

Histone deacetylase HDA6 enhances brassinosteroid signaling by inhibiting the BIN2 kinase

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Glycogen synthase kinase 3 (GSK3)-like kinases play important roles in brassinosteroid (BR), abscisic acid, and auxin signaling to regulate many aspects of plant development and stress responses. The *Arabidopsis thaliana* GSK3-like kinase BR-INSENSITIVE 2 (BIN2) acts as a key negative regulator in the BR signaling pathway, but the mechanisms regulating BIN2 function remain unclear. Here we report that the histone deacetylase HDA6 can interact with and deacetylate BIN2 to repress its kinase activity. The *hda6* mutant showed a BR-repressed phenotype in the dark and was less sensitive to BR biosynthesis inhibitors. Genetic analysis indicated that HDA6 regulates BR signaling through BIN2. Furthermore, we identified K189 of BIN2 as an acetylated site, which can be deacetylated by HDA6 to influence BIN2 activity. Glucose can affect the acetylation level of BIN2 in plants, indicating a connection to cellular energy status. These findings provide significant insights into the regulation of GSK3-like kinases in plant growth and development.

HDA6 | BIN2 | brassinosteroid signaling | deacetylation | development

Brasinosteroids (BRs), plant-specific steroid hormones, play important roles in development and stress responses (1). In the *Arabidopsis thaliana* BR signaling pathway, perception of BRs through the plasma membrane receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1) (2) and coreceptor BRI1-ASSOCIATED KINASE 1 (BAK1) (3) results in release of the negative regulator BRI1 KINASE INHIBITOR 1 (BKI1) (4) from the plasma membrane. BRI1 can phosphorylate downstream kinases, such as BR SIGNALING KINASES (BSKs) and CONSTITUTIVE DIFFERENTIAL GROWTH 1 (CDG1) (5). These kinases regulate the Kelch-repeat phosphatase BRI1 SUPPRESSOR 1 (BSU1), which may dephosphorylate BR-INSENSITIVE 2 (BIN2) to inhibit its kinase activity (6) and lead to the accumulation of the dephosphorylated BRI1-EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLE RESISTANT 1 (BZR1) (7–9). As the active form, dephosphorylated BES1 regulates BR-responsive gene expression, and the longer form, BES1-L, has stronger activity to promote BR signaling (10).

In the BR signaling pathway, the negative regulator BIN2 is one of 10 glycogen synthase kinase 3 (GSK3)-like kinases (GSK3s) encoded in the *A. thaliana* genome. BIN2 plays key roles in linking multiple signaling pathways to regulate many aspects of plant development (11–14). The gain-of-function mutant *bin2-1* showed a BR-deficient phenotype, including dark green dwarf stature, epinastic round leaves, delayed flowering and senescence, male infertility, activated stress responses, and hypersensitivity to abscisic acid (ABA) (11, 15–17). BIN2 can also regulate auxin signaling by phosphorylating ARF2 to repress its DNA binding activity (18). In addition, BIN2 can enhance ABA signaling by phosphorylating SnRK2.2 and SnRK2.3 to promote their kinase activity (19). BIN2 and its homologs can phosphorylate EGL3 and TTG to promote root hair formation (20), phosphorylate PIF4 to prepare it for degradation in the regulation of hypocotyl elongation (21), and phosphorylate YDA to inhibit its phosphorylation of MAKK4 in the regulation of stomatal development (22).

The regulatory mechanisms by which BRs and other signals regulate BIN2 activity and stability remain unclear. Although in BR signaling it is reported that BIN2 can be regulated by

dephosphorylation of the Y200 residue (6), the functions of many other phosphorylation sites remain unknown. In addition, overexpression of Y200 phosphatase *BSU1* only partially rescued the BRI1 mutant *bri1-116* (6), indicating that other mechanisms may also be involved in the regulation of BIN2 activity. Furthermore, BRs can regulate BIN2 degradation mediated by the 26S proteasome by unknown mechanisms (23). Besides BRs, other signals can also regulate BIN2 activity. For example, BIN2 can be recruited to the plasma membrane by OCTOPUS, a polar-localized plasma membrane-associated protein functioning in phloem differentiation; this relocalization inhibits the activity of BIN2 in the nucleus (24).

In mammals, GSK3-like kinases can be regulated by multiple modifications. Most substrates of GSK3s must be phosphorylated by other kinases to be recognized by the phospho-pocket of GSK3, which includes the R96, R180, and K205 amino acids (25). However, if the N-terminal Ser-9/21 residues of GSK3s are phosphorylated, the N terminus can bind to the pocket to inhibit GSK3 activity (26). Furthermore, phosphorylation of Tyr-279-GSK3 α and Tyr-216-GSK3 β is important for full activity of GSK3 α and - β (26). Other sites on GSK3 β , such as Ser-48, -389, and -390, can also be phosphorylated, but their functions remain unknown (27). Besides phosphorylation, other modifications also regulate GSK3 activities, such as cleavage by calpain and by matrix metalloproteinase 2 leading to activated GSK3 fragments, mono-ADP ribosylation leading to GSK3 inhibition, and citrullination leading to nuclear localization of GSK3 (27, 28). Recent work reported that mammalian GSK3 β can be acetylated, and depletion of the deacetylase led to reduced phosphorylation and enhanced kinase activity of GSK3 β (29). Lysine acetylation is an important posttranslational modification that neutralizes the positively charged lysine residue. Acetylation of many nonhistone proteins plays an important role in regulating biological processes such as the cell cycle, splicing, nuclear transport, metabolism, aging, and several diseases (30–34).

