Short Article

Developmental Cell

Brassinosteroid Signaling Regulates Leaf Erectness in *Oryza sativa* via the Control of a Specific U-Type Cyclin and Cell Proliferation

Highlights

- Abaxial sclerenchyma cell number in rice lamina joints affects leaf erectness
- Brassinosteroids (BRs) inhibit abaxial sclerenchyma cell proliferation
- CYC U4;1 is a U-type cyclin controlling abaxial sclerenchyma cell proliferation
- BR signaling regulates both CYC U4;1 gene expression and protein activity

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In Brief

Brassinosteroids (BRs) control leaf erectness in monocots. Sun et al. uncover the underlying mechanism in rice, showing that BR inhibits the proliferation of a specific abaxial cell population in the lamina joint regions. The authors identify a U-type cyclin (*CYC U4;1*) whose activity and expression are controlled by BR signaling.

Sun et al., 2015, Developmental Cell *34*, 220–228
July 27, 2015
http://dx.doi.org/10.1016/j.devcel.2015.05.019

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Brassinosteroid Signaling Regulates Leaf Erectness in *Oryza sativa* via the Control of a Specific U-Type Cyclin and Cell Proliferation

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http://dx.doi.org/10.1016/j.devcel.2015.05.019

SUMMARY

Leaf erectness is key in determining plant architecture and yield, particularly in cereal crops. Brassinosteroids (BRs) play a unique role in controlling this trait in monocots, but the underlying cellular and molecular mechanisms remain big mysteries. Here we report that the abaxial sclerenchyma cell number of rice lamina joints (LJs) is closely related to leaf erectness, and BR signaling tightly regulates their proliferation. We identified a rice U-type cyclin *CYC U4;1* enriched in rice LJs, with its expression accompanying LJ development. Genetic and biochemical studies demonstrated that CYC U4;1 plays a positive role in promoting leaf erectness by controlling the abaxial sclerenchyma cell proliferation. Furthermore, BR signaling inhibits the abaxial sclerenchyma cell division by coordinately regulating *CYC U4;1* expression through BES1 and CYC U4;1 protein activity through GSK3 kinases. These results support a key role of the cyclin *CYC U4;1* in mediating BR-regulated cell division to control leaf erectness.

INTRODUCTION

Brassinosteroids (BRs) are essential plant steroid hormones in regulating many aspects of plant development, such as cell elongation, vascular differentiation, stomata development, and male fertility (Yang et al., 2011). In monocots, BRs play a unique and crucial role in determining leaf erectness (Chono et al., 2003; Sakamoto et al., 2006; Hartwig et al., 2011), an important trait closely related to crop yields, as erect leaves allow great penetration of light to lower canopy and enable plantings with high density to increase leaf area index (Sinclair and Sheehy, 1999; Sakamoto, 2006). The elevated BR content or enhanced BR signaling promotes lamina joint (LJ) inclination (LJI) in rice, while the BR-deficient or insensitive rice mutants exhibit erect leaves (Hong et al., 2004; Tong and Chu, 2012). A previous study showed that the applied BRs can enhance LJI via inducing greater expansion of adaxial cells than dorsal cells in LJs (Cao and Chen, 1995). Besides BRs, the leaf inclination is also regulated by other signals (Zhao et al., 2010; Ning et al., 2011). However, the underlying cellular and molecular mechanisms of BR regulating rice leaf erectness are long-sought mysteries. We found that groups of sclerenchyma cells at the abaxial side in the rice LJ regions are closely related to leaf erectness, and BR signaling tightly inhibits their proliferation. We identified a U-type cyclin *CYC U4;1* highly expressed in LJs and its overexpression in rice led to erect leaves by promoting these sclerenchyma cell division. In addition, further genetic and biochemical experiments demonstrated that the BR-activated transcription factor BES1 (bri1-EMS-suppressor 1) (Yin et al., 2002) directly binds to the *CYC U4;1* promoter to inhibit its expression. Moreover, a GSK3-like kinase, BIN2 (Brassinosteriod-Insensitive 2), a critical negative regulator in the BR signaling pathway (Li and Nam, 2002), interacts with and phosphorylates CYC U4;1 to activate the CYC U4;1/CDKA complex to promote DNA replication. Thus, our findings support a fascinating model in which BR signaling regulates abaxial sclerenchyma cell proliferation in rice LJs to control leaf erectness via coordinately regulating both expression and activation of CYC U4;1.

RESULTS AND DISCUSSION

BR Signaling Regulates Leaf Erectness by Inhibiting LJ's Abaxial Sclerenchyma Cell Proliferation

To systematically examine the effect of BRs on the morphology and anatomy of the LJ regions, the excised LJs from the dark-grown rice seedlings (T65) were treated with 1 μM 24-epibrassinolide (eBL). We found that the leaf angle (the angle between leaf blade and the upward stem, as shown in Figure S1A) was opened to 130° under eBL treatment as compared with 30° under mock treatment (Figure S1B), and two edges (defined as L1), and both adaxial and abaxial middle regions (defined as L2 and L3, respectively) of LJs were dramatically expanded after eBL treatment (Figures S1C and S1D). Longitudinal sections of LJs showed that eBL treatment did not significantly alter cell length of the BR-perceptional mutant *d61-1* (Figure S1E) but greatly enhanced adaxial cell expansion of the WT T65 and a BR-deficient mutant *d2-2* (Figures S1E–S1H), which is similar to a

