate two transcription factors belonging to the BRASSINAZOLE RESISTANT (BZR) family [2] (Figure 1). Mutants affected in the BR production have been reported to display a mild stomatal clustering and an excess of stomata in *in vitro*-grown leaves [3]. However, no detailed analyses of stomatal phenotypes of BR-related mutants have been carried out so far. Recently, molecular links between stomata and BR signalling pathways have been identified by two research groups [4,5]. One group discovered that BIN2, a negative regulator of the BR signalling, can inactivate the MAPKKK YDA by phosphorylation in its autoregulatory domain [4]. The overabundance of stomata and stomatal clustering in BR-related mutants might be due to an increased activity of SPCH caused by the inactive YDA in these plants. By contrast, evidence has been provided that similar BR mutants, grown under different conditions, not only completely lack stomatal clustering in cotyledons and leaves, but also display a slightly decreased stomatal index in leaves and a strongly reduced number of stomata in hypocotyls [5]. This promotive effect of BRs on stomatal and epidermal development results from the counteraction of the BIN2-mediated phosphorylation and subsequent degradation of SPCH. BIN2 phosphorylates residues of SPCH already described as MAPK targets as well as novel phosphorylation sites in the N-terminal region of SPCH, which lead to excessive epidermal cell proliferation when mutated. Excess of BRs increases the number of stomata in hypocotyls, but does not enhance stomata production in cotyledons or leaves, except for plants overproducing SPCH within its native domain, suggesting that MAPKs and BIN2 redundantly control phosphorylation of SPCH [5]. These results are in contrast to the repressive effect of excessive BRs on stomatal formation in cotyledons of the BR-biosynthetic mutant *de-etiolated 2* and the similar effect caused by bikinin (a BIN2 kinase inhibitor that mimics the BR effects on shoot growth) in cotyledons of wild-type, *tmm* and *er;erl1;erl2* triple mutant [4]. In agreement with the observed phosphorylation of YDA by BIN2, bikinin fails to affect the ectopic stomatal production in the mutants *yda-Y295* and *scream-D* (a dominant mutation in the bHLH transcription factor SCREAM) and in plants containing inactive MPK3 and MPK6 [4]. However, a second allele of *yda* (*yda-5*) was responsive to BRs for stomatal development in hypocotyls [5]. In addition, BRs and bikinin both prevent the YDA-regulated activation of MPK3 and MPK6 by flagellin 22 (flg22) [4]. However, expression of the two MAPKs and YDA is not restricted to the stomatal lineage and, besides YDA, the MAPKKK MEKK1 has also been shown to mediate the activation of MPK3 and MPK6 in response to flg22 [6]. Clearly, additional experiments, involving identical genotypes, tissues, growth conditions, and treatments are needed to elucidate the precise role of BRs in stomatal development.