In this study, to explore the regulatory mechanisms of BIN2 in *Arabidopsis*, we used a mass spectrometry approach and *BIN2-FLAG* transgenic *Arabidopsis* to identify a number of BIN2-interacting

Significance

The brassinosteroid (BR) signaling pathway has important functions in plant development, but the mechanisms that control its key regulator BR-INSENSITIVE 2 (BIN2) remain poorly understood. We found that the histone deacetylase HDA6 can interact with and deacetylate BIN2 to inhibit its activity, providing significant insights into the repression of BIN2 and the function of the histone deacetylase in modifying nonhistone proteins. We also found that this repression may be related to energy status in plants.

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proteins, including the deacetylase HDA6. We used various genetic and biochemical approaches to confirm that HDA6 can interact with BIN2. Interestingly, we did not find that BIN2 phosphorylates HDA6; rather, we found that HDA6 can deacetylate BIN2 to repress its kinase activity and enhance BR signaling in *Arabidopsis*. The BIN2 acetylation site is in the conserved phospho-binding pocket of GSK3-like kinases and plays an essential role in regulating BIN2 kinase activity, likely under conditions of energy limitation.

Results

HDA6 Interacts with the BIN2 Kinase. BIN2 functions as a key negative regulator in the BR signaling pathway and plays an important role in linking many other signaling pathways (11, 19, 35). To identify substrates or regulators of BIN2 kinase, we immunoprecipitated BIN2-FLAG from 35S::BIN2-FLAG transgenic *Arabidopsis* plants. We then used mass spectrometry to identify a number of potential BIN2-interacting proteins (19), including HDA6. The expression of HDA6 and subcellular localization of HDA6 are very similar to those of BIN2 (36); also, the *hda6* knockout mutants and *bin2-1* gain-of-function mutant have similar phenotypes, such as late flowering (35, 37), sensitivity to ABA (16, 38), delayed senescence (35, 37), and more root hairs (20, 39). Therefore, we focused on HDA6 for further studies. To confirm the interaction of BIN2 with HDA6, we first used bimolecular fluorescence complementation (BiFC) in the pavement cells of *Nicotiana benthamiana* and found that BIN2 and HDA6 can interact in the cytoplasm and nucleus (Fig. 1A). Second, we purified the recombinant proteins HDA6-GST and BIN2-His and the kinase-dead BIN2^{K69R}-His from *Escherichia coli* to conduct GST-pull down assays. We observed that HDA6-GST can directly interact with both forms of BIN2-His (Fig. 1B). Third, we created transgenic plants expressing HDA6-YFP and crossed them with BIN2-FLAG plants to perform coimmunoprecipitation (co-IP), which showed that HDA6-YFP can interact with BIN2-FLAG in planta (Fig. 1C). Taken together, these results suggested that BIN2 can interact with HDA6 in vitro and in vivo.

HDA6 Can Deacetylate BIN2. To investigate the biochemical outcomes of the HDA6-BIN2 interaction, we first conducted in vitro kinase assays to test whether BIN2 can phosphorylate HDA6, because some HDACs in mammals, such as HDAC4, HDAC5, HDAC7, and HDAC9, can be phosphorylated to regulate their subcellular localization and shuttling between the nucleus and cytoplasm (40). However, we did not observe significant phosphorylation of HDA6 by BIN2 (Fig. S1A).

Mammalian GSK3 β , a close homolog of BIN2, can be acetylated, and the deacetylation of GSK3 β can promote dephosphorylation of Ser9 to activate GSK3 β kinase activity (29). Therefore, we speculated that BIN2 may be modified by acetylation, and that HDA6 may deacetylate BIN2 to regulate its function. Thus, we purified BIN2-His recombinant protein from *E. coli* cultured with or without the deacetylation inhibitors trichostatin A (TSA) and β -nicotinamide (NAM). We detected the acetylation status of BIN2-His with an anti-acetyl-lysine antibody. We found that the acetylation level of BIN2-His from the cultures treated with TSA and NAM was higher than that of BIN2-His from the untreated culture (Fig. 1D), indicating that BIN2-His can be acetylated in *E. coli*. To test whether HDA6 can deacetylate BIN2 in *E. coli*, we transformed the *E. coli* strain containing the BIN2-His construct with the HDA6-GST construct. We found that the acetylation level of BIN2-His significantly decreased in the cells producing BIN2-His and HDA6-GST, compared with cells producing only BIN2-His (Fig. 1E and F). To test whether HDA6 can deacetylate BIN2 in planta, we detected the acetylation level of BIN2-FLAG from transgenic plants harboring BIN2-FLAG alone or both BIN2-FLAG and HDA6-YFP, which we produced by crossing the HDA6-YFP line with BIN2-FLAG plants. We found that the acetylation level of BIN2-FLAG from the HDA6-YFP BIN2-FLAG plants was lower than that of BIN2-FLAG from the plants expressing only BIN2-FLAG (Fig. 1G). These experiments demonstrated that HDA6 can deacetylate BIN2 both in vitro and in vivo.