previous report (Cao and Chen, 1995). In addition, eBL treatment did not significantly influence the cell number measured with the longitudinal sections (represented by adaxial epidermal cells between the dashed lines shown in Figure S1E) and the cell layer number measured with the transverse sections of the WT (m1 regions, shown in Figures 1A, S1I, and S1J), indicating that the exogenous eBL greatly promoted LJ's parenchyma cell expansion but did not affect adaxial cell number. We then compared the transverse sections of the second LJs from *d61-1* and *d2-2* with that from T65 and interestingly observed that the BR-attenuated mutants with more erect leaves had increased sclerenchyma cell layers in m2 regions (abaxial sclerenchyma cells between epidermis and the middle vascular bundles [vbs] of LJs) than T65 (Figures 1A–1C). To further determine the role of other BR signaling components in LJ development, we created transgenic rice expressing a negative regulator *Atbin2-1*, a gain-of-function mutation of *AtBIN2* in *Arabidopsis* (Li and Nam, 2002), and a positive regulator *bes1-D*, a gain-of-function mutation of *AtBES1* (Yin et al., 2002), driven by a rice LJ-specific promoter *OsDWARF4*/*DWF4* (Sakamoto et al., 2006) (Figure S2A). The *pDWF4::bin2-1* rice displayed an enhanced leaf erectness, whereas the *pDWF4::bes1-D* rice showed greatly enlarged leaf angles (Figures 1D and S3C). Similar to *d61-1*, the *pDWF4::bin2-1* plants were also insensitive to eBL in LJI assay, whereas the *pDWF4::bes1-D* plants were hypersensitive to BRs (Figure S2B). The LJ transverse sections indicated that the *pDWF4::bes1-D* rice had more expanded cells, but with similar m1 cell layers as compared with its WT Nipponbare (Ni) (Figures 1E–1G), but their m2 sclerenchyma cell layer number was slightly reduced (Figure 1H). In contrast, the *pDWF4::bin2-1* plants had similar cell size and cell layer number in m1 regions compared with Ni, but had more m2 sclerenchyma cell layers than Ni and the *pDWF4::bes1-D* plants (Figures 1E–1H). Additionally, sclerenchyma cells with heavy lignin deposition in the m2 region of the *d61-1* mutant, as shown by phloroglucinol-HCl staining, apparently provide strong physical support to ensure leaf erectness (Figure 1I).

CYC U4;1 Regulates Leaf Joint Development by Controlling Abaxial Sclerenchyma Cell Proliferation

To understand how BRs regulate the sclerenchyma cell proliferation in the LJ regions, we conducted microarray analysis (see the detail in Supplemental Information) to identify the LJ-enriched genes potentially involved in cell division. We initially found four cyclin-like genes *Os04g46660*, *Os12g39830*, *Os10g41430*, and *Os09g29100*, which were 104-, 12-, 8.5-, and 7.9-fold upregulated in LJs compared with leaf blades, respectively. qRT-PCR analysis further confirmed their enhanced expression in LJs (Figure S2C). Sequence analysis indicated that the *Os04g46660* and *Os10g41430* encode U-type cyclins and the *Os12g39830* and *Os09g29100* encode the D-type cyclins. However, the two D-type cyclins were downregulated in the LJs of *d61-1* and *d2-2* (Figure S2D), which was not consistent with the LJs phenotype of the *d61-1* and *d2-2* with more sclerenchyma cell layers. Therefore, we focused on the *Os10g41430*, belonging to the CycU4 subgroup, and *Os04g46660*, belonging to the CycU2 subgroup (Wang et al., 2004). However, overexpression of *Os04g46660* (*pCYCU2::CYCU2*) did not lead to enhanced leaf erectness (Figure S2E); we focused our study on *Os10g41430* (renamed as *CYC U4;1*). Because the U-type cyclin is a cyclin with unknown function in cell cycle, we analyzed the expression level of the U-type cyclin in the cell cycle by synchronizing the induced rice calli with hydroxyurea, a reversible inhibitor of the G1/S-phase for 24 hr and then releasing. We could not detect the expression of *CYC U4;1* in the calli based on the β-glucuronidase (GUS) reporter line, and we also found the *CYC U2* (*Os04g46660*) has strong expression in the calli (Figure 2A). Therefore, we analyzed the expression levels of the *CYC U2*, *CYC D3* (a G1/S stage-specific marker), and *CYC B2* (a G2/M stage-specific marker) (Li et al., 2011) in the released calli. The results revealed that the *CYC U2* had highest expression at 3 hr after release, earlier than the *CYC D3* induced at 6 hr after release, indicating that *CYC U2* may function in the early G1/S phase responsible for G1-to-S transition; the transcription of *CYC B2* was induced at 15 hr after release, and the expression of *CYC D3* was also slightly induced at this stage, indicating their role in G2/M phase (Inzé and De Veylder, 2006; Schnittger et al., 2002) (Figure 2B). To detect whether the G1/S phase gene *CYC U4;1* is involved in DNA replication, we synchronized the transgenic BY-2 cells using aphidicolin, an inhibitor of DNA polymerase usually used to arrest the cells at the G1/S boundary. We found that the *35S::CYC U4;1* transgenic BY-2 cells showed the 4C peak (8 hr after release from the aphidicolin) earlier than the control BY-2 cells (10 hr after release from the aphidicolin). Furthermore, the *35S::CYC U4;1* transgenic BY-2 cells complete DNA replication (10 hr after release from the aphidicolin) earlier than the empty-vector control (14 hr after release from the aphidicolin) (Figure 2C). These results suggested that the *CYC U4;1* participates in the cell cycle at the early G1/S stage. The expression of the *GUS* reporter driven by the *CYC U4;1* promoter in rice indicated that *CYC U4;1* was highly expressed in the LJs (Figure S2F), and its expression was accompanied with the LJs developmental process (Figure 2D). Because the first true leaf of rice is incomplete leaf with unexpanded blade and the leaves from the second true leaf to the flag leaf are complete leaves consisting of sheath and blade (Hoshikawa, 1989), we analyzed the *CYC U4;1* expression pattern in the LJ of the second true leaf. We defined the developmental process of the second true leaf joint into four stages. Stage 1 occurs 2 days after germination. The first true leaf is packaged in the coleoptile, and the border is between the blade and the sheath of the second true leaf is visible. Stage 2 occurs 3 days after germination. The first true leaf emerges from the coleoptile. In stage 3, the tip of the second true leaf emerges from the sheath of the first true leaf. In stage 4, the leaf joint of the second true leaf grows out from the first true leaf sheath. We did not detect the GUS signal in the joint border between the blade and the sheath at stage 1, and we detected the initiation of the *CYC U4;1* expression in the abaxial side of the second true leaf joint at stage 2, and the GUS signal was detected in the outer side of the veins, which is similar to that of stage 3. Interestingly, at stage 4, the GUS signal was detected in an enlarged area of the LJ regions, and it was weaker in the outer side of the main vein than stages 2 and 3. The LJ sections of the *pCYC U4::GUS* and the in situ hybridization assay all indicated that *CYC U4;1* was highly expressed in the sclerenchyma cells between the vb and the outer epidermis in abaxial collar (Figures 2E and S2G). The *RNAi-CYC U4;1* knockdown lines exhibited greatly enlarged leaf angles compared with Ni (Figures

