

LATERAL ORGAN BOUNDARIES defines a new family of DNA-binding transcription factors and can interact with specific bHLH proteins

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Received June 25, 2007; Revised September 15, 2007; Accepted September 17, 2007

ABSTRACT

Conserved in a variety of evolutionarily divergent plant species, *LOB DOMAIN (LBD)* genes define a large, plant-specific family of largely unknown function. *LBD* genes have been implicated in a variety of developmental processes in plants, although to date, relatively few members have been assigned functions. *LBD* proteins have previously been predicted to be transcription factors, however supporting evidence has only been circumstantial. To address the biochemical function of *LBD* proteins, we identified a 6-bp consensus motif recognized by a wide cross-section of *LBD* proteins, and showed that **LATERAL ORGAN BOUNDARIES (LOB)**, the founding member of the family, is a transcriptional activator in yeast. Thus, the *LBD* genes encode a novel class of DNA-binding transcription factors. Post-translational regulation of transcription factors is often crucial for control of gene expression. In our study, we demonstrate that members of the basic helix–loop–helix (bHLH) family of transcription factors are capable of interacting with *LOB*. The expression patterns of *bHLH048* and *LOB* overlap at lateral organ boundaries. Interestingly, the interaction of *bHLH048* with *LOB* results in reduced affinity of *LOB* for the consensus DNA motif. Thus, our studies suggest that *bHLH048* post-translationally regulates the function of *LOB* at lateral organ boundaries.

INTRODUCTION

The *Arabidopsis LATERAL ORGAN BOUNDARIES (LOB)* gene is expressed at the boundaries of lateral

organs during vegetative and reproductive plant development (1). *LOB* encodes a protein containing a conserved amino acid domain of unknown function, termed the *LOB* domain. The *LOB* domain contains a motif resembling a zinc finger and another similar to a leucine zipper. However, both motifs have atypical spacing and until now, the biochemical function of the *LOB* domain has not been defined, although proteins containing *LOB* domains have been assumed to be transcription factors (2). The *Arabidopsis* genome encodes 43 proteins containing a *LOB* domain. *LOB DOMAIN (LBD)* genes are found only in plants, suggesting they may regulate plant-specific processes (1).

While the biological role of *LBD* genes is poorly understood, a number of developmental functions have recently been described. *ramosa2 (ra2)*, the presumed maize ortholog of *LOB*, has been shown to regulate inflorescence architecture (3). *ra2* is expressed in axillary meristem anlagen rather than organ boundaries, suggesting that the functions of *ra2* and *LOB* may have diverged. The *Arabidopsis LBD* gene *ASYMMETRIC LEAVES2 (AS2)* functions in the specification of adaxial/abaxial organ polarity and also negatively regulates expression of *KNOX* transcription factors in lateral organs (4–8). The maize *AS2* ortholog, *indeterminate gametophyte*, is also required to prevent *KNOX* gene expression in lateral organs and in addition, functions to limit proliferation of the female gametophyte (9). *JAGGED LATERAL ORGANS* is essential for *Arabidopsis* embryo development (10). Several *LBD* genes have been implicated in the regulation of auxin signaling. *Crown rootless1 (CrI1)/Adventitious rootless1 (ArI1)* regulates crown root formation in rice and is directly regulated by an AUXIN RESPONSE FACTOR (ARF) protein (11,12). The maize *CrI1* ortholog, *rootless concerning crown and seminal roots*, is similarly induced by auxin and regulates formation of

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post-embryonic shoot-borne roots as well as embryonic seminal roots (13). The orthologous *Arabidopsis LBD* genes are also needed for lateral root formation and are directly regulated by ARF transcription factors (14,15). Thus, *LBD* genes are emerging as important regulators of developmental processes.

The LOB domain is predicted to contain a secondary structure that resembles a DNA-binding zinc finger (1). The region between the Cys pairs however, is only six amino acids long, relatively short in comparison with those found in other Cys₂/Cys₂ type zinc-finger transcription factors, which typically have significantly longer fingers, ranging from 16 to 21 amino acids in length (16). Therefore, structural analysis of the LOB domain does not reveal a possible role in gene regulation. In this study, we show that *LBD* genes encode DNA-binding proteins that recognize a 6-bp consensus DNA motif. We also provide evidence that a hybrid protein containing LOB fused to the GAL4 DNA-binding domain (BD) can activate transcription in yeast, and that LOB is post-translationally regulated by members of the basic helix-loop-helix (bHLH) family of transcription factors. These data demonstrate that the LBD proteins constitute a novel class of DNA-binding transcription factors.

MATERIALS AND METHODS

Construction of 35S:GFP-LOB construct

The *LOB* coding region was amplified from cDNA, cloned into pGEM T-Easy (Promega, Madison, WI) and sequenced to confirm its integrity (see Supplementary Data—Oligonucleotide Sequences for primer sequences). *LOB* was cloned in-frame into the binary vector pCAMBIA 1300 containing a 35S:GFP cassette (a gift from Natasha Raikhel) to create a GFP-LOB fusion protein of ~48 kDa. This construct was introduced into wild-type *Ler Arabidopsis* plants by floral dip (17).

Recombinant proteins for SAAB and EMSAs

cDNAs were cloned in-frame into the pET21a vector containing N-terminal T7 and C-terminal His tags (Invitrogen, Carlsbad, CA, USA) (see Supplementary Data—Oligonucleotide Sequences for primer sequences). Plasmids were transformed into *Escherichia coli* BL21(DE3) cells for protein overexpression according to manufacturer's protocols (Stratagene, La Jolla, CA, USA). Bacteria were disrupted by sonication and His-tagged proteins were purified from soluble fractions using a Ni²⁺ column (Novagen, Madison, WI). Eluted proteins were desalted through a Sepharose column (Amersham Biosciences Corp., Piscataway, NJ) in 1× EMSA buffer (25 mM Tris-HCl pH 8.8, 50 mM KCl, 1 mM DTT, 2 mM EDTA, 10 mM MgCl₂, 20% glycerol, 0.5% NP-40). Desalted proteins were quantified on a Coomassie Blue-stained denaturing gel compared to a bovine serum albumin (BSA) standard (EMD, San Diego, CA).