HDA6 Plays a Positive Role in BR Signaling. To explore the function of BIN2 deacetylation by HDA6, we conducted a series of biochemical and genetic experiments to test the effect of HDA6 on BIN2 function and the BR signaling outputs. First, we checked whether the HDA6 knockout mutant or the HDA6 overexpression line showed altered BR-related phenotypes. Using the hypocotyl elongation assay, we discovered that the *axe1-5* mutant, which has an HDA6 loss-of-function mutation (41), had shorter hypocotyls compared with wild-type Col-0, and that the HDA6-YFP plants had longer hypocotyls (Fig. 2A and B). We further measured the transcript levels of the BR-responsive genes *CPD* (CONSTITUTIVE PHOTOMORPHOGENIC DWARF) and *DWF4* (DWARF4) by quantitative real-time RT-PCR (qRT-PCR) and found that their expression was up-regulated in the *axe1-5* mutant and down-regulated in the HDA6-YFP line, compared with the wild-type (Fig. 2C). We also found that more dephosphorylated BES1 accumulated in the HDA6-YFP plants, similar to the *BRI1-GFP* overexpression line (Fig. 2E). Finally, we treated the HDA6-YFP

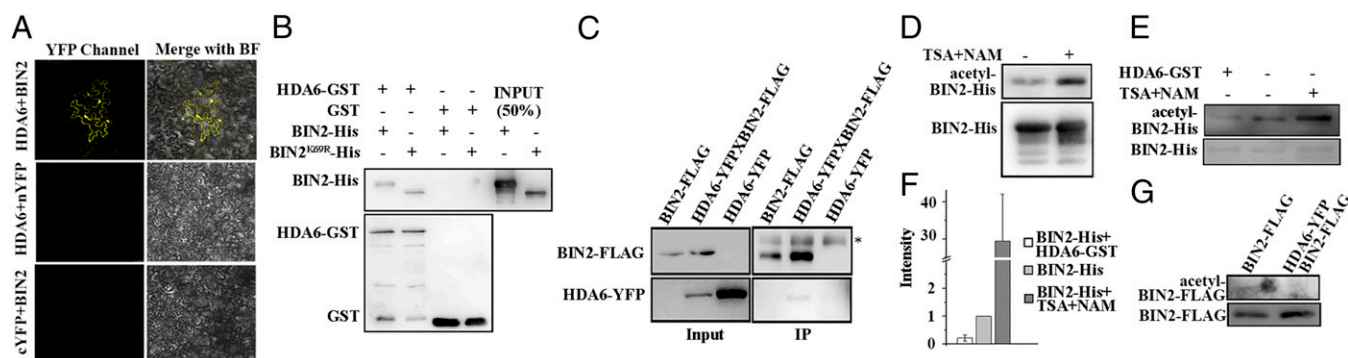


Fig. 1. HDA6 interacts with and deacetylates BIN2. (A) HDA6 interacts with BIN2 in BiFC assays. The nYFP-BIN2 or nYFP and HDA6-cYFP or cYFP constructs were cotransformed into the pavement cells of *N. benthamiana*. BF, bright field; magnification, 20 \times . (B) HDA6-GST interacts with BIN2-His in GST-pull down assays. (C) HDA6-YFP coimmunoprecipitated by BIN2-FLAG in plants. The asterisk indicates a nonspecific band. (D) The recombinant BIN2-His protein purified from *E. coli* was acetylated. The *E. coli* strain containing the BIN2-His construct was cultured in LB medium with or without 5 nM TSA and 5 mM NAM. The acetylation level of the purified BIN2-His was detected with an anti-acetyl-Lys antibody. (E) BIN2-His can be deacetylated by HDA6 in vitro. The acetylation status of BIN2-His was determined with an anti-acetyl-Lys antibody. (F) Statistical analysis of the relative acetylation level of BIN2-His. (G) BIN2-FLAG is acetylated in plants. BIN2-FLAG and HDA6-YFP BIN2-FLAG (crossed from BIN2-FLAG and HDA6-YFP) plants were grown on soil for 2 wk. The acetylation level of the immunoprecipitated BIN2-FLAG protein was detected with an antiacetyl-Lys antibody. Error bars represent SE.

seedlings with the BR synthesis inhibitors brassinazole 220 (BRZ220) or propiconazole and found that, like the *BRI1-GFP* overexpression line, the *HDA6-YFP* plants showed less growth inhibition than the wild-type plants (Fig. 2D). These data suggested that HDA6 plays a positive role in the BR signaling pathway.

HDA6 Regulates BR Signaling Through GSK3s. To test whether the regulation of BR signaling by HDA6 acts through BIN2 and its homologs, we first used the hypocotyl elongation assay to examine the effect of the deacetylation inhibitor TSA on *BRI1-GFP*, *bin2-3 bil1 bil2*, and *BES1-D* plants. We found that the *BRI1-GFP* line, which overexpresses the BR receptor BRI1, was more sensitive to TSA compared with the wild-type. By contrast, the *bin2-3 bil1 bil2* triple mutant, which knocks out BIN2 and its close homologs BIL1 and BIL2, and the *BES1-D* line, which is a gain-of-function mutant of BR transcription factors, were less sensitive to TSA in hypocotyl elongation compared with their wild-type counterparts (Fig. 3A–C), indicating that HDA6 may function downstream of the BR receptor BRI1 and upstream or through BIN2 to modulate BR signaling.

