Regulation of Brassinosteroid Signaling by a GSK3/SHAGGY-Like Kinase

Jianming Li* and Kyoung Hee Nam

GSK3/SHAGGY is a highly conserved serine/threonine kinase implicated in many signaling pathways in eukaryotes. Although many GSK3/SHAGGY-like kinases have been identified in plants, little is known about their functions in plant growth and development. Here we show that the *Arabidopsis BRASSINOSTEROID-INSENSITIVE 2* (*BIN2*) gene encodes a GSK3/SHAGGY-like kinase. Gain-of-function mutations within its coding sequence or its overexpression inhibit brassinosteroid (BR) signaling, resulting in plants that resemble BR-deficient and BR-response mutants. In contrast, reduced *BIN2* expression via cosuppression partially rescues a weak BR-signaling mutation. Thus, BIN2 acts as a negative regulator to control steroid signaling in plants.

BRs are a unique class of plant steroids that play important roles throughout the plant life cycle (*1*). Plants that are defective in BR biosynthesis or perception display characteristic mutant phenotypes that include a dwarf stature, delayed flowering and senescence, reduced apical dominance and male fertility, and photomorphogenesis in the dark (*2–5*). Previous genetic screens for BR-response mutants in *Arabidopsis* have led to the identification of only one BR-signaling gene, *BRI1*, which encodes a critical component of a membrane BR receptor (*4–8*). How BR signals are transduced inside plant cells is still a mystery.

To better understand the molecular mechanism(s) of the plant steroid signaling, we have analyzed various dwarf and semidwarf mutants that were collected during our previous genetic screen for *bri1* mutants (*5*) and have identified a new genetic locus, *BIN2*, whose gain-of-function mutations resulted in plants that display *bri1*-like phenotypes. Detailed genetic and physiological studies suggested that *bin2* mutations might define a negative regulator in a BR signal transduction pathway important for plant growth (*9*).

We have cloned the *BIN2* gene using a map-based cloning strategy. Fine mapping analysis delimited the *BIN2* locus to a 13-kb fragment of a completely sequenced bacterial artificial chromosomal (BAC) clone that was mapped to the middle of chromosome IV between markers CH42 and AG (Fig. 1A). Sequence analysis of the three genes in the region from two independently isolated *bin2* alleles identified mutations in a gene that encodes a previously reported cytoplasmic serine/threonine kinase, ASKη (*10*) (Fig. 1B). The catalytic domain of ASKη displays 70% sequence identity to those of the mammalian GSK3β (*11*) and the *Drosophila* SHAGGY protein kinase (*12*) (Fig. 1C). GSK3/SHAGGY kinases are a group of highly conserved serine/threonine kinases implicated in numerous signaling pathways, controlling metabolism, cell fate determination, and tissue patterning in organisms ranging from yeast to mammals (*13*). Homologs have been identified in several plant species and there are 10 GSK3/SHAGGY-like kinase genes in the *Arabidopsis* genome (*14*). However, little is known about their functions in plant growth and development. A few molecular and biochemical studies have suggested possible roles for plant GSK3/SHAGGY-like kinases in osmotic stress response (*15*), wound signaling (*16*), and flower meristem patterning (*17*).

Because the two *bin2* alleles that we have isolated contain gain-of-function mutations (*9*), we could not perform a rescue experiment to further prove that the mutated kinase gene is indeed the *BIN2* gene. Instead, we cloned a 6.2-kb genomic fragment containing the complete *ASKη* gene, recreated the *bin2-1* mutation by site-directed mutagenesis (*18*), and transformed the mutated gene into the wild-type *Arabidopsis* plants. As shown in Fig. 2A, such a mutated *ASKη* gene recapitulated the phenotype of the homozygous *bin2* mutant, providing proof that the *ASKη* gene is indeed the *BIN2* gene. In agreement with its gain-of-function nature, a second site mutation in which Lys 69 was replaced by

