



# CsBRC1 inhibits axillary bud outgrowth by directly repressing the auxin efflux carrier *CsPIN3* in cucumber

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**Shoot branching is an important agronomic trait that directly determines plant architecture and affects crop productivity. To promote crop yield and quality, axillary branches need to be manually removed during cucumber production for fresh market and thus are undesirable. Auxin is well known as the primary signal imposing for apical dominance and acts as a repressor for lateral bud outgrowth indirectly. The *TEOSINTE BRANCHED1/CYCLOIDEA/PCF (TCP)* family gene *BRANCHED1 (BRC1)* has been shown to be the central integrator for multiple environmental and developmental factors that functions locally to inhibit shoot branching. However, the direct molecular link between auxin and BRC1 remains elusive. Here we find that cucumber *BRANCHED1 (CsBRC1)* is expressed in axillary buds and displays a higher expression level in cultivated cucumber than in its wild ancestor. Knockdown of *CsBRC1* by RNAi leads to increased bud outgrowth and reduced auxin accumulation in buds. We further show that CsBRC1 directly binds to the auxin efflux carrier *PIN-FORMED (CsPIN3)* and negatively regulates its expression in vitro and in vivo. Elevated expression of *CsPIN3* driven by the *CsBRC1* promoter results in highly branched cucumber with decreased auxin levels in lateral buds. Therefore, our data suggest that CsBRC1 inhibits lateral bud outgrowth by direct suppression of *CsPIN3* functioning and thus auxin accumulation in axillary buds in cucumber, providing a strategy to breed for cultivars with varying degrees of shoot branching grown in different cucumber production systems.**

auxin transport | axillary bud outgrowth | CsBRC1 | CsPIN3 | cucumber

**S**hoot branching is an important agronomic trait that directly determines plant architecture and affects crop productivity. In flowering plants, the development of lateral shoots starts from axillary meristem (AM) initiation at the leaf axils. The AMs then develop into axillary buds comprising a few leaf primordia and a meristem, which either remain dormant at this stage or grow out to form a branch (1, 2). Excess of branches often compete for nutrient allocation and light harvesting and generally have inverse effects on crop yield. In fact, suppression of shoot branching is an important selection trait during crop domestication (3). Cucumber (*Cucumis sativus* L.), a worldwide cultivated vegetable crop that is consumed freshly or processed into pickles, is domesticated from its highly branched wild ancestor *Cucumis sativus* var. *hardwickii* (4). Despite the fact that lateral branches are greatly suppressed in cultivated cucumber as compared to *hardwickii*, the degree of branch suppression varies depending on cultivars, geographic regions, and market types. To promote crop yield and quality, extra branches need to be manually removed during cucumber production, especially for fresh market, which is labor intensive and time-consuming. Therefore, it is of great importance for cucumber production and breeding to understand the regulatory mechanisms of lateral branch arrest.

Axillary bud arrest or outgrowth is a plastic developmental process regulated by diverse environmental and developmental

factors (5). Auxin is synthesized in the shoot apex and acts as a repressor for branching and serves as the primary signal imposing for apical dominance (6). The main shoot tip produces a massive amount of auxin, which moves downward within the stem and represses axillary bud outgrowth. Removal of the main shoot tip by decapitation leads to the outgrowth of axillary buds into branches (7, 8). Two nonexclusive hypotheses explain indirect regulation of shoot branching by auxin. According to the second messenger hypothesis, auxin acts outside the axillary buds and regulates the production of second messengers, cytokinins (CK) and strigolactones (SL), to control shoot branching (9, 10). A more recently proposed model is the auxin transport canalization-based hypothesis (11, 12), in which auxin flows between an auxin source and an auxin sink leading to up-regulation and polarization of auxin transporters to canalize auxin transport into cell files (11, 13–15). The shoot apex and axillary buds are auxin sources that compete for sink strength of the stem to transport auxin rootward (11, 16). The shoot apex continuously produces auxin and transports down the stem, which reduces

## Significance

Lateral branch suppression has been selected during crop domestication, and axillary branches need to be manually removed during fresh cucumber production. Auxin is a key repressor for shoot branching underlying apical dominance. The *TEOSINTE BRANCHED1 (TB1)/BRANCHED1 (BRC1)* gene acts as the focal point for multiple signals to inhibit branching. However, the relationship between auxin and BRC1 remains elusive. Here cucumber *BRANCHED1 (CsBRC1)* is found to inhibit shoot branching by directly repressing the auxin efflux carrier *CsPIN3*. Compared to its wild ancestor, cultivated cucumber displays reduced branches, higher *CsBRC1* expression, reduced *CsPIN3* expression, and higher auxin accumulation in buds. In this study, we find a regulatory pathway of CsBRC1–CsPIN3–auxin transport in suppressing shoot branching in cucumber.

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stem sink strength and prevents auxin export from lateral buds, hence resulting in bud arrest (15).

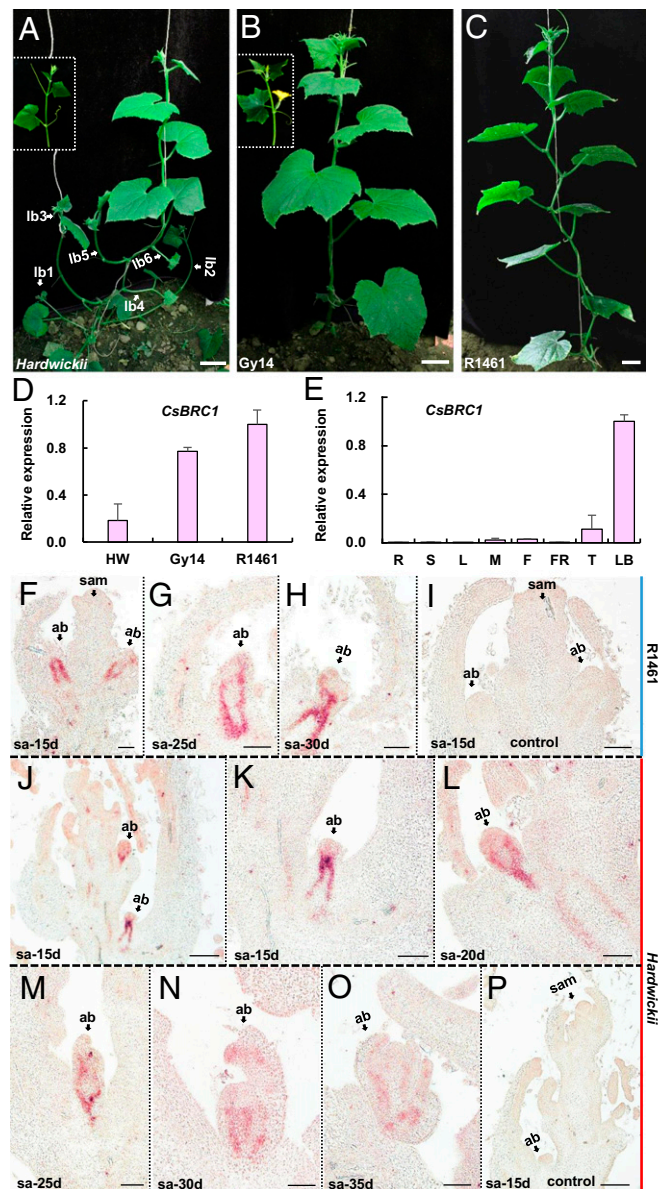
The founder member of the class II TCP (TEOSINTE BRANCHED1/CYCLOIDEA/PCF) family of transcription factors, known as TEOSINTE BRANCHED1 (TB1)-like in monocots and BRANCHED1 (BRC1)-like in eudicots, has been shown to be an integrator of multiple internal and external signals that act inside the axillary buds to suppress shoot branching (17–20). The biosynthesis of CK can be repressed by auxin, resulting in increased expression of *TB1/BRC1* to inhibit shoot branching in rice and pea (21, 22). On the contrary, auxin stimulates SL biosynthesis to promote transcription of *TB1/BRC1* and repress shoot branching in *Arabidopsis* and pea (18, 22). *BRC1* transcription is auxin responsive in *Arabidopsis*; *BRC1* determines bud activation potential but is dispensable for bud growth inhibition (11). Sugar, an important nutrient substance, plays a pivotal role in axillary bud outgrowth through repressing *TB1/BRC1* expression in wheat (23–25). Shade, generated by increased planting density or by decreased ratio of red to far-red light, induces the expression of *TB1/BRC1* and leads to axillary bud dormancy (26–28). Therefore, *TB1/BRC1* serves as the focal point for hormonal, nutritional, and environmental signals to inhibit lateral bud outgrowth.