To further test whether the HDA6-enhanced BR signaling was mediated by BIN2 or other components in the BR signaling pathway, we conducted genetic analysis, generating double or multiple mutants of HDA6-related and BR-related mutants and overexpression lines. We first generated double mutants of *axe1-5* or *HDA6-YFP* with the BR receptor mutant *bri1-301*. We found that the *axe1-5 bri1-301* seedlings were smaller than the *bri1-301* seedlings, and that the *HDA6-YFP bri1-301* seedlings were larger than the *bri1-301* seedlings (Fig. 3D). The expression of the BR-responsive genes *CPD* and *DWF4* was enhanced in the *axe1-5 bri1-301* double mutants but repressed in the *HDA6-YFP bri1-301* double mutants, compared with *bri1-301* (Fig. 3F). Because *bri1-301* is a weak allele of *bri1*, to mimic a null allele of *bri1*, we grew *bri1-301*, *axe1-5 bri1-301*, and *HDA6-YFP bri1-301* on medium containing a BR biosynthetic inhibitor, BRZ220, and measured the hypocotyl length of the dark-grown seedlings. We found that the hypocotyl length of *axe1-5 bri1-301* is still shorter than that of *bri1-301*, and that the hypocotyl length of *HDA6-YFP bri1-301* is still longer than that of *bri1-301* (Fig. S1B). These results support that HDA6 acts downstream of BRI1 to modulate BR signaling. We further generated the *axe1-5 bin2-1* double mutant and the *HDA6-YFP bin2-1* line, and observed that the *axe1-5 bin2-1* seedlings were smaller but the *HDA6-YFP bin2-1*

seedlings were larger than *bin2-1* (Fig. 3E). Similarly, the expression of *CPD* and *DWF4* in the *axe1-5 bin2-1* plants was enhanced, compared with that in *bin2-1*, but was suppressed in *HDA6-YFP bin2-1* plants (Fig. 3G).

Because *bin2-1* is a gain-of-function mutant of *BIN2*, the above results indicate that HDA6 may function downstream of *BIN2*, or HDA6 may regulate the activity of the mutant *BIN2* protein. To distinguish these possibilities, we further generated *HDA6-RNAi* lines in *Ws-2* and crossed one line to the *bin2-3 bil1 bil2* triple-knockout background to gain *HDA6-RNAi bin2-3 bil1 bil2*. We found that the *HDA6-RNAi bin2-3 bil1 bil2* plants showed similar phenotypes in both seedling growth and *CPD* and *DWF4* expression to *bin2-3 bil1 bil2*, compared with the *HDA6-RNAi* plants (Fig. 3H and I), suggesting that without *BIN2* and its homologs the effect of HDA6 on BR signaling was reduced. We also found that the *HDA6-RNAi bin2-3 bil1 bil2* seedlings were less sensitive to the BR biosynthetic inhibitor BRZ in hypocotyl elongation inhibition than the *HDA6-RNAi* seedlings but similar to *bin2-3 bil1 bil2* plants in the dark (Fig. 3J), suggesting that HDA6 regulates BR signaling largely through *BIN2* and its homologs in the dark. Furthermore, we found that HDA6 can still deacetylate the *bin2-1* protein (Fig. S1C). The *bin2-3 bil1 bil2* mutant had lower *HDA6* transcript levels than the wild-type *Ws-2* (Fig. 3I), implying feedback inhibition of *HDA6* expression by the GSK3-like kinase family. Taken together, these observations demonstrated that HDA6 enhances BR signaling through *BIN2* and other GSK3-like kinases.

HDA6 Deacetylates the K189 Residue of BIN2 to Inhibit BIN2 Kinase Activity. To investigate how HDA6 enhanced BR signaling, we tested whether *BIN2* that had been deacetylated by HDA6 had altered kinase activity. We used the phosphorylation status of *BES1* as a biochemical marker to evaluate whether HDA6 influences the kinase activity of *BIN2* (4, 42). Therefore, we conducted *BES1*-MBP phosphorylation assays using *BIN2*-His deacetylated by HDA6, normal *BIN2*-His, and hyperacetylated *BIN2*-His produced in *E. coli* treated with TSA and NAM. We found that the acetylated *BIN2*-His had the highest kinase activity, based on its ability to phosphorylate *BES1*-MBP, and that the deacetylated *BIN2*-His had the lowest kinase activity (Fig. 4A). Therefore, we concluded that HDA6 can deacetylate *BIN2* to inhibit its kinase activity.

To understand which acetylated/deacetylated residues of *BIN2* are important for its kinase activity, we used mass spectrometry