Selection and amplification binding assay

Following Ni²⁺-column purification, recombinant T7 and His-tagged LOB-domain (LD) protein (amino acids 1 through 113) was further purified by incubation with a mix of Protein A magnetic beads and T7 Ab (Novagen, Madison, WI) against the N-terminal T7-epitope tag. A total of 200 ng of doubly purified T7-LD-His protein were used to perform the SAAB assay, as previously described (18). The pool of ds oligonucleotides used to perform the SAAB assay contained the sequence 5'-GAGAGGATCCAGTCAGCATG(N)₂₀CTCAGCCTCGAGAATTCCAA-3'.

Electrophoretic mobility shift assays

Ni²⁺-purified proteins were first pre-incubated with 10–50 ng of poly (dI-dC) (Sigma-Aldrich, Steinheim, Germany) in a 1× EMSA binding buffer for 15 min at room temperature to allow for protein-protein interactions. The reactions to assay heterodimerization included 200 μg of BSA to stabilize interactions. Following the addition of 40 fmol of labeled probe, the DNA-protein mixture was incubated at 4°C for 45 min. All protein-DNA complexes were resolved on an 8% (70:1) native polyacrylamide gel (25 mM Tris-HCl, 0.25 M glycine, 2 mM EDTA), except AS2 complexes, which were resolved on a 10% (70:1) polyacrylamide gel. Gels were dried and exposed to X-ray film. EMSAs were performed using two probe sequences recovered from the SAAB assay, annotated as AH3 and AH4. For all proteins except LBD4, AH3 was used. LBD4 EMSAs were performed with AH4, as non-specific binding to AH3 was observed (see Supplementary Data—Oligonucleotide Sequences for probe sequences). Wild-type and mutant probes were generated by annealing complementary oligonucleotides resulting in the formation of dsDNAs with a 5' overhang of 5 Gs on each end. The overhangs were filled in with radioactive ³²P-dCTP and Klenow (NEB, Ipswich, MA) to create labeled probe, or with non-radioactive dCTP to create unlabeled competitor DNA. Probes were purified over a Sephadex G-25 spin column (Amersham Biosciences, UK Ltd, Buckinghamshire, England) and diluted to 40 fmol/μl.

Yeast two-hybrid screen

The full-length *LOB* and *AS2* coding regions were cloned into pAS2-1 (Clontech, Mountain View, CA) to create fusions to the Gal4 DNA-binding domain. The full-length *AS1* coding region was cloned into pACT2 (Clontech, Mountain View, CA) to create a fusion to the Gal4 activation domain. The Gal4 activation domain tagged library was provided by Hong Ma (19). Plasmids were transformed into the yeast strain AH109 (Clontech, Mountain View, CA) for the library screen and into Y190 (Invitrogen, Carlsbad, CA) for subsequent confirmation and reporter gene analysis, using a lithium acetate protocol (20). Yeast cells containing both Gal4 plasmids were plated on -Leu -Trp media to select for the plasmids, and -Leu -Trp -Ade media for stringent selection of protein-protein interactions. Yeast cells expressing

putative interacting proteins were plated on selection -Leu -Trp -His media supplemented with varying concentrations of 3-aminotriazole (3-AT), a competitive inhibitor of the HIS3 gene product, to further analyze the strength of the interactions. Crude plasmid preparations were made from the positive yeast clones with a lyticase isolation protocol (Clontech, Mountain View, CA) and transformed to *E. coli* KC8 (Clontech, Mountain View, CA) for subsequent plasmid isolation and sequencing.

Yeast transcriptional-activation assay

LOB-BD and empty-BD plasmids were transformed into yeast strain AH109 (Clontech, Mountain View, CA) using a lithium acetate protocol (20) and plated on -Trp media to select for the presence of the plasmid. Yeast cells transformed with either LOB-BD or empty-BD plasmids were suspended in 100 μ l of water and 10 μ l of the cell suspension was spotted on -Trp -His plates supplemented with 0, 3 or 5 mM 3-aminotriazole (3-AT) (Sigma-Aldrich, Steinheim, Germany), to eliminate residual expression of the leaky HIS3 reporter gene. For each construct, 16 independent transformants were tested. Yeast were allowed to grow for 6 days on 5 mM 3-AT -Trp -His plates, or 3 days on 3 mM 3-AT -Trp -His plates, and photographed using a Leica MZ12 stereomicroscope. Cell suspensions were simultaneously spotted on -Trp plates for growth in the absence of reporter gene selection.

Pull-down assays

A pGEX-4T-1 vector (Amersham Biosciences, Piscataway, NJ) was converted to a Gateway (Invitrogen, Carlsbad, CA) destination vector for expression of bHLH proteins according to manufacturer's instructions. Entry clones for bHLH cDNAs were provided by the *Arabidopsis* Biological Resource Center (2). Recombinant GST proteins were synthesized in *E. coli* strain BL21(DE3) (Stratagene, La Jolla, CA). Constructs in the pET21a (Invitrogen, Carlsbad, CA) vector containing fusions of the entire LOB coding region or shorter LOB domain sequence were used as templates for *in vitro* transcription and translation using the TNT-T7 system (Promega, Madison, WI). Pull-down experiments were performed using the MagneGST Pull-Down System (Promega, Madison, WI), according to manufacturer's instructions, with addition of 100 μ M lysozyme (Sigma-Aldrich) to the lysis reaction and a sonication step. BSA (EMD, San Diego, CA) at a concentration of 1% was also included in the pull-down reaction.

