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RESEARCH PAPER

Transcriptional repression of *BODENLOS* by HD-ZIP transcription factor HB5 in *Arabidopsis thaliana*

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Abstract

In *Arabidopsis thaliana*, the phytohormone auxin is an important patterning agent during embryogenesis and post-embryonic development, exerting effects through transcriptional regulation. The main determinants of the transcriptional auxin response machinery are AUXIN RESPONSE FACTOR (ARF) transcription factors and AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) inhibitors. Although members of these two protein families are major developmental regulators, the transcriptional regulation of the genes encoding them has not been well explored. For example, apart from auxin-linked regulatory inputs, factors regulating the expression of the *AUX/IAA BODENLOS* (*BDL*)/*IAA12* are not known. Here, it was shown that the HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIP) transcription factor HOMEOBOX PROTEIN 5 (HB5) negatively regulates *BDL* expression, which may contribute to the spatial control of *BDL* expression. As such, HB5 and probably other class I HD-ZIP proteins, appear to modulate BDL-dependent auxin response.

Key words: *Arabidopsis*, auxin, BODENLOS (BDL), embryo, HOMEOBOX PROTEIN 5 (HB5), transcriptional regulation.

Introduction

The proper distribution of auxin as well as the adequate translation of its accumulation into developmental outputs is crucial for normal plant development. During early embryogenesis, auxin is transported from the basal to the apical cell(s) where it induces embryo proper development. Later in embryogenesis, the auxin flux is reversed and auxin accumulates in the hypophysis, triggering root meristem initiation. In addition, cotyledon initiation, which establishes the bilaterally symmetric apical part of the embryo, has also been shown to depend on auxin transport and/or response (Vanneste and Friml, 2009; Lau *et al*., 2012).

Generally, the transcriptional auxin response is controlled by AUXIN RESPONSE FACTOR (ARF) transcription factors and AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) proteins. The latter interact with ARFs and inhibit transcriptional induction by (activating) ARFs. This inhibition is relieved by the auxin-facilitated degradation of AUX/IAAs by the 26S proteasome via interaction with the ubiquitin-ligating SCF$^{TIR1/AFB}$ complex (Lau *et al*., 2008; Chapman and Estelle, 2009). For example, BODENLOS (BDL)/IAA12 and its interacting ARF partner MONOPTEROS (MP)/ARF5 play a pivotal role during the earliest stages of embryonic development. Most prominently, both stabilizing *bdl* and loss-of-function *mp* mutants lack a seedling root and frequently display cotyledon defects (Berleth and Jürgens, 1993; Hardtke and Berleth, 1998; Hamann *et al*., 1999; Hamann *et al*., 2002).

Abbreviations: ABA, abscisic acid; EMSA, electrophoretic mobility shift assay; GUS, β-glucuronidase.

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AUX/IAAs, as negative regulators of (activating) ARFs, are probably not only subject to auxin-mediated degradation for proper plant development but also to spatio-temporal control of expression. The latter is reflected in the restricted expression domain of, for example, *IAA18* (Ploense *et al.*, 2009), *SOLITARY ROOT* (*SLR*)/*IAA14* (Fukaki *et al.*, 2002; Vanneste *et al.*, 2005), *IAA28* (De Rybel *et al.*, 2010) or *BDL* (Hamann *et al.*, 2002). While *BDL* and *MP* seem to be expressed in the whole embryo proper early on, their expression is restricted during further embryo development (Hamann *et al.*, 2002). How this is brought about is not fully known. We recently showed that MP itself is an important regulator of *BDL* expression (Lau *et al.*, 2011), and therefore *BDL* expression might mainly follow *MP* expression. However, *MP* is expressed more broadly than *BDL*. This is most apparent at later embryonic stages and also during post-embryonic development when *MP* is expressed at the basal pole of the embryo and in the root columella cells, respectively, while *BDL* is not expressed there (Hamann *et al.*, 2002; Weijers *et al.*, 2006), implying that there are factors besides MP that regulate *BDL* expression.

The HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIP)-encoding superfamily of homeobox genes is unique to plants and consists of more than 40 members in *Arabidopsis*, which have been divided into four subfamilies (Ruberti *et al.*, 1991; Schena and Davis, 1992; Ariel *et al.*, 2007). HD-ZIP family members are involved in the regulation of meristem activity and patterning, and also in various physiological responses (Harris *et al.*, 2011; Zúñiga-Mayo *et al.*, 2012). In these situations, links between plant hormones and HD-ZIPs have been reported, but these connections are often in the context of drought stress or shade avoidance (Himmelbach *et al.*, 2002; Sorin *et al.*, 2009; Son *et al.*, 2010; Harris *et al.*, 2011).

