Involvement of the N-terminal B-box Domain of Arabidopsis BBX32 Protein in Interaction with Soybean BBX62 Protein

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Background: AtBBX32 is a member of the B-box protein family from A. thaliana. Its molecular mechanism is poorly understood.

Results: We demonstrate functional interactions of AtBBX32 with soybean BBX62 (GmBBX62).

Conclusions: Our data offer novel insight into the role of B-box domains in mediating protein-protein interactions between different plant B-box proteins.

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Background: Arabidopsis thaliana BBX32 (AtBBX32) represses light signaling in A. thaliana and that expression of AtBBX32 in soybean increases grain yield in multiple locations and multiyear field trials. The BBX32 protein is a member of the B-box zinc finger family from A. thaliana and contains a single conserved Zn$^{2+}$-binding B-box domain at the N terminus. Although the B-box domain is predicted to be involved in protein-protein interactions, the mechanism of interaction is poorly understood. Here, we provide in vitro and in vivo evidence demonstrating the physical and functional interactions of AtBBX32 with another B-box protein, soybean BBX62 (GmBBX62). Deletion analysis and characterization of the purified B-box domain indicate that the N-terminal B-box region of AtBBX32 interacts with GmBBX62. Computational modeling and site-directed mutagenesis of the AtBBX32 B-box region identified specific residues as critical for mediating the interaction between AtBBX32 and GmBBX62. This study defines the plant B-box as a protein interaction domain and offers novel insight into its role in mediating specific protein-protein interactions between different plant B-box proteins.

Previous studies have demonstrated that Arabidopsis thaliana BBX32 (AtBBX32) represses light signaling in A. thaliana and that expression of AtBBX32 in soybean increases grain yield in multiple locations and multiyear field trials. The BBX32 protein is a member of the B-box zinc finger family from A. thaliana and contains a single conserved Zn$^{2+}$-binding B-box domain at the N terminus. Although the B-box domain is predicted to be involved in protein-protein interactions, the mechanism of interaction is poorly understood. Here, we provide in vitro and in vivo evidence demonstrating the physical and functional interactions of AtBBX32 with another B-box protein, soybean BBX62 (GmBBX62). Deletion analysis and characterization of the purified B-box domain indicate that the N-terminal B-box region of AtBBX32 interacts with GmBBX62. Computational modeling and site-directed mutagenesis of the AtBBX32 B-box region identified specific residues as critical for mediating the interaction between AtBBX32 and GmBBX62. This study defines the plant B-box as a protein interaction domain and offers novel insight into its role in mediating specific protein-protein interactions between different plant B-box proteins.

The Arabidopsis thaliana BBX32 (AtBBX32, At3g21150) gene belongs to a family of 32 proteins that contain Cys-rich zinc-binding B-box domains. The first B-box domain protein described in A. thaliana was the transcriptional regulator CONSTANS (1, 2). This family represents a subgroup of zinc finger proteins that contain one or more B-box domains with specific tertiary structures that are stabilized by binding Zn$^{2+}$ ions. AtBBX32 contains a single B-box domain at the N terminus. Unlike CONSTANS and CONSTANS-like proteins, AtBBX32 lacks a conserved CCT domain at the C terminus, which has been shown to bind DNA (2, 3).

B-box domain-containing proteins have been identified in both di- and mono-cotyledenous plants (2, 4–6). It has been proposed that the B-box domain is involved in the mediation of protein-protein interactions, including both heterodimerization of B-box family members and interactions with described DNA-binding proteins (4, 5). Plant B-box proteins play a role in light-regulated transcription and development through their interactions with key regulators of the light-signaling pathway (5, 7, 8). For example, SALT TOLERANCE HOMOLOG 2 (STH2, AtBBX21) interacts via the B-box domain with HY5, an early light-responsive transcriptional regulator, providing evidence for the role of B-box domains in modulating light signaling through protein-protein interaction (5). Using a transient expression assay in protoplasts, the same authors demonstrated that a functional B-box domain plays a direct role in activating transcription in plants. Mutations of conserved residues in CONSTANS B-boxes caused late flowering (9), further demonstrating an important biological function of the B-box domain in plant development. More recently, overexpression of AtBBX32 in A. thaliana has been shown to repress HY5-associated light signaling through a mechanism involving AtBBX32 protein-protein interaction (10).

The B-box domain has been classified into two subgroups, B-box-1 and B-box-2, both of which have seven or eight conserved Cys and His residues to coordinate two Zn$^{2+}$ atoms in a RING-like fold (11). B-boxes are usually found as single domains or as tandem repeats. All A. thaliana B-box proteins have at least one B-box with an Asp as the fourth zinc-coordinating residue (5). The consensus sequence of this conserved B-box is CXCX$_2$CXC$_{7–9}$CCX$_4$DXX$_1$CX$_4$CX$_2$H$_7$X$_2$H$_2$ (2). Involvement of conserved Cys and His residues in zinc ligation was illustrated in one-dimensional $^1$H NMR studies of the B-box domain from Xenopus nuclear factor-7 (XNF-7) (12, 13). However, there has been little biochemical and biophysical characterization of B-box domains from plant B-box proteins.

Transgenic soybean lines expressing AtBBX32 show increased grain yield in multiple environments tested for a few years (14). Increased yield is driven by changes in the growth and reproductive development of AtBBX32-expressing soy-
bean lines as a result of AtBBX32-mediated changes in soybean gene expression during the transition from night to day. These data suggest that AtBBX32 plays a role in regulating diurnal processes in soybean through the modulation of gene expression specifically at dawn (14). Because AtBBX32 does not contain known DNA binding domains, this regulation is likely the result of an AtBBX32-containing protein complex. To explore the biochemical nature of AtBBX32-containing transcriptional complexes in transgenic soybean plants, we have employed genetic and biochemical approaches to identify soybean proteins that directly interact with AtBBX32. In this study, we have identified and biochemically characterized the interaction between AtBBX32 and GmBBX62, a B-box protein from soybean. Computational modeling and mutational analysis of AtBBX32 reveals that the B-box domain is necessary for binding with GmBBX62 and that the metal-binding residues are critical for this interaction in vitro. We also show that a salt bridge formed by Asp-28 and Arg-43, and the predicted interface residues His-15 and Arg-44 of AtBBX32, play an important role in mediating the interaction between AtBBX32 and GmBBX62. Our data offer novel insight into the role of B-box domains in mediating specific protein-protein interactions between different plant B-box proteins, and we demonstrate that ectopically expressed AtBBX32 protein can interact with an existing protein network in soybean.