Promoter:GUS lines and histochemical localization of GUS activity

The *pLOB:GUS* line has been described previously (1). To examine the expression pattern of *bHLH048*, a 1.9 kb fragment containing the entire intergenic region upstream of *bHLH048* was amplified (see Supplementary Data—Oligonucleotide Sequences for primer sequences) and cloned into the binary vector pCB308 (21) using introduced restriction sites to generate a translational fusion between the first eight amino acids of *bHLH048* and GUS. Plant tissues were stained for GUS activity in

5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid and were cleared in 70% (v/v) ethanol as previously described (1).

RESULTS AND DISCUSSION

LOB localizes to the nucleus

To determine the subcellular localization of LOB, an N-terminal fusion to green fluorescent protein (GFP), under control of the ubiquitously expressed Cauliflower Mosaic Virus 35S promoter, was generated. The resulting *35S:GFP-LOB* construct was transformed into wild-type *Arabidopsis* plants. Twenty-eight transgenic plants with high levels of GFP fluorescence showed a dwarf phenotype with minimal fertility (data not shown), similar to the phenotype resulting from ectopic expression of LOB (1). Thus, the GFP-LOB fusion protein appears to be functional. GFP fluorescence was examined in the roots of plants expressing either *35S:GFP* or *35S:GFP-LOB*. *35S:GFP* transgenic plants exhibited GFP fluorescence localized to both cytoplasm and nucleus (Figure 1A) similar to previous reports (22). In contrast, GFP fluorescence was observed primarily in the nucleus of *35S:GFP-LOB* plants, although faint fluorescence was also detected in the cytoplasm (Figure 1A). These data indicate the GFP-LOB fusion protein was mainly targeted to the nucleus, consistent with its predicted role as a transcription factor.

Identification of a DNA-sequence motif bound by LOB

Proteins containing the LOB domain have been classified as transcription factors (2) although a recognizable DNA-binding domain has not been reported (1). To determine whether the LOB domain specifically associates with

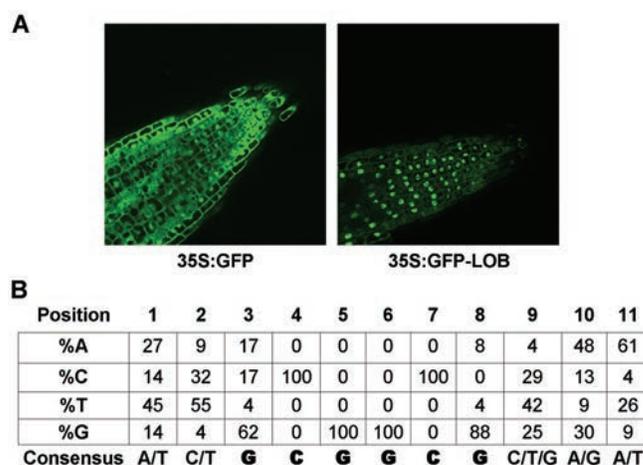


Figure 1. Subcellular localization of LOB and results of selection and amplification binding (SAAB) assay. (A) Root tip of transgenic *Arabidopsis* expressing either *35S:GFP* or *35S:GFP-LOB*. The GFP-LOB translational fusion localized to the nucleus, whereas GFP alone was observed in both the cytoplasm and the nucleus. (B) Summary of the consensus sequence obtained from analysis of 24 unique oligonucleotides identified in a SAAB assay. The LOB domain of LOB recognized the hexamer GCGGCG, with some degeneracy permitted at the 5' and 3' nucleotides.

a DNA motif, we performed a selection and amplification binding (SAAB) assay (23,24). A truncated LOB protein containing only the conserved LOB domain (amino acids 1 through 113) fused to an amino-terminal T7-epitope tag and a C-terminal poly His tag ('T7-LD-His', hereafter 'LD') was incubated with a pool of double-stranded degenerate oligonucleotides. Protein-DNA complexes were coimmunoprecipitated with anti-T7 antibodies. The eluted DNA molecules were amplified by PCR and the resulting products were incubated with LD protein for another cycle of selection. After six rounds of selection and amplification, products of the final PCR reaction were cloned and sequenced. Of 35 clones sequenced, 27 were unique and 24 contained a 6-bp consensus sequence 5'-(G)CGGC(G)-3' we have termed the LBD motif (Figure 1B). Within this consensus, the 4-nt core sequence CGGC was absolutely conserved, with G being the most common nucleotide at both the 5' and 3' flanking positions. Of the 24 unique sequences recovered, 12 contained Gs in both the 5' and 3' positions (GCGGCG), 9 contained a G only at the 3' position (HCGGCG) and 3 contained a G only at the 5' position (GCGGCW). These results indicate that a G must be present at either the 5' or 3' position flanking the CGGC core for LD binding.