Moreover, multiple regulatory pathways have been dissected for the *TB1/BRC1*-mediated suppression of shoot branching (5). The transcriptional repressor TIE1 (TCP interactor containing EAR motif protein 1) positively regulates shoot branching by directly repressing *BRC1* activity in *Arabidopsis*, while the transcription factor IDEAL PLANT ARCHITECTURE1 (IPA1) promotes the expression of *OsTB1* by directly binding to its promoter region in rice (29, 30). *BRC1* can directly bind to 3 homeodomain leucine zipper protein (HD-ZIP)-encoding genes, *HOMEODOMAIN PROTEIN 21*, *HOMEODOMAIN PROTEIN 40*, and *HOMEODOMAIN PROTEIN 53*, and positively regulates their expression in *Arabidopsis*. *BRC1*, together with these 3 downstream genes, promotes the transcription of *9-CIS-EPOXICAROTENOID DIOXIGENASE 3*, which encodes a key enzyme in abscisic acid (ABA) biosynthesis, and leads to ABA accumulation and branching suppression (9). Besides, maize *TB1* was found to be a key domestication gene that directly activates the expression of *TASSELS REPLACE UPPER EARS1 (TRU1)* to mediate axillary bud dormancy (31). Modern maize with suppressed lateral branches largely resulted from overexpression of *TB1* by retrotransposon insertion in the promoter during domestication from its wild ancestor teosinte (32).

Although the function of *BRC1* in lateral bud arrest is widely conserved in the plant kingdom, the direct molecular link between *BRC1* and auxin remains unknown. Here we show that the cucumber *BRC1* (*CsBRC1*) directly binds to the auxin efflux carrier *PIN-FORMED3 (CsPIN3)* and negatively regulates its expression, resulting in auxin accumulation in axillary buds and arrest of bud outgrowth.

## Results

**Expression of *CsBRC1* Is Negatively Correlated with Lateral Branch Outgrowth in Cucumber.** Compared to the highly branched wild ancestor *Cucumis sativus* var. *hardwickii* (Fig. 1A), the lateral branch is suppressed at different levels in cultivated cucumber varieties. The sequenced pickling cucumber Gy14 line displays moderate branching with lateral branch suppression mainly occurring in the early growth period (Fig. 1B), whereas East Asian line R1461 shows very few branches with bud outgrowth arrested throughout the life cycle (Fig. 1C). To identify which genes account for lateral branch suppression during cucumber domestication, we performed expression analysis with cucumber homologous genes to *BRANCHED 1 (CsBRC1)*, *LATERAL SUPPRESSOR (CsLS)*, *BLIND (CsBLIND)*, and *MORE AXILLARY BRANCHES 1, 2, 3, and 4 (CsMAX1 to CsMAX4)* in *hardwickii*, Gy14, and R1461 (Fig. 1D and *SI Appendix*, Fig. S1). The expression of *CsBRC1* (*Csa1G020890*) was negatively correlated with branching and displayed the greatest change between *hardwickii* and R1461 (Fig. 1D) and thus was chosen for further characterization in this study.



**Fig. 1.** Expression of *CsBRC1* in cultivated cucumbers and its wild ancestor *Cucumis sativus* var. *hardwickii*. (A–C) Morphology of highly branched cucumber *hardwickii* (A), moderately branched cucumber line Gy14 (B), and rarely branched line R1461 (C). lb, lateral branch. (D and E) Expression analyses of *CsBRC1* by qRT-PCR in 3 cucumber lines (D) and in different organs of cucumber line R1461 (E). Values are means of 3 independent biological replicates from different plants. Error bars represent  $\pm$ SD. HW, *hardwickii*; R, roots; S, stems; L, leaves; M, male buds; F, female buds; FR, fruits; T, shoot apex; LB, lateral buds. (F–P) In situ hybridization of *CsBRC1* in cultivated cucumber R1461 (F–I) and wild ancestor *hardwickii* (J–P) during seedling development from 15 to 35 d after planting. K is the magnified image of the bottom axillary bud in J. sa, shoot apex; sam, shoot apical meristem; ab, axillary bud. (Scale bars represent 5 cm in A–C, 100  $\mu$ m in F–I and K–O, and 200  $\mu$ m in J and P.)

*CsBRC1* contains 2 exons and encodes a 342-amino-acid-long putative TCP transcription factor with a typical bHLH motif (*SI Appendix*, Fig. S2). There is no difference in the coding sequence of *CsBRC1* among the 3 cucumber varieties *hardwickii*, Gy14, and R1461. Subcellular localization analysis indicated that *CsBRC1* was localized in the nucleus (*SI Appendix*, Fig. S3). Quantitative real-time PCR indicated that *CsBRC1* was highly enriched in axillary buds (Fig. 1E). In situ hybridization further showed that the

transcripts of *CsBRC1* were specifically accumulated in vascular tissues of axillary buds (Fig. 1 *F–P*). In the cultivated cucumber R1461, the expression of *CsBRC1* remained at high levels at axillary buds during the development from 15- to 30-d seedlings, and no outgrowth of axillary buds was detected (Fig. 1 *F–H*). However, in the wild ancestor *hardwickii*, the *CsBRC1* signals became weaker as the axillary buds grew up over time (Fig. 1 *J–O*). No signal was detected upon hybridization with the sense probe of *CsBRC1* (Fig. 1 *I* and *P*).