**EXPERIMENTAL PROCEDURES**

Yeast Two-hybrid Screening—Yeast two-hybrid screening was performed by Hybrigenics Services, S.A.S., Paris, France.

The coding sequence for the N-terminal amino acids 1–74 of AtBBX32 (At3g21150) or full-length AtBBX32 was PCR-amplified and cloned into pB27 as a C-terminal fusion to LexA (N-LexA-AtBBX32-C). The resulting plasmid was used as bait to screen a random-primed (N-LexA-AtBBX32-C). The resulting plasmid was used as bait to screen a random-primed mRNA isolated from soybean leaf and root tissues. The library contains 10 million independent fragments in yeast with an average fragment size of 800–1000 nucleotides. Vector pB27 is derived from the original pBTM116 plasmid. Seventy-nine million clones were screened using a mating Vector pB27 is derived from the original pBTM116 plasmid yeast with an average fragment size of 800–1000 nucleotides.

**Preparation of RNA and Selected B-box Gene Expression Analysis**—RNA was prepared according to the manufacturer’s instructions. RNA yield was determined using a NanoDrop-1000 spectrophotometer, and RNA integrity was visualized using the Agilent 2100 Bioanalyzer. RNA amplification for labeling was performed according to the manufacturer’s recommendations using the TargetAmp 1-round Biotin-aRNA amplification kit from EpiCentre (Madison, WI). Twelve micromolars of labeled RNA was then fragmented according to the standard protocols for gene expression analysis provided by Affymetrix. Fragmented cRNA samples were hybridized to Affymetrix microarrays according to the manufacturers’ standard protocol.

**Signal intensities** were normalized using RMA (Robust Multi-Array Algorithm) and Partek software (St. Louis, MO). Intensity values were subsequently transformed into log2 scale. Expression profiles for the probe sets representing selected soybean B-box proteins were processed using the cluster and correlation analysis tools in Tibco Spotfire. Profiles for those B-box proteins that were represented by more than one probe set were averaged together. A standard Pearson correlation analysis was run in Spotfire to compute all the pairwise correlation coefficients shown in **supplemental Table S1**, with the rows being arranged in the order determined by a hierarchical cluster analysis using the UPGMA clustering method with cosine correlation as the distance metric.

**Soybean Proteoplast Isolation and Transformation**—Soybean protoplasts were isolated from 4- to 6-mm cotyledons and transformed as described by Abel and Theologis (19). Approximately 1 × 10^6 protoplasts were transformed in 15-ml Falcon conical tubes with 90 μg of Qiagen prepped plasmid DNA listed in **supplemental Table S2**. Protoplasts were then incubated 18–24 h at 22 °C. Protoplasts were harvested at 150 × g for 3 min and lysed with lysis buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 1% Triton X-100 and Complete Protease Inhibitor (Roche Applied Science)) for 1 h on ice with vortexing every 15 min. The lysate was centrifuged for 5 min at 3000 × g, and soluble fractions were retained for use in Luminex-based co-immunoprecipitation (co-IP) assays.

**Luminex Co-immunoprecipitation Assay**—The assay was carried out using the miniaturized sandwich immunoassay and co-IP method with modifications (20). This combination of co-IP and sandwich immunoassay allows the relative quantification of components of the complex. Briefly, antibodies for GFP (MBL International, D153-3) and FLAG (Bethyl Laboratories, A190-101A) were covalently coupled to carboxylated fluorescent microspheres (Luminex) according to the manufacturer’s protocol. Fifty μl of protoplast cell lysate (above) was aliquoted to three different wells in a 96-well clear flat-bottom plate (Bio-Rad) for Luminex assays. To each sample, 50 μl of conjugated beads was added to each appropriate well and incubated for 30 min with shaking. Forward and reverse co-IPs were performed with anti-GFP antibody-conjugated beads and anti-FLAG antibody-conjugated beads. Beads were captured for 2 min and washed three times with PBST (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KHPO_4, and 0.05% Tween 20). Biotinylated GFP (ab6658, 1:1000; Abcam) and FLAG (A190-101B, 1:1000; Bethyl) antibodies were added to appropriate wells. The plate was incubated on a shaker for 30 min. After washing, 100 μl of reporter NeutrAvidin (R)-phycoceryth-
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rin at a 1:1000 dilution was added to each well and incubated for 30 min with subsequent washing. Each well was resuspended in 100 μl of PBST buffer containing 0.5% BSA before plates were analyzed using FLEX MAP three-dimensional system (Luminex Corp, Austin, TX). Approximately 100 beads were measured per sample to determine the median fluorescence intensity.

Computational Modeling of B-box Structures of AtBBX32 and Interacting GmBBX62—The structural template was selected out of an NMR ensemble (Protein Data Bank code 2D8V) of B-box structures. The sequence similarity between the template and that of AtBBX32 is 27% after adjusting the metal-binding residues. The “interactive modeling” option provided by ICM-pro 3.6-1 (21) was used to build a homology model by tethering the predicted metal-binding residues to the template. Side-chain minimization was run via Cartesian minimization for up to 300 steps using a conjugate gradient algorithm for all but metal-binding residues. The AtBBX32 and GmBBX62 B-box protein domain sequences are 45% identical. The AtBBX32 model structure was then used to build the GmBBX62 model. To build the protein-protein interaction model, several dimeric structures, including B-box and RING domains, were identified from the Protein Data Bank. The modeled monomeric B-box structures of AtBBX32 and GmBBX62 were superimposed on the template structures with side chain minimization as described earlier.