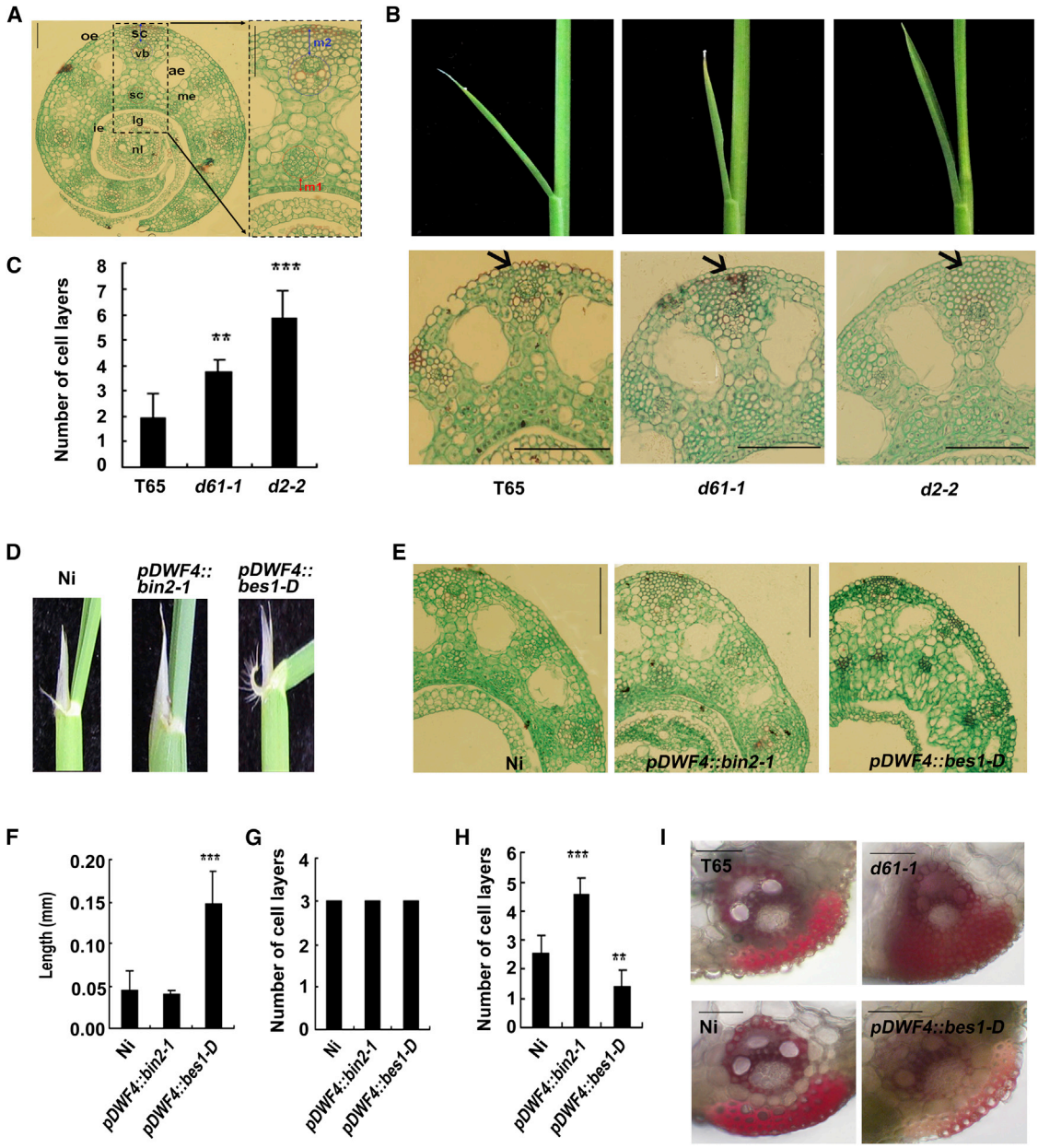

Figure 1. BR Signaling Inhibits LJ's Abaxial Sclerenchyma Cell Proliferation

(A) Anatomy of the LJs from the etiolated seedlings. m1, the region between the adaxial epidermis and the sclerenchyma in the middle area of the cross section; m2, the region between the abaxial epidermis and the abaxial central vb of the cross-section; ae, aerenchyma; ie, inner epidermis; lg, ligule; me, mesophyll cells; nl, newly emerged leaf; oe, outer epidermis; sc, sclerenchyma cell. Scale bars represent 0.25 mm.

(B) The morphology and the cross-sections of the second LJs of T65, *d2-2*, and *d61-1*. Scale bars represent 0.25 mm. Arrows point to the m2 regions.

(C) The number of sclerenchyma cell layers in the m2 regions of the second LJs (shown in Figure 1B). Error bars are SD (n = 15). p values were determined by Student's t test. The significant levels are as follows: $^{**}p < 0.01$; $^{***}p < 0.001$.

(D) The LJ morphology of Ni, the *pDWF4::bin2-1*, and the *pDWF4::bes1-D* lines.

(E) Cross-sections of the second LJs of Ni, the *pDWF4::bin2-1*, and the *pDWF4::bes1-D* lines. Scale bars represent 0.25 mm.

