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Brassinosteroid regulates stomatal development by GSK3-mediated inhibition of a MAPK pathway

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Abstract

Plants must coordinately regulate biochemistry and anatomy to optimize photosynthesis and water use efficiency. The formation of stomata, epidermal pores facilitating gas exchange, is highly coordinated with other aspects of photosynthetic development. However, the signaling pathways controlling stomata development are not fully understood[1,2], although Mitogen Activated Protein Kinase (MAPK) signaling is known to play key roles. Here we demonstrate that brassinosteroid (BR) regulates stomatal development by activating the MAPKKK, YODA. Genetic analyses indicate that receptor kinase-mediated BR signaling inhibits development through the GSK3-like kinase BIN2, and BIN2 acts upstream of YODA but downstream of the known ERECTA family of stomatal receptor kinases. Complementary *in vitro* and *in vivo* assays show that BIN2 phosphorylates YODA to inhibit YODA phosphorylation of its substrate MKK4, and activities of downstream MAPKs are reduced in BR-deficient mutants but increased by treatment with either BR or GSK3-kinase inhibitor. Our results indicate that BR inhibits stomatal development by alleviating GSK3-mediated inhibition of the MAPK module, providing two key links; that of a plant MAPKKK to its upstream regulators and BR to a specific developmental output.

In both animals and plants, steroid hormones play important roles in coordinating development and metabolism[4]. In contrast to animal steroid hormones, which act through nuclear receptor transcription factors[4], the plant steroid hormone brassinosteroid (BR) binds to the extracellular domain of the membrane-bound receptor kinase BRI1. This activates intracellular signal transduction mediated by BSK1 kinase, BSU1 phosphatase, BIN2 (GSK3-like) kinase, PP2A phosphatase, and BZR1-family transcription factors[3,7,8,9,10,11,12]. When BR levels are low, BZR1 is inactivated due to phosphorylation by the GSK3-like kinase BIN2[13,14]. BR signaling leads to inactivation of BIN2, and PP2A-mediated

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Author Contributions T.W.K. performed all experiments. T.W.K, and Z.W designed the experiments, analyzed data and wrote the manuscript. M.M cloned cDNAs of BSL2 and BSL3, and BSL2pro-BSL2. D.C.B. contributed materials and wrote the manuscript.

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dephosphorylation and activation of BZR1[3,11,12] (Supplementary Fig. 1A). Although the BR signaling pathway is well characterized, its connections to other signaling and developmental pathways are not fully understood.

Stomata are epidermal pores that control gas exchange between the plant and the atmosphere and are critical for maintaining photosynthetic and water-use efficiency in the plant. The density and distribution of stomata in the epidermis of aerial organs is modulated by intrinsic developmental programs, by hormones, and by environmental factors such as light, humidity, and carbon dioxide[1,2,15,16]. The genetically-defined signaling pathway that regulates stomatal development includes peptide ligands, a receptor protein (TMM), the ERECTA family (ERf) of receptor-like kinases (ER, ERL1 and ERL2) and a MAPK module (MAPKKK: YDA, MAPKKs: MKK4/5/7/9, and MAPKs: MPK3/6)[17]. Potential downstream targets include bHLH transcription factors SPEECHLESS (SPCH), MUTE, FAMA, ICE1/SCRM and SCRM2, with SPCH being negatively regulated by direct MPK3/6-mediated phosphoregulation[18,19] (Supplementary Fig. 1B). The MAPK pathway could potentially integrate environmental and hormonal inputs to optimized stomatal production, but nothing is known about the nature of these signals and their biochemical mechanisms of MAPK pathway regulation.