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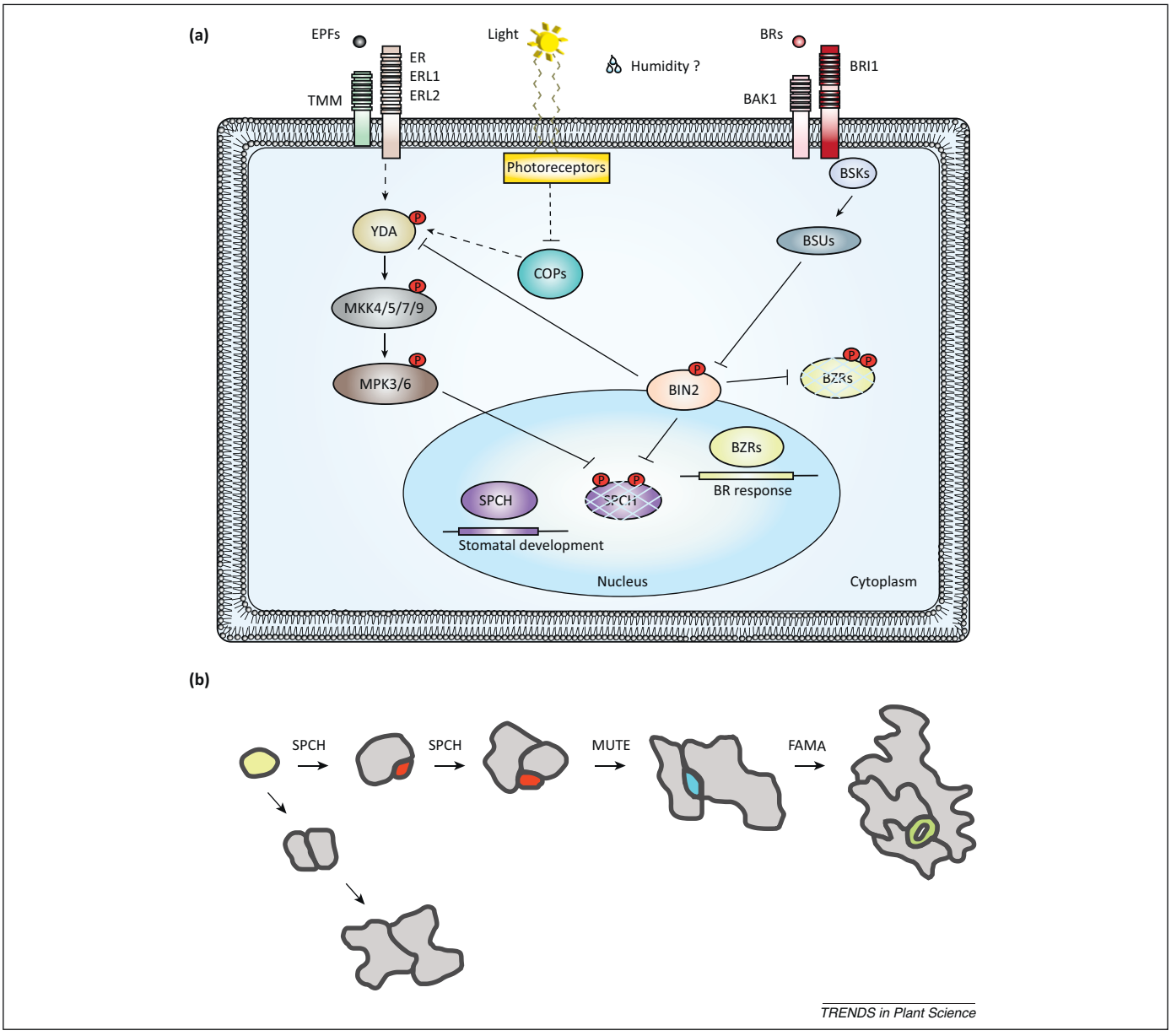

Figure 1. Plant brassinosteroid (BR) regulation of stomatal development. (**a**) An inhibitory cascade activated by binding of EPIDERMAL PATTERNING FACTOR (EPF) peptides to the TOO MANY MOUTHS (TMM)/ERECTA (ER) receptors activates a mitogen-activated protein kinase (MAPK) cascade that results in phosphorylation and subsequent inactivation and degradation of the basic helix–loop–helix (bHLH) transcription factor SPEECHLESS (SPCH). BRs can regulate this signalling cascade in opposite ways: either they inhibit the stomatal development by relieving the BR INSENSITIVE 2 (BIN2) phosphorylation of YDA on its autoregulatory domain [4] or they promote it by inhibiting the BIN2 phosphorylation of SPCH, thus increasing its stability [5]. BR mutants display light phenotypes in the dark similar to the constitutive photomorphogenesis (*cop*) mutants (*cop1*, *det1*/*fus2*, *cop10*, *cop8*, *cop11* and *cop12*/*fus12*) that show stomatal clustering when grown under these conditions. COP1 acts downstream of photoreceptors, phytochromes and cryptochromes (yellow box) and upstream of YDA. Additional environmental factors, such as humidity, could also affect stomatal development via a still uncharacterised mechanism. (**b**) Meristemoid mother cells (yellow) present in developing leaf epidermis can either differentiate into pavement cells after dividing symmetrically, or undergo a SPCH-dependent division that creates a triangular meristemoid (red), thus initiating a stomatal lineage. After null to three amplifying (also SPCH-regulated) divisions, the meristemoid differentiates into a guard mother cell (blue) that, in turn, divides symmetrically to generate stomata. The last two steps depend on two bHLH transcription factors closely related to SPCH, designated MUTE and FAMA.