LBD proteins specifically bind the LBD motif *in vitro*

Electrophoretic mobility shift assays (EMSAs) were used to verify the DNA-binding results obtained from the SAAB assay. Complementary oligonucleotides identical to one clone identified in the SAAB assay, which contained the full-length LBD motif, were synthesized (see Materials and Methods section). As two central guanines were absolutely conserved in the 24 unique sequences obtained in the SAAB assay, we reasoned that they were likely essential for binding. Therefore, to determine the LD DNA-binding specificity, complementary oligonucleotides containing a mutant version of the motif, in which the central guanines were replaced by adenines to create a GCAACG sequence, were also synthesized (see Materials and Methods section). In EMSAs, the LD protein bound only to labeled probe containing the wild-type motif and not to the mutated version (Figure 2A, lanes 3 versus 6). To verify this association with the wild-type motif, we used antibodies directed against the T7 epitope fused to the N-terminus of LD. LD was pre-incubated with T7 antibodies prior to probe addition. Separation of this DNA-binding reaction by EMSA showed that addition of the T7 antibodies reduced the migration of the LD-DNA complex, confirming that the LD associated with the wild-type probe (Figure 2A, lanes 3 versus 4). The T7 antibodies alone did not associate with either labeled probe (Figure 2A, lanes 5 and 8). Taken together, these results showed that the LD directly associates with the wild-type LBD motif. In order to verify the specificity of LD DNA binding, increasing amounts of unlabeled wild-type or mutant dsDNA were added to the binding reactions containing radiolabeled wild-type probe. In the presence of increasing amounts of unlabeled wild-type dsDNA, the intensity of the LD-DNA complex decreased,

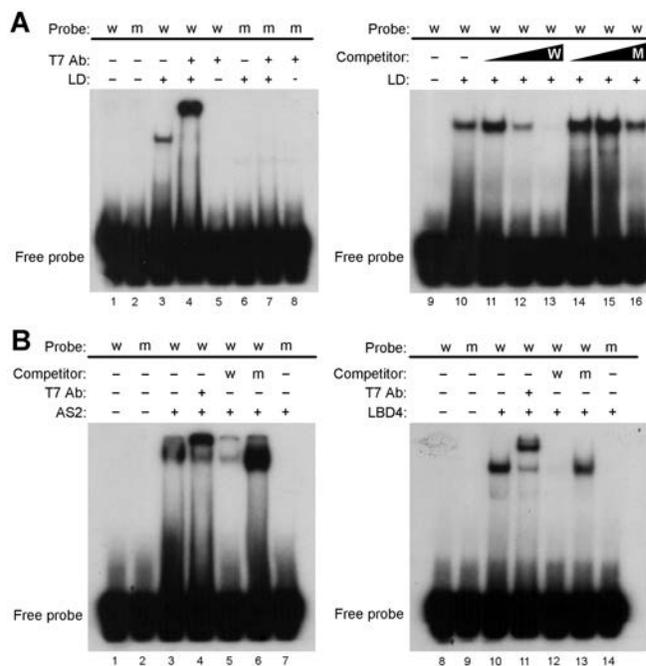


Figure 2. LOB and other LBD proteins specifically bind the LBD motif. (A) DNA-binding assays show that the LD binds specifically to a probe containing the wild-type GCGGCG motif and not to a probe containing the mutated sequence GCAACG (lanes 3, 4 versus 6, 7). Incubation with antibody against the T7 epitope on the LD resulted in the appearance of a super-shifted band (lane 4). Binding was specific, as demonstrated by competition with increasing amounts of unlabeled wild-type (W) or mutant (M) DNA at 50-, 250- or 1250-fold excess over labeled probe (lanes 10–16). (B) AS2 and LBD4, two other LBD proteins, also bound specifically to the wild-type motif (lanes 3 versus 7; lanes 10 versus 14). A 500-fold excess of unlabeled wild-type (w) DNA competed this interaction while mutant (m) DNA did not (lanes 3 versus 5; lanes 10 versus 12).

indicating that wild-type unlabeled DNA effectively competed for LD DNA binding (Figure 2A, lanes 10–13). In contrast, unlabeled mutant dsDNA had little effect on the binding of LD protein to the labeled probe in competition experiments, even at concentrations of 1250× over the radiolabeled wild-type probe (Figure 2A, lanes 14–16). These data demonstrate that the LD specifically recognizes the wild-type GCGGCG LBD motif. Similar binding to the LBD motif was observed using full-length LOB protein, although in initial experiments the protein-DNA complexes did not resolve as well, prompting our decision to use the LD in these EMSAs (data not shown).

To determine whether other LBD proteins are capable of binding the LBD motif, DNA-binding assays were performed with two additional recombinant proteins, T7-AS2-His and T7-LBD4-His. Both proteins bound to the wild-type labeled probe, but not to a mutant labeled probe (Figure 2B, lanes 3 versus 7 and lanes 10 versus 14). To verify these interactions, T7 antibodies were added to the AS2 and LBD4 DNA-binding reactions. EMSA results showed that in both cases, the addition of T7 antibodies resulted in a slower migrating protein-DNA complex, demonstrating that AS2 and LBD4 associate with the wild-type radiolabeled probe (Figure 2B,

lanes 4 and 11). The binding of both AS2 and LBD4 to labeled wild-type probe was efficiently competed with unlabeled wild-type dsDNA, while the mutant dsDNA competitor had little to no effect on DNA binding (Figure 2B, lanes 5 versus 6 and lanes 12 versus 13). Together, these EMSA results showed that AS2 and LBD4 also specifically associate with the LBD motif *in vitro*.

As DNA-binding proteins often bind DNA as dimers (25), we tested for dimer formation during DNA-binding reactions with LOB using two recombinant variants of LOB protein: (1) full-length LOB and (2) truncated LD. Different ratios of full-length LOB and LD were mixed prior to their addition to a DNA-binding reaction containing wild-type probe. LD and full-length LOB both bound to the LBD motif but displayed differences in mobility in EMSAs (Figure 3A, lanes 2 versus 6). When both proteins were included in the DNA-binding reaction, a DNA-protein complex of intermediate mobility was observed (Figure 3A, lanes 3–5 versus lanes 2 and 6), indicating the presence of LOB-LD dimers. These results indicate that homodimers of LOB and LD bind the LBD motif *in vitro*, and confirms the LOB domain is sufficient to mediate protein-protein interactions. With this dimerization data in mind, the absence of additional faster-mobility complexes (e.g. Figure 3A, lanes 2 and 6) suggests that monomers of LOB and LD are either unable to bind the motif, or do so with reduced affinity that is below the limits of detection using EMSAs.