Excess stomata have been observed in some BR-deficient mutants[5]. To elucidate the function of BR in regulating stomatal development, we examined the distribution of stomata on leaves of BR-deficient and BR-signaling mutants. In wild-type *Arabidopsis*, stomata are always distributed with at least one pavement cell between them (Fig. 1a). BR deficiency causes stomatal clusters (Fig. 1b and 1c), whereas treatment with brassinolide (BL, the most active form of BR) reduces stomatal density (Fig. 1d), indicating that BR represses stomatal development. The BR-insensitive mutants *bri1-116*, quadruple *amiRNA BSL2,3;bsu1;bsl1* (*bsu-q*)[11], dominant *bin2-1*, and BIN2-overexpressing plants also exhibit stomatal clustering (Fig. 1e–h), and overproduce stomatal precursors (meristemoids and guard mother cells) (Fig. 1u and Supplementary Fig. 2). In contrast to the weak stomatal clustering phenotype of the *det2-1* and *bri1-116* mutants, *bsu-q* showed large stomatal clusters on hypocotyls (Supplementary Fig. 4) and cotyledon surfaces consisting almost entirely of stomata (Fig. 1f, u and Supplementary Fig. 2, 3). Surprisingly, the hyperactive *bzr1-1D* mutation[12,20] did not affect stomatal development or suppress the stomatal phenotypes of *bri1-116*, *bsu-q* and *bin2-1*, although it suppressed their dwarf phenotypes (Fig. 1i–n and Supplementary Fig. 5). These results indicate that BR regulation of stomatal development is mediated by upstream signaling components including BRI1, BSU1, and BIN2, but is independent of the BIN2-substrate BZR1.

Consistent with increased stomatal development in BR-insensitive mutants, fewer stomata were observed in cotyledons of plants overexpressing some of the positive BR-signaling components of the BSU1 family (Fig. 1q, u and Supplementary Fig. 6) and in *bin2-3;bil1;bil2* loss-of-function mutants lacking 3/7 BR-signaling GSK3-like kinases (Fig. 1o, p, u and Supplementary Fig. 2). We used bikinin, a highly specific inhibitor for the 7 *Arabidopsis* GSK3-like kinases that appear to be involved in BR signaling[21,11,22], to further investigate the function of BR-related GSK3-like kinases in stomatal development. When added to the growth medium, bikinin decreased stomatal production in wild-type plants,

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fully suppressed the stomatal clustering phenotypes of *bin2-1*, and significantly suppressed the severe stomatal phenotypes of *bsu-q* (Fig. 1r–u). These results confirm that increased activity of the GSK3-like kinases is responsible for enhanced stomatal production in BR-deficient and BR-insensitive mutants.

We examined genetic interactions between BR mutants and known stomatal mutants. Expression of constitutively-active YDA (*CA-YDA*) can completely eliminate stomatal development[23] (Fig. 2a), likely via activation of a MAP kinase pathway that phosphorylates and inactivates SPCH[17,18]. Expression of *CA-YDA* completely suppressed stomatal development of the *bri1-116*, *bsu-q* and *bin2-1* mutants (Fig. 2b–d). Loss of SPCH was also completely epistatic to *bsu-q* in that a *bsu-q;spch-3* (null) mutant lacked stomata and precursors (Fig. 2e, f), indicating that the BR signaling components act upstream of the canonical stomatal MAP kinase pathway. Bikinin effectively suppressed the weak stomatal clustering phenotype of *tmm* and partially suppressed the severe phenotype of *er;erl1;erl2* triple mutants (Fig. 2g, h and Supplementary Fig. 7 and 8), but had no significant effect on the phenotypes of the *yda* mutant, on plants overexpressing the pathogen effector HOPAI1 (which inactivates MPK3 and MPK6, Ref. 24), or the *scrm-D* gain-of-function mutant[25] (Fig. 2i–k, and Supplementary Fig. 8). The BR biosynthetic inhibitor brassinazole also significantly enhanced the stomatal phenotypes of *tmm*, but did not further increase stomata in *er;erl1;erl2*, likely because the *er;erl1;erl2* surfaces are already nearly confluent with stomata (Supplementary Fig. 9). These results strongly indicate that GSK3-like kinases act downstream of the ER and TMM receptors, but upstream of the YDA MAPKKK.