**Knockdown of *CsBRC1* Resulted in Increased Outgrowth of Axillary Bud in Cucumber.** To gain insight into *CsBRC1* function in lateral branch outgrowth in cucumber, we created a double-stranded RNAi construct containing the 585-bp coding sequence of *CsBRC1* and transformed into the cucumber inbred line R1461 via *Agrobacterium*-mediated cotyledon transformation (33, 34). Twelve *CsBRC1*-RNAi lines were obtained, and 3 representative lines (21, 26, and 29) with different severities were chosen for further characterization (Fig. 2). Quantitative real-time PCR showed that the expression of *CsBRC1* was reduced by 64% ~78% in the transgenic *CsBRC1*-RNAi lines as compared to the empty vector control (WT) (Fig. 2*A*). In situ hybridization confirmed that the transcripts of *CsBRC1* were dramatically decreased in lateral buds of *CsBRC1*-RNAi lines (Fig. 2 *B* and *C*). Consistent with its negative correlation with shoot branching (Fig. 1), *CsBRC1*-RNAi plants produced many more lateral branches with great increase in length (Fig. 2 *D–F*). In cucumber production, lateral branches longer than 4 cm are considered as manageable branches to be manually removed by farmers or technicians; we thus set 4 cm as the cut-off value of branch outgrowth. Using this standard, only 2 to 3 branches were formed out of the 20 examined nodes in WT plants, whereas 12 to 15 branches were produced in *CsBRC1*-RNAi transgenic lines (Fig. 2*E*). Similarly, the branch length was greatly increased, with a maximum of 5.5 cm in WT but of 18.3 to 30 cm in the *CsBRC1*-RNAi transgenic lines (Fig. 2*F*). These data suggested that *CsBRC1* acts as an important repressor for axillary bud outgrowth in cucumber.

***CsBRC1* Inhibits Branching through Regulating Auxin Pathway in Cucumber.** To identify the downstream targets and regulatory network of *CsBRC1* in cucumber, transcriptome profiling by RNA-Seq was performed with the lateral buds from WT and *CsBRC1*-RNAi line 21. Using a false discovery rate (FDR) less than 0.05 and the fold change larger than 2 as thresholds, 1,680 up-regulated and 1,171 down-regulated genes were identified as differentially expressed genes (DEGs) (*SI Appendix, Table S1* and *Dataset S1*) (35). Gene Ontology (GO) term enrichment analysis of the up-regulated genes in the *CsBRC1*-RNAi line indicated that the term “auxin efflux transmembrane transporter activity” was significantly enriched (*SI Appendix, Fig. S4*). Heat map analysis indicated that 23 genes related to auxin response, and 2 auxin transporters and 1 auxin signaling gene were differentially expressed in *CsBRC1*-RNAi vs. WT plants (Fig. 3*A*). Quantitative real-time PCR confirmed that 2 auxin efflux carrier genes, *CsPIN1b* (*Csa1G025070*) and *CsPIN3* (*Csa5G576590*), were significantly up-regulated in *CsBRC1*-RNAi lines (Fig. 3*B*), with 7.4 fold and 4.4 fold higher in the *CsBRC1*-RNAi lines as compared to that in WT, respectively. Phylogenetic analysis indicated that there are 3 *CsPIN1* genes (*CsPIN1a*, *CsPIN1b*, and *CsPIN1c*) in cucumber, and *CsPIN3* is closely related to *CsPIN4* (*SI Appendix, Fig. S5*).

Next, we measured the auxin level in lateral buds of WT and *CsBRC1*-RNAi lines. As expected, auxin concentration was reduced in *CsBRC1*-RNAi lines (Fig. 3*C*), consistent with the repressing role of auxin in lateral branch outgrowth (5). Immune colloidal gold assay of 3-Indole acetic acid (IAA) further showed the reduced auxin accumulation in lateral buds of *CsBRC1*-RNAi plants (Fig. 3 *D–F*), suggesting that decrease of auxin level may be responsible for the increased axillary branch outgrowth in *CsBRC1*-RNAi lines.

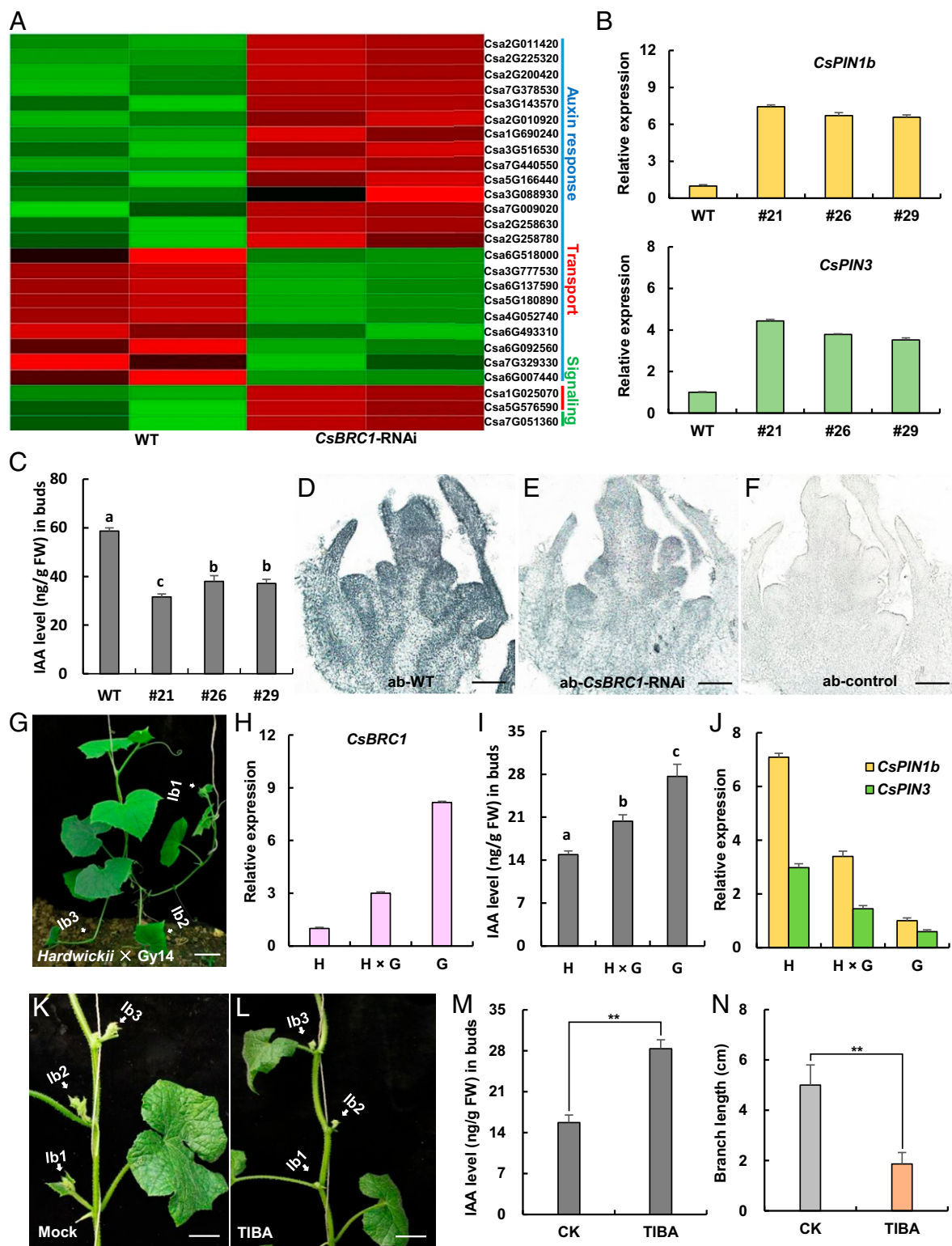
We next performed a cross experiment using *hardwickii* (more branches) and Gy14 (less branches) as parents. The resultant

F1 plants displayed an intermediate phenotype (more branches than Gy14 but less than *hardwickii*) (Fig. 3*G*). Consistently, the expression of *CsBRC1* and the auxin level in F1 plants were between the values of 2 parents (Fig. 3 *H* and *I*). Further, the expression of *CsPIN1b* and *CsPIN3* displayed the same trend (Fig. 3*J*), suggesting that there may be a dosage effect of auxin on lateral branch suppression. To explore whether auxin efflux leads to increased axillary bud outgrowth, 0.5 mM 2,3,5-Triiodobenzoic acid (TIBA), a well-known auxin transport inhibitor (36), was applied to the axillary buds of *hardwickii* (Fig. 3 *K* and *L*). As compared to the mock treatment, the auxin content of the TIBA-treated axillary buds was significantly increased, and consequently, the outgrowth of axillary bud was dramatically inhibited (Fig. 3 *M* and *N*).