(F) The length of m1 (shown in Figure 1E). Error bars are SD (n = 15). Student's t test: $^{***}p < 0.001$.

(G) The number of cell layers in the m1 region (shown in Figure 1E). Error bars are SD (SD = 0; n = 15).

(H) The number of sclerenchyma cell layers in the m2 region (shown in Figure 1E). Error bars are SD (n = 15). Student's t test: $^{**}p < 0.01$; $^{***}p < 0.001$.

(I) Cross-sections of the third LJs stained with phloroglucinol-HCl (red color) (Zhou et al., 2009). Scale bars represent 0.1 mm.

See also Figures S1 and S2.

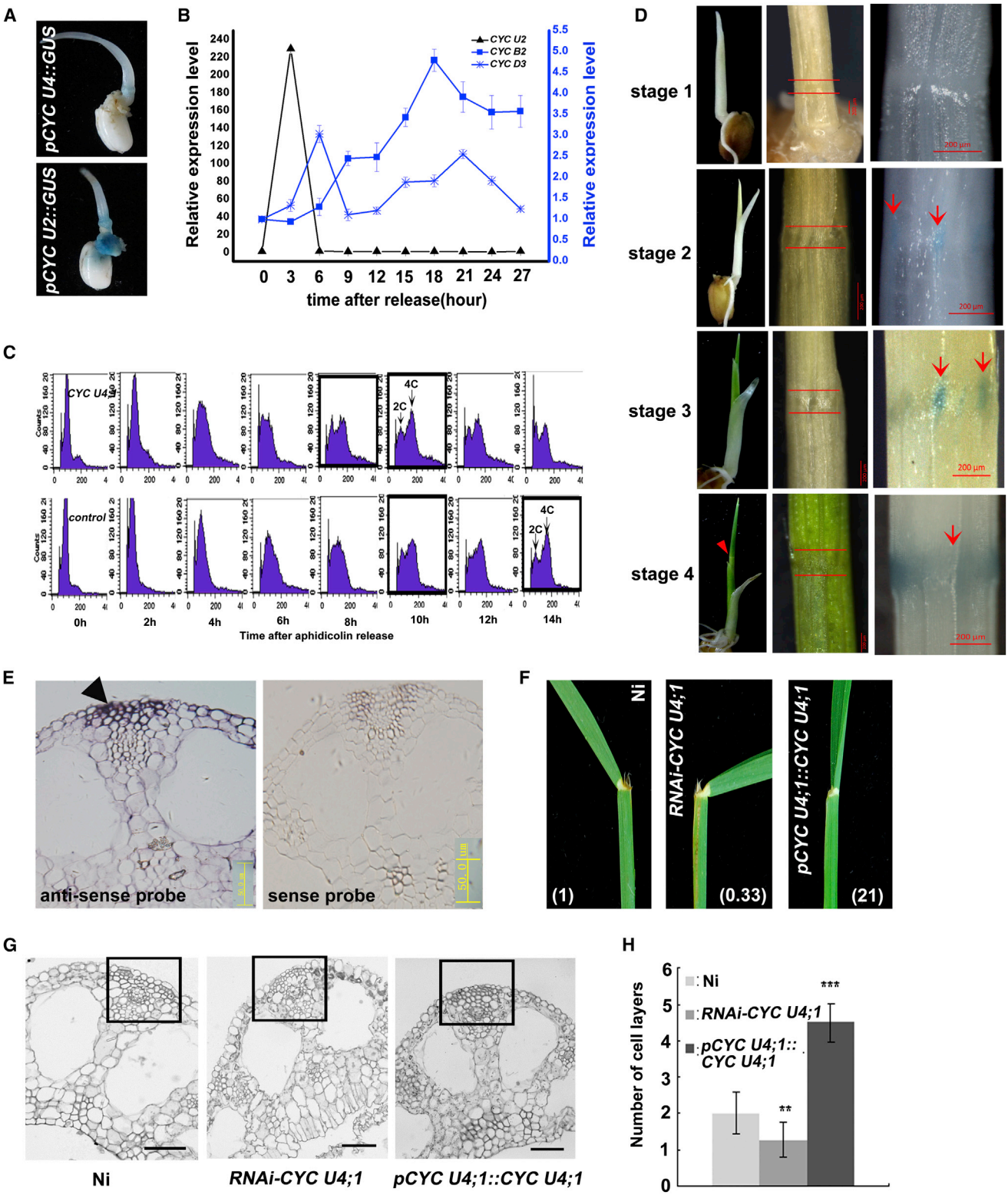

Figure 2. *CYC U4;1* Regulates Leaf Erectness by Controlling Abaxial Sclerenchyma Cell Proliferation

(A) The expression pattern of GUS reporters driven by the promoters of *CYC U4* and *CYC U2*, respectively, in the 7-day-old dark-grown callus.

(B) The expression level of *CYC U2*, *CYC D3*, and *CYC B2* in Ni calli after released from hydroxyurea. The expression levels are normalized to expression level at 0 hr after release (n = 3). The *CYC U2* refers to the black vertical ordinate, and *CYC D3* and *CYC B2* refer to the blue vertical ordinate.

(C) Flow cytometric analysis of the synchronized transgenic BY-2 cell culture; 0–14 hr indicated the hours after release from the aphidicolin.