DNA Constructs—Full-length AtBBX32 (At3g21150) cDNA from A. thaliana and GmBBX62 cDNA from soybean (G. max) were cloned into a pET-based expression vector containing a T7 promoter. The expressed recombinant GmBBX62 carries an N-terminal FLAG tag. The resulting plasmids were designated as pMON102126 for AtBBX32 and pMON93920 for FLAG::GmBBX62, respectively. The sequence integrity in these plasmids was confirmed by DNA sequence analysis.

Site-directed mutagenesis of AtBBX32 was performed via a method of overlap extension PCR described by Qi et al. (22), using pMON102126 carrying wild-type AtBBX32 (supplemental Table S3). The oligonucleotide primer pairs used for mutating AtBBX32 proteins are listed in supplemental Table S3. The PCR products encoding the desired full-length mutants were gel-purified, digested, and then cloned into the corresponding sites of pET-based vector containing the T7 promoter, and the resulting mutant plasmids are listed in supplemental Table S2. Deletion mutants ΔN17 and Δ53–73 were also generated via a PCR-based method using the oligonucleotide primers listed in supplemental Table S3. All generated plasmids were confirmed to carry the desired mutation by DNA sequencing.

Expression and Purification of Recombinant FLAG::GmBBX62—The chimeric plasmid containing FLAG-tagged GmBBX62 (pMON93920) was transformed into Escherichia coli BL21 (DE3) cells, and transformants were selected on LB (Luria-Bertani) medium containing kanamycin (50 μg/ml). For induction of gene expression, transformants selected from single colonies were grown overnight in 2 ml of LB selection liquid medium at 37 °C and subcultured to 200 ml of auto-induction medium (23) supplemented with kanamycin for 2 h at 37 °C and then for 48 h at 16 °C with 230 rpm shaking. Cells were harvested by centrifugation for 20 min at 7000 × g, and the cell pellets were used for the protein purification.

Washed cell pellets were resuspended in Lysis Buffer (Sigma B7435, Cellytic B) at 10 ml/g of cell paste in the presence of protease inhibitor mixture tablets without EDTA (Roche Applied Science), rlysozyme (1 KU/ml, Novagen), and benzoinase (25 units/ml, Novagen). Resuspended cells were incubated for 30 min at 4 °C on a rotating mixer and then at room temperature for 30 min to extract soluble proteins. The lysate was centrifuged at 21,000 × g at 4 °C for 15 min. The resulting supernatant was used for the protein purification via affinity chromatography with anti-FLAG M2 affinity gel (Sigma A2220) according to the supplier’s instructions.

The identity of purified protein was confirmed by SDS-PAGE stained with Coomassie Blue, Western blotting with anti-FLAG antibody (Sigma F2555), and mass spectrometry analysis (data not shown).

Expression and Purification of B-box Domain of AtBBX32—cDNA fragments encoding the B-box region (residues 1–52) of AtBBX32 (AtBBX32(1–52) as well as the two N-terminal domain variants carrying a single mutation C5A (AtBBX32(1–52)-C5A) and double mutations H15A/R44A (AtBBX32(1–52)-H15A/R44A) were cloned into pET-based vector containing the T7 promoter (Novagen). To enhance the soluble expression of the N-terminal AtBBX32 B-box fragments, His₁₀-tagged maltose-binding protein (MBP)² was fused in-frame to AtBBX32(1–52) and both variants. A tobacco vein mottling virus protease site was engineered between the MBP-His₁₀ fusion tag and the target protein. The resulting plasmids containing AtBBX32(1–52), AtBBX32(1–52)-C5A, and AtBBX32(1–52)-H15A/R44A were designated pMON116242, pMON144787, and pMON144789, respectively. MBP-His₁₀-tobacco vein mottling virus tag and the target protein. The resulting plasmids were transformed into BL21(DE3) E. coli cells and grown overnight at 37 °C in 100 ml of LB medium with kanamycin (50 μg/ml). Overnight cultures were diluted 20-fold (2 liter final volume) into fresh TB media containing kanamycin (50 μg/ml) and grown to an A₆₀₀ of 1.0. After addition of 0.5 mM isopropyl 1-thio-β-D-galactopyranoside, the cells were grown at 15 °C for 24 h. Cells were harvested by centrifugation at 6000 rpm for 10 min. The cell pellet was resuspended in lysis buffer (300 mM NaCl, 15 mM imidazole, 1 mM benzamidine, and 0.5 μg/ml lysozyme) and incubated at room temperature for 20 min and then centrifuged for 30 min at 11,000 rpm. Supernatant was loaded onto the 5-ml HisTrap Column (GE Healthcare) equilibrated with washing buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 50 mM imidazole). After washing the column extensively with washing buffer, MBP-His₁₀-tagged N-terminal AtBBX32 fragment was eluted with elution buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 250 mM imidazole). Protein fractions were pooled, treated with tobacco vein mottling virus protease for removal of the MBP-His₁₀ tag, and dialyzed against 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM DTT for 16 h at 4 °C.

The cleaved protein mixture was then loaded onto the 5-ml

² The abbreviation used is: MBP, maltose-binding protein.
HisTag column to remove His-tagged MBP and uncut His-tagged MBP fusion protein. Flow-through containing the tag-free N-terminal AtBBX32 fragment was collected and loaded onto amylase resin to complete removal of the remaining MBP. Protein fractions containing tag-free AtBBX32(1–52) fragments were collected, concentrated, and further purified by gel filtration using a Superdex-75 16/60 column (GE Healthcare) in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM DTT. Purified protein fractions were pooled and concentrated for use in the protein characterization.

The purified AtBBX32(1–52) fragments appear >90% pure as assessed by SDS-PAGE and visualization by Coomassie Blue staining (supplemental Fig. S2). The identity of the truncated protein was also confirmed by Western blotting with anti-AtBBX32 polyclonal antibody (data not shown). Proteins were quantified with Bradford reagent (Bio-Rad) using bovine serum albumin as standard.