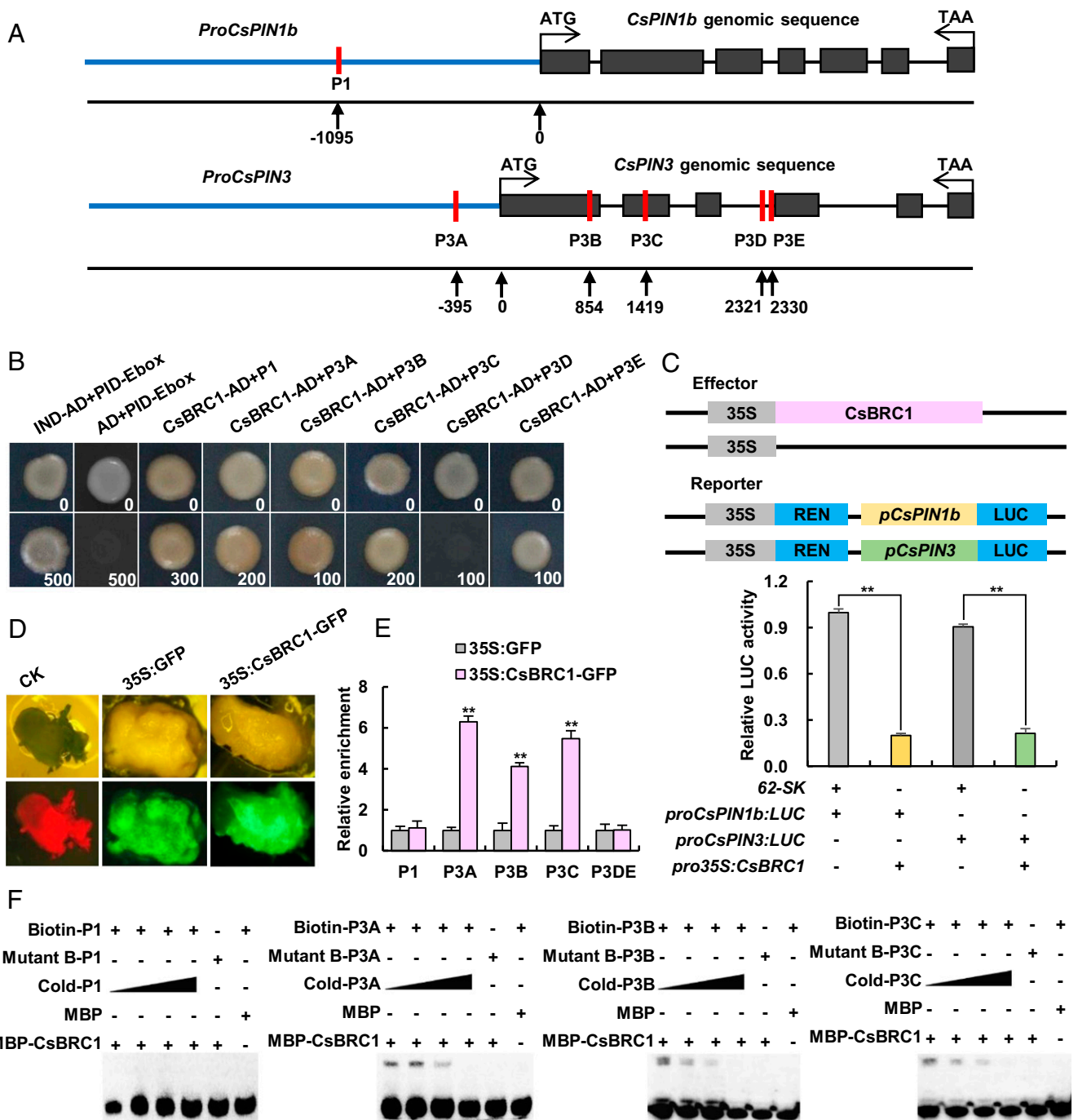
***CsBRC1* Directly Binds to the Auxin Efflux Carrier *CsPIN3* In Vitro and In Vivo.** Previous studies showed that transcription factors of the TCP family directly bind to GGNCCCAC elements (9, 37). To further dissect the regulatory role of *CsBRC1* on the auxin pathway, *cis*-acting elements on the genome sequences of *PIN* genes were analyzed. One putative element (P1) in the *CsPIN1b* promoter and 5 putative elements (P3A–P3E) in the *CsPIN3* genome sequence were identified (Fig. 4*A*). No GGNCCCAC element was found on other *PIN* genes in cucumber. To explore whether *CsBRC1* could bind to *CsPIN1b* and *CsPIN3* directly, we carried out yeast 1-hybrid assay. The results showed that *CsBRC1* bound to the P1 element in *CsPIN1b* and 4 (P3A, P3B, P3C, and P3E) out of the 5 elements of *CsPIN3* (Fig. 4*B*). A dual-luciferase (LUC) transactivation assay in tobacco leaves was then performed. The relative intensity of LUC signals significantly decreased upon cotransformation of 35S:*CsBRC1* with *ProCsPIN1b:LUC* or *ProCsPIN3b3:LUC*, as compared to the vector control (Fig. 4*C*), suggesting that *CsBRC1* may repress expression of *CsPIN1b* and *CsPIN3*. To verify such interactions in vivo, 35S:*CsBRC1-GFP* and 35S:*GFP* were separately transformed into cucumber calli from hypocotyl explants for chromatin immunoprecipitation (ChIP) assay (Fig. 4*D*). ChIP-qPCR results showed that the P3A, P3B, and P3C elements in *CsPIN3* were significantly enriched by antibodies recognizing *CsBRC1-GFP* protein, while P3DE (containing P3D and P3E) in *CsPIN3* and the P1 element in *CsPIN1b* were not (Fig. 4*E*). As expected, electrophoretic mobility shift assay (EMSA) indicated binding of P3A, P3B, and P3C elements in *CsPIN3* by *CsBRC1*; trinucleotide mutations could abolish such binding activities (Fig. 4*F*). Together, *CsBRC1* directly binds to *CsPIN3* to repress its expression in vitro and in vivo.