YDA contains 84 putative GSK3 phosphorylation sites (Ser/Thr-x-x-x-Ser/Thr). Many of these sites are conserved in YDA's two rice homologs, Os02g0666300 and Os04g0559800, and these homologues also share a highly conserved sequence just N-terminal of the kinase domain. Importantly, YDA can be made constitutively active when part of this region (AA 185–322, Fig. 3a) is deleted[23]. The region deleted in CA-YDA contains 23 putative GSK3 phosphorylation sites including successive phosphorylation sites as found in the known BIN2 target BZR1 (Fig. 3a and Supplementary Fig. 10).

We tested whether BIN2 directly interacts with and phosphorylates YDA. MBP-YDA was detected in an overlay assay by using GST-BIN2 and anti-GST antibody (Fig. 3b), demonstrating direct YDA binding to BIN2 *in vitro*. BIN2 also interacted with YDA and CA-YDA in yeast two-hybrid assays (Fig. 3c). *In vitro* kinase assays demonstrated that BIN2 phosphorylated YDA, but YDA did not phosphorylate a kinase-inactive BIN2 mutant or other BR signaling components (Fig. 3d and Supplementary Fig. 11). BIN2 strongly phosphorylated the region deleted in CA-YDA (Fig. 3e), suggesting that BIN2 might inhibit YDA by phosphorylating its auto-regulatory domain.

BIN2 phosphorylation of BZR1 causes mobility shifts of the phosphorylated BZR1 band in SDS-PAGE gels[14]. Like BZR1, YDA phosphorylated by BIN2 *in vitro* also exhibited slower mobility (Fig. 3d and Supplementary Fig. 11). Consistent with the *in vitro* data, bikinin treatment of *Arabidopsis* seedlings increased the mobility of YDA-myc in SDS-PAGE (Fig. 3f). When transiently expressed in *N. benthamiana* leaf cells, both YDA-myc and CA-YDA-myc were co-immunoprecipitated by anti-GFP antibody when co-expressed with BIN2-YFP

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but not when expressed alone (Fig. 3g), demonstrating interaction between BIN2 and YDA *in vivo*. Furthermore, co-expression of BIN2 retarded mobility of YDA, but not of CA-YDA bands in immunoblots (Fig. 3g). These results confirm that BIN2 mainly phosphorylates the YDA N-terminal regulatory domain.

Finally, we tested whether BIN2 phosphorylation of YDA affects YDA kinase activity and whether BR and bikinin affect MAPK activity in plants. YDA was pre-incubated with BIN2 and ATP, or a kinase-inactive mutant BIN2 (mBIN2) as control, and then purified and further incubated with MKK4 (its known substrate), bikinin, and ^{32}P-γ-ATP. Pre-incubation with BIN2, but not with mBIN2, decreased YDA phosphorylation of MKK4 (Fig. 3h, Supplementary Fig. 12), indicating that BIN2 phosphorylation inhibits YDA activity. Consistent with BIN2 inactivation of YDA, the kinase activities of MPK3 and MPK6 were reduced in the *det2* mutant but increased by treatment with bikinin or brassinolide (Fig. 3i and 3j).

Taken together, our genetic and biochemical analyses demonstrate that BR negatively regulates stomatal development by inhibiting the BIN2-mediated phosphorylation and inactivation of YDA (Fig. 3k). When BR levels are low, active BIN2 directly phosphorylates and inactivates YDA; reduced MAP kinase pathway activity can derepress SPCH, allowing SPCH to initiate stomatal development. BR signaling through BRI1, BSK1, and BSU1 inactivates the GSK3s, resulting in activation of the MAP kinase pathway and inhibition of stomatal production (Fig. 3k).