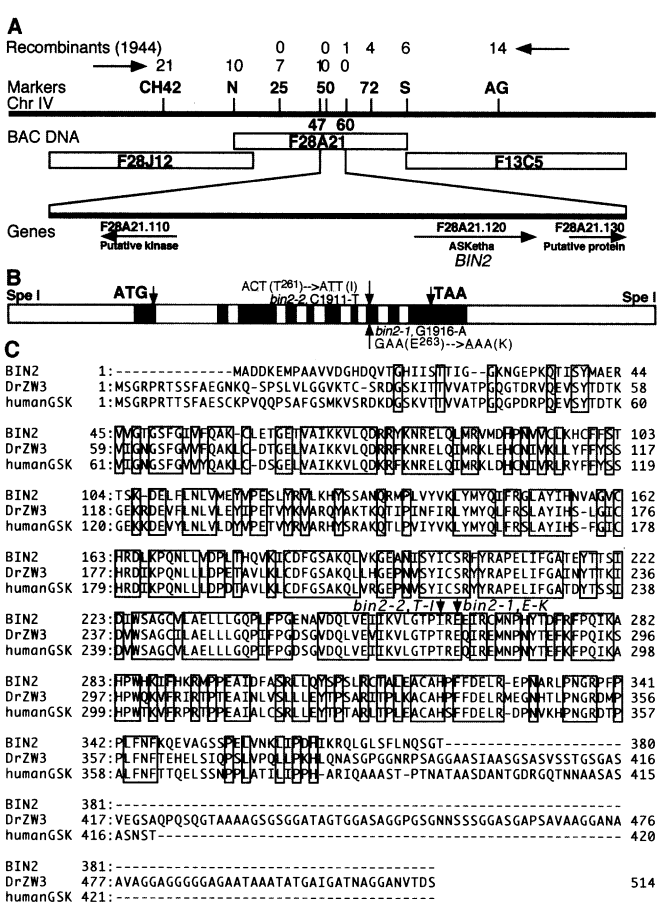

Fig. 1. Molecular analysis of the *BIN2* gene. **(A)** Mapping of the *BIN2* gene on chromosome IV. *BIN2* was previously mapped between markers CH42 and AG (*9*). Three overlapping BAC clones, F28J12, F28A21, and F13C5, were known to cover this region (*26*). A published F21A28 sequence (AL035526) was used to generate new molecular markers N, 25, 47, 50, 60, 72, and S that were used to narrow the *BIN2* gene to a 13-kb fragment that contains three predicted genes. Numbers of recombinants for each marker are shown on the top. **(B)** The structure of the *BIN2* gene and the positions of two *bin2* mutations. The *BIN2* gene is composed of 10 exons (black box) and 9 introns (open box). The start ATG and the termination codons are indicated, and the positions of the two *bin2* mutations are relative to the start ATG codon. **(C)** Sequence alignment of the BIN2 protein with the human GSK3β and the *Drosophila* SHAGGY kinases. The identical amino acids are boxed (*19*). The positions and molecular nature of the two *bin2* mutations are indicated.

Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI 48109–1048, USA.

*To whom correspondence should be addressed. E-mail: jian@umich.edu

Arg (K69R) (*19*) that was known to destroy the GSK/SHAGGY kinase activity (*20*) eliminated the phenotypic effect of the *bin2-1* mutation, although the K69R mutation itself had no effect on the morphology of the transgenic plants (Fig. 2A).