Measurement of Circular Dichroism (CD) Spectra—CD spectra of freshly purified AtBBX32(1–52), AtBBX32(1–52)-C5A, and AtBBX32(1–52)-H15A/R44A in 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl were measured at room temperature in an Olis DSM 20 CD spectrophotometer with 1-mm path length quartz cuvette at a protein concentration of 0.3–0.8 mg/ml. Each spectrum was obtained as the average of three scans to optimize the signal to noise and was background-corrected by subtracting the CD spectrum of the buffer solution over the range of 260–200 nm. The data were reported in terms of mean residue molar ellipticity \( \theta \), which is defined as \( \theta = 100 \theta_{\text{obs}}/l_c \), where \( \theta_{\text{obs}} \) is observed ellipticity in degrees; \( c \) is the concentration in residue moles/liter, and \( l \) is the length of the light path in centimeters.

In Vitro Transcription and Translation of Wild-type and Mutant AtBBX32 Proteins—Wild-type (WT) and mutant AtBBX32 proteins were produced by in vitro translation using the RTS 100 Wheat Germ CECF kit (5 Prime, Inc., Gaithersburg, MD). The DNA templates used for expression of these proteins are listed in supplemental Table S3. Briefly, 35 \( \mu \)l of reaction mixture was prepared as described in the kit protocol and added to 2 \( \mu \)g of plasmid DNA in a volume of 15 \( \mu \)l of RNase-free double distilled H_2O. In vitro translation was carried out using RTS ProteoMaster (Roche Applied Science) for 24 h at 900 rpm at 20 °C. The soluble faction represents the supernatant after centrifuging the reaction mixture for 15 min at 10,000 \( \times \) g at 4 °C. Soluble proteins were confirmed by Western blotting analysis (data not shown).

WT or mutant AtBBX32 protein in soluble fractions was quantified by a sandwich ELISA method. Briefly, a 96-well Nunc Maxi-Sorp microtiter plate (Nunc 439454) was coated with anti-AtBBX32 polyclonal antibody (500 ng/well) in 100 \( \mu \)l of coating buffer (50 mM sodium carbonate/bicarbonate buffer, 150 mM NaCl, pH 9.6) overnight at 4 °C. The coated plate was washed three times with 250 \( \mu \)l/well of PBST and blocked with PBST containing 1% BSA at room temperature (RT) for 2 h. After washing with PBST, the plate was incubated with various concentrations of WT or mutant AtBBX32 protein in 100 \( \mu \)l/well of PBST containing 0.5% BSA at 4 °C overnight. After washing, biotinylated AtBBX32 antibody (1 mg/ml, Thermo Scientific, catalog no. 21335 Sulfo-NHS-LC-Biotin) was added at a 1:1000 dilution in PBST containing 0.5% BSA for 1 h of incubation at 37 °C. The plate was then incubated with streptavidin-conjugated horseradish peroxidase (HRP, 1:3000; Pierce) in PBST at 37 °C for 1 h. The enzyme reaction was initiated by adding 100 \( \mu \)l/well of the substrate 3,3’,5,5’-tetramethylbenzidine (Sigma). The reaction ran for 7 min and was terminated by the addition of 100 \( \mu \)l 3 \( \mu \)l phosphoric acid. The protein was quantified by measuring the absorbance at 450 nm/650 nm using a SpectraMax plate reader (Molecular Devices, CA) and the SoftMax Pro data analysis program (Molecular Devices, CA).

The concentrations of AtBBX32 mutants were interpolated and normalized by a working curve generated by WT AtBBX32 protein in the same assay. The normalized concentrations of mutant proteins were used to calculate the relative binding activity (% WT AtBBX32) for Figs. 4–6 and 8 using the ELISA-based protein-protein interaction assay below.

In Vitro AtBBX32-GmBBX62 Binding Assays—A commonly used in vitro pulldown assay was first conducted to examine the direct interaction between AtBBX32 and GmBBX62. Unless otherwise indicated, the assay was carried out at 4 °C. Briefly, 80 \( \mu \)l of anti-FLAG M2 affinity resin (A2220, Sigma) was washed three times with 1 ml of 1 X TBS buffer containing 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl to remove glycerol. The washed resin was then equilibrated with 500 \( \mu \)l of HEPES binding buffer containing 25 mM HEPES, pH 7.8, 10 mM MgCl_2, 5 mM ATP, 50 \( \mu \)M ZnSO4, 50 \( \mu \)M MG132, 50 mM NaCl, 0.2% Nonidet P-40, and Complete Protease Inhibitor without EDTA (Roche Applied Science). Purified FLAG::GmBBX62 was added to the resuspended resin in 200 \( \mu \)l of HEPES binding buffer. All samples including controls were incubated on a roller shaker for 1 h at RT. AtBBX32 produced from wheat germ continuous exchange cell-free system was added to FLAG::GmBBX62 affinity-bound anti-FLAG M2 beads in a final volume of 450 \( \mu \)l of HEPES binding buffer, and the reactions were incubated for 2 h at room temperature on a roller shaker. To remove nonspecifically bound proteins, the resin was washed six times with 0.4 ml of 25 mM HEPES buffer, pH 7.6, containing 5 mM MgCl_2, 150 mM NaCl, 0.2% Nonidet P-40, and protease inhibitors. The bound protein complex was suspended in 100 \( \mu \)l of SDS loading buffer and boiled for 5 min. Twenty five microliters of the eluted solution was subjected to 4–20% SDS-PAGE (Bio-Rad). AtBBX32 in the protein complex can be identified by Western blot analysis using anti-AtBBX32 antibody.