This study supports a role of BR as a master regulator that coordinates both physiological and developmental aspects of plant growth. Previous studies have demonstrated key functions of BR in inhibiting photomorphogenesis and photosynthetic gene expression[6,26,27]. Here we add a role for BR in stomatal production, which must be coordinated with other developmental processes to optimize photosynthetic and water-use efficiency. Interestingly, BR represses light-responsive gene expression and chloroplast development mainly through the BZR1-mediated transcriptional network[26,27], but represses stomatal development through a BZR1-independent GSK3-MAPK crosstalk mechanism. Both GSK3 and MAPK are highly conserved in all eukaryotes, but it remains to be seen whether GSK3s directly inactivate MAPKKKs in animals. This GSK3-MAPK connection has the potential to act in multiple receptor kinase-mediated signaling pathways, mediating crosstalk between these pathways in plants. The stronger stomata-clustering phenotype of *bsu-q* and suppression of *er;erl1;erl2* stomata phenotypes by bikinin raise a possibility that members of the BSU1 and GSK3 families mediate signaling by the ERf receptor kinases. The signals from BRI1 and ERf must be partitioned differently downstream, however, such that BRI1 controls GSK3 regulation of both BZR1 and YDA but ERf mainly controls the GSK3 inactivation of YDA (Fig. 3k), because *er;erl1;erl2* had no obvious effect on BR-regulated BZR1 phosphorylation (Supplementary Fig. 13). Similar mechanisms and components might also be used by additional signaling pathways, such as the innate immunity pathway downstream of the FLS2 receptor kinase, which shares with BRI1 the BAK1 co-receptor[28] and downstream components MPK3/6 (Ref. 24). In support of such an idea, overexpression of a GSK3-like kinase reduced the pathogen-induced activation of MPK3 and MPK6 (Ref. 29). How signaling specificity is maintained when multiple

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pathways share the same components is an outstanding question for future study, and the studies of the BR model system will likely shed light on the hundreds of plant receptor kinases and their crosstalk during plant responses to complex endogenous and environmental cues.

METHODS SUMMARY

Stomatal quantification

Cotyledons of 8-day-old seedlings were cleared in ethanol:acetic acid and mounted on slides in Hoyer's solution (as in Ref. 23). Two to four images at 400× magnification (180 μm^2) were captured per cotyledon from central regions of abaxial leaves. Guard cells, meristemoids, GMCs, and pavement cells were counted. Statistical analysis was performed by Sigmaplot software (Systat Software Inc. San Jose, CA). For treatment with bikinin[21] (4-[(5-bromopyridin-2-yl)amino]-4-oxobutanoic acid, ChemBridge Corporation, San Diego, CA), seedlings were grown on 1/2 MS medium containing DMSO or 30 μM bikinin (+10 μM estradiol for HOPAI1 inducible lines) for 8 days before stomata were analyzed.

Biochemical assays

To test the bikinin effect on YDA-myc phosphorylation, homozygous *YDA-4myc* plants were grown on 1/2 MS medium containing 2 μM BRZ for 5 days and treated with 30 μM bikinin or 2 μM BRZ solution for 30 min with gentle agitation. Yeast two-hybrid, *in vitro* interaction and kinase assays[11,14], and in-gel kinase assays[30] were as described previously. Details of methods are available as Supplementary Information available online.

METHODS

Materials and growth conditions

All mutants are in the Columbia ecotype except *yda* Y295 (C24 ecotype)[23], CA-YDA (Ler ecotype)[23], and *bin2-3;bil1;bil2* triple mutant obtained from Dr. Jianming Li (Ws ecotype)[31]. The *erecta* triple *er105;erl1-2;erl2-1* (Ref. 32) and *scrm-D* (Ref 25) were obtained from Dr. Keiko Torii. Dr. Jian-Min Zhou kindly provided seeds of estradiol-inducible *HOPAI1* transgenic plants[24]. For all analyses, *Arabidopsis* seedlings were grown on MS agar medium for 8 days under continuous light in Percival growth chamber at 22 °C.

Plasmids

For cloning MBP-185/322, a partial cDNA was amplified from YDA cDNA clone using primers (forward; 5'-caccAGTAACAAAAACTCAGCTGAGATGTTT-3', reverse; 5'-AGAGCTAG GACCAGGGCTTGTCATTCT-3'), cloned into pENTR-SD-DTOPO vector (Invitrogen, Carlsbad, CA) and then subcloned into the gateway compatible pMALc2 vector (New England Biolab, Beverly, MA). For expression in plants, cDNA entry clones of YDA and CA-YDA were subcloned into a Gateway compatible 35S: :4myc-6His vector constructed in the pCAMBIA 1390 vector. *BSL2* cDNA in the pENTR vector was subcloned into Gateway compatible pEarley-101 vector[33] to generate *35S: :BSL2-YFP*.