We next examined whether LOB can activate transcription in a heterologous yeast system, using a modified yeast-one-hybrid approach. This approach uses a hybrid protein containing a fusion to the Gal4 DNA-binding domain to test for transcriptional activation of a reporter gene driven by Gal4 UAS sequences. Transcription activation, which is separable from DNA-binding activity (26), has previously been demonstrated using this approach (27–29). We fused full-length LOB in-frame to the Gal4 DNA-binding domain (LOB-BD) and transformed it into the yeast reporter strain AH109. Yeast cells carrying either the empty BD construct or the LOB-BD construct exhibited vigorous growth on -Trp media, which selected for the presence of the plasmid. Yeast cells expressing the LOB-BD fusion protein also exhibited vigorous growth on -Trp -His media at concentrations of up to 5 mM 3-AT (Figure 3B, bottom row). In contrast, yeast carrying the empty-BD construct, while able to grow on -His media in the absence of 3-AT, showed virtually no growth in the presence of 3-AT, which provides a more stringent selection (Figure 3B, top row). These results demonstrate that the LOB-BD fusion protein was able to activate transcription in yeast. These data, coupled with the sequence-specific interaction of LOB with a *cis*-element *in vitro*, support the idea that LOB is likely to function as a transcriptional activator. The LBD family thus represents a new class of DNA-binding transcription factors, although their effects on transcription are likely to vary from protein to protein, perhaps determined by their divergent C-termini.

The *in vitro* DNA-binding and heterologous transcriptional activation studies presented here indicate that

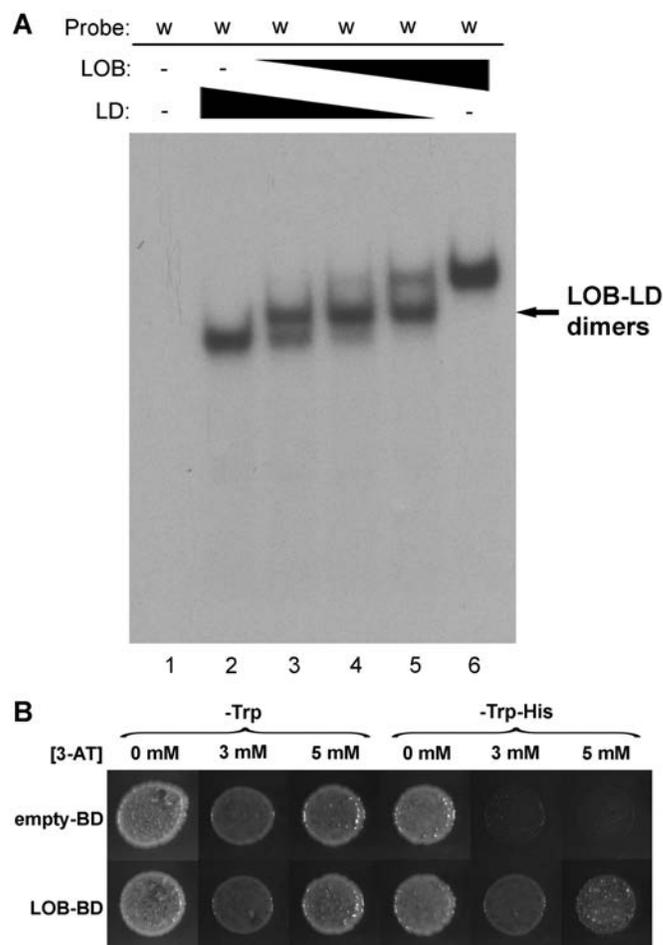


Figure 3. LOB binds the LBD motif as a dimer and is a transcriptional activator in yeast. (A) LD and LOB proteins were pre-incubated at ratios of 60 ng:0 ng, 45 ng:15 ng, 30 ng:30 ng, 15 ng:45 ng and 0 ng:60 ng (lanes 2–6, respectively), then added to a DNA-binding reaction containing wild-type probe. LD and LOB alone bound to the LBD motif but displayed differences in mobility in EMSAs. The presence of both proteins in the DNA-binding reaction resulted in the formation of a DNA-protein complex of intermediate mobility (lanes 3–5). Free probe (lane 1) was run off the gel to achieve maximal separation of the proteins. (B) LOB fused to the Gal4 DNA-binding domain (LOB-BD) and the empty vector (empty-BD) were transformed separately into yeast strain AH109 and plated on -Trp media to select for transformants. LOB-BD transformants showed growth when plated on 3 mM or 5 mM 3-AT -Trp -His selective plates (bottom row), while clones carrying the empty-BD did not (top row), demonstrating that LOB can function as a transcriptional activator in yeast.

the LBD protein family represents a new class of DNA-binding transcription factors that recognize the *cis*-element GCGGCG. The DNA-binding activity is present within the LOB domain, which is conserved throughout the 43 *Arabidopsis* family members as well as LBD proteins in other plant species (1,3,9,11–13,30). DNA-binding activity appears to be conserved throughout LBD proteins, as additional members of the family, LBD3, LBD16 and LBD41, were also found to bind the LBD motif with varying affinity (data not shown). DNA binding was conserved for quite distantly related LBD proteins, including the class II protein LBD41, suggesting that all proteins in this family are likely to bind to the

same DNA motif, similar to other DNA-binding transcription factors (18,31–33), although this may reflect limitations of the *in vitro*-based methodology. Target specificity is likely conferred by a combination of spatial and temporal regulation of expression, modification of activity by the divergent C-termini, and combinatorial control through interaction with other nuclear-localized proteins. Indeed, the *Arabidopsis LBD* genes have diverse expression patterns (1,8,10,15).