To evaluate the in vitro binding activity of a number of AtBBX32 mutants compared with WT AtBBX32 via statistical analysis of multiple reproducible data sets, we have developed a quantitative high throughput ELISA-based AtBBX32-GmBBX62 binding assay. This 96-well plate assay is a modification of methods described by Bagnasco et al. (24) and Craig et al. (25). Briefly, a saturating concentration of purified FLAG::GmBBX62 (300 ng/well) in 100 \( \mu \)l of buffer (25 mM HEPES, pH 7.6, 5 mM MgCl_2, 50 \( \mu \)M ZnSO4, 150 mM NaCl) was coated on 96-well plates (Nunc catalog no. 439454) overnight at 4 °C. The coated plate was washed three times with 300 \( \mu \)l of binding buffer (25 mM HEPES, pH 7.6, 5 mM MgCl_2, 50 \( \mu \)M ZnSO4, 50 mM NaCl, 0.2% Nonidet P-40, 5% glycerol, 5 mM ATP, and Complete Protease Inhibitor without EDTA (Roche Applied Science) at 4 °C. The plate was then incubated with streptavidin-conjugated horseradish peroxidase (HRP, 1:3000; Pierce) in PBST at 37 °C for 1 h. The enzyme reaction was initiated by adding 100 \( \mu \)l/well of the substrate 3,3’,5,5’-tetramethylbenzidine (Sigma). The reaction ran for 7 min and was terminated by the addition of 100 \( \mu \)l 3 \( \mu \)l phosphoric acid. The protein was quantified by measuring the absorbance at 450 nm/650 nm using a SpectraMax plate reader (Molecular Devices, CA) and the SoftMax Pro data analysis program (Molecular Devices, CA).
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Science). GmBBX62-bound plates were blocked with 300 μl of blocking buffer (binding buffer containing 0.5% BSA) for 2 h at RT with rocking. Following blocking, AtBBX32 or mutant forms of the protein were diluted in binding buffer, added to the blocked wells, and followed by incubation for 1 h at RT with rocking or for 16 h at 4 °C for the purified AtBBX32(1–52), AtBBX32(1–52)-CSA, or AtBBX32(1–52)-H15A/R44A. For each assay, the same amount of the mutant protein and AtBBX32 was used, based on the normalized concentration for the wheat germ protein extracts as described above and a Bradford method-determined protein concentration for the purified N-terminal AtBBX32(1–52) fragments. Wells were washed three times with 300 μl/well binding buffer without ATP and glycerol. Bound AtBBX32 or mutant protein was incubated with biotinylated goat anti-AtBBX32 polyclonal antibody diluted 1:1000 in PBST for 1 h at RT. The plate was washed and incubated with streptavidin-conjugated HRP at 1:3000 in PBST at 37 °C for 1 h at RT. The interacted AtBBX32 or mutant protein was quantified by measuring the absorbance at 450/650 nm, as described above.

**Immunoblot Analysis**—Immunoblotting experiments were performed using protein samples from the pulldown assay or in vitro translation. Protein samples were separated on a 4–20% Tris-HCl Criterion Precast Gel (Bio-Rad) and electrotransferred onto a nitrocellulose membrane (Bio-Rad) for immunoblot detection. The membrane was blocked for 1 h in PBST containing 5% nonfat dry milk, washed three times with PBST, and incubated for 1 h with a primary antibody (anti-FLAG or anti-AtBBX32 antibody) in PBST containing 1% nonfat dry milk. After washing, the membranes were incubated for 1 h with goat anti-rabbit IgG conjugated to HRP (Pierce). The blots were developed using the SuperSignal West Femto kit (Pierce). The anti-FLAG (F2555, Sigma), anti-AtBBX32, and horseradish peroxidase-conjugated anti-rabbit secondary antibodies were used at 1:2500, 1:2000, and 1:3000 dilutions in PBST, respectively. Goat anti-AtBBX32 polyclonal antibody was produced by Open Biosystems using denatured AtBBX32 purified from AtBBX32-expressed E. coli cells.

**Statistical Analysis of AtBBX32-GmBBX62 Binding Data**—Statistical analysis was performed using SAS 9.2 for Windows, SAS Institute Inc. The BBX32 activity assay data were analyzed by analysis of variance model, fitting treatment as a fixed effect. Point estimates and associated 95% confidence intervals were provided for the comparisons among treatments. A least significant difference test was used to determine significance in the binding difference. All p values reported are for the two-tailed test, and the statistically significant difference was defined as p < 0.05.

**RESULTS AND DISCUSSION**

**Predicted Structural Features of AtBBX32 Protein**—The coding region of AtBBX32 is 675 bp in length and encodes a protein of 225 amino acids. We previously identified two homologs of AtBBX32, GmBBX52 and GmBBX53, from the 62-member B-box family in soybean. GmBBX52 and GmBBX53 share 32 and 32.5% amino acid sequence identity to AtBBX32, respectively, across the entire protein (supplemental Fig. S1) (14). AtBBX32 shares a much higher degree of identity (54.9%) with GmBBX52 and GmBBX53 within the B-box region, but it shows a lower degree of identity to the soy homologs in the C-terminal region. AtBBX32 is predicted to contain one B-box with an Asp as the fourth zinc-coordinating residue at its N terminus. The consensus sequence of the AtBBX32 B-box is a CXXCHX(CX)2CX(CX)2CX(CX)2CHXH(H). It is closest to type 1 B-box domains (consensus sequence, CX4CX6–8CX6–8CX2H2–4C(H)X7–10H) (11), but it has an Asp substituted for the fourth Cys (5). The predicted metal-binding residues are Cys-5, Cys-8, Cys-16, Asp-19, Cys-24, Cys-27, His-32, and His-41. This consensus sequence and the same predicted metal binding residues are also present in the soybean homologs.

Although AtBBX32 and the two soybean homologs share only a moderate level of sequence similarity, experimental evidence suggests that these proteins are functionally equivalent; constitutive expression of GmBBX52 or GmBBX53 in soybean results in an increase in grain yield and modulation of clock gene expression, similar to AtBBX32 transgenic soybean (14).