In this study, we investigate the transcriptional control of the well-characterized *AUX/IAA* gene *BDL*. We identify HOMEOBOX PROTEIN 5 (HB5)/ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 5 (ATHB5), a member of the HD-ZIP I subfamily, as a negative regulator of *BDL* expression. HB5 might as such contribute to the spatial regulation of *BDL* expression, although there appear to be additional negative regulatory influences.

Materials and methods

Plant material and growth conditions

Plants were grown under long-day conditions (16 h light, 8 h dark) at 22 to 24 °C. Seedlings used for expression or phenotypic analyses were grown from surface-sterilized seeds on vertical half-strength MS agar plates containing 10 g l^{-1} of sucrose. *Arabidopsis thaliana* ecotype Columbia, Landsberg *erecta* or Wassilewskija was used. The *HB5* allele *hb5-1* as well as transgenic lines *pBDL::bdl:GUS*, *pBDL::NLS:3×GFP*, *LTP::LhG4*, and *UAS::bdl* have been described previously (Baroux *et al.*, 2001; Johannesson *et al.*, 2003; Dharmasiri *et al.*, 2005; Weijers *et al.*, 2006; De Smet *et al.*, 2010).

Transient activity assays

Transient activity assays were performed as described previously (Lau *et al.*, 2011).

Cloning and constructs

pGreenII/BAR $pBDL_{921}$*::bdl:GUS*, *pGreenII/BAR* $pBDL_{245}$*::bdl:GUS*, and *pGreenII/BAR* $pBDL_{195}$*::bdl:GUS* were constructed by replacing the *BDL* promoter of *pGreenII/BAR pBDL::bdl:GUS* (Dharmasiri *et al.*, 2005) by *Eco*RI/*Pst*I-cut fragments amplified with primers 5'-CCGAATTCAACATAATGATGAATATCTCATCACC-3' and 5'-GGCTGCAGACAACAAGAAGAGAAAGAG-3' (for *pGreenII/BAR* $pBDL_{921}$*::bdl:GUS*), 5'-CCGAATTCGCCATTACAAGACATATGGG-3' and 5'-GGCTGCAGACAACAAGAAGAGAAAGAG-3' (for *pGreenII/BAR* $pBDL_{245}$*::bdl:GUS*), and 5'-CCGAATTCAAATCCTCCTCTCTCTCTC-3' and 5'-GGCTGCAGACAACAAGAAGAGAAAGAG-3' (for *pGreenII/BAR* $pBDL_{195}$*::bdl:GUS*).

To clone *GIIK p3×PF:m35S::NLS:3×GFP*, first *GIIK m35S::NLS:3×GFP* was generated by amplifying *m35S* from *pGreen II 35Smini::NLS:3×EGFP::nost* (Takada and Jürgens, 2007) with primers 5'-TTTCTGCAGCTTCGCAAGACCCTTCCTCTATATAAG-3' and 5'-AAGGATCCATCCCCCGTGTTCTCTCCAAATGAAATG-3', cutting the amplified *m35S* with *Pst*I and *Bam*HI, and inserting the *Pst*I/*Bam*HI-cut *m35S* fragment into *Pst*I/*Bam*HI-cut *pGreenII KAN NLS:3×GFP* (Schlereth *et al.*, 2010). Then *3×PF* was amplified from ligated annealed linkers 5'-AATTCGCCATTACAAGACATATGGGTCCCAATTCTCATCACTCTCTCCACCACCG-3' and 5'-AATTCGGTGGTGGAGAGAGTGATGAGAATTGGGACCCATATGTCTTGTAATGGCG-3' with primers 5'-CGATAAGCTTGATATCCTCGAGGCCATTACAAGAC-3' and 5'-CTTTATTATTTTAATTAAATACTGCAGGGTGGTGGAGAG-3', cut with *Xho*I and *Pst*I, and inserted into *Xho*I/*Pst*I-cut *GIIK m35S::NLS:3×GFP*.

LucTrap p3×PF:m35S was generated by amplifying the *3×PF* fragment from ligated annealed linkers 5'-AATTCGCCATTACAAGACATATGGGTCCCAATTCTCATCACTCTCTCCACCACCG-3' and 5'-AATTCGGTGGTGGAGAGAGTGATGAGAATTGGGACCCATATGTCTTGTAATGGCG-3' with primers 5'-CGATAAGCTTGATATCGGATCCGCCATTACAAGAC-3' and 5'-CTTTATTATTTTAATTAAATAGAGCTCGGTGGTGGAGAG-3', cutting the amplified fragment with *Bam*HI and *Sac*I, and inserting it into *Bam*HI/*Sac*I-cut *LucTrap m35S* (Lau *et al.*, 2011).