LOB interacts with members of the bHLH family

To gain further insight into the biochemical function of LOB, we performed a yeast two-hybrid screen to identify interacting proteins. Full-length LOB was fused in-frame to the Gal4 DNA-binding domain (LOB-BD) and used as bait to screen a yeast two-hybrid library prepared from young flower bud cDNA (19). From $\sim 10^6$ transformants screened, 47 positives were identified. Following elimination of known and likely false positives, seven predicted nuclear proteins remained as potential interactors with LOB. These included several different RNA-binding proteins, a CHROMOd domain helicase and transcription factors of the bZIP and bHLH families. Of all identified positives, the clone containing *bHLH048* (*At2g42300*) promoted the most vigorous growth on minimal selective media lacking both histidine and adenine (Figure 4A and data not shown). Growth of yeast containing LOB-BD and *bHLH048*-AD was equivalent to that of yeast coexpressing AS2-BD and AS1-AD, which have previously been shown to interact (34,35). Under these stringent selection conditions, which require activation of two independent reporter genes, yeast cells containing the LOB-BD fusion construct together with the AD empty vector did not grow on selective media (Figure 4A).

To verify the interaction between LOB and *bHLH048* observed in yeast-two-hybrid experiments, we assayed for interaction between these proteins *in vitro* using a pull-down system. *bHLH048* and other closely related bHLH proteins were expressed in *E. coli* as N-terminal fusions to glutathione S-transferase (GST). Recombinant bHLH proteins were incubated with ^{35}S -methionine (Met)-labeled, *in vitro* translated LOB protein. The protein mixtures were affinity purified with glutathione-coupled particles, separated by SDS/PAGE and analyzed by autoradiography to determine whether radiolabeled LOB protein copurified. While a low-level of interaction was observed with many of the bHLHs, the strongest interactions were observed between LOB and *bHLH048* or the closely related *bHLH060* (*At3g57800*) (Figure 4B, upper panel). In control experiments, little to no radiolabeled LOB associated with GST.

To examine whether the LOB domain alone could mediate this protein–protein interaction, *bHLH048* and *bHLH060* were tested for their ability to interact with ^{35}S -Met labeled, *in vitro* translated LOB domain (amino acids 1 through 113). The LD bound to both proteins in this assay, but with an apparently lower affinity than full-length LOB. Little to no association with GST was observed (Figure 4B, lower panel). These data indicate

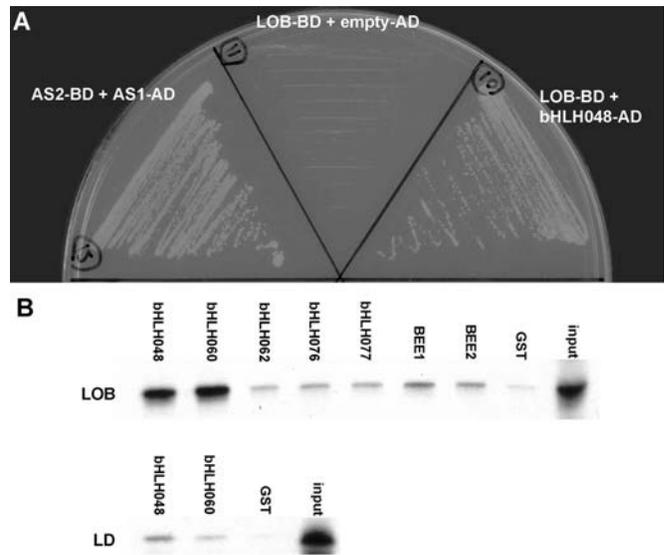


Figure 4. LOB interacts with members of the bHLH family of transcription factors. (A) Yeast two-hybrid assay: LOB fused to the Gal4 DNA-binding domain (LOB-BD) interacts with *bHLH048* fused to the Gal4 activation domain (*bHLH048*-AD) to permit growth on minimal media lacking histidine and adenine, but does not interact with the activation domain alone (empty-AD). A yeast strain coexpressing AS2-BD and AS1-AD was included as a positive control, as AS1 and AS2 have been previously shown to interact (34,35). (B) Pull-down assay with different recombinant GST-tagged bHLH proteins from the *bHLH048* subclade and full-length *in vitro* transcribed/translated ^{35}S -labeled LOB protein (upper panel). The strongest interactions were between LOB and *bHLH048* or the closely related *bHLH060*. *bHLH048* and *bHLH060* also interact with the LD alone (lower panel). *In vitro* synthesized ^{35}S -labeled input protein is shown in the far right lanes.

that, in addition to mediating homodimerization, the LD is sufficient to mediate protein–protein interactions between LOB and other proteins. The weaker interaction between LD and the bHLHs may indicate that the LOB C-terminus functions to stabilize or enhance protein–protein interactions, or could reflect structural differences between the truncated and full-length proteins.