**Enhanced Expression of *CsPIN3* Driven by the *CsBRC1* Promoter Led to Excessive Shoot Branching in Cucumber.** The expression patterns of *CsPIN3* were analyzed by qRT-PCR and in situ hybridization (Fig. 5 *A–E*). *CsPIN3* was highly expressed in the young stem and lateral buds (Fig. 5*A*). In situ hybridization further showed that *CsPIN3* transcripts specifically accumulated in lateral buds, leaf primordia, and stems in cucumber (Fig. 5 *B–E*). We next transformed *CsPIN1b* and *CsPIN3*, driven by the *CsBRC1* promoter, into cucumber inbred line R1461. Three representative *ProCsBRC1:CsPIN1b* transgenic lines (PIN1b-11, PIN1b-16, and PIN1b-24) and 3 *ProCsBRC1:CsPIN3* transgenic lines (PIN3-7, PIN3-13, and PIN3-15) were chosen for further characterization (Fig. 5 *F–N*). qRT-PCR showed that the expression of *CsPIN1b* and *CsPIN3* were significantly increased in the transgenic lines as compared to that in lines harboring the empty vector (WT) (Fig. 5 *F* and *G*). Importantly, enhanced expression of *CsPIN3* resulted in more lateral branches with a great increase in length, while elevated expression of *CsPIN1b* displayed no such significant difference as compared to WT (Fig. 5 *H–J*). Quantification data showed that only 3 to 5 branches were formed out of the 20 examined nodes in WT plants, while 11 to 14 branches were produced in *CsPIN3* transgenic lines (PIN3-7, PIN3-13, and PIN3-15) (Fig. 5 *K* and *L*). Similarly, the maximum of lateral branch length in WT was 7.0 cm, while those of *CsPIN3* transgenic lines were 27 to 38 cm (Fig. 5*M*). Moreover, the auxin level was significantly reduced in lateral buds of *CsPIN3* transgenic lines





**Fig. 3.** *CsBRC1* regulates auxin transport in cucumber. (A) Heat map showing the expression levels of cucumber homologs of auxin pathway genes in WT and *CsBRC1*-RNAi lines. Red boxes mean up-regulation, and green boxes indicate down-regulation. Blue, red, and green lines represent genes involved in auxin response, transport, and signaling, respectively. (B) Expression verification by qRT-PCR of the 2 auxin efflux carrier genes *CsPIN1b* and *CsPIN3* identified by RNA-seq analysis. (C) The content of endogenous auxin was greatly reduced in the axillary buds of *CsBRC1*-RNAi lines. (D–F) Immuno-gold localization of IAA in elongated axillary buds at the same node of WT and *CsBRC1*-RNAi plants. ab, axillary bud. (G) A representative image of an F1 plant resulted from a cross between *hardwickii* and Gy14. lb, lateral branch. (H–J) The expression of *CsBRC1* (H), auxin content (I), and the expression of *CsPIN1b/3* (J) in F1 plant as well as in the 2 parents (*hardwickii* and Gy14). (K and L) Images showing the axillary bud outgrowth after auxin inhibitor TIBA treatment of *hardwickii* lateral buds. (M) The IAA level was significantly increased upon TIBA treatment. (N) The branch length was significantly shorter in TIBA-treated plants than mock. Values are means of 3 biological replicates of lateral buds (B, C, H–J, and M) or 6 repeats of lateral branches (N) from different plants. Error bars represent  $\pm$ SD. Significance analysis was conducted with the 1-way ANOVA in C and I ( $P < 0.01$ ) and the 2-tailed Student's *t* test in M and N (\*\* $P < 0.01$ ). (Scale bars represent 100  $\mu$ m in D–F and 5 cm in G, K, and L.)