Stomatal clustering in BR mutants: a photomorphogenetic connection?

To unravel the reason for the opposite effects of BRs on stomatal development [4,5], similar combinations of genotypes and chemical treatments should be examined and compared. Nevertheless, it is striking that the identical alleles *bri1-116* and *bin2-1* display either an excess of stomata [4] or no change or reduction, depending on the observed organs [5]. Could the differences in phenotypes be connected to different growth conditions? Remarkably, the overabundance of stomata and clustering phenotypes in leaves and cotyledons of BR mutants is observed only in plants grown in Murashige and Skoog medium *in vitro* and completely absent in plants grown in soil (our observations). Under the *in vitro* growth conditions [5], excess and clustering of stomata appear progressively only after day 8 and increase with age. When plants are grown under continuous light or when sucrose is supplemented to the growth medium (our observations; Montaña Mena and Carmen Fenoll, personal communication), these phenotypes are enhanced and start to develop earlier. Clearly, BRs prevent overproliferation of stomata in leaves and

cotyledons by counteracting an unidentified factor present under *in vitro* growth conditions. Probably, the phosphorylation of YDA mediated by BIN2 plays a role in this regulation, but phosphorylation of SPCH causes the observed promotive effects of BRs on stomatal development [5].

Which mechanism is involved in shifting the balance towards increasing or decreasing the number of stomata in BR mutants according to the environment? A possible clue might be found in the observed constitutive photomorphogenesis (COP) phenotypes of BR loss-of-function mutants. Interestingly, *cop* mutants not directly connected with BR signalling, namely *cop1*, *det1/fus2* [7], *cop10*, *cop8*, *cop11,* and *cop12/fus12* [8,9], also display stomatal clustering and possibly overproliferation of stomata when grown *in vitro*. In the case of *cop10-1*, just as for *in vitro*-grown BR mutants, clusters are absent at the very early stages of growth but appear progressively later [8]. COP1 regulates the stomatal development upstream of YDA and downstream of different photoreceptors, which are expressed in the stomatal lineage [7]. Thus, YDA possibly acts as a node integrating signals from light, BRs, and perhaps other environmental stimuli, such as humidity [10] (Figure 1). Given that under *in vitro* growth conditions humidity is typically close to 100%, it is likely that high humidity can also contribute to the excess of stomata in BR mutants grown under these conditions.

Future outlook

It is evident that BRs regulate stomatal development at more than one level. Because excess of stomata and clustering observed in *in vitro*-grown BR mutants occurs also in several other *cop* mutants, it would be interesting to find out whether there are themes common to all of them, such as, for example, dependence of the stomatal phenotype on *in vitro* growth conditions, epistatic relationship with YDA, and light regulation of stomata. Other well-studied MAPKKKs require a complex control system with multiple steps for activation, suggesting that a more in-depth analysis of the YDA regulation is needed. By contrast, the promotive effect of BRs on stomatal and epidermal development by counteracting the BIN2-mediated phosphorylation of SPCH, which occurs under natural growth conditions, might satisfy the enhanced carbon demand imposed by BRs on plant growth. Further studies on the role of BIN2 phosphorylation of SPCH, particularly on the novel regulatory residues located outside the MAPK target domain, and of YDA regulation will help to clarify the contribution of BR regulation of stomatal development on plant fitness.

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