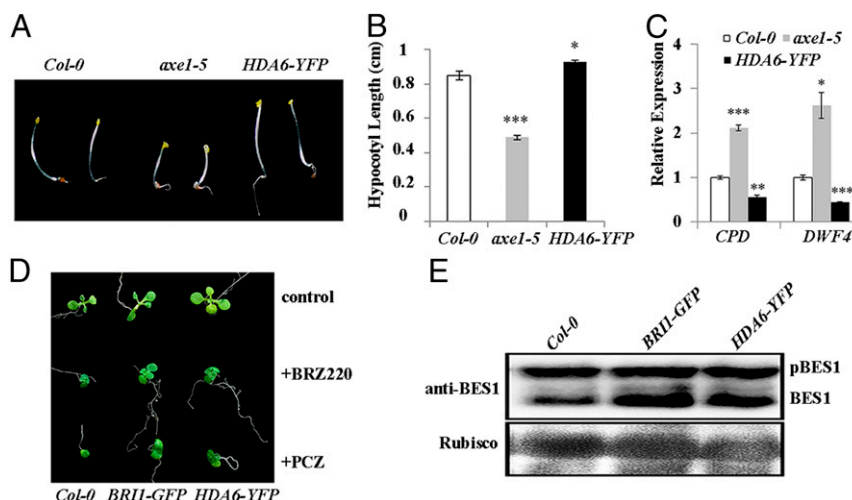


Fig. 2. HDA6 positively regulates BR signaling. (A) Dark-grown hypocotyl phenotypes of Col-0, the *axe1-5* mutant, and the *HDA6-YFP* overexpression line. (B) Hypocotyl lengths of the dark-grown Col-0 ($n = 25$), *axe1-5* ($n = 25$), and *HDA6-YFP* ($n = 25$). (C) Expression levels of the BR-responsive genes *CPD* and *DWF4*. Their expression level in Col-0 was defined as "1." (D) The seedling phenotype of Col-0, *BRI1-GFP*, and *HDA6-YFP* plants grown on medium containing 1 μ M BR synthetic inhibitor BRZ220 and propiconazole (PCZ). (E) Phosphorylation status of *BES1* in Col-0, *BRI1-GFP*, and *HDA6-YFP* plants. *BES1* was detected by an anti-*BES1* antibody. Error bars represent SE. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

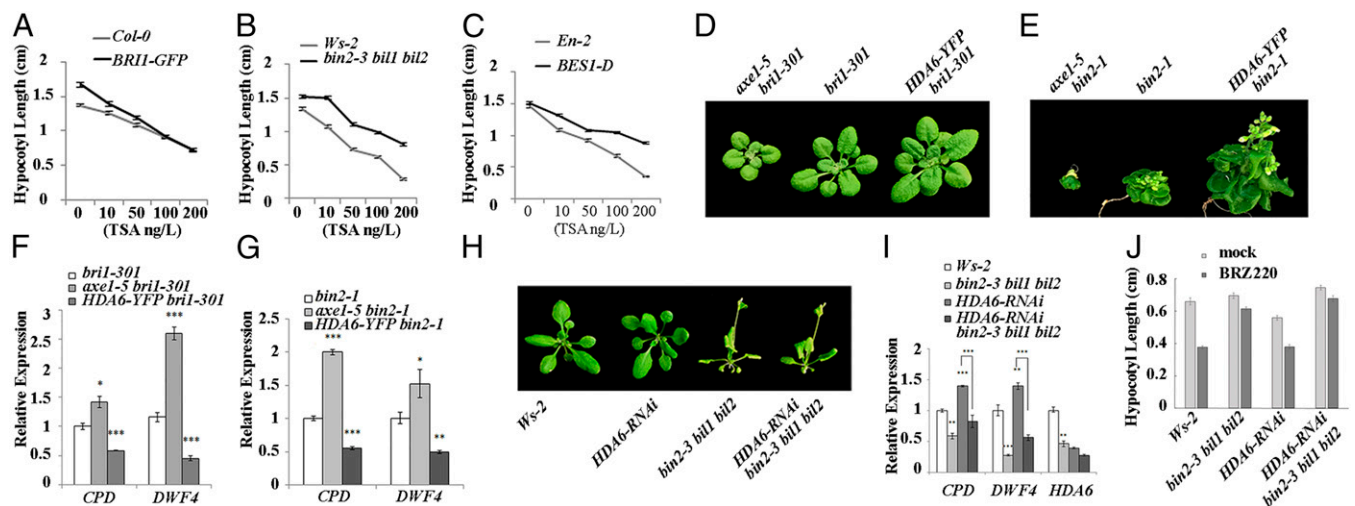


Fig. 3. HDA6 regulates BR signaling through BIN2 and its homologs. (A–C) Hypocotyl length of Col-0 and the *BR11-GFP* line (A), *Ws-2* and *bin2-3 bil1 bil2* plants (B), and *En-2* and *bes1-D* plants (C). Plants were grown in the dark on medium containing different concentrations of TSA. (D) *HDA6* partially rescued the dwarf phenotype of *bri1-301*. (E) *HDA6* can partially suppress the dwarf phenotype of *bin2-1*. (F) Expression levels of the BR-responsive genes *CPD* and *DWF4* in *bri1-301*, *axe1-5 bri1-301*, and *HDA6-YFP bri1-301*. Their expression in *bri1-301* was defined as 1. (G) Expression levels of the BR-responsive genes *CPD* and *DWF4* in *bin2-1*, *axe1-5 bin2-1*, and *HDA6-YFP bin2-1*. Their expression in *bin2-1* was defined as 1. (H) *HDA6-RNAi* cannot suppress the seedling phenotype of the *bin2-3 bil1 bil2* triple mutant. (I) Expression levels of *CPD*, *DWF4*, and *HDA6* in *bin2-3 bil1 bil2* and *HDA6-RNAi bin2-3 bil1 bil2* plants. Their expression level in *Ws-2* was defined as 1. (J) *HDA6-RNAi* hardly affects hypocotyl elongation in the *bin2-3 bil1 bil2* background growing on medium containing 1 μM BRZ220 in the dark. Error bars represent SE. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