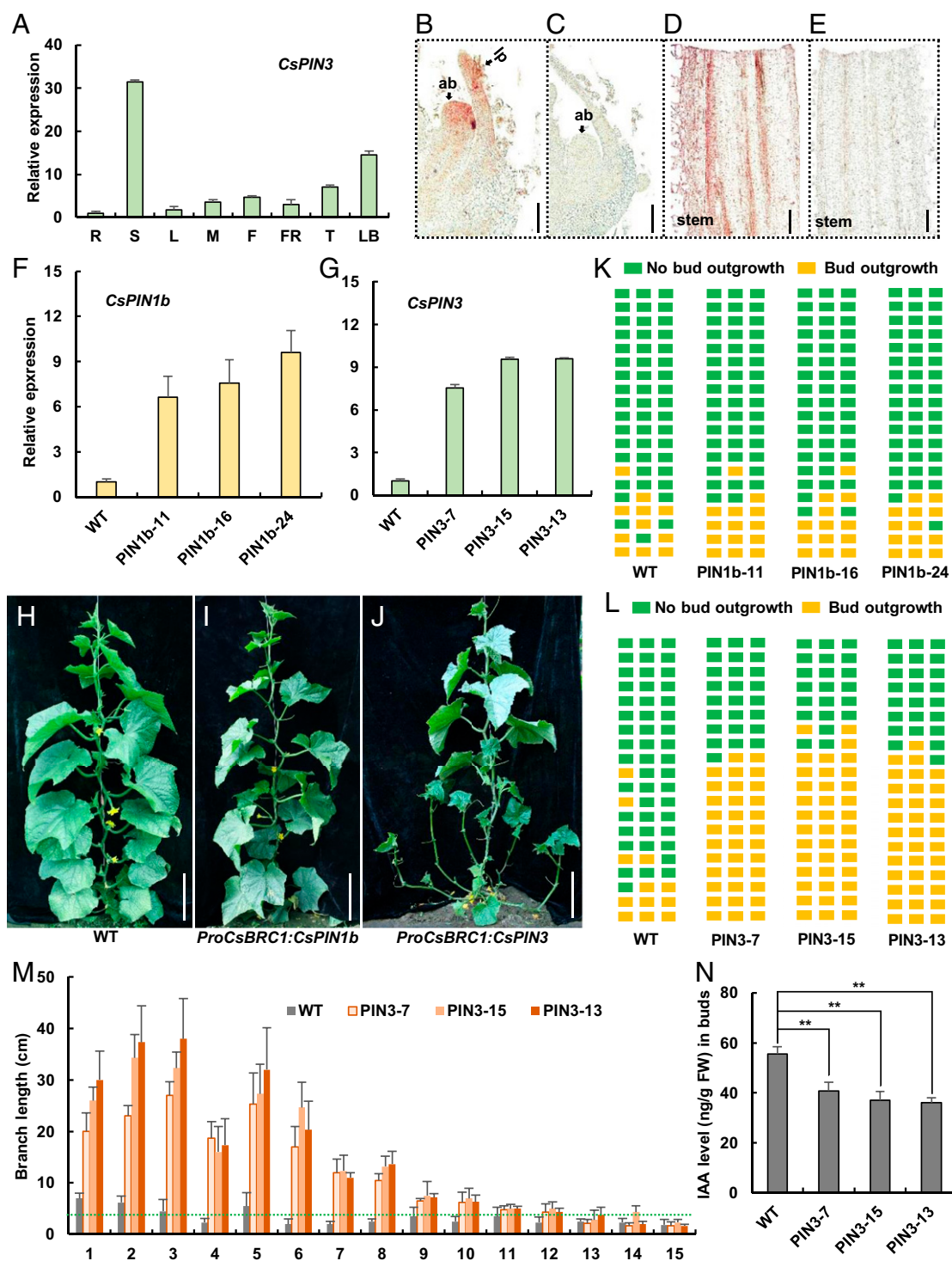


**Fig. 4.** CsBRC1 directly binds to the auxin efflux carrier gene *CsPIN3* to repress its expression. (A) Schematic diagram of the putative *cis*-elements in *CsPIN1b* (P1) and *CsPIN3* (P3A–P3E) used for CsBRC1 binding assay. (B) Yeast 1-hybrid assay showed binding of CsBRC1 to P1, P3A, P3B, P3C, and P3E. The numbers in the right corner indicate the basal concentration of Aureobasidin A (AbA; ng/mL). (C) Luciferase activity measurement in tobacco leaves after coexpression of *Pro35S:CsBRC1* and *ProCsPIN1b:LUC* or *ProCsPIN3:LUC*. The empty vector 62-SK was used as the control. Four independent transfection experiments were performed. (D) Callus images from cucumber hypocotyl explants transfected with empty control, *Pro35S:GFP*, and *Pro35S:CsBRC1-GFP* used in ChIP assay. (E) ChIP-qPCR assay showed that CsBRC1 binds to P3A, P3B, and P3C regions of *CsPIN3*. Three technical replicates and 3 biological replicates from different calli were performed. Error bars represent  $\pm$ SD. (F) EMSA assay showed that CsBRC1 binds to P3A, P3B, and P3C regions of *CsPIN3*. Significance analysis was conducted with the 2-tailed Student's *t* tests (\*\* $P < 0.01$ ).

(Fig. 5N). On the basis of the canalization hypothesis (12), we found that *CsPIN3*, but not *CsPIN1b*, plays an essential role in auxin export during bud outgrowth in cucumber.

**CsBRC1 Shows Different Responses to Light Intensity in Wild and Cultivated Cucumbers.** Increase in planting density is an important trait that has been selected for high yield during crop do-

mestication (26), which results in some degree of shading from neighboring plants. Recent studies showed that *BRC1* promotes branch suppression in response to shade in *Arabidopsis*, sorghum, and potato (26–28). To investigate whether *BRC1* responds to variations in light intensity related to shading in wild and cultivated cucumbers, we performed light intensity treatment (by blocking 90% light) to wild cucumber *hardwickii* and inbred line R1461. Transcript



**Fig. 5.** Phenotype analysis of overexpression of *CsPIN1b* and *CsPIN3* driven by the promoter of *CsBRC1*. (A) Expression analysis of *CsPIN3* by qRT-PCR in different organs of cucumber inbred line R1461. Values are means of 3 biological replicates from different plants. Error bars represent  $\pm$ SD. R, roots; S, stems; L, leaves; M, male buds; F, female buds; FR, fruits; T, shoot apex; LB, lateral buds. (B–E) In situ hybridization of *CsPIN3* in axillary buds (B) and the stem (D). The sense *CsPIN3* probe was hybridized as the negative control (C and E). ab, axillary bud; lp, leaf primordium. (F and G) Expression analysis of *CsPIN1b* (F) and *CsPIN3* (G) by qRT-PCR in control (WT), *ProCsBRC1:CsPIN1b* (PIN1b-11, PIN1b-16, and PIN1b-24), and *ProCsBRC1:CsPIN3* transgenic plants (PIN3-7, PIN3-15, and PIN3-13). Values are means of 3 biological replicates of lateral buds from different plants. (H–J) Representative images of WT (H), *ProCsBRC1:CsPIN1b* (I), and *ProCsBRC1:CsPIN3* transgenic plants (J) 60 d after planting grown under the same greenhouse condition. (K and L) Diagrammatic data showed the position of axillary bud outgrowth in *CsPIN1b* (K) and *CsPIN3* (L) transgenic cucumber lines. Each layer represents a node in cucumber. Green squares represent no bud outgrowth, and yellow squares represent bud outgrowth (longer than 4 cm). (M) The length of each lateral bud in WT and *ProCsBRC1:CsPIN3* transgenic lines from the 1st to 15th nodes. Bud length higher than the green dotted line indicates bud outgrowth. Values are means of lateral branch length from the same node of 3 independent plants. (N) The content of endogenous IAA was greatly reduced in the axillary buds of *ProCsBRC1:CsPIN3* transgenic lines. Values are means of 3 biological replicates of lateral buds from independent plants. Error bars represent  $\pm$ SD. Significance analysis was conducted with the 2-tailed Student's *t* tests (\*\**P* < 0.01). (Scale bars represent 100  $\mu$ m in B and C, 200  $\mu$ m in D and E, and 20 cm in H–J.)

levels of *CsBRC1* were increased by 2 to 3 fold in axillary buds of line R1461 upon 10 to 20 h shading (*SI Appendix*, Fig. S6A). In contrast, *CsBRC1* expression was not induced by shading in wild cucumber *hardwickii* (*SI Appendix*, Fig. S6A). Promoter analysis indicated that R1461 possesses 2 additional light responsive elements (an AE box and a GATA motif) as compared to that in *hardwickii* (*SI Appendix*, Fig. S6B), which may be the reason for different responses to reduced light intensity between *hardwickii* and R1461.

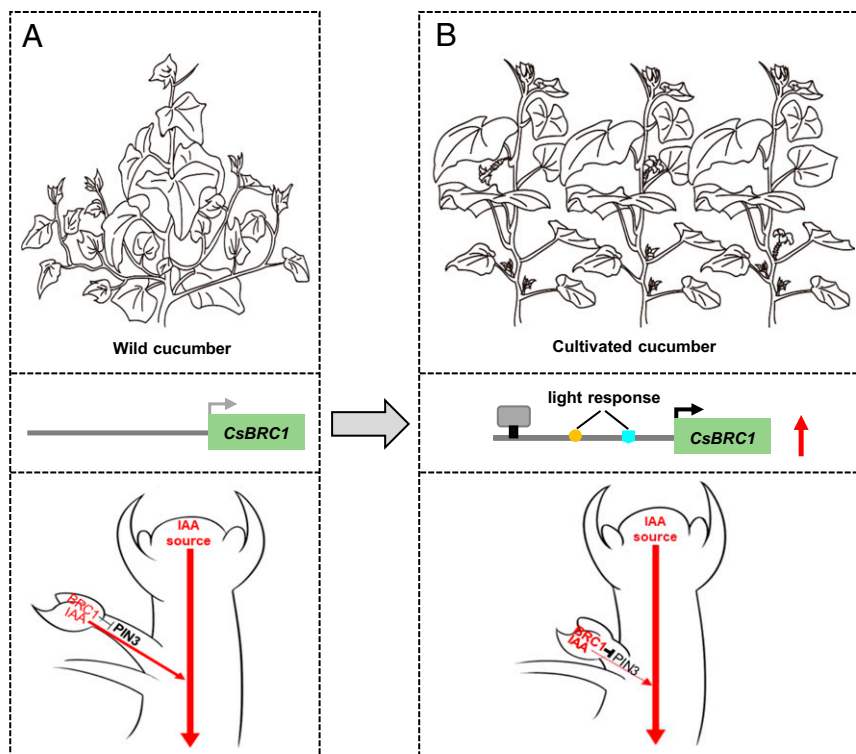
## Discussion

Apical dominance is a widely found phenomenon in plants where a main shoot dominates and inhibits the outgrowth of lateral branches, and auxin has been shown to be the key inhibitory signal produced by shoot tip responsible for apical dominance (7, 38). Auxin transport canalization hypothesis is one of the leading theories explaining apical dominance (11, 39). According to the theory, polar auxin transport from shoot apex to root leads to the auxin concentration in the main stem and thus reduces its sink strength, which will inhibit auxin flow from axillary buds to main stem, resulting in auxin accumulation in lateral buds and subsequent suppression of lateral branches (11, 16). On the other side, TB1/BRC1 has been well characterized to be the central integrator for multiple environmental and developmental factors inhibiting shoot branching (18, 22). *BRC1* transcription in lateral buds is responsive to auxin application in *Arabidopsis* (11). However, the direct molecular link between BRC1 and auxin remains elusive.