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Overlay assay

To test interaction of YDA and BIN2 *in vitro*, a gel blot separating MBP, MBP-CDG1 (a protein kinase used as a negative control), and MBP-YDA was incubated with 20 μg GST-BIN2 in 5% non-fat dry milk/PBS buffer and washed four times. The blot was then probed with HRP-conjugated anti-GST antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

In vitro kinase assay

Induction and purification of proteins expressed from *E.coli* was performed as described previously[14]. For Figure 3d and 3e, 1 μg of GST-BIN2 or 0.5 μg of MBP-BIN2 was incubated with 1 μg of MBP-YDA or MBP-185/322 in the kinase buffer (20 mM Tris, pH 7.5, 1 mM $MgCl_2$, 100 mM NaCl and 1 mM DTT) containing 100 μM ATP and 10 μCi ^{32}P-γ-ATP at 30°C for 3 hrs. To examine whether BIN2 inhibits YDA activity, equal amounts of MBP-YDA were pre-incubated with GST-BIN2 or GST-mBIN2 (M115A) for 2 hrs. Pre-incubated MBP-YDA was subsequently purified using glutathione beads and amylose beads to remove GST-BIN2 or GST-mBIN2. Purified YDA was further incubated with GST-mMKK4 (K108R), 10 μCi ^{32}P-γ-ATP, and 10 μM bikinin (to inhibit any residual BIN2) at 30°C for 3 hrs. YDA kinase activity toward mMKK4 was analyzed by SDS-PAGE followed by autoradiography.

In-gel kinase assay

The in-gel kinase assay was performed as described previously[30], with the following modifications. Total proteins were extracted with buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 5% Glycerol, 1% Triton X-100, 1 mM PMSF, 1 μM E-64, 1μM bestatin, 1 μM pepstatin and 2 μM leupeptin. Supernatant obtained from 12,000 rpm centrifugation was quantified by Bradford protein assay. Equal amount of protein (40 μg) was loaded on 10% SDS-PAGE gel embedded with 0.2 mg/mL of myelin basic protein. After electrophoresis, SDS was removed by incubation with washing buffer (25 mM Tris, pH 7.5, 0.5 mM DTT, 5 mM NaF, 0.1 mM Na_3VO_4, 0.5 mg/mL BSA, and 0.1% Triton X-100) with three buffer exchanges at RT 1.5 hr. The gel was incubated with renaturation buffer (25 mM Tris, pH 7.5, 0.5 mM DTT, 5 mM NaF, and 0.1 mM Na_3VO_4) at 4°C overnight with four buffer exchanges. After pre-incubation with 100 mL of kinase reaction buffer without ATP for 30 min, the gel was incubated with 30 mL of kinase reaction buffer (25 mM Tris, pH 7.5, 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4, 200 nM ATP, and 50 μCi ^{32}P-γ-ATP) for 1.5 hr. The gel was washed with solution containing 5% trichloroacetic acid (w/v) and 1% potassium pyrophosphate (w/v) four times for 2–3 hrs. Dried gel was exposed with phosphor screen followed by phosphoimager analysis.

Transient interaction assays and creation of transgenic plants

Agrobacterium GV3101 strains transformed with *35S*: :*CA-YDA-4myc-6His* or *35S*: :*YDA-4myc-6His* constructs were co-infiltrated with or without *35S-BIN2-YFP* expressing *Agrobacterium* into *Nicotiana benthamiana* leaves as described previously[11]. After 36 hrs, protein extracts were prepared from *N. benthamiana* leaves in IP buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 5% Glycerol, 1% Triton X-100, 1 mM PMSF, 1 μM E-64, 1μM bestatin, 1 μM pepstatin and 2 μM leupeptin. Supernatant obtained

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from 20,000g centrifugation was incubated with anti-GFP antibody bound protein A beads for 1 hr. Beads were washed 5 times with IP buffer containing 0.2% Triton X-100. Immunoprecipitated proteins were eluted with 2× SDS Laemmli buffer, separated on SDS-PAGE and subjected to immunoblotting using anti-myc antibody (abcam, Cambridge, MA) and anti-GFP antibody.