to identify its potential acetylation sites using recombinant BIN2-His protein produced in *E. coli* treated with TSA and NAM. We found three potential acetylation sites, K5, K189, and K347, of BIN2 (Fig. S2). We mutated each K to R and produced these BIN2 mutant proteins in *E. coli* treated with TSA and NAM. The acetylation level of BIN2^{K189R}-His substantially decreased compared with wild-type BIN2 (Fig. 4B), and BIN2^{K189R}-His could not be deacetylated by HDA6 in vitro (Fig. 4C). Moreover, the kinase activity of BIN2^{K189R}-His was dramatically reduced, based on BIN2^{K189R}-His phosphorylation of BES1-MBP (Fig. 4D), indicating that K189 is a crucial site for BIN2 acetylation and activity. BIN2^{K189R}-His also had decreased autophosphorylation activity (Fig. 4B), consistent with its decreased kinase activity.

To further investigate whether the K189 site can be acetylated and has important functions in plants, we detected the acetylation level of BIN2-FLAG and BIN2^{K189R}-FLAG with and without TSA treatments in the transgenic plants. The results showed that the acetylation level of BIN2^{K189R}-FLAG was substantially lower than that of BIN2-FLAG, and TSA treatment did not significantly enhance the acetylation level of BIN2^{K189R}-FLAG (Fig. 4E and F), indicating that K189 is an important acetylation site in vivo. Previous studies showed that overexpression of the wild-type BIN2 in *Arabidopsis* did not lead to a strong BR-deficient phenotype, but that overexpression of the *bin2-1* (BIN2^{E263K}) protein can cause a severe BR-deficient phenotype, for unknown reasons (36, 43). To investigate whether BIN2^{K189R} had altered activity in vivo, we made constructs to express *bin2-1*-FLAG and *bin2-1*^{K189R}-FLAG and transferred these constructs into *Ws-2*. We speculated that if the acetylation of K189 is important for BIN2 kinase activity, the K189R mutation should cause a phenotype different from the dwarf phenotype shown by plants expressing *bin2-1*-FLAG. Indeed, our results showed that the *bin2-1*^{K189R}-FLAG plants were similar to the wild-type, rather than the *bin2-1*-FLAG overexpression lines (Fig. 4G). In addition, the BR marker genes *CPD* and *DWF4* and the BES1 phosphorylation status in the *bin2-1*^{K189R}-FLAG plants were similar to that in the *BIN2-FLAG* and *BIN2*^{K189R}-FLAG plants rather than that in the *bin2-1*-FLAG plants (Fig. 4H and I), indicating that *bin2-1*^{K189R} had reduced kinase activity. Furthermore, in vitro acetylation assays indicated that *bin2-1*^{K189R}-His had lower acetylation levels (Fig. 4J) and lower kinase activity to phosphorylate BES1-MBP (Fig. 4K), compared with *bin2-1*-His. Therefore,

we concluded that the acetylation site K189 of BIN2 is essential for its kinase activity in vivo.

Discussion

This study has provided several lines of evidence to support the idea that HDA6 plays a positive role in the BR signaling pathway. First, the *HDA6* loss-of-function mutant *axe1-5* and the *HDA6* overexpression line had altered hypocotyl elongation phenotypes in the dark and altered expression of BR marker genes. Second, the triple knockout of *bin2-3 bil1 bil2* and the *bes1-D* gain-of-function mutant showed reduced sensitivity to the deacetylase inhibitor TSA in hypocotyl elongation. Third, *axe1-5* enhanced the dwarf phenotype of *bri1-301*, and the *HDA6* overexpression line partially rescued its dwarf phenotype. Fourth, *HDA6* can directly interact with and deacetylate BIN2 to repress its kinase activity.

We provide genetic and biochemical data to demonstrate that the deacetylation of the K189 residue of BIN2 by HDA6 is important to regulate BIN2 activity in *Arabidopsis*. The acetylated K189 of BIN2 is equivalent to K205 of GSK3β in mammals (11), which is one of three positively charged amino acids (R96, R180, and K205) constituting the binding pocket for a phosphoserine residue in GSK3β (25). BIN2 K189 is a novel acetylation site found in plants. Acetylation neutralizes this positively charged side chain of K189, likely to inhibit phospho-binding by BIN2. The K189R mutation nullified the dwarf phenotype of the *bin2-1*-FLAG transgene, suggesting that the acetylation of K189 is important for the kinase activity of BIN2. In many other kinases like CDK2 and ERK2, the residue equivalent to K189 is arginine rather than lysine (25); therefore, K189 acetylation may play a unique role in GSK3s. In animals, this pocket is occupied by the phosphorylated N terminus of GSK3β to take the place of the primed phosphorylated substrate to repress GSK3β activity (25). However, BIN2 has no priming phosphorylation or conserved N terminus to repress its activity in plants (23). Therefore, other phospho-residues may inhibit BIN2 activity by binding to the pocket after BIN2 deacetylation.