Here we found that *CsBRC1* was specifically expressed in axillary buds to inhibit bud outgrowth in cucumber (Figs. 1–2). Further, *CsPIN3* contains 5 *CsBRC1*-binding elements in its genome sequence (Fig. 4), and its expression was significantly up-regulated in *CsBRC1*-RNAi lines (Fig. 3). More importantly, both *in vitro* and *in vivo* biochemical assays suggested that *CsBRC1* directly binds to the auxin transporter *CsPIN3* and down-regulates its expression (Fig. 4). Elevated expression of *CsPIN3* driven by the *CsBRC1* promoter results in excessive shoot branching in cucumber (Fig. 5). We propose that *CsBRC1* functions as a key repressor for shoot branching by directly inhibiting *CsPIN3* activity, which down-

regulates auxin efflux from buds, hence bud outgrowth arrests in cucumber (Fig. 6A and B).

Considerable work has showed that PIN proteins are responsible for polar auxin transport underlying multiple aspects of plant development (40). The PIN1 protein is polarized on cell membrane and involved in plant organogenesis and leaf shape formation, as well as root development (41–44). PIN3 protein is expressed in gravity-sensing tissues and involved in differential growth in root (45), acts as a target for the CK signaling pathway to promote shoot branching (46), and mediates connective auxin transport facilitating communication between shoot apices to control shoot branching (40). Triple mutants of *PIN3*, *PIN4*, and *PIN7* (*pin3 pin4 pin7*) show no marked difference in shoot branching, although triple mutation (*pin3 pin4 pin7*) partially suppresses excessive branching of SL-deficient mutants. Further, involvement of PIN3, PIN4, and PIN7 in regulation of shoot branching is independent of the BRC1 pathway in *Arabidopsis* (47). However, *CsPIN3* is featured by its multiple *cis* elements for *CsBRC1* binding in cucumber, and *CsBRC1* indeed binds to *CsPIN3* for transcriptional suppression, as indicated by a whole set of biochemical, transcriptomic, and transgenic data (Figs. 3–5). Enhanced expression of *CsPIN3* dramatically stimulated bud outgrowth (Fig. 5). Thus, there may exist divergent regulatory mechanisms shaping shoot architecture in 2 different dicots *Arabidopsis* and cucumber. Such regulatory divergence may be attributed to following facts. First, the promoter and genic region of *CsPIN3* in cucumber harbors *cis* elements for *CsBRC1* binding (Fig. 4), while *PIN3* does not contain BRC1 binding elements in *Arabidopsis*. Second, *CsPIN3* transcripts were enriched in lateral buds of cucumber (Fig. 5) in contrast to no *PIN3* transcription reported in lateral buds in *Arabidopsis* (48). Third, 2 dicots have dramatically different shoot branching patterns. In *Arabidopsis*, the shoot apical meristem produces rosette leaves with compact internodes during vegetative growth. The inflorescence meristem gives rise to a few cauline leaves and then flowers with elongated internodes during the reproductive stage (49). Between 2 types of branching (rosette and cauline) in *Arabidopsis*, BRC1 and PIN3 play a negative and positive role,



**Fig. 6.** A working model of *CsBRC1* in repressing axillary bud outgrowth in cucumber. The main shoot tip produces massive auxin, which moves downward in the stem. In wild cucumber, low *BRC1* expression is unable to inhibit the *PIN3*-mediated auxin efflux from lateral buds, which leads to bud outgrowth (A). During domestication, insertion of 2 light response elements in *CsBRC1* promoter may favor elevation of *CsBRC1* expression and adaption to shade response from increased planting density. Alternatively, an unknown regulator (indicated as a gray box) promotes the transcription of *CsBRC1*. The elevated *CsBRC1* expression inhibits *PIN3*-mediated auxin efflux from lateral buds. Consequently, auxin accumulates in axillary buds and no bud outgrowth in cultivated cucumber (B).



respectively, in regulating rosette branching (18, 40, 47). In cucumber, however, the shoot apical meristem produces leaves with elongated internodes throughout the life cycle, and only 1 type of shoot branches (similar to cauline branches in *Arabidopsis*) emerges from the leaf axil of each node. Thus, genetic and developmental divergence enables *CsBRC1* to inhibit cucumber branching by repressing *CsPIN3* transcription and diminishing auxin export from lateral buds to the main stem (Figs. 2–5). Our finding paves a path to dissect *BRC1*-auxin interplay in shoot branching and yielding in closely related horticultural crops.

During crop domestication, a set of common traits have been selected and fixed to meet human needs and modern agronomic practices, such as reduced lateral branching, lack of shattering, greater fruit and seed size, and more determinate growth (50). *TB1* is one of the few domestication genes that regulates many of the domestication traits including shoot branching, prolificacy, inflorescence sex, and glume hardness that distinguish maize from its wild ancestor teosinte (51). In cucumber, cultivated cucumber displays reduced branching, larger fruit size, non-bitterness, and increased planting density as compared to wild cucumber *hardwickii* (4). What genes or regulatory networks underlying above domestication traits are largely unknown. Here we found that the expression of *CsBRC1*, a repressor for shoot branching, was higher in cultivated cucumber lines R1461 and Gy14 than that in wild cucumber *hardwickii* (Fig. 1). Further, our data showed that there were 2 additional light-responsive elements in the promoter of *CsBRC1* in cultivated cucumber other than in *hardwickii*, which might be potentially related to enhanced *CsBRC1* expression when light intensity was dramatically reduced (*SI Appendix*, Fig. S6). Increase in planting density is a common feature during crop domestication (52). To maximize light harvesting and avoid shade from neighboring vegetation, plants have evolved the shade avoidance syndrome, among which suppression of shoot branching is one of the key developmental responses. In wild cucumber, low *CsBRC1* expression allows higher levels of *PIN3*-mediated auxin efflux from buds to the stem, which leads to axillary bud outgrowth (Fig. 6A). In cultivated cucumber, 2 inserted light response *cis* elements in the *CsBRC1* promoter during domestication possibly favor elevation of *CsBRC1* expression and plant competition for light interception in the densely planting system. Another possibility is that yet-to-be-identified upstream regulators promote *CsBRC1* transcription in cultivated cucumber. Considering that *CsBRC1* expression varies among cultivated cucumber cultivars (Fig. 1), high *CsBRC1* expression reduces *CsPIN3*-mediated auxin efflux from lateral buds and thus suppresses axillary bud outgrowth (Fig. 6B); our work suggests a strategy, via modulating *CsBRC1* expression, to breed for cultivars with varying degrees of shoot branching grown in different cucumber production systems.

## Materials and Methods

**Plant Materials and Growth Conditions.** Cucumber (*Cucumis sativus* L.) inbred lines R1461 and Gy14, as well as wild ancestor *hardwickii*, were used in this study. The cucumber seeds were germinated at 28 °C in dark and then grown in a growth chamber at 16 h day (25 °C) and 8 h night (18 °C) until 2 true-leaf stage. Seedlings were then transferred to the greenhouse of China Agricultural University in Beijing. Water and pest control were performed according to standard protocol.