For transgenic *Arabidopsis* plants, wild-type *Arabidopsis* was transformed with *Agrobacterium* containing *35S*: :*YDA-4myc-6His* or *35S*: :*BSL2-YFP* construct by floral dip. Hygromycin or Basta-resistant T1 plants were screened by immunoblot using anti-myc or anti-GFP antibody, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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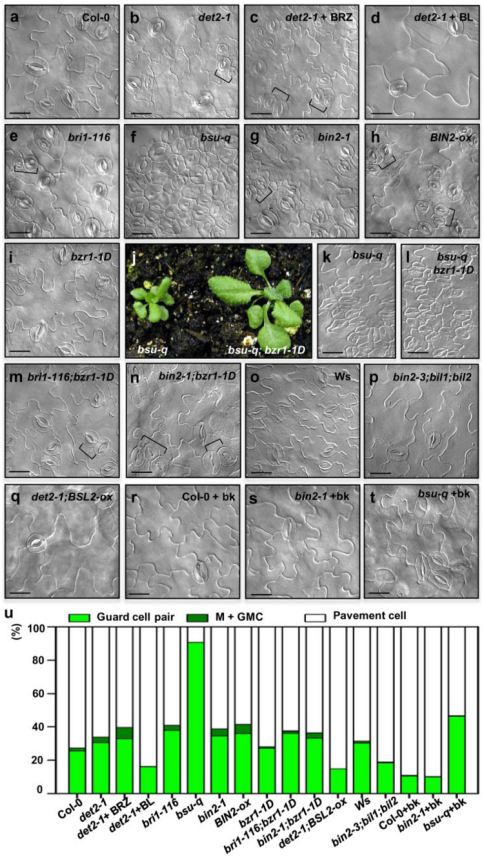

Figure. 1. BR negatively regulates stomatal development

a–i and **k–t, DIC** Images of 8-dpg abaxial cotyledon epidermis or 4-week-old leaf epidermis (**k–l**) with indicated genotypes (Col-0 and Ws are wild type controls), grown on medium ±BRZ (2 μM), brassinolide (BL, 50 nM), or bikinin (bk, 30 μM 30). **j**, Growth phenotype of 4-week-old *bsu-q* and *bsu-q;bzr1-1D* mutants. **u**, Quantification of epidermal cell types of the indicated 8-dpg mutants, expressed as % ratios. M; meristemoid, GMC; Guard Mother Cell. Brackets indicate clustered stomata. Scale bars = 50 μm.

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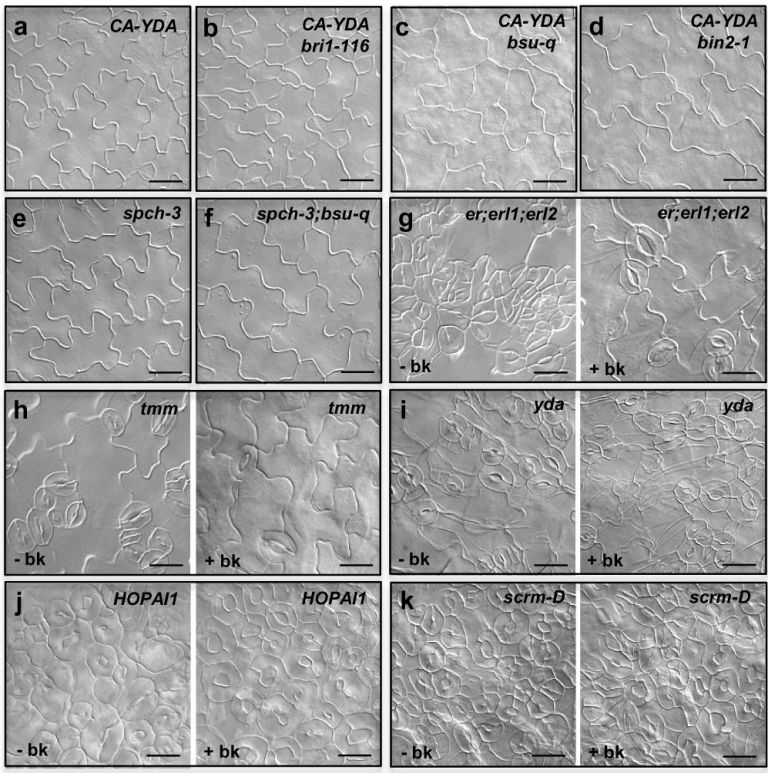

Figure. 2. *Arabidopsis* GSK3s act downstream of ERf and TMM but upstream of YDA in the stomatal development signaling pathway

a–d, CA-YDA expression eliminates stomata in *bri1-116*, *bsu-q* and *bin2-1*. **e–f**, Loss of *SPCH* eliminates stomata in *bsu-q* mutants. **g–k**, Representative stomatal phenotypes of leaf epidermis of *er;erl1;erl2* (**g**), *tmm* (**h**), *yda* (**i**), *HOPAI1* (**j**), and *scrm-D* (**k**) plants grown in the absence (−bk) or presence (+bk) of 30 μM bikinin. Scale bars = 50 μm.

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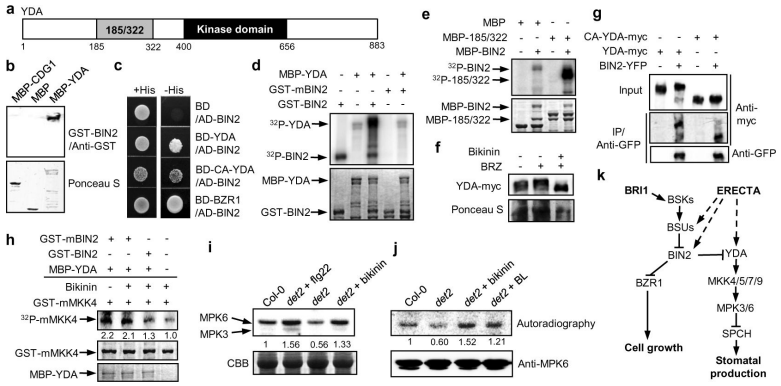

Figure. 3. BIN2 inhibits YDA kinase activity through phosphorylation

a, Domain structure of YDA. **b**, Gel blot of indicated proteins (MBP-CDG1 is a negative control) sequentially probed with GST-BIN2 and anti-GST-HRP antibody. **c**, Yeast two-hybrid assays of indicated proteins. **d–e**, *In vitro* kinase assays of BIN2 phosphorylation of YDA or YDA–185–322. Upper panel shows autoradiography and bottom, protein staining. mBIN2 is kinase-inactive. **f**, *YDA-myc* plants grown for 5 days on medium containing 2 μM BRZ ±30 μM bikinin, and analyzed by anti-myc immunoblot. **g**, Proteins transiently expressed in *N. benthamiana* leaves, immunoprecipitated with anti-GFP antibody, and immunobloted with anti-myc or anti-GFP antibody. **h**, YDA pre-incubated by BIN2 or mBIN2 (kinase-inactive mutant) and ATP was purified, then incubated with mMKK4 and ^{32}P-γ-ATP, ±bikinin. Numbers indicate relative signal levels normalized to loading control. **i–j**, MPK6 and MPK3 activities in seedlings treated with flg22 (10 nM, positive control), bikinin (30 μM), or BL (100 nM) for 30 min (**i**) or 2 hr (**j**), analyzed by in-gel kinase assays. Numbers indicate relative signal levels (upper panel) normalized to the loading control (CBB or MPK6 immunoblot). **k**, A model for regulation of stomatal development by two receptor kinase-mediated signal transduction pathways. When BR levels are low, BIN2 phosphorylates and inactivates YDA, increasing stomatal production. BR signaling through BRI1 inactivates BIN2, leading to activation of YDA and downstream MAPKs and suppression of stomatal development. ERECTA is genetically upstream of YDA; a biochemical link is yet unknown, but BSU1 and BIN2 or their homologs are strong candidates for intermediates (dashed line).