**Identification of GmBBX62 as an AtBBX32-interacting Protein**—It has been shown that expression of AtBBX32 in soybean can improve soybean agronomic performance (14). To gain insight into the molecular function of AtBBX32 in transgenic soybean, we sought to identify endogenous soybean proteins that interact with AtBBX32. We performed yeast two-hybrid screens using either full-length AtBBX32 or its N-terminal B-box region (amino acids 1–74) as bait against a G. max cDNA library. We identified 40 putative interactors out of about 79 million screened cDNA clones according to confidence scores as described under “Experimental Procedures.” Of these, 12 putative binding partners were further screened for interaction with AtBBX32 via a protein pulldown assay (data not shown). The soybean B-box protein GmBBX62 was confirmed to interact physically with AtBBX32 and was selected as a target for more in-depth characterization of its interaction with AtBBX32. AtBBX32-GmBBX62 interactions were detected using both AtBBX32 full-length and N-terminal bait constructs in yeast two-hybrid screens, suggesting that AtBBX32 interacts with soybean BBX62 through the N-terminal B-box region. Bioinformatic analysis and structure modeling indicate that like AtBBX32, GmBBX62 is a B-box protein that encodes a single B-box domain but lacks a CCT domain (14).

To establish the hypothesis that GmBBX62 is likely to be a biologically relevant interacting partner of AtBBX32 in transgenic soybean plants, we examined the expression patterns of GmBBX62 and the soybean homologs of AtBBX32, GmBBX52, and GmBBX53, and we tested their ability to physically interact. GmBBX62 showed high expression correlation with GmBBX52 and GmBBX53 (supplemental Table S1) and demonstrated physical interaction with GmBBX53 in a yeast two-hybrid screen (data not shown), suggesting that it may be directly involved in an existing protein complex in soybean with which AtBBX32 also associates.

**AtBBX32 and GmBBX62 Are Physically Associated in Vivo**—To further validate the interaction between AtBBX32 and GmBBX62, we tested the ability of AtBBX32 to associate with GmBBX62 in vivo. AtBBX32::GFP and FLAG::GmBBX62 fusion proteins were transiently co-expressed in soybean pro-
toplasts. A co-IP experiment was performed with the co-expressed soybean protoplast extracts using an antibody against GFP. The protein complex was captured via the immobilized GFP antibody, and the associated components within the complex were detected via anti-FLAG antibody and visualized using phycoerythrin-conjugated reporter molecules. As shown in Fig. 1A, the median fluorescence intensity from cell extracts co-expressing AtBBX32::GFP and FLAG::GmBBX62 is significantly higher (6-fold) than the control samples, including nontransfected cells and cell extracts lacking either AtBBX32::GFP or FLAG::GmBBX62. As expected, low fluorescence signals for the single gene construct samples did not show a significant difference from nontransfected cell extract, suggesting that these signals represent nonspecific binding. We also performed a reciprocal co-IP experiment with anti-FLAG antibody-immobilized beads. The presence of AtBBX32::GFP within the complex was detected by anti-GFP antibody. Similarly, the fluorescence intensity for the co-expressed cell extract exhibited a 2.5-fold increase compared with the controls (Fig. 1B). The results indicate that AtBBX32 and GmBBX62 physically interact in soybean cells, further demonstrating the biological relevance of GmBBX62 as a partner of AtBBX32.

**AtBBX32 Binds GmBBX62 in Vitro**—In addition to demonstrating that AtBBX32 and GmBBX62 bind in vivo, we sought to further characterize the interaction biochemically. To that end, we assayed the binding of AtBBX32 to GmBBX62 using in vitro pulldown and ELISA-based protein-protein interaction assays.

Production of soluble B-box proteins is challenging from heterologous expression systems, such as in *E. coli* (12). We observed that soluble full-length AtBBX32 protein can be expressed by coupled transcription/translation in a wheat germ cell-free system. Wheat germ-based eukaryotic cell-free systems have been shown to be applicable for both functional and structural analyses of proteins (26–30). Therefore, full-length WT and mutant AtBBX32 proteins for use in the pulldown- and ELISA-based protein binding assays described below were produced using a wheat germ cell-free system.

**Pulldown Assay**—Purified FLAG::GmBBX62 was bound to anti-FLAG M2-agarose resin and incubated with soluble protein extracts from wheat germ lysate expressing AtBBX32. The beads were pelleted and washed, and the bound protein complexes were eluted using SDS-PAGE loading buffer. The eluted protein complexes were identified by Western blotting analysis. As expected, FLAG::GmBBX62 was detected with anti-FLAG antibody from FLAG::GmBBX62 beads but not from the negative controls (Fig. 2B), demonstrating that FLAG::GmBBX62 can bind to AtBBX32 in vitro.

**ELISA-based Protein-Protein Binding Assay**—Plate-based protein-protein binding assays provide quantitative, rapid, and reproducible analysis of multiple samples for characterization of protein-protein interactions in vitro (20, 24, 25). To enable quantitative and statistical analyses of the effects of mutations in AtBBX32 on the AtBBX32-GmBBX62 interaction, we developed an ELISA-based Assay. We conducted a titration experiment with various concentrations of AtBBX32. Purified FLAG::GmBBX62 was immobilized in 96-well plates and then incubated with wheat germ-produced soluble AtBBX32. As shown in Fig. 3, AtBBX32 and GmBBX62 showed dose-response binding using different amounts of AtBBX32 at a fixed concentration (300 ng/well) of purified FLAG::GmBBX62. A dose response was observed for the entire concentration range tested in the experiment. As expected, this dose-response binding was absent for empty vector negative control samples. Statistical analysis revealed that binding sig-
Although there is no plant B-box structure reported, several B-box structures from other species have been determined (34). We generated a computational model of the structure of the AtBBX32 B-box using an NMR structure ensemble (Protein Data Bank code 2D8V) of a type-I B-box from mouse as a structural template, although its structure shows low sequence identity (29%) with AtBBX32 B-box. As shown in Fig. 4A, the structural model successfully recapitulates the two metal-binding sites in the B-box of AtBBX32. The first metal-binding site consists of conserved residues Cys-5, Cys-8, Cys-24, and Cys-27. The second metal-binding site contains conserved residues, Cys-16, Asp-19, His-32, and His-41. If the B-box domain is required for binding of AtBBX32 to GmBBX62, a mutation to any of these residues, which is likely to disrupt the structure of the B-box, may lead to reduction in the protein binding activity.