Protein acetylation, which plays a major role in metabolic regulation in mammalian systems, may also be important in regulating plant development under different energy conditions. Almost all of the important enzymes in several mammalian metabolic pathways, such as glycolysis, gluconeogenesis, the TCA cycle,

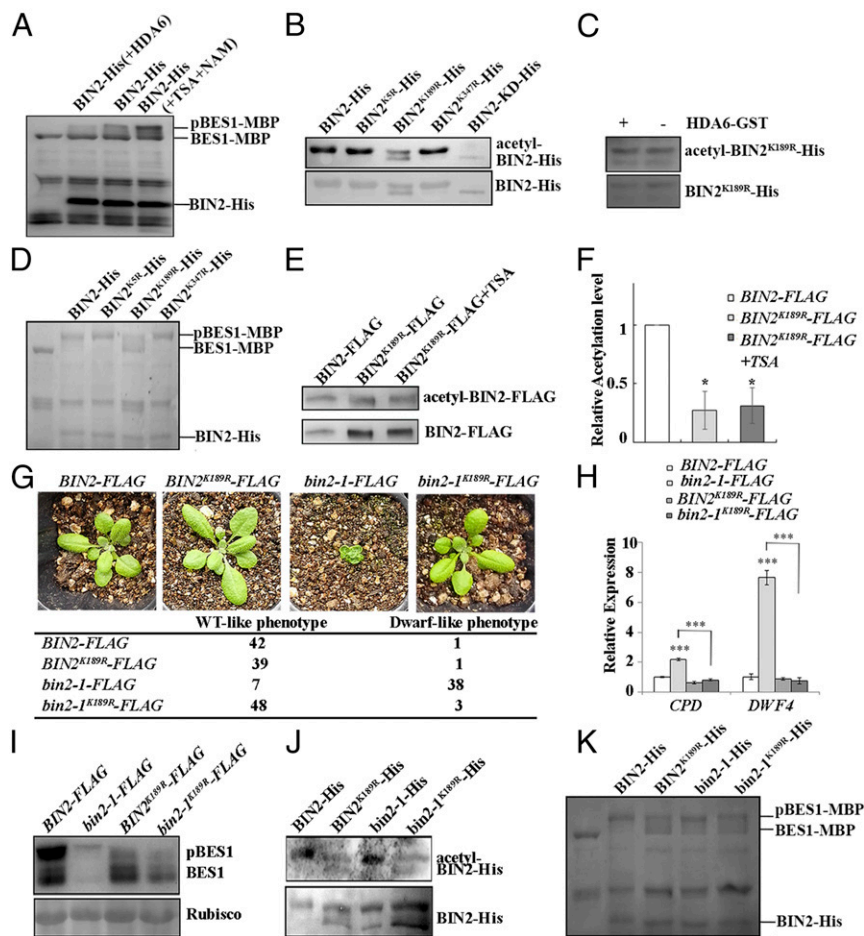


Fig. 4. HDA6 deacetylates BIN2 to inhibit its kinase activity. (A) Acetylated BIN2 has higher kinase activity to phosphorylate BES1-MBP. (B) Acetylation levels of the mutated BIN2 proteins on the predicted acetylation residues. *BIN2^{K189R}* showed reduced acetylation level and autophosphorylation activity compared with other forms of BIN2. *BIN2^{K69R}* showed unphosphorylated BIN2. (C) Additional HDA6 did not change the acetylation level of *BIN2^{K189R}*. (This acetylation band of *BIN2^{K189R}* may be caused by nonspecific recognition of the antibody or acetylation of BIN2 by other enzymes on other residues.) (D) BES1-MBP phosphorylation by BIN2, *BIN2^{K5R}*, *BIN2^{K189R}*, and *BIN2^{K347R}*. (E) Acetylation level of *BIN2-FLAG* and *BIN2^{K189R}-FLAG* from plants with or without TSA treatments. (F) Statistical analysis of the relative acetylation level of *BIN2-FLAG* and *BIN2^{K189R}-FLAG* from plants with or without TSA treatments. The acetylation level of *BIN2-FLAG* was defined as 1. (G) *bin2-1^{K189R}-FLAG* overexpression rescued the dwarf phenotype of *bin2-1-FLAG* overexpression. (H) Expression levels of the BR-responsive genes *CPD* and *DWF4* in *BIN2-FLAG*, *BIN2^{K189R}-FLAG*, *bin2-1-FLAG*, and *bin2-1^{K189R}-FLAG* plants. (I) BES1 phosphorylation status in *BIN2-FLAG*, *BIN2^{K189R}-FLAG*, *bin2-1-FLAG*, and *bin2-1^{K189R}-FLAG* plants detected by an anti-BES1 antibody. (J) Acetylation levels of *BIN2-His*, *BIN2^{K189R}-His*, *bin2-1-His*, and *bin2-1^{K189R}-His* proteins purified from *E. coli*. (K) Phosphorylation of BES1-MBP by *BIN2-His*, *BIN2^{K189R}-His*, *bin2-1-His*, and *bin2-1^{K189R}-His* protein. Error bars represent SE. * $P < 0.05$ and *** $P < 0.001$.