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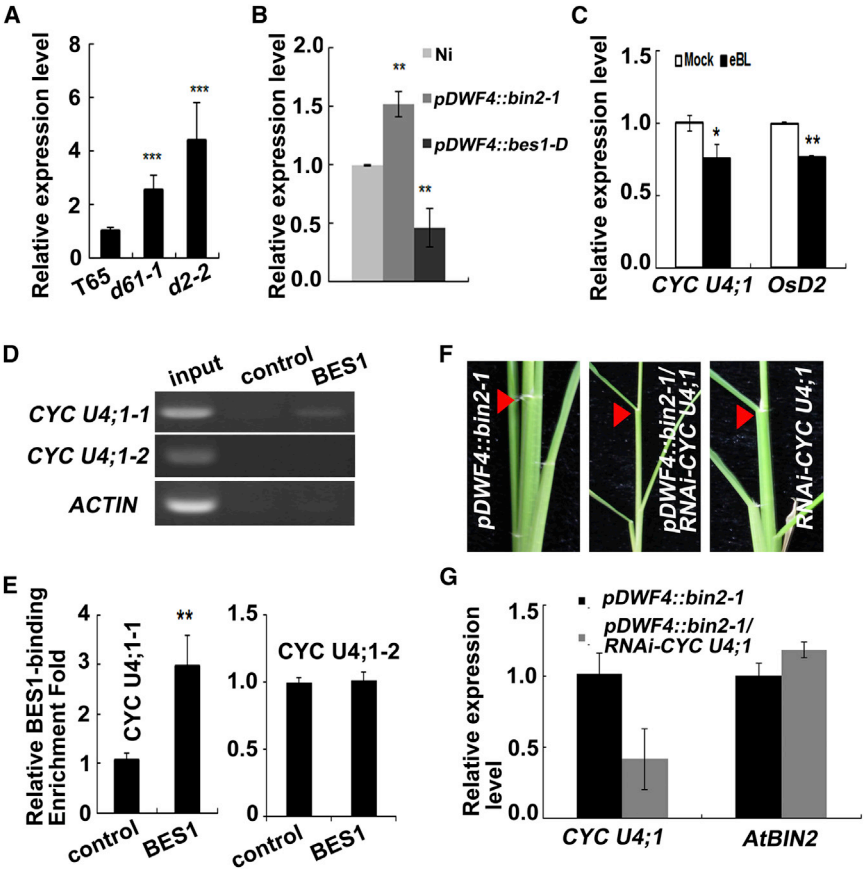


Figure 3. BR Signaling Regulates Leaf Erectness through the BES1-Mediated *CYC U4;1* Expression

(A) Relative expression levels of *CYC U4;1* in the LJs of T65, *d61-1*, and *d2-2*.

(B) Relative expression levels of *CYC U4;1* in the LJs of Ni, the *pDWF4::bin2-1* line, and the *pDWF4::bes1-D* line.

(C) The expression of *CYC U4;1* is inhibited by eBL treatment. *OsD2*, an ortholog of *AtDWARF1*, is used as a BR-responsive gene (Hong et al., 2003).

(D and E) The ChIP assay detected by semi-quantitative PCR (D) and real-time qRT-PCR (E) indicates that AtBES1 directly binds to the promoter region of *CYC U4;1*. The *CYC U4;1-1* and *CYC U4;1-2* are the different regions in the *CYC U4;1* promoter. The control of the ChIP is no-antibody.

(F) The *pDWF4::bin2-1/RNAi-CYC U4;1* has large leaf angles similar to the *RNAi-CYC U4;1* line. The red arrowheads indicate leaf angle.

(G) The relative expression level of *AtBIN2* and *CYC U4;1* in the lines shown in (F).

2F and S2H), as the *RNAi-CYC U4;1* line had more expanded m1 parenchyma cells (Figure 2G) and had one to two layers of m2 sclerenchyma cells (Figures 2G and 2H), which is similar to *pDWF4::bes1-D*. In contrast, the transgenic rice overexpressing *pCYC U4;1::CYC U4;1* exhibited more erect leaves with more m2 sclerenchyma cell layers than the *RNAi-CYC U4;1* line and Ni (Figures 2F–2H and S2I). Taken together, it is suggested that *CYC U4;1* promotes LJ sclerenchyma precursor cell proliferation, resulting in leaf erectness.

BR Signaling Regulates the *CYC U4;1* Expression through BES1

To investigate whether *CYC U4;1* mediates the BR-regulated leaf erectness, we first measured its expression in the LJs of the BR-related mutants and found that *CYC U4;1* was greatly upregulated in *d61-1* and *d2-2* compared with T65 (Figure 3A). Furthermore, its expression was also enhanced in the *pDWF4::bin2-1*, but was reduced in the *pDWF4::bes1-D* lines (Figure 3B). Consistently, its expression in the LJs was suppressed by eBL (Figure 3C). It is well known that BES1 acts as a transcription factor in the downstream of the BR signaling pathway to directly regulate gene expression involved in plant growth and development (Sun et al., 2010; Yu et al., 2011). Therefore, we conducted chromatin immunoprecipitation (ChIP) experiment using BES1 antibody and found that BES1 can directly bind to the specific promoter region of *CYC U4;1* (Figures 3D and 3E), suggesting that BRs may activate BES1 to inhibit *CYC U4;1* expression to control abaxial sclerenchyma cell proliferation. Furthermore, the transgenic lines of *pDWF4::bin2-1/RNAi-CYC U4;1* exhibited similar leaf angle with that of the *RNAi-CYC U4;1* line (Figures 3F and 3G), supporting that *CYC U4;1* acts as a downstream target of BR signaling to regulate leaf erectness.

BIN2 Promotes CYC U4;1 Activity

In addition to its expression, cyclin can also be regulated at protein levels in a cell cycle-dependent manner, which is crucial for

(D) Expression pattern of the *GUS* reporter driven by the *CYC U4;1* promoter at different developmental stages of the second true leaf joint. Each developmental stage was defined in the text. The red arrowhead indicates the leaf joint of the second true leaf. The red arrows indicate the GUS signal position. The regions between the red lines indicate the leaf joint regions.