Interaction with *bHLH048* inhibits the DNA-binding activity of LOB

Numerous studies have shown that protein–protein interactions can affect the DNA-binding affinity of transcription factors (18,36,37). With this in mind, we examined whether association with *bHLH048* altered the DNA-binding ability of LOB. LD protein was pre-incubated with increasing amounts of recombinant T7-*bHLH048*-His and the mixture added to an EMSA reaction containing labeled wild-type probe. Addition of increasing amounts of *bHLH048* resulted in a concomitant reduction in the association of LD with the wild-type probe (Figure 5A, lanes 2–5). This reduction in DNA-binding activity was also observed when *bHLH048* was pre-incubated with the LD-T7Ab complex (Figure 5A, lanes 6–9). Control experiments confirmed that *bHLH048* did not bind to the wild type or mutant labeled probe in the presence or absence of T7 antibody (Figure 5B,

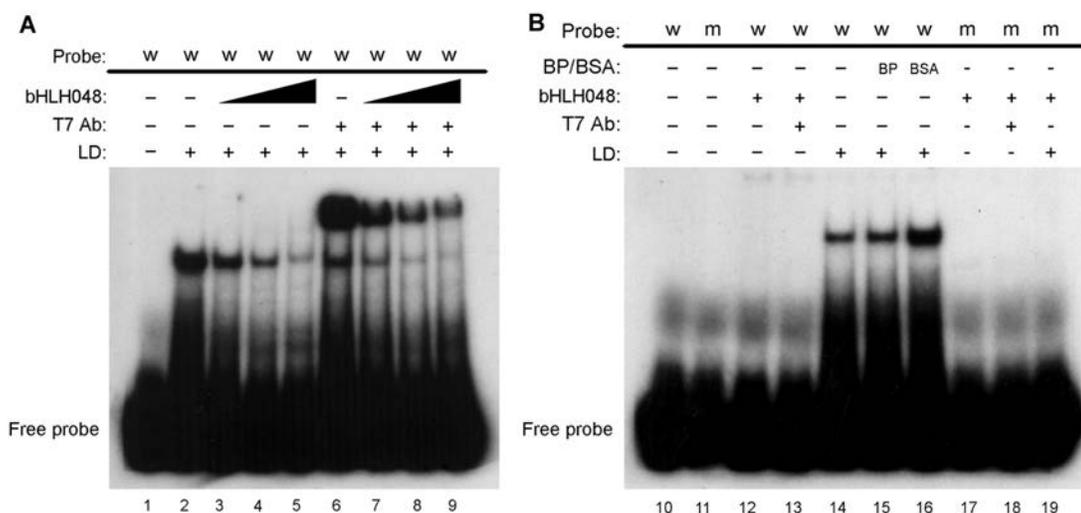


Figure 5. bHLH048 specifically reduces the binding affinity of the LD for the LBD motif. (A) Pre-incubation of 25, 50 or 100 ng of bHLH048 (lanes 3–5) with 25 ng of LD increasingly disrupted DNA binding. The LD–T7–antibody complex showed a similar reduction in DNA-binding affinity (lanes 6–9). (B) bHLH048 did not bind wild-type (w) or mutant (m) labeled probe (lanes 12, 13 and 17, 18). Pre-incubation with 50 ng of BP or BSA did not reduce the DNA-binding affinity of the LD (lanes 14 versus 15 and 16). LD–bHLH048 complexes did not recognize the mutant probe (lane 19).

lanes 12, 13, 17, 18). Furthermore, incubation with two unrelated proteins that were not expected to interact with the LD–BSA or the homeobox transcription factor BREVIPEDICELLUS (BP)—did not result in a reduction in DNA-binding ability (Figure 5B, lanes 14–16), indicating that inhibition is a specific consequence of interaction with bHLH048.

Results from above indicate that the association of bHLH048 with LD dramatically reduces the DNA-binding affinity of LD for the LBD motif. Two simple models can be used to explain these results. The binding of bHLH048 with LD (1) prevents the association of LD with DNA or (2) alters the DNA-binding specificity such that the bHLH048–LD complex recognizes a new DNA motif. To address the latter model, the SAAB assay was used to determine if association with bHLH048 alters the DNA-binding specificity of the LD. For this experiment, bHLH048 was fused to the V5 epitope tag at the C-terminus (bHLH048–V5), which was used to immunoprecipitate the fusion protein with V5-antibody magnetic beads. In this SAAB assay, recombinant bHLH048–V5 was mixed with T7–LD–His then immobilized on V5-antibody magnetic beads. Western blot analysis using T7-antibodies demonstrated that LD protein coimmunoprecipitated with bHLH048–V5 (Supplementary Data, Figure S1). Results obtained from SAAB assays using the LD–bHLH048–V5 complex showed that two discrete populations of DNA motifs were identified (Supplementary Data, Figure S2). One of the motifs contained a palindromic G-box sequence (Supplementary Data, Figure S2B), a motif that is bound by a subset of bHLH proteins (31). The G-box was likely bound by bHLH048–V5, since this motif was identified in SAAB assays performed with bHLH048 alone (Supplementary Data, Figure S2A). The other consensus sequence identified was identical to the LBD motif

(Supplementary Data, Figure S2C). These two motifs were never found on the same DNA fragment, suggesting that LD–bHLH048 complexes did not simultaneously bind to the G-box and LBD motifs. Moreover, a new DNA motif was not retrieved in SAAB assays performed with LD–bHLH048 complexes. In control experiments in which T7–LD–His protein alone was used in the V5-antibody SAAB assay, no DNA was recovered, indicating that this recombinant LD fusion protein did not interact with the anti-V5 antibody non-specifically (data not shown). Taken together, our studies indicate that the LOB–bHLH048 interaction inhibits the ability of LOB to bind the LBD motif and does not alter binding site specificity.