the urea cycle, fatty acid metabolism, and glycogen metabolism, can be acetylated (31). Animal GSK3s are closely related to energy responses. For example, GSK3s were named due to their role in phosphorylating glycogen synthase to inhibit glycogen synthesis, which is closely related to energy status in mammals (12). In addition, under starvation conditions, GSK3s phosphorylate TSC2 to inhibit the mTOR pathway, which promotes protein and lipid synthesis (44), and, under energy-rich conditions, GSK3s phosphorylate and inhibit AMPK, which inhibits protein and fatty acid synthesis (45). Therefore, GSK3s act as critical players to regulate many anabolic and catabolic pathways in mammals (45). To test whether energy affects the acetylation of BIN2, we added glucose and sucrose to the medium to observe any changes of the BIN2 acetylation level and plant growth. We found that the short hypocotyl phenotype caused by TSA treatment can be rescued by additional glucose or sucrose in the growth medium (Fig. S3A), and the applied glucose can enhance BIN2 acetylation (Fig. S3B). We also found that additional ATP can reduce the interaction between HDA6 and BIN2 in vitro (Fig. S3C), which may cause the increase in BIN2 acetylation. Indeed, we only observed the BR-related phenotype of the *HDA6* mutants and overexpression lines in sugar-free medium or in BR-deficient mutant backgrounds (Figs. 2A and 3D–F and J). Therefore, the repression of BIN2 by HDA6-dependent acetylation may only occur under energy-limiting conditions.

Based on previous work and our findings, we propose a model to illustrate how HDA6 regulates BR signaling through GSK3s in *Arabidopsis*. HDA6 can interact with BIN2 and deacetylate BIN2 on K189. The acetylated form of BIN2 has stronger activity to repress BR signaling. Under certain growth conditions (most likely those involving low energy levels), HDA6 is activated to deacetylate and inhibit BIN2; this enhances BR signaling. Meanwhile, BR signaling also represses HDA6 transcription in a feedback

regulatory mechanism (Fig. S4). Besides the regulation of HDA6 to deacetylation BIN2, HDA6 may also function downstream of BIN2, because *CPD/DWF4* expression was increased in *HDA6-RNAi bin2-3 bil1 bil2* plants compared to *bin2-3 bil1 bil2* (Fig. 3I, the fourth lane and the second lane of marker genes *CPD* and *DWF4*). We then tested the physical interaction between HDA6 and the downstream transcription factor BES1 and found that they can interact with each other (Fig. S5), suggesting that HDA6 may also directly regulate BES1. Further study is needed to investigate how HDA6 regulates BES1 activity. In addition, how energy affects the regulation of BIN2 acetylation status and activity in plants also requires further investigation.

Materials and Methods

Plant Materials and Growth Conditions. *A. thaliana* plants for genetic analysis, RNA samples for genetic analysis, acetylation levels in vivo, and BES1 phosphorylation status were grown on 1/2× Murashige-Skoog medium for 9–10 d and then transferred to soil (sand:nutrient soil:roseite 9:3:1) to grow at 23 °C with a 16-h light/8-h dark cycle for an additional 2 wk. Hypocotyl length and RNA samples for qRT-PCR were from seedlings grown at 23 °C in the dark for 3 d. Plant materials used in this research are listed in Table S1.

Bimolecular Fluorescence Complementation. *HDA6-cYFP* and *BIN2-nYFP* constructs in agrobacteria were injected into the leaves of *N. benthamiana* and grown for 2–3 d. *HDA6-cYFP* cotransfected with *nYFP* and *BIN2-nYFP* cotransfected with *cYFP* were used as controls. Pavement cells around the injected tobacco leaves were observed using a confocal laser-scanning microscope (Leica SP8).

In Vitro Pull-Down Assay. Details of in vitro pull-down assay and co-IP assay can be found in SI Materials and Methods.

Detection of BIN2 Acetylation Status. We detected BIN2 acetylation levels using an acetyl-lysine antibody (Cell Signaling Technology; 9681).

Gene Expression Analysis by Quantitative Real-Time PCR. We extracted total RNA with an RNAPure Plant Kit (Tiangen) and used a Reverse Transcriptase M-MLV Kit (TaKaRa) to generate first-strand cDNAs. cDNAs were combined with SYBR Master Mix (Invitrogen) for qRT-PCR, which was performed with an Eppendorf iCycler. Primers for qRT-PCR are listed in Table S2.

Generation of Transgenic *Arabidopsis* RNAi Lines. The *HDA6-RNAi* construct has been described in Cai et al. (19), and the primers used in this paper are listed in Table S3. The construct was transferred to *Ws-2* plants to obtain *HDA6-RNAi* lines. Then, a line showing strong repression of *HDA6* was chosen by qRT-PCR to cross to *bin2-3 bil1 bil2* plants to obtain the *HDA6-RNAi bin2-3 bil1 bil2* line.

In Vitro Phosphorylation Assay of BES1. For phosphorylation assays, 2 μ g of BES1-MBP and 0.5 μ g of each form of BIN2-His were reacted in 1 \times kinase buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 12 mM MgCl₂, 1 mM DTT, and 1 mM ATP) at

37 °C for 30 min. Reactions were terminated by adding 5 \times SDS loading buffer and boiling at 95 °C for 10 min and then loaded onto SDS/PAGE.

Generation of Point Mutants of BIN2. Details can be found in *SI Materials and Methods*.

Identification of the Acetylation Site of BIN2 by Liquid Chromatography/Tandem Mass Spectrometry. *E. coli* cells were transformed with the *BIN2-His* vector for expressing BIN2 protein, and then 5 nM TSA and 5 mM NAM were added to the medium. Cells were harvested after 16 h of treatment and BIN2-His protein was purified. BIN2 protein was purified by SDS/PAGE and subjected to in-solution alkylation/tryptic digestion followed by liquid chromatography/tandem mass spectrometry (LC-MS/MS) as described by Cai et al. (19).

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