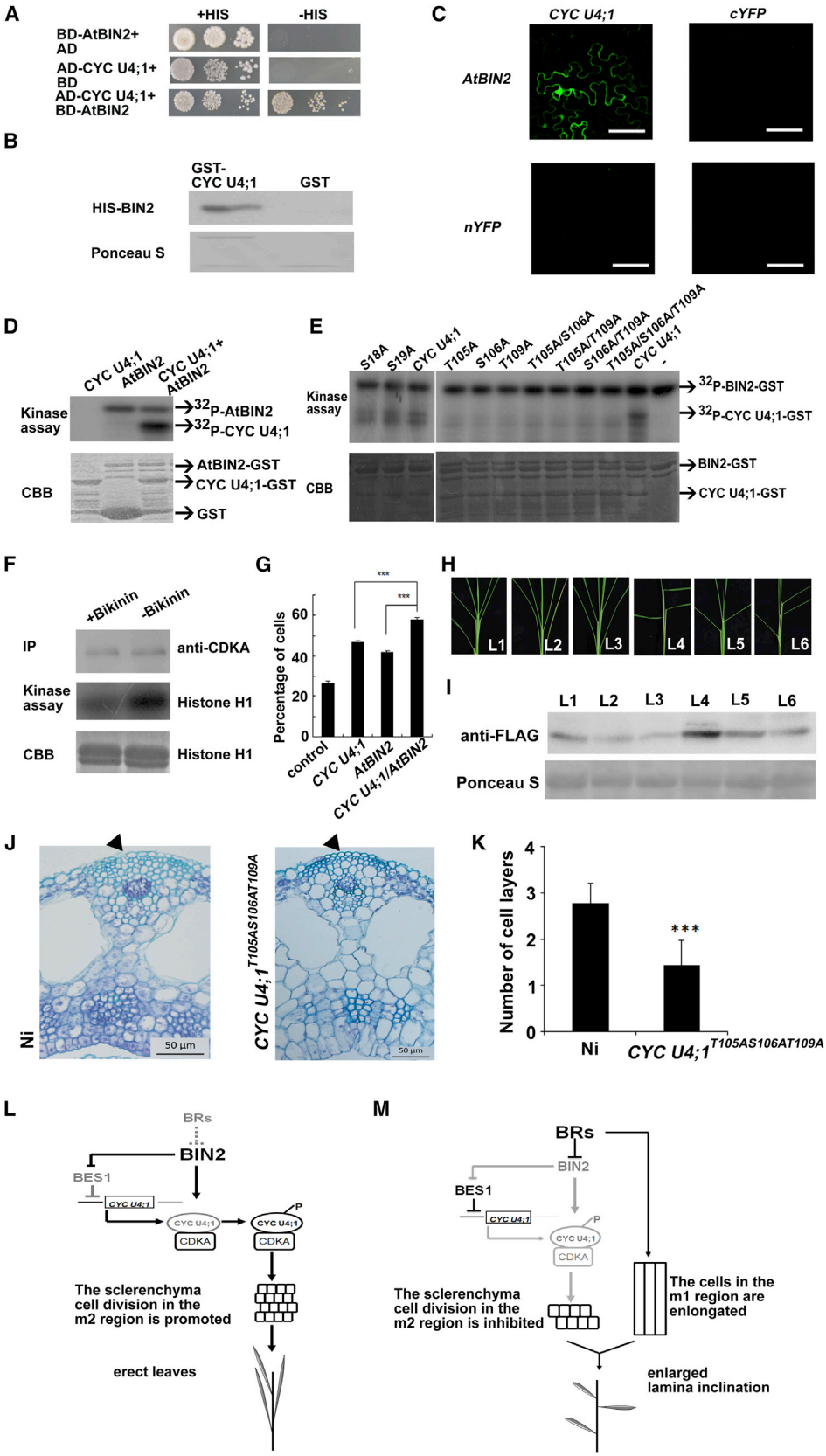
(E) Expression pattern of *CYC U4;1* in the LJs. In situ hybridization with an anti-sense probe (left) and a sense probe (right) of *CYC U4;1*. The black arrow head indicates heavy signal in the sclerenchyma cells. Scale bars represent 0.05 mm.

(F) The LJ morphology of Ni, the *RNAi-CYC U4;1* line, and the *pCYC U4;1::CYC U4;1* line. Numbers in the parenthesis indicate the relative expression levels of the corresponding genes in the transgenic lines compared with the WT Ni.

(G) Cross-sections of the second LJs of Ni, the *RNAi-CYC U4;1* line, and the *pCYC U4;1::CYC U4;1* line. The black box indicated the m2 region. Scale bars represent 0.02mm.

(H) The number of sclerenchyma cell layers in the m2 regions (shown in Figure 2G).

Error bars are SD ($n > 7$). Student's t test: $^{**}p < 0.01$; $^{***}p < 0.001$. See also Figure S2.



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cell-cycle progresses in mammals (Takahashi-Yanaga and Sasaguri, 2008) and plants (Criqui et al., 2000). The cell cycle-dependent cyclin regulation, such as activity, subcellular localization, or stability, is closely related to its phosphorylation by CDK, GSK3, MAPK, and/or Ser/Thr phosphatase (Welcker et al., 2003; Minella et al., 2008; Qi et al., 2012). However, such regulation is still largely unknown in plants (Inzé and De Veylder, 2006). BIN2 is a GSK3-like kinase and plays a critical role in BR signaling. Interestingly, when we tested whether BIN2 could interact with CYC U4;1, we found that CYC U4;1 can strongly interact with AtBIN2 in vitro and in vivo (Figures 4A–4C). CYC U4;1 can also be phosphorylated by AtBIN2 kinase (Figure 4D). In mammals, the cyclin phosphorylated by GSK3 kinases is usually related to their degradation or nuclear export (Welcker et al., 2003; Alt et al., 2000). However, we found that the protein level of CYC U4;1-FLAG in the *bin2-1*, a gain of function mutation of a GSK3-like kinase in *Arabidopsis*, was similar to that in WT (Figure S3A), and its subcellular localization was not regulated by BIN2 kinase (Figure S3B), suggesting that the phosphorylated modification of CYC U4;1 by BIN2 may be related to cyclin activation, which may be different from the mechanism in mammals.