LucTrap $4×PF_{36bp}$*:m35S* was generated by amplifying $4×PF_{36bp}$ from ligated annealed linkers 5'-GGCCCGCCATTACAAGACATATGGGTCCCAATTCTCATCACG-3' and 5'-GGCCCGTGATGAGAATTGGGACCCATATGTCTTGTAATGGCG-3' with primers 5'-GTCGACCTCGAGGGGGGGATCCGCCATTACAAGACA-3' and 5'-AGGGCGAATTGGGTACCGAGCTCGTGATGAGAATTG-3', cutting this fragment with *Bam*HI and *Sac*I, and inserting it into *Bam*HI/*Sac*I-cut *LucTrap m35S* (Lau *et al.*, 2011).

pHB5::HB5:3×GFP was cloned by first inserting *Eco*RI/*Pst*I-digested *pHB5*, which was amplified with primers 5'-CCGAATTCAGCATTGGATAAAGGTGTTTGG-3' and 5'-CCCTGCAGCTTGTTTGGTCGGAACA-3', into *Eco*RI/*Pst*I-cut *pGreenIIKAN 3×GFP* (Schlereth *et al.*, 2010), to yield *GIIK pHB5::3×GFP*, and then inserting a *Pst*I/*Bam*HI-cut genomic *HB5* fragment from the start codon to the last codon before the stop codon, which was amplified with primers 5'-CCCTGCAGATGAAGAGATCACGTGGAA-3' and 5'-CCGGATCCCGAATTCCACTGATCGGAG-3', into *Pst*I/*Bam*HI-cut *GIIK pHB5::3×GFP*.

To generate *GIIK pRPS5A::HB5*, *JIT60 HB5*, and *pRSET A HB5*, *HB5* was amplified from cDNA with primers 5'-CCCTGCAGATGAAGAGATCACGTGGAA-3' and 5'-CCGGATCCTTACGAATTCCACTGATCGGAG-3', cut with *Pst*I and *Bam*HI, and inserted into either *Pst*I/*Bam*HI-cut *GIIK RPS5A-tNOS* (Weijers *et al.*, 2006), *Pst*I/*Bam*HI-cut *pJIT60* (Schwechheimer *et al.*, 1998) or *pRSET A* (Invitrogen), in which the multiple cloning site had been modified to allow insertion of *Pst*I/*Bam*HI-cut *HB5*.

JIT60 HB6 was generated by amplifying *HB6* from cDNA with primers 5'-AAAGTCGACATGATGAAGAGATTAAGTAGTTCAGATTCAGTG-3' and 5'-TTGGATCCTCAATTCCAATGATCAACGGTGGAGTAC-3' and inserting the *Sal*I/*Bam*HI-digested fragment into *Sal*I/*Bam*HI-digested *pJIT60* (Schwechheimer *et al.*, 1998).

pBluescript 2×PF$_{36bp}$ was generated by inserting two copies of annealed linkers 5'-GGCCCGCCATTACAAGACATATGGGTCCCAATTCTCATCACG-3' and 5'-GGCCCGTGATGAGAATTGGGACCCATATGTCTTGTAATGGCG-3' into *Bsp*120I-cut *pBluescript*, and *pBluescript 4×mPF$_{36bp}$* by inserting four copies of annealed linkers 5'-GGCCCGCCCTCACAAGACATCTGGGTCCTAGTGCTCATCACG-3' and 5'-GGCCCGTGATGAGCACTAGGACCCAGATGTCTTGTGAGGGCG-3' into *Bsp*120I-cut *pBluescript*.

pHIS3NX PF was created by inserting annealed linkers 5'-AATTCGCCATTACAAGACATATGGGTCCCAATTCTCATCACTCTCTCCACCACCG-3' and 5'-AATTCGGTGGTGGAGAGAGTGATGAGAATTGGGACCCATATGTCTTGTAATGGCG-3' into *Eco*RI-cut *pHIS3NX* (Ouwerkerk and Meijer, 2001). To create *pINT1 PF HIS3*, *PF HIS3* was cut out from *pHIS3NX PF* with *Not*I and *Xba*I, and inserted into *Not*I/*Xba*I-cut *pINT1* (Ouwerkerk and Meijer, 2001). *JIT60 MP* and *LucTrap pBDL* have been described previously (Lau *et al*., 2011).