We tested this hypothesis using deletion and site-directed mutational analysis of AtBBX32. The deletion of N-terminal 1–17 amino acids (ΔN17) eliminated both predicted metal-binding sites and resulted in a loss of the binding to GmBBX62 (Fig. 4B). Similarly, when we disrupted predicted metal binding by individually substituting the metal binding residues Cys-5, Cys-16, and Asp-19 with alanine, we found a dramatic reduction in the binding activity (Fig. 4B). However, a 19-amino acid deletion outside the B-box of AtBBX32 (Δ53–72) did not significantly affect either the predicted metal binding domain or the binding to GmBBX62 (Fig. 4B). These results demonstrate that putative Zn$^{2+}$-binding residues of the B-box region are critical in the binding of AtBBX32 to GmBBX62.

To further examine whether nonmetal-binding residues within the B-box region also play a role in the interaction between AtBBX32 and GmBBX62, additional site-directed mutants were generated based on the modeled structure and tested via the ELISA binding assay. Based on the model, we identified an intra-molecular salt bridge (Asp-28 to Arg-43) located near the second metal-binding site (Fig. 5A). The formation of salt bridges has been reported to stabilize local structures and to contribute to unfolding kinetics by introducing electrostatic attraction (35–37). It can provide conformational specificity and contribute to molecular recognition and catalysis (36). The disruption of a salt bridge would be predicted to impair protein folding and stability, and thereby interfere with protein-protein interaction. In this study, several site-directed mutants were tested to validate this prediction. A D28A substitution in AtBBX32 should remove the negative charge and therefore the salt bridge. A D28R substitution should disrupt the salt bridge via introducing a repulsive interaction. Both AtBBX32 mutants showed a 40–50% reduction in binding activity with GmBBX62 (Fig. 5B). Similarly, substitution of Arg-43 to histidine should decrease the electrostatic attraction and is predicted to destabilize the structure. Substitution of Arg-43 with histidine (R43H) resulted in a 77% reduction in the binding to GmBBX62 (Fig. 5B). Based on the structural model (Fig. 5A), Asp-28 is in the buried region and Arg-43 side chain points into the buried region that forms a salt bridge with Asp-28. When mutating Asp-28 to alanine, a small hydrophobic residue is still buried inside but no longer forming a salt bridge interaction with Arg-43. The smaller residue in the hydrophobic core destabilizes the structure. The R43H mutation, however, likely moves the R43H side chain to the surface, causing a
buried single charge (Asp-28) in the hydrophobic core. This change can be more significant than that of leaving a small hydrophobic core. This could be a reason why R43H had a greater reduction in the binding activity than that for Asp-28 mutants (Fig. 5B).

By swapping the charges, the double mutation variant of D28R/R43D would be expected to restore the salt bridge and therefore stability. Consistent with this hypothesis, the D28R/R43D mutant retained 85% of WT AtBBX32 binding activity (Fig. 5B), further supporting an important role for the Asp-28 to Arg-43 salt bridge in maintaining the structural integrity of B-box and the binding of AtBBX32 to GmBBX62.

A double mutation of D19A/D28A in AtBBX32 would be expected to cause a greater decrease in protein stability via simultaneous disruption of the predicted metal-binding motif and salt bridge, and accordingly it showed the highest (82%) reduction in binding activity, compared with a 41, 50, or 77% reduction for single mutants D28A, D19A, or R43H (Figs. 4B and 5B). Taken together, we provide structural modeling and biochemical evidence demonstrating that the B-box region in AtBBX32 plays a critical role in mediating a physical interaction between AtBBX32 and GmBBX62 in vitro.

Identification of Interface Residues, His-15 and Arg-44 in Modeled AtBBX32-GmBBX62 Complex—Identification of the protein-protein binding interface and detection of specific amino acid residues that can contribute to the specificity and affinity of protein interactions are essential for better understanding complex formation and protein function (38). In an effort to identify the interface of AtBBX32-GmBBX62 interaction, we constructed structural models of the AtBBX32-GmBBX62 complex. Several structural templates were chosen from the Protein Data Bank. The templates include B-box heterodimers and a RING heterodimer. Both the RING and B-box domains contain cysteine-rich zinc-binding motifs and mediate protein-protein interactions (34). The B-box models of AtBBX32 and GmBBX62 were superimposed onto the selected structure templates. The GmBBX62 B-box structure was modeled after the AtBBX32 B-box model because these two B-box region sequences (1–51 amino acids) are 45% identical. Interfaces that assume different orientations were predicted. As shown in the model of the protein complex (Fig. 6A), residue His-15 of AtBBX32 appears to be at a predicted interface. Mutating His-15 to alanine is expected to decrease the interaction of AtBBX32 with GmBBX62 due to a reduction in size of the side chain at the interface; this is confirmed through the binding assay showing 27% reduction in the binding (Fig. 6B).

From the model (Fig. 6A), residue Arg-44 of AtBBX32 also appears to be at the interface interacting with residue Glu-17 of GmBBX62. This possible inter-molecular salt bridge is predicted to stabilize the protein-protein interaction. The mutation R44A is proposed to remove the predicted salt bridge, therefore decreasing protein-protein interaction. Similar to H15A, mutation of Arg-44 to alanine resulted in a significant
29% reduction ($p < 0.05$) in the binding to GmBBX62 (Fig. 6B), supporting the significance of the predicted salt bridge at the binding interface. Further sequence alignment among soybean B-box homolog proteins reveals that the Arg-44 is unique to the BBX32 subclade, and Glu-17 is unique to the GmBBX62 subclade. These unique residues may impact specificity of the AtBBX32-GmBBX62 interaction.