We predicted the putative BIN2 kinase phosphorylation motifs in the CYC U4;1 and found one typical phosphorylation motif ($T^{105}S^{106}T^{109}$) by GSK3. We then mutated each site to Ala and constructed the mutant proteins designated as T105A, S106A, T109A, T105AS106A, S106AT109A, T105AT109A, and T105AS106AT109A, respectively, for BIN2 kinase assay. We also mutated S18 and S19, which are not located in a typical GSK3 recognition motif, as controls. We found that all of the mutant proteins related to the motif $T^{105}S^{106}T^{109}$ cannot be phosphorylated by BIN2, indicating that the relevant sites might interfere with the structure of CYC U4;1 related to the phosphorylation activity by BIN2 kinase, and the mutant proteins related to S^{18} and S^{19} can still be phosphorylated by BIN2 (Figure 4E). These phosphorylation sites $T^{105}S^{106}T^{109}$ are in the cyclin box domain, which may be related to its activity during cell cycle. To test this hypothesis, we created transgenic *Arabidopsis* plants overexpressing FLAG-tagged *CYC U4;1* driven by the cauliflower mosaic virus (CaMV) 35S promoter. The immunoprecipitated proteins from the crude extracts of the *CYC U4;1-FLAG* plants with anti-FLAG gel were quantified with the CDKA, a cyclin-dependent kinase in the G1-to-S and G2-to-M transitions (Nowack et al., 2012) (Figure 4F). The immunoprecipitated CYC U4;1/CDKA complex from the *CYC U4;1* transgenic plants without bikinin (a specific inhibitor of GSK3-like kinases) treatment had higher kinase activity (assayed using histone H1 as a substrate) than that from plants treated with 30 μM bikinin (De Rybel et al., 2009) (Figure 4F).

To explore the effect of the CYC U4;1 phosphorylation by BIN2 on cell-cycle regulation, we generated BY-2 suspension cell lines transformed with *35S::CYC U4;1*, *pAtBIN2::AtBIN2*, and *35S::CYC U4;1-GFP/pAtBIN2::AtBIN2-FLAG* respectively. DNA content analysis indicated that the *35S::CYC U4;1* BY-2 cells contained more 4C DNA than the WT. Interestingly, the *35S::CYC U4;1/pAtBIN2::AtBIN2* cell lines had higher 4C DNA content than the corresponding single transgenic lines (Figure 4G), which suggests that CYC U4;1 phosphorylated by BIN2 kinase can enhance its activity to promote 4C DNA content. Furthermore, we generated the transgenic rice with the mutant gene *CYC U4;1*^{T105AS106AT109A} to determine the function of phosphorylation in vivo. The results indicated that the enlarged leaf angle of transgenic rice is positively related with the CYC U4;1^{T105AS106AT109A} protein level (Figures 4H and 4I). The *pCYC U4::CYC 4;1*^{T105AS106AT109A} rice had more expanded m1 parenchyma cells and had one to two layers of m2 sclerenchyma cells (Figures 4J and 4K), which is similar to the *RNAi-CYC U4;1* lines. These results suggest that the function of the mutant CYC U4;1^{T105AS106AT109A} protein is weaker than the WT CYC U4;1 protein.

These data support the regulation mechanism of leaf erectness by BRs. When BR signaling is attenuated in LJs, the active BIN2 can inhibit BES1 activity to release the inhibitory effect on *CYC U4;1* transcription, and the active BIN2 can also phosphorylate CYC U4;1 to enhance the activity of CYC U4;1/CDKA complex, which accelerate abaxial sclerenchyma cell proliferation, resulting in erect leaves (Figure 4L). In contrast, the enhanced BR signaling is accompanied by the adaxial cell expansion and the decreased abaxial sclerenchyma cell number to enhance lamina inclination (Figure 4M). Although it is reported that cyclin gene expression can be modulated by plant growth factors, such

Figure 4. BIN2 Interacts with, Phosphorylates, and Stimulates CYC U4;1 Activity

(A) Interaction between CYC U4;1 and BIN2 in yeast two-hybrid assays.

(B) Interaction between CYC U4;1-GST and AtBIN2-HIS in vitro.

(C) Interactions of AtBIN2 with CYC U4;1 in BiFC assays. Scale bars represent 0.1 mm.

(D) AtBIN2 phosphorylates CYC U4;1 in vitro. The upper shows autoradiography, and the bottom shows Coommassi Blue staining.

(E) The phosphorylation of the various mutant proteins of CYC U4;1 by BIN2 in vitro.

(F) Kinase assays of the immunoprecipitated CYC U4;1/CDKA;1 complexes on histone H1.

(G) The cell percentage of 4C ploidy distribution of the indicated transgenic tobacco BY-2 suspension cell lines.

(H) The phenotypes of the transgenic lines with *CYC U4;1*^{T105AS106AT109A}. The L1–L6 indicated the independent T1 transgenic lines.

(I) The protein levels of CYC U4;1-FLAG in the transgenic rice (shown in Figure 4H).

(J) Cross-sections of the second LJs of Ni and the *CYC U4;1*^{T105AS106AT109A} line. The black arrowheads indicate sclerenchyma cells in m2 region.

(K) The number of sclerenchyma cell layers in the m2 regions (shown in Figure 4J).

Error bars are SD (n = 10). Student's t test: ***$p < 0.001$.

(L and M) The model to illustrate how BR signaling regulates rice leaf erectness through CYC U4;1. (L) Without BR, the active BIN2 can phosphorylate BES1 to release the transcriptional inhibition of *CYC U4;1* by BES1. In the mean time, the active BIN2 kinase can also phosphorylate CYC U4;1 to activate the CYC U4;1/CDKA complex. Both together enhance abaxial sclerenchyma cell division and leaf erectness. (M) With the enhanced BR signaling, the transcription and phosphorylation of CYC U4;1 are inhibited, which leads to the reduced abaxial sclerenchyma cell proliferation and the enlarged adaxial cells in m1. These together increased lamina inclination. The gray color indicates inactive states and the black color indicates active states.