Fig. 2. *bin2-1* is likely a hypermorphic mutation. (**A**) Recapitulation of the *bin2* phenotype by a mutated *ASKη* gene containing the *bin2-1* mutation. Shown here (from left to right) are a wild-type *Arabidopsis* seedling, transgenic plants expressing the wild-type *ASKη* gene, or mutated *ASKη* gene containing *bin2-1*, K69R, or *bin2-1*, K69R double mutation, and *bin2* homozygous mutant. (**B**) The *bin2-1* mutation leads to a more active BIN2 kinase in *E. coli*. Transphosphorylation activities toward a GSK3β peptide substrate of the wild-type GST-BIN2 fusion protein and its three mutant counterparts containing *bin2-1*, K69R, or *bin2-1*, K69R double mutation were measured, by using an in vitro phosphorylation assay (*21*), and expressed as a percentage of the specific activity of the wild-type GST-BIN2 fusion protein. Average activity ($n = 3$) and standard deviation are indicated. (**C** and **D**) *BIN2* overexpression gives rise to an allelic series of *bin2* phenotype. (C) Seedlings grown on synthetic medium; (D) the same plants grown in soil under similar growth conditions. (**E**) Analysis of *BIN2* expression of the *BIN2* transgenic plants. Total RNAs (5 μg each) isolated from wild-type plants, *bin2-1*, and *BIN2* transgenic plants were separated by a formaldehyde-containing agarose gel, blotted to nylon membranes, and hybridized first with a full-length *BIN2* probe and then with an 18*S* rDNA probe.

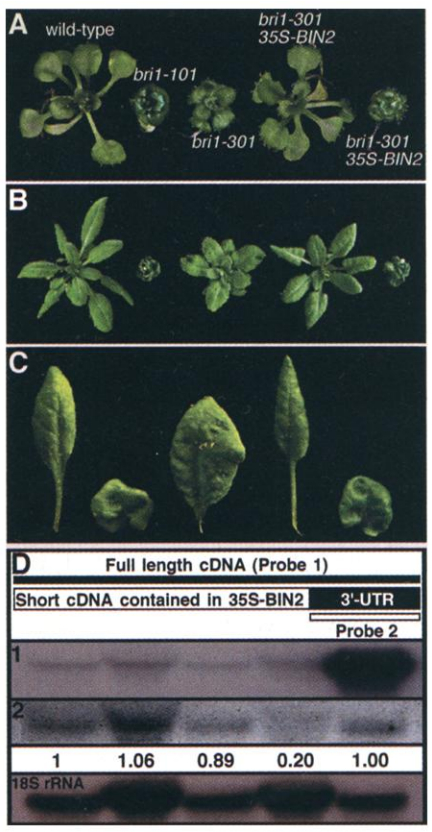

Fig. 3. A reduction in *BIN2* gene expression partially suppressed a weak *bri1* mutation. (**A**) Seedlings grown on synthetic medium [from left to right in (A to D)], wild-type, *bri1-101*, *bri1-301*, suppressed, and enhanced *bri1-301* transgenic plants containing the *35S-BIN2* cDNA transgene). (**B**) Four-week-old seedlings grown in soil under similar growth conditions. (**C**) Leaf shape comparison. (**D**) Northern analysis of *BIN2* gene expression. Total RNAs (5 μg each) isolated from wild-type plants, *bri1-101*, *bri1-301*, suppressed *bri1* plants, and extreme dwarf mutants were separated by a formaldehyde-containing agarose gel, blotted to a nylon membrane, and hybridized first with a full-length *BIN2* probe (probe 1 to detect the total *BIN2* messages), and then with probe 2 (derived from the 3′-untranslated region of the BIN2 cDNA to detect the endogenous *BIN2* messages). After stripping off probe 2, the same filter was hybridized with an 18*S* rDNA probe to control equal loading of total RNAs for each sample. Hybridization signals were quantified by a Bio-Rad GS-363 Molecular Imager System and normalized relative to respective 18*S* rRNA signals. The numbers listed in (D) are relative signal strength calculated as a ratio between a normalized *BIN2* signal of a given sample and the normalized *BIN2* signal of the wild-type plants.