As interacting proteins must have overlapping expression domains, we compared the expression patterns of *LOB* and *bHLH048* using promoter–*GUS* fusion constructs (see Materials and Methods section). As previously described (1), *LOB* expression was observed at organ boundaries throughout development (Figure 6A, C and E). In general, the expression of *bHLH048* was broader than that of *LOB*, but showed significant overlap. Strong *GUS* activity was observed in the shoot apex and lateral root primordia of *pbHLH048:GUS* seedlings (Figure 6B and D). *GUS* expression was also observed in flower pedicels, the base of floral organs and in the stigma (Figure 6F). The observed overlap in expression patterns is consistent with our hypothesis that bHLH048 buffers the impact of LOB on plant development through an inhibition of its DNA-binding ability.

We have demonstrated that LOB interacts with members of the bHLH family and this interaction reduces the affinity of LOB for the LBD motif. Its interacting partner, bHLH048, lacks conserved features required for DNA binding and has been categorized as a non-DNA-binding Group D HLH (31). Group D HLHs are

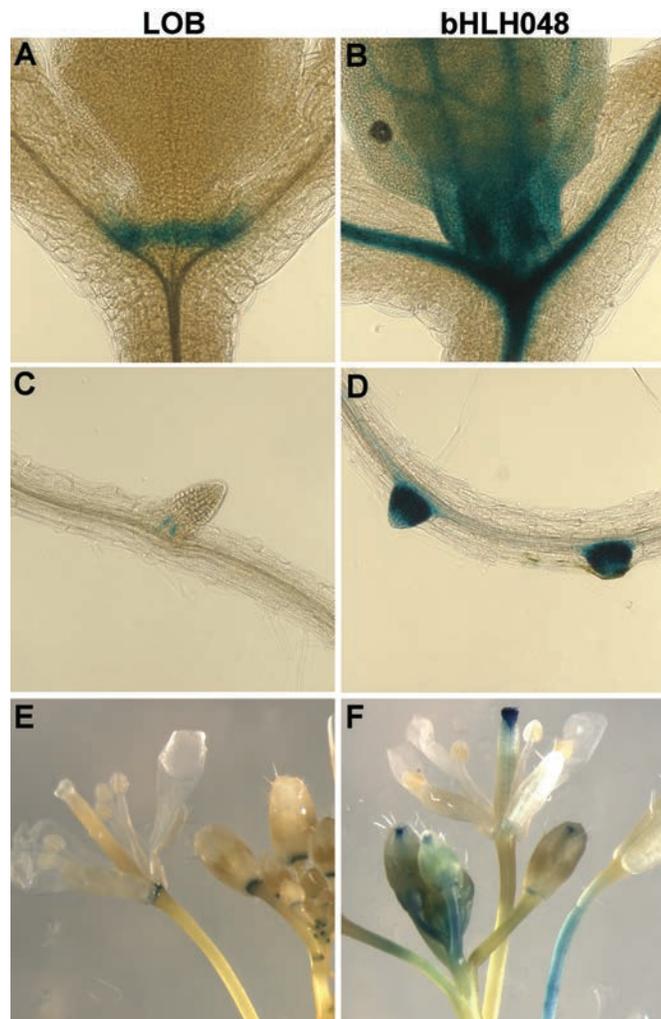


Figure 6. *bHLH048* and *LOB* expression patterns are distinct, but share regions of overlap. (A, C and E) *pLOB:GUS* drives expression in the boundaries of all lateral organs throughout plant development, as previously described (1). (B, D and F) Transgenic plants expressing *pbHLH048:GUS* exhibited GUS activity in the vasculature of leaves and throughout the shoot apex (B), the vasculature of the primary root and incipient lateral roots (D), the base of floral organs (F) and in the stigma, style and ovules of developing flowers (F).

proposed to function by binding to other transcription factors and inhibiting their association with DNA. A classic example of this is the interaction between the HLH Id and the bHLH MyoD in mammals. Heterodimers of Id and MyoD show greatly reduced affinity for the E-box motif, relative to that observed with homodimers of MyoD (38,39). Thus, the observed ability of bHLH048 to inhibit the DNA-binding activity of partner proteins is consistent with its predicted function as an HLH transcription factor. SAAB reactions with bHLH048-V5 proteins recovered oligonucleotides containing G-box motifs, demonstrating that bHLH048 is capable of binding to DNA (Supplementary Data, Figure S2A and S2B). However, only small amounts of DNA were recovered and sequence analysis indicated that the recovered oligonucleotides were low in complexity, suggesting that bHLH048 binds to DNA with low affinity

in vitro. Whether this protein is also capable of binding to DNA *in vivo* remains to be determined.

CONCLUSIONS

The above studies demonstrate that the LBD family of proteins specifically recognizes a 6-bp consensus motif, GCGGCG, and that at least one member, LOB, can activate transcription in yeast. These data define the *LBD* gene family as a novel, plant-specific class of DNA-binding transcription factors. The conserved LOB domain was found to be sufficient for DNA-binding activity and capable of mediating interactions with other proteins, including the bHLH protein bHLH048. Interestingly, interaction between LD and bHLH048 greatly reduced the affinity of the LD for the LBD motif. Interaction of these two transcription factors may represent a post-translational checkpoint, where the activity of LOB is refined through modulation of its DNA-binding activity by bHLH048. Future work is needed to determine whether post-translational control of DNA binding represents a more general mechanism of regulation of the LBD proteins.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank Hong Ma for the gift of the yeast two-hybrid library, Natasha Raikhel for the gift of the GFP vector and Howard Judelson for the gift of yeast strain AH109. We thank Benjamin Aronson and Gina Broitman-Maduro for helpful suggestions and the Arabidopsis Biological Resource Center for providing clones. This work was funded by National Science Foundation grants (IBN-0318822 and IOS-0420202 to P.S.). Funding to pay the Open Access publication charges for this article was provided by NSF grant (IOS-0420202).

Conflict of interest statement. None declared.

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