Plant transformation

Plants were transformed by the floral dip method (Clough and Bent, 1998). Primary transformants were selected on half-strength MS agar plates containing either 15 mg l^{-1} of phosphinothricin or 62.5 mg l^{-1} of kanamycin.

Electrophoretic mobility shift assay (EMSA)

Recombinant HB5 was obtained by expressing *pRSET A HB5* in *Escherichia coli* BL21(DE3)pLysS; the recombinant HB5 was purified. The *PF$_{36bp}$* and *mPF$_{36bp}$* probes for the EMSA were cut out from *pBluescript 2×PF$_{36bp}$* and *pBluescript 4×mPF$_{36bp}$*, respectively, with *Bsp*120I and purified; overhangs were filled with radiolabelled dNTPs by the Klenow fragment. Binding reactions were performed in a total volume of 10 µl, combining 10 000 cpm of radiolabelled probe, 1 µl of binding buffer [250 mM HEPES (pH 7.6), 10 mM EDTA pH 8, 50 % (w/v) glycerol, 10 mM KCl] and 200 ng poly(dI-dC) in the presence or absence of recombinant HB5. Samples were incubated for 30 min at room temperature and loaded on a pre-run polyacrylamide gel.

Yeast one-hybrid screen

A yeast one-hybrid screen was performed as described previously (Ouwerkerk and Meijer, 2001). The *PF* sequence was used as the bait sequence in *pINT1 PF HIS3*.

Expression analysis

For β-glucuronidase (GUS) staining, seedlings were fixed in 90% acetone for 20 min at –20 °C and then washed twice for 10 min in washing buffer [0.1 M phosphate (pH 7.0), 10 mM EDTA, 2 mM $K_3Fe(CN)_6$]. Subsequently, seedlings were incubated in staining buffer [0.1 M phosphate (pH 7.0), 10 mM EDTA, 1 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6.3H_2O$, 1 mg ml^{-1} 5-bromo-4-chloro-3-indolyl glucuronide] in a desiccator for 10 min at room temperature, and then overnight at 37 °C. GUS-stained seedlings were fixed in a 3:1 mixture of ethanol and acetic acid for 1 h, and then washed in 75, 50, and 25% ethanol for 10 min each. For microscopy, seedlings were mounted in chloral hydrate solution (8:3:1 mixture of chloral hydrate, water, and glycerol). GFP fluorescence was analysed in whole-mount preparations of embryos or seedlings. Propidium iodide was used at a concentration of 10 µg ml^{-1}.

Image acquisition

Images were acquired using a confocal laser-scanning microscope (TCS-SP2; Leica), a Zeiss Axiophot microscope or a digital camera (Coolpix 990; Nikon). Images were processed using Adobe Photoshop software.

Data mining

Protein and genomic sequence information used in this study were retrieved from the Arabidopsis Information Resource (http://www.arabidopsis.org), Joint Genome Insitute (http://genome.jgi-psf.org), Brassica Genome Gateway (http://brassica.bbsrc.ac.uk), Plant Transcription Factor Database (http://plntfdb.bio.uni-potsdam.de) and NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence details are given in Supplementary List S1 at *JXB* online.

Phylogenetic analysis

Protein sequences were aligned using CLC DNA Workbench 5.7.1 with the following settings: gap open cost: 10, gap extension cost: 1, end gap cost: as any other, alignment: very accurate. The phylogenetic tree was created using the unweighted pair group method with arithmetic mean (UPGMA) algorithm and a bootstrap analysis with 1000 replicates within CLC DNA Workbench 5.7.1.

Analysis of 5'-upstream sequences

A 1 kb 5'-upstream region was used for each gene of interest and uploaded into mVISTA (http://genome.lbl.gov/vista/mvista/submit.shtml). Analyses were performed using the standard settings (using MLAGAN alignment). Subsequently, individual comparisons against the 1 kb 5'-upstream region of *IAA12*/*BDL* from *A. thaliana* were optimized for each sequence by adjusting the conservation parameters ('0' for minimum *y*-value on the VISTA plot, '50' for minimum length for a conserved non-coding region, and a minimum conservation identity as indicated for the respective comparison as a percentage). A sequence logo was made from manually optimized 50 bp promoter sequence alignments (generated by CLC DNA Workbench 5.7.1) using WebLogo 3 (http://weblogo.threeplusone.com/) (Schneider and Stephens, 1990; Crooks *et al*., 2004).

Statistics

Details of the statistical tests used are given in Supplementary Table S1 at *JXB* online.

Results

Conserved regulatory fragment important for BDL expression

To gain insight into the transcriptional control of *BDL* