LETTER

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

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Plants must coordinate the regulation of biochemistry and anatomy to optimize photosynthesis and water-use efficiency. The formation of stomata, epidermal pores that facilitate gas exchange, is highly coordinated with other aspects of photosynthetic development. The signalling pathways controlling stomata development are not fully understood[1,2], although mitogen-activated protein kinase (MAPK) signalling is known to have key roles. Here we demonstrate in *Arabidopsis* that brassinosteroid regulates stomatal development by activating the MAPK kinase kinase (MAPKKK) YDA (also known as YODA). Genetic analyses indicate that receptor kinase-mediated brassinosteroid signalling inhibits stomatal development through the glycogen synthase kinase 3 (GSK3)-like kinase BIN2, and BIN2 acts upstream of YDA but downstream of the ERECTA family of receptor kinases. Complementary *in vitro* and *in vivo* assays show that BIN2 phosphorylates YDA to inhibit YDA phosphorylation of its substrate MKK4, and that activities of downstream MAPKs are reduced in brassinosteroid-deficient mutants but increased by treatment with either brassinosteroid or GSK3-kinase inhibitor. Our results indicate that brassinosteroid inhibits stomatal development by alleviating GSK3-mediated inhibition of this MAPK module, providing two key links; that of a plant MAPKKK to its upstream regulators and of brassinosteroid to a specific developmental output.

In animals and plants, steroid hormones have important roles in coordinating development and metabolism[3]. In contrast to animal steroid hormones, which act through nuclear receptor transcription factors[3], the plant steroid hormone brassinosteroid binds to the extracellular domain of the membrane-bound receptor kinase brassinosteroid insensitive 1 (BRI1). This activates intracellular signal transduction mediated by the serine/threonine protein kinase BSK1, the protein phosphatase BSU1, the GSK3-like BIN2 kinase, PP2A phosphatase and BRASSINAZOLE RESISTANT 1 (BZR1) family transcription factors[4–10]. When brassinosteroid levels are low, BZR1 is inactivated owing to phosphorylation by BIN2 (refs 11, 12). Brassinosteroid signalling leads to inactivation of BIN2, and PP2A-mediated dephosphorylation and activation of BZR1 (refs 4, 9, 10) (Supplementary Fig. 1a). Although the brassinosteroid signalling pathway has been characterized, its connections to other signalling 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[1,2,13,14]. The genetically defined signalling pathway that regulates stomatal development includes peptide ligands, a receptor protein (TMM), the ERECTA family of receptor-like kinases (ER, ERL1 and ERL2) and a MAPK module comprised of the MAPK kinase kinase (MAPKKK) YDA, the MAPK kinases (MAPKKs) MKK4, MKK5, MKK7 and MKK9, and MAPKs MPK3 and MPK6 (ref 15). Potential downstream targets include basic helix–loop–helix (bHLH) transcription factors SPEECHLESS (SPCH), MUTE, FAMA, ICE1 (also known as SCRM) and SCRM2, with SPCH being negatively regulated by direct MPK3- and MPK6-mediated phosphorylation[16,17] (Supplementary Fig. 1b). It is possible that the MAPK pathway integrates environmental and hormonal inputs to optimize 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 brassinosteroid-deficient mutants[18]. To elucidate the function of brassinosteroid in regulating stomatal development, we examined the distribution of stomata on leaves of brassinosteroid-deficient and brassinosteroid-signalling mutants. In wild-type *Arabidopsis*, stomata are always distributed with at least one pavement cell between them (Fig. 1a). Brassinosteroid deficiency causes stomatal clusters (Fig. 1b, c), whereas treatment with brassinolide (the most active form of brassinosteroid) reduces stomatal density (Fig. 1d), indicating that brassinosteroid represses stomatal development. The brassinosteroid-insensitive mutants *bri1-116*, quadruple *amiRNA-BSL2,3 bsu1 bsl1* (*bsu-q*)[9], dominant *bin2-1* and plants that overexpress *BIN2* 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 Figs 2 and 3). Surprisingly, the hyperactive *bzr1-1D* mutation[10,19] 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 brassinosteroid regulation of stomatal development is mediated by upstream signalling components that include BRI1, BSU1 and BIN2, but that it is independent of the BIN2 substrate BZR1.

Consistent with increased stomatal development in brassinosteroid-insensitive mutants, fewer stomata were observed in cotyledons of plants overexpressing some of the positive brassinosteroid-signalling 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 brassinosteroid-signalling GSK3-like kinases (Fig. 1o, p, u and Supplementary Fig. 2). We used bikinin (4-[(5-bromopyridin-2-yl) amino]-4-oxobutanoic acid, ChemBridge Corporation), a highly specific inhibitor for the 7 *Arabidopsis* GSK3-like kinases that appear to be involved in brassinosteroid signalling[9,20,21], to investigate further the function of brassinosteroid-related GSK3-like kinases in stomatal development. When added to the growth medium, bikinin decreased stomatal production in wild-type plants, fully suppressed the stomatal clustering phenotypes of *bin2-1* and partially 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 brassinosteroid-deficient and brassinosteroid-insensitive mutants.

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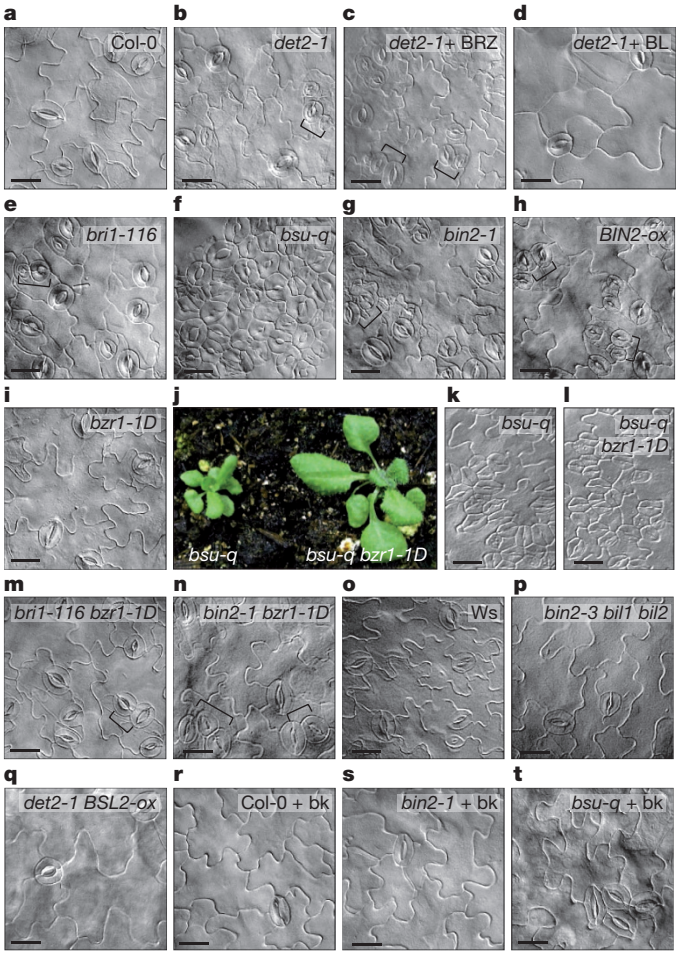

Figure 1 | Brassinosteroid negatively regulates stomatal development. **a–i**, **k–t**, Differential interference contrast (DIC) microscopy images of abaxial cotyledon epidermis of 8-day-old seedlings or leaf epidermis of 4-week-old plants (**k**, **l**) with indicated genotypes (Col-0 and Ws are wild-type controls), grown on medium ± BRZ (2 μM), brassinolide (BL, 50 nM), or bikinin (bk, 30 μM). **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-day-old mutants, expressed as percentage of total cells. GMC, guard mother cell; M, meristemoid. Brackets in **b**, **c**, **e**, **g**, **h**, **m**, **n** indicate clustered stomata. Scale bars, 50 μm.

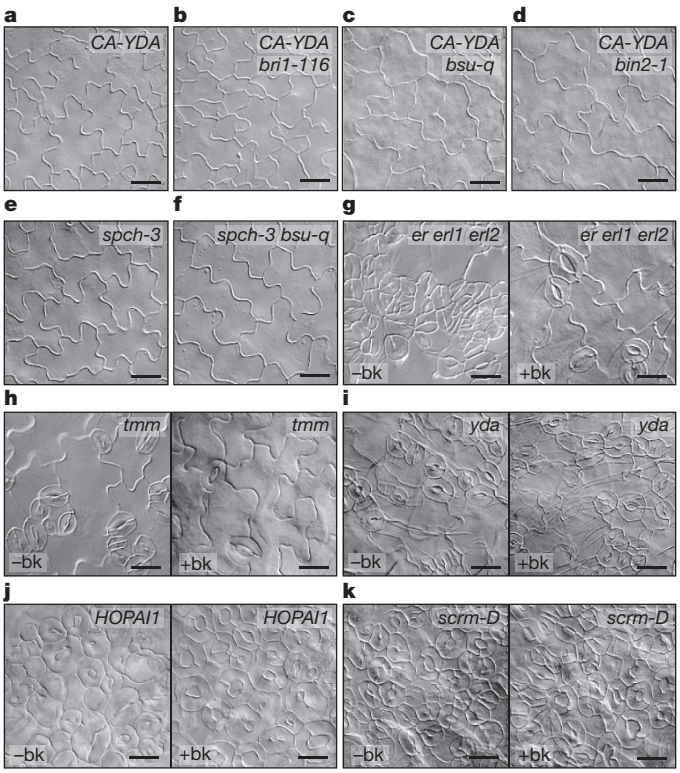

Figure 2 | *Arabidopsis* GSK3 acts downstream of ERECTA family and TMM but upstream of YDA in the stomatal development signalling pathway. **a–d**, CA-YDA expression eliminates stomata in *bri1-116, bsu-q* and *bin2-1*. **e, f**, Loss of *SPCH* eliminates stomata in *bsu-q* mutants. **g–k**, Representative 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.

We examined genetic interactions between brassinosteroid mutants and known stomatal mutants. Expression of constitutively active YDA (CA-YDA) can completely eliminate stomatal development[22] (Fig. 2a), probably through activation of a MAP kinase pathway that phosphorylates and inactivates SPCH[15,16]. 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 brassinosteroid signalling 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 Figs 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)[23] or on the *scrm-D* gain-of-function mutant[24] (Fig. 2i–k and Supplementary Fig. 8). The brassinosteroid biosynthetic inhibitor brassinazole also significantly enhanced the stomatal phenotypes of *tmm*, but did not further increase stomata in *er erl1 erl2*, probably 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 the two rice homologues of YDA, Os02g0666300 and Os04g0559800, and these homologues also share a highly conserved sequence just amino-terminal of the kinase domain. Importantly, YDA can be made constitutively active when part of this region (amino acids 185–322; Fig. 3a) is deleted[22]. The region that is deleted in CA-YDA contains 23 putative GSK3 phosphorylation sites, including successive phosphorylation sites that are similar to sites found in the known BIN2 target BZR1 (Fig. 3a and Supplementary Fig. 10).

We tested whether BIN2 directly interacts with and phosphorylates YDA. Maltose binding protein (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

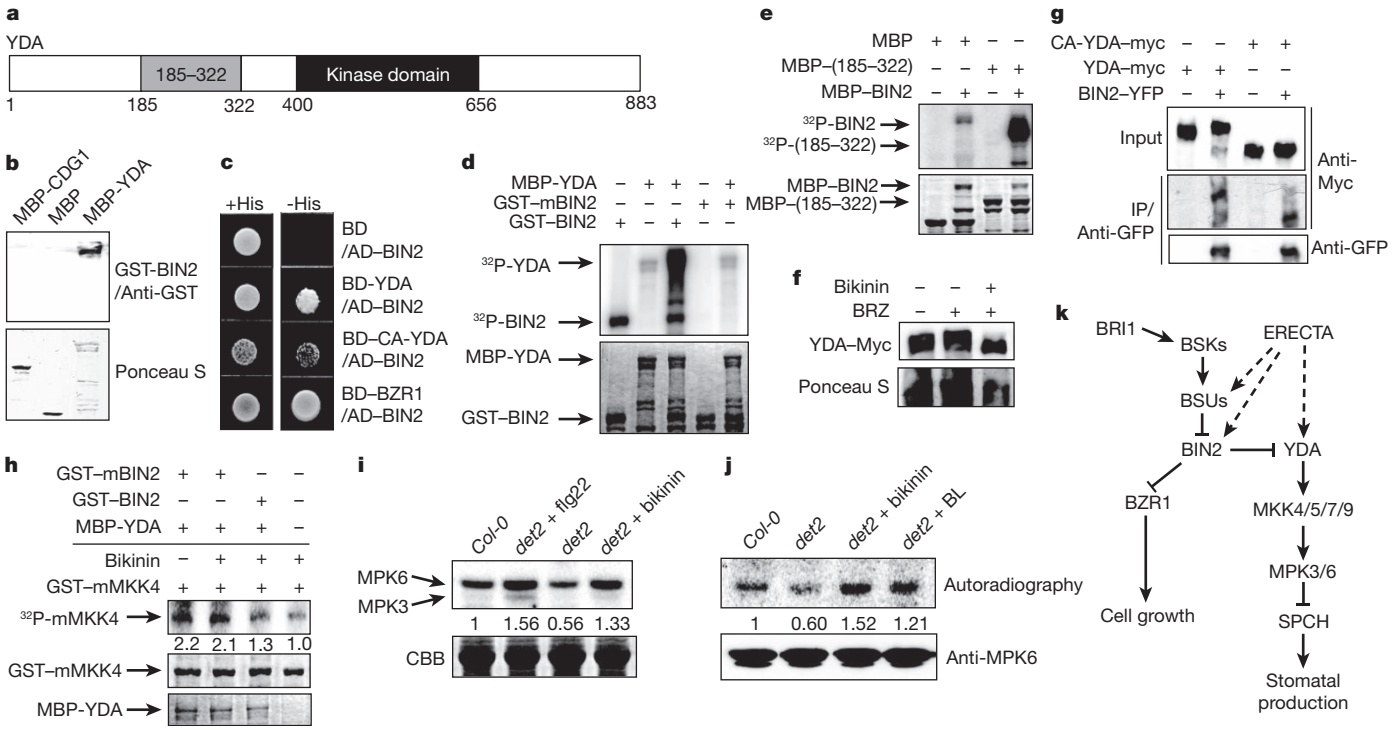

Figure 3 | BIN2 inhibits YDA kinase activity through phosphorylation. **a**, Domain structure of YDA. **b**, Gel blot of indicated proteins (MBP–CDG1 is a negative control) sequentially probed with GST–BIN2 and anti-GST–HRP antibody. **c**, Yeast two-hybrid assays of indicated proteins. **d**, **e**, *In vitro* kinase assays of BIN2 phosphorylation of YDA or YDA fragment containing amino acids 185-322 (185-322). Upper panel shows autoradiography and bottom panel shows protein staining. Mutant BIN2 (mBIN2) is kinase inactive. **f**, *YDA–Myc* plants grown for 5 days on medium containing 2 μM BRZ ± 30 μM bikinin and analysed by anti-Myc immunoblot. **g**, Proteins transiently expressed in *N. benthamiana* leaves, immunoprecipitated (IP) with anti-YFP antibody, and immunoblotted with anti-Myc or anti-YFP antibody. **h**, YDA pre-incubated with BIN2 or mBIN2 (kinase-inactive mutant) and ATP was purified then incubated with mutant MKK4 (mMMK4) and [^{32}P]γATP, ± 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 h (**j**), analysed 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 brassinosteroid levels are low, BIN2 phosphorylates and inactivates YDA, increasing stomatal production. Brassinosteroid signalling through BRI1 inactivates BIN2, leading to activation of YDA and downstream MAPK proteins, and suppression of stomatal development. ERECTA is genetically upstream of YDA; a biochemical link is not known, but BSU1 and BIN2 or their homologues are strong candidates for intermediates (dashed line).

assays showed that BIN2 phosphorylated YDA, but YDA did not phosphorylate a kinase-inactive BIN2 mutant or other brassinosteroid signalling components (Fig. 3d and Supplementary Fig. 11). BIN2 strongly phosphorylated the region deleted in CA-YDA (Fig. 3e), indicating that BIN2 might inhibit YDA by phosphorylating its autoregulatory domain.

BIN2 phosphorylation of BZR1 causes mobility shifts of the phosphorylated BZR1 band in SDS–polyacrylamide gel electrophoresis (SDS–PAGE) gels[12]. Like BZR1, YDA that was 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 *Nicotiana benthamiana* leaf cells, both YDA–Myc and CA-YDA–Myc were co-immunoprecipitated by anti-yellow fluorescent protein (YFP) antibody when co-expressed with BIN2–YFP but not when expressed alone (Fig. 3g), demonstrating that there is an 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 brassinosteroid and bikinin affect MAPK activity in plants. YDA was pre-incubated with BIN2 and ATP, or with a kinase-inactive mutant BIN2 as a control, and then purified and further incubated with MKK4 (its known substrate), bikinin and [^{32}P]γATP. Pre-incubation with BIN2, but not with mutant BIN2, decreased YDA phosphorylation of MKK4 (Fig. 3h and 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 brassinosteroid negatively regulates stomatal development by inhibiting the BIN2-mediated phosphorylation and inactivation of YDA (Fig. 3k). When brassinosteroid levels are low, active BIN2 directly phosphorylates and inactivates YDA; reduced MAP kinase pathway activity can de-repress SPCH, allowing SPCH to initiate stomatal development. Brassinosteroid signalling through BRI1, BSK1 and BSU1 inactivates GSK3, resulting in activation of the MAP kinase pathway and inhibition of stomatal production (Fig. 3k).

This study supports a role of brassinosteroid as a master regulator that coordinates both physiological and developmental aspects of plant growth. Previous studies have demonstrated key functions of brassinosteroid in inhibiting photomorphogenesis and photosynthetic gene expression[25–27]. Here we find a role for brassinosteroid in stomatal production, which must be coordinated with other developmental processes to optimize photosynthetic and water-use efficiency. Notably, brassinosteroid 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 GSK3 directly inactivates MAPKKK

proteins in animals. This GSK3–MAPK connection has the potential to act in multiple receptor kinase-mediated signalling 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 signalling by the ERECTA family receptor kinases. However, the signals from BRI1 and ERECTA family must be partitioned differently downstream so that BRI1 controls GSK3 regulation of both BZR1 and YDA but ERECTA family mainly controls the GSK3 inactivation of YDA (Fig. 3k), because *er erl1 erl2* had no obvious effect on brassinosteroid-regulated BZR1 phosphorylation (Supplementary Fig. 13). Similar mechanisms and components might also be used by additional signalling pathways, such as the innate immunity pathway downstream of the FLS2 receptor kinase, which shares the BAK1 co-receptor[28] and downstream components MPK3 and MPK6 with BRI1 (ref. 23). In support of such an idea, overexpression of a GSK3-like kinase reduced the pathogen-induced activation of MPK3 and MPK6 (ref. 29). How signalling specificity is maintained when multiple pathways share the same components is a question for future study, and studies of the brassinosteroid model system will probably 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 with acetic acid (ratio of 19:1, v/v) and mounted on slides in Hoyer's solution (see ref. 22). 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). For treatment with bikinin[20], seedlings were grown on half-strength Murashige and Skoog (MS) medium containing dimethylsulphoxide (DMSO) or 30 μM bikinin (+10 μM oestradiol for HOPAI1-inducible lines) for 8 days before stomata were analysed.