See also Figure S3.

as cytokinins, auxins, BRs, sucrose, or gibberellins, the regulation of cyclin activation is barely reported (Inzé and De Veylder, 2006). Thus, this study provides significant insight into the direct regulation of cyclin activation by BR signaling. In addition, the cellular and molecular mechanisms of BR controlling leaf erectness through CYC U4;1 provide a way to improve the yield of the major cereal crop.

The BR-regulated plant developmental processes are mediated through cell division, cell expansion, and cell differentiation, but most of these studies suggested that BR signaling mainly promotes cell division and tissue-specific cell differentiation during the root and leaf development (Hacham et al., 2011; González-García et al., 2011; Miyazawa et al., 2003; Zhiponova et al., 2013). Our study provided strong evidence to support that BR signaling inhibits abaxial sclerenchyma cell division in LJs by regulating expression and activity of a tissue-specific cyclin *CYC U4;1*. A large number of cyclin genes are present in plants, and their tissue-specific regulation by various signaling may play important role in regulating diverse developmental processes (Wang et al., 2004).

Some BR signaling components in rice had been characterized from the orthologs of the known BR signaling components in *Arabidopsis* (Zhang et al., 2014), and the function of *AtBIN2/OsGSK2* and *AtBES1/OsBZR1* was demonstrated to be conserved in the rice and *Arabidopsis* (Koh et al., 2007; Bai et al., 2007; Tong et al., 2012). In this study, the LJ phenotypes of the *pDWF4::Atbin2-1* and the *pDWF4::Atbes1-D* transgenic rice are very similar to that of *pOsGSK2::OsGSK2-1* (rice ortholog of AtBIN2) and *pOsBZR1::Osbzr1-D* (rice ortholog of AtBES1) transgenic rice, respectively (Figure S3C). In addition, we found that the OsGSK2 can interact with CYC U4 in yeast (Figure S3D), and the phenotype of the *CYC U4;1*^{T105AS106AT109A} transgenic rice supports that the phosphorylation sites by AtBIN2 are conserved in rice. Therefore, it is very likely that the regulation of *AtBIN2* and *AtBES1* on *CYC U4;1* should be conserved in rice.

In this study, we found that the *CYC U4;1-RNAi* rice exhibited the large leaf angle with the increased adaxial cell size in LJs, which is similar to BR treatment, and also that the phenotype of *CYC U4;1* transgenic rice is similar to the BR-insensitive transgenic line *pDWF4::bin2-1*. We speculated that CYC U4;1 might also regulate the BR signaling, which is similar to the mechanism of the Wnt signaling regulated by cyclin Y (Davidson and Niehrs, 2010). Therefore, it is still needed to explore the mechanisms how *CYC U4;1* regulates other aspects of rice development, such as the dwarfism caused by the *CYC U4;1* overexpression.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions

The rice materials include the BR-deficient mutant *d2-2* (Hong et al., 2003), the BR-insensitive rice mutant *d61-1* (Yamamuro et al., 2000), and their WT background T65 (*Oryza sativa* L. cv. Taichung 65). The BR-insensitive mutant *bin2-1* is a gain-of-function mutation (E263K) of *BIN2* (Li et al., 2001). Ni was used as the host for all the rice transformation. The *Arabidopsis* ecotype Col-0 was used for transformation. Generation of transgenic lines was described in the Supplemental Experimental Procedures.

Microarray Experiments

Total RNA was extracted from the leaf blades and the LJs of 3-week-old Ni seedlings for microarray analysis. The detailed procedure was described in the Supplemental Experimental Procedures.

Paraffin Sections

Rice LJs were fixed in FAA solution (50% ethanol, 5% acetic acid, and 10% formaldehyde in water), dehydrated in a graded butanol/ethanol series, and embedded in paraffin (Leica) (Ye et al., 2010). Microtome sections (7 μm) were applied onto poly-L-lysine-coated slides. The sections were de-paraffinized in xylene, dehydrated through a graded ethanol series, and stained with Safranin O/Fast Green staining. The sections were observed under a light microscope (Olympus, Japan).

In Vitro Kinase Assay

The ORFs of *AtBIN2*, *CYC U4;1* were cloned into pGEX4T-1 vector, respectively, and the recombinant fusion proteins were expressed in *E. coli* (BL21) and purified with Glutathione Resin. The detailed procedure was described in the Supplemental Experimental Procedures.

Synchronization of Cell Culture and Flow Cytometric Analysis

Synchronization of rice calli and BY-2 suspension cells were performed as described previously (Qi et al., 2012; Kumagai-Sano et al., 2006). The detailed procedure was described in the Supplemental Experimental Procedures.

Kinase Assays for H1

Total protein was extracted from the *CYC U4;1-OE* seedlings (*Arabidopsis*) grown on the 1/2MS medium with or without 30 μM Bikinin, and the protein extracts were immunoprecipitated with the FLAG affinity gel; the immunoprecipitates (IPs) were examined by immunoblotting with the anti-CDKA;1 antibody. The IPs were subjected to kinase assays using histone H1 as the substrate (Cockcroft et al., 2000). Because BIN2 can phosphorylate the H1, we added the 50 μM Bikinin in the both kinase assay incubation of the IPs and H1 to inhibit the activity of the co-immunoprecipitated BIN2.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.05.019.

ACKNOWLEDGMENTS

We thank Makoto Matsuoka (Nagoya University) for providing the rice mutants of *d61-1* and *d2-2*; D. Luo, J. Yang, and X. Liu for assistance on in situ hybridization; and H. Saiyin for assistance on preparing paraffin sections. This work was supported by grant 2012CB114304 of the Ministry of Science and Technology of China (to X.W. and S.S.) and grants 91317302 and 31271300 (to X.W.), 31271684 (to S.S.), and 30800595 (to D.C.) of the National Natural Science Foundation of China.

Received: January 10, 2015
Revised: April 6, 2015
Accepted: May 20, 2015
Published: July 16, 2015

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