To investigate the biochemical effect of the *bin2-1* mutation, we expressed the wild-type BIN2 and its three mutant counterparts containing *bin2-1*, K69R, and *bin2-1*, K69R double mutations, respectively, as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli* and measured their kinase activities using a synthetic peptide that contains only one unphosphorylated serine residue as a substrate (*21*). As indicated in Fig. 2B, although both K69R mutated proteins display almost no kinase activity, the GST-BIN2 fusion protein containing the *bin2-1* mutation alone shows a statistically significant increase (33 ± 12%, $n = 3$) in kinase activity over the wild-type fusion protein, suggesting that *bin2-1* might be a hypermorphic mutation. If this were true, we would expect that overexpression of the wild-type *BIN2* gene driven by its native promoter, a "transgenic hypermorph," could also lead to the *bin2* phenotype. Indeed, ~10% of the transgenic plants containing the wild-type *BIN2* genomic construct exhibited an allelic series of *bin2* phenotypes (Fig. 2, C and D). Northern analysis with total RNAs isolated from representative transgenic plants revealed a correlation between *BIN2* expression level and phenotypic severity (Fig. 2E). *BIN2* overexpression can lead to increased biosynthesis of BIN2 proteins, resulting in an increase in BIN2 kinase activity. In both cases, an increased BIN2 kinase activity could phosphorylate and inactivate more positive BR signaling proteins to block BR signal transduction, giving rise to a *bri1*-like phenotype.

To further prove that BIN2 acts negatively to regulate BR signaling, we generated transgenic plants in a weak *bri1* mutant background [*bri1-301* (*22*)] using either a sense or antisense *BIN2* cDNA driven by the strong, constitutively active cauliflower mosaic virus 35*S* promoter. If BIN2 acted as a negative regulator in BRI1-mediated BR signaling, we would expect that a reduction in *BIN2* expression could, at least partially, suppress the weak *bri1* mutation, whereas its overexpression could lead to a stronger *bri1* phenotype. Although we did not observe any phenotypic alteration with the *35S-BIN2* antisense construct, we identified three phenotypic classes of transgenic plants containing the *35S-BIN2* sense construct, including plants resembling the parental *bri1-301* mutant, lines showing a suppressed *bri1* phenotype, and extreme dwarfs similar to *bri1-101*, a strong *bri1* mutant (Fig. 3, A to C). We suspected that the enhanced *bri1* phenotype was likely caused by *BIN2* overexpression driven by the strong 35*S* promoter, whereas the suppressed *bri1* phenotype was due to a reduced *BIN2* expression caused by cosuppression.

To test this prediction, we performed a Northern blot analysis with total RNAs isolated from wild-type plants, the two *bri1*

mutants, one representative each of the suppressed and enhanced *bri1-301* transgenic plants containing the *35S-BIN2* transgene. Using a full-length *BIN2* cDNA as a probe, we observed a ~50-fold accumulation of *BIN2* transcripts in the enhanced *bri1-301* line compared with the wild-type control (Fig. 3D). However, no difference in hybridization signal was observed between the suppressed line and the other three tested plants, most likely due to cross-hybridization of the full-length *BIN2* probe with other *Arabidopsis GSK3/SHAGGY-like* genes. To see if the suppressed *bri1-301* line accumulated fewer *BIN2* messages, we used a gene-specific probe corresponding to the 3′-untranslated region of the *BIN2* cDNA that was not included in the *35S-BIN2* transgene. As shown in Fig. 3D, the suppressed line accumulated ~20% of the amount of *BIN2* transcripts in the wild-type control. Because such a cosuppressed line is equivalent to a reduction-of-function *bin2* mutant, this result provides a further support for BIN2 being a negative regulator in a BRI1-mediated BR signaling pathway.

Our results indicate that a GSK/SHAGGY-like kinase plays an important role in a steroid signal transduction pathway. In animals, GSK3/SHAGGY is a constitutively active kinase that negatively regulates a variety of substrates by phosphorylation, and the inhibition of its kinase activity constitutes a key event in many signal transduction pathways (*13*). It is well established that GSK3/SHAGGY kinase itself is negatively regulated by protein phosphorylation or protein-protein interaction in response to a variety of stimuli (*13*). If BIN2 functions as a negative regulator in BR signaling, a crucial step in this signal transduction pathway would be to inhibit the BIN2 kinase activity in response to BR signals, which are thought to be perceived by BRI1, a leucine-rich-repeat–receptor–like kinase (*5–8*). Thus, it is quite tempting to speculate that BRI1 might be one of the upstream kinases that could phosphorylate and inactivate BIN2 to relieve its inhibitory effect on downstream BR signaling components. However, repeated experiments using yeast two-hybrid analysis and in vitro protein interaction assay failed to demonstrate a direct physical interaction between BRI1 and BIN2, suggesting the existence of additional regulators that are involved in suppressing BIN2 activity. We hypothesize that, in the absence of BRs, BIN2 is a constitutively active kinase that can phosphorylate and inactivate positive BR signaling proteins to block BR signal transduction. The identification of upstream regulators and downstream targets of the BIN2 kinase is crucial to understand the molecular mechanisms of steroid signaling in plants.

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18. A 6.2-kb Spe I fragment was cloned from BAC F28A21 into the pPZP212 vector (*23*). To create the *bin2-1* mutation, two complementary primers (CACCAACTCGAAAAGAAATCCGTTG and CAACGGATTTCTTTTCGAGTTGGTG) were used for site-directed mutagenesis using the Stratagene QuikChange site-directed mutagenesis kit. The primer set (CCGTGGCGA TAAGGAAGGTTTTGC and GCAAAACCTTCCTTATCGCCACGG) was used to create K69R mutation with DNAs of wild-type and the *bin2-1*–mutated *BIN2* genomic constructs.
19. Single letter abbreviations for amino acid residues: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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21. To express BIN2 protein in *E. coli*, a BIN2 cDNA was cloned into the pGEX-KG vector (*24*), and the resulting plasmid was used to create the *bin2-1*, K69R, or *bin2-1*, K69R double mutation, as described above. The induction and protein purification was carried out according to a published protocol (*24*). The transphosphorylation activity of each GST-BIN2 fusion protein was assayed as described (*25*) by using the CREB phosphopeptide [KRREILSRRPS(p)YR, New England Biolab] as a substrate.
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Requirement of the Activation-Induced Deaminase (*AID*) Gene for Immunoglobulin Gene Conversion

Hiroshi Arakawa, Jessica Hauschild, Jean-Marie Buerstedde*

Three phenotypically distinct processes—somatic hypermutation, gene conversion, and switch recombination—remodel the functionally rearranged immunoglobulin (Ig) loci in B cells. Somatic hypermutation and switch recombination have recently been shown to depend on the activation-induced deaminase (*AID*) gene product. Here, we show that the disruption of the *AID* gene in the chicken B cell line DT40 completely blocks Ig gene conversion and that this block can be complemented by reintroduction of the *AID* complementary DNA. This demonstrates that the *AID* master gene controls all B cell–specific modifications of vertebrate Ig genes.

The formation of a large antigen receptor repertoire by DNA rearrangement and hypermutation is unique to the immune system. Early lymphocytes first assemble their antigen receptor genes from different V, D, and J segments by site-specific V(D)J recombination. B cells then further modify the rearranged V segments by untemplated hypermutation (*1*) or pseudogene templated gene conversion (*2*). Some species (such as sheep) exclusively use somatic hypermutation for V segment diversification, whereas others (such as chickens, rabbits, cattle, and pigs) rely predominantly on Ig gene conversion (*3*). Concomitant with antigen stimulation, the Ig heavy chain locus is further reshuffled by

Department of Cellular Immunology, Heinrich-Pette-Institute, Martinistrasse 52, 20251 Hamburg, Germany.

*To whom correspondence should be addressed. E-mail: buersted@hpi.uni-hamburg.de