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 (*BRASSINAZOLE*, an inhibitor of BR synthesis) 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[9,12], and in-gel kinase assays[30] were carried out as described previously. Details of methods are available in the Supplementary Methods.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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

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METHODS

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

Stomatal quantification. Cotyledons of 8-day-old seedlings were cleared in ethanol with acetic acid and mounted on slides in Hoyer's solution (see ref. 22). 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). For treatment with bikinin[20], seedlings were grown on half-strength MS medium containing DMSO or 30 μM bikinin (+10 μM estradiol for HOPAI1-inducible lines) for 8 days before stomata were analysed.

Plasmids. For cloning MBP-185/322, a partial cDNA was amplified from a YDA cDNA clone using primers (forward; 5′-caccAGTAACAAAAACTCAGCTG AGATGTTT-3′, reverse; 5′-AGAGCTAG GACCAGGGCTTGTCATTCT-3′), cloned into pENTR-SD-D-TOPO vector (Invitrogen) and then subcloned into the gateway-compatible pMALc2 vector (New England Biolab). 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*.

Overlay assay. To test the 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).

***In vitro* kinase assay.** Induction and purification of proteins expressed from *Escherichia coli* was performed as described previously[12]. For Fig. 3d, e, 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]γATP at 30 °C for 3 h. To examine whether BIN2 inhibits YDA activity, equal amounts of MBP–YDA were pre-incubated with GST–BIN2 or GST–mBIN2 (M115A) for 2 h. Pre-incubated MBP–YDA was subsequently purified using glutathione beads and amylose beads to remove GST–BIN2 or GST–mBIN2. Purified YDA was then incubated with GST–mMKK4 (K108R), 10 μCi [^{32}P]γATP and 10 μM bikinin (to inhibit any residual BIN2) at 30 °C for 3 h. YDA kinase activity towards mMKK4 was analysed by SDS–PAGE followed by autoradiography.

In-gel kinase assay. The in-gel kinase assay was performed as described previously[30], with some 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 phenylmethylsulphonyl fluoride (PMSF), 1 μM E-64, 1 μM bestatin, 1 μM pepstatin and 2 μM leupeptin. Supernatant obtained from 12,000 r.p.m. centrifugation was quantified by Bradford protein assay. Equal amounts of protein (40 μg) were loaded on 10% SDS–PAGE gel embedded with 0.2 mg ml^{-1} 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^{-1} bovine serum albumin and 0.1% Triton X-100) with three buffer exchanges at 22 °C for 1.5 h. 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 h. The gel was washed with solution containing 5% trichloroacetic acid (w/v) and 1% potassium pyrophosphate (w/v) four times for 2–3 h. Dried gel was exposed with phosphor screen followed by phospho-imager analysis.

Transient interaction assays and analysis of bikinin effects on YDA in transgenic plants. *Agrobacterium* GV3101 strains transformed with *35S::CA-YDA-4Myc-6His* or *35S::YDA-4Myc-6His* constructs were alone or co-infiltrated with *35S-BIN2-YFP* expressing *Agrobacterium* into *N. benthamiana* leaves as described previously[9]. After 36 h, protein extracts were prepared from *N. benthamiana* leaves in immunoprecipitation buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 5% Glycerol, 1% Triton X-100, 1 mM PMSF, 1 μM E-64, 1 μM bestatin, 1 μM pepstatin and 2 μM leupeptin. Supernatant obtained from 20,000*g* centrifugation was incubated with anti-YFP-antibody-bound protein A beads for 1 h. Beads were washed 5 times with immunoprecipitation 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) and anti-YFP 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-YFP antibody, respectively.

To test the bikinin effect on YDA–Myc phosphorylation, homozygous *YDA-4Myc* plants were grown on half-strength 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. YDA-Myc was analysed by immunoblot.

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