Mutations designed to impact the binding interface between AtBBX32 and GmBBX62, including the single mutations H15A and R44A, and the double mutation H15A/R44A, reduce the binding activity only by about 30%, although the reduction is significant at the $\alpha$ level of 0.05 (Fig. 6B). In a comparison, mutations to destabilize the B-box structure, both at the predicted metal-binding motif and the intramolecular salt bridge, tend to decrease protein-protein interactions more drastically than those destabilizing the binding interface. These results suggest that interactions at the binding interface may play a bigger role in providing appropriate specificity to the interaction, as opposed to contributing to the overall stability of the structures. The decrease in binding energy brought by a single mutation at the interface may be partially compensated by backbone relaxation and changing the side chain confor-
tions from residues surrounding the mutated position. Future structure-function studies on AtBBX32-GmBBX62 complex would provide clearer insight into the molecular determinants of the protein recognition.

Further Confirmation of the B-box Binding Activity Using Purified AtBBX32-B-box Domains—To further confirm B-box domain binding activity between AtBBX32 and GmBBX62, the relevant B-box regions were produced, purified, and analyzed at the secondary structure level. The B-box domains (residues 1–52) from WT AtBBX32 and the C5A and H15A/R44A protein variants were designated AtBBX32(1–52), AtBBX32(1–52)-C5A, and AtBBX32(1–52)-H15A/R44A, respectively.

Circular Dichroism (CD) spectroscopy was used to analyze the secondary structure of the purified B-box domains. The far-UV CD spectrum of the wild-type N-terminal AtBBX32(1–52) fragment shows a well-defined secondary structure with a double minima at 208 and 222 nm, characteristic of the presence of substantial \(\alpha\)-helical structure (Fig. 7, solid line), and consistent with the modeled structure. AtBBX32(1–52)-H15A/R44A, which contains mutations predicted to affect the binding interface between AtBBX32 and GmBBX62, has a CD spectrum (Fig. 7, dashed line) that also shows an \(\alpha\)-helical signature, suggesting a well-folded structure that is consistent with the structural modeling presented above. The mutant H15A/R44A B-box shows an overall secondary structure similar to that of wild-type B-box AtBBX32(1–52), with a slight increase at 222 nm (Fig. 7). In contrast, the CD spectrum of mutant AtBBX32(1–52)-C5A shows a loss of minima at 208 and 222 nm (Fig. 7, dotted line), indicating disruption of the secondary structure of the B-box domain, as consistent with structural prediction.

The in vitro binding of the wild-type and mutant B-box domains with GmBBX62 is consistent with binding activity obtained using full-length protein (Figs. 4 and 6). AtBBX32(1–52) shows binding to GmBBX62 in an ELISA binding assay (Fig. 8), demonstrating that the B-box domain alone is sufficient to interact with GmBBX62. As shown in Fig. 8, both AtBBX32(1–52)-C5A and AtBBX32(1–52)-H15A/R44A protein variants show significant reduction (\(p < 0.05\)) in binding activity with GmBBX62 relative to wild-type AtBBX32(1–52). These results indicate that either a disruption of the secondary structure of the B-box, due to a loss of a zinc coordination residue, or a loss of critical residues in the binding interface can impact AtBBX32-GmBBX62 interaction. To our knowledge, this study is the first detailed biochemical and secondary structural characterization of a purified B-box domain from a plant B-box protein.

Summary—Protein complexes are dynamic structures that assemble, store, and transduce biological information. Protein-protein interactions are integral to virtually all processes in a living cell. Elucidating both individual protein associations and complex protein interaction networks adds to the understanding of the molecular mechanisms of biological functions. Previous studies have shown that AtBBX32 plays a role in HY5-associated light signaling in *A. thaliana* (10) and that constitutive expression of AtBBX32 in soybean results in a consistent yield increase (14). It has been proposed that AtBBX32 functions as part of a multiprotein complex as a transcriptional accessory protein (10). However, no biochemical function has been definitively ascribed to the B-box motif in AtBBX32. In this study, we have carried out yeast two-hybrid screens to identify proteins that are likely to constitute components of
AtBBX32 complexes in transgenic soybean, and we have performed protein computational modeling and mutational analysis to begin to elucidate the role of the B-box region.

We identified a biologically relevant partner protein, GmBBX62, from soybean and characterized its interaction with AtBBX32. The results obtained from an in vivo co-IP assay clearly demonstrate the biochemical association of AtBBX32 with GmBBX62 in extracts of soybean protoplasts in which the two proteins were co-expressed. Data from in vitro protein pulldown and ELISA-based assays provide additional support for a physical interaction between these two proteins. Deletion of the first 17 amino acids at the N terminus of AtBBX32 diminishes the binding to GmBBX62 by 80%. However, deletion of a 19-amino acid sequence (residues 53–72) that lies outside the B-box region appears to have no effect on binding to GmBBX62. Substitution of metal-binding residues Cys-5, Cys-16, or Asp-19 to alanine results in a dramatic reduction in the binding of AtBBX32 to GmBBX62. A similar reduction in AtBBX32 binding activity was observed when the predicted Asp-28 to Arg-43 salt bridge was disrupted by mutating Asp-28 to alanine or arginine or mutating Arg-43 to histidine. These results suggest an essential role of the B-box region in mediating AtBBX32-GmBBX62 interactions. Furthermore, computational modeling of the AtBBX32-GmBBX62 complex and mutational analysis indicate an important role for predicted interface residues His-15 and Arg-44 of AtBBX32 in mediating specificity in the interaction between the two proteins and further supports the hypothesis that the two proteins interact via their B-box domains.

In conclusion, we have provided in vivo and in vitro biochemical evidence demonstrating that AtBBX32 interacts physically and functionally with GmBBX62. This interaction is mediated through the AtBBX32 B-box domain. To our knowledge, this study represents the first report of a detailed biochemical and biophysical characterization of plant B-box protein interactions via protein structural modeling and mutational analysis. Given that the expression of AtBBX32 can improve soybean grain yield, we propose that the interaction between AtBBX32 and GmBBX62 plays a role in modulation of soybean reproductive development.

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