**Gene Cloning and Phylogenetic Analysis.** The complete coding sequence of *CsBRC1*, *CsPIN1b*, and *CsPIN3* was amplified from the axillary buds using gene-specific primers. The full-length amino acid sequence of cucumber *CsBRC1*, *CsPIN1b*, and *CsPIN3* homologs were performed by BLASTP at Cucurbit Genomics Database (<http://cucurbitgenomics.org>). The phylogenetic tree was generated with MEGA5 software, using the neighbor-joining method with 1,000 bootstrap replicates (53). Gene information for protein alignment and phylogenetic analysis is listed in *SI Appendix*, Table S2.

**Expression Analysis by Quantitative Real-Time PCR.** The cucumber root, stem, leaf, flower bud, fruit, shoot tip, and axillary bud were used for total RNA extraction and cDNA synthesis. The qRT-PCR analysis was performed using ABI PRISM 7500 Real-Time PCR System (Applied Biosystems). The cucumber *UBIQUITIN* (*Csa000874*) was used as internal controls. Three biological and

3 technical replicates (3 × 3) were performed for each gene. The primer information is listed in *SI Appendix*, Table S3.

**Subcellular Localization.** The full-length coding sequence of *CsBRC1* without the termination codon was cloned into the plasmid pCambia1300 to fuse with GFP. Subcellular localization was performed in the abaxial sides of tobacco leaves (4 to 6 wk old) as described previously (54). Fluorescence images were taken with a confocal laser-scanning microscope (Leica SP8) excited at a 488-nm wavelength. The primers are listed in *SI Appendix*, Table S3.

**In Situ Hybridization.** Cucumber shoot apices of 15-, 20-, 25-, 30-, and 35-d-old seedlings were fixed in 3.7% formal-acetic-alcohol. Sample fixation, sectioning, and hybridization were performed as described (55). The *CsBRC1* probe was 501-bp and the *CsPIN3* probe was 1,020-bp linearized fragments from the unique region of the corresponding coding sequence. The primers are listed in *SI Appendix*, Table S3.

**Cucumber Transformation.** A 585-bp specific fragment of the *CsBRC1* coding sequence was used for generating the *CsBRC1*-RNAi construct, and the empty PFGC-1008 vector was used as transformation control. The recombinant construct was introduced into *Agrobacterium* strain GV3101 and transformed into cucumber inbred line R1461 using a cotyledon transformation method as described (33, 34). To generate *ProCsBRC1:CsPIN1b* and *ProCsBRC1:CsPIN3*, the full-length coding sequence of *CsPIN1b* and *CsPIN3* was driven by the 2-kb promoter sequence of *CsBRC1*, and the recombinant construct was transformed into cucumber line R1461 as described above. The primers are listed in *SI Appendix*, Table S3.

**RNA-Seq Sample Preparation and Sequencing.** The lateral branches from the same node of WT and transgenic cucumber plants were collected for RNA-seq analysis. Two biological replicates were performed for each sample. Illumina HiSeq 2000 platform was used to generate 100-bp paired-end reads. Bioinformatic analyses of RNA-Seq data were performed as previously described (56).

**Extraction and Quantification of Endogenous Auxins.** About 0.1 g samples were harvested from axillary buds of WT and *CsBRC1*-RNAi transgenic plants and used for measurement of auxin content. The extraction and quantification of endogenous auxin were performed using ELISAs according to methods previously described (57). Three biological repeats were performed for each sample.

**Immuno-Gold Localization of IAA.** Cucumber axillary buds were fixed and incubated with anti-IAA monoclonal antibody as previously described (57). The primary antibody (IAA | indole-3-acetic acid [N1]) and secondary antibody (Anti-Rabbit IgG–Gold antibody produced in goat) were used in this experiment. Images were obtained using a light microscope (D72; Olympus).

**Auxin Transport Inhibitor Treatment.** Axillary buds (about 2 mm) at the same nodes of wild cucumber *hardwickii* were treated with 0.5 mM 2, 3, 5-Triiodobenzoic acid (TIBA) as auxin transport inhibitor. The treatment was repeated every 72 h until 15 d. Three biological repeats were performed for each treatment.

**Yeast 1-Hybrid Assay.** The coding region sequence of *CsBRC1* was cloned into prey vector (pGADT7), and the oligonucleotides of *CsBRC1*-binding sites (P1 in *CsPIN1* and P3A–P3E in *CsPIN3*) were synthesized and ligated into the pAbAi vectors (Clontech). The linearized pAbAi vectors containing the binding sites were transformed into the yeast strain Y1H Gold according to the manufacturer's manual. Then pGADT7-*CsBRC1* was transformed into the Y1H Gold strain with *CsBRC1* binding sites and selected by AbA (Aureobasidin A) on SD/-LEU (Synthetic Dropout Medium/-Leucine) medium (Clontech). Interaction of IND-AD with the PID-E box was used as a positive control (57), and the empty AD and PID-E box served as a negative control. The primers are listed in *SI Appendix*, Table S3.

**Dual Luciferase (LUC) Assay for Protein–DNA Interactions in Tobacco Leaves.** Promoters of *CsPIN1b* and *CsPIN3* (2,000 bp) were cloned into the transient expression vector pGreenII 0800-Luc. The *CsBRC1* coding sequence was cloned into pGreenII 62-SK as the effector. Tobacco (*Nicotiana benthamiana*) leaves were used for coexpression studies as previously described (57). The empty vector pGreenII 62-SK and the *proCsPIN1b*-LUC or *proCsPIN3*-LUC vector were used as negative controls. The dual luciferase assay reagents (Promega) were used for examining the firefly luciferase and renilla luciferase. The primers for all constructs are listed in *SI Appendix*, Table S3.

**Electrophoretic Mobility Shift Assay.** The full-length CsBRC1 protein sequence was fused with MBP tag and introduced into *Escherichia coli* BL21 (DE3). Hot probes harboring elements (P1 in *CsPIN1* and P3A-P3C in *CsPIN3*) were synthesized and labeled with biotin. The recombinant protein was purified by using maltose. An electrophoretic mobility shift assay (EMSA) was performed using the Light Shift Chemiluminescent EMSA kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The primers are listed in *SI Appendix, Table S3*.

**ChIP-qPCR Assay.** The recombinant pCAMBIA1300-CsBRC1-GFP construct and pCAMBIA1300-GFP constructs were transformed into cucumber calli of hypocotyl explants, and the ChIP assays were performed as described in the EpiQuik Plant ChIP Kit (Epigentek) with anti-GFP antibodies (Sigma-Aldrich). Probes of P1 and P3A-P3DE were analyzed to assess their enrichment. For each ChIP-qPCR assay, 3 biological repeats and 3 technical replicates (3 × 3) were included. The primers are listed in *SI Appendix, Table S3*.

**Shade Treatment.** The *hardwickii* and R1461 plants with 15 nodes were selected for shade treatment. Half of the plants were shaded for 48 h (frame covered with a thick black fabric shading cloth which could block 90% of light). The other half of the plants placed under normal light conditions (10 h day/14 h night) were used as control. Axillary buds at the same node in plants were harvested to detect the expression level of *CsBRC1* after 10 or 20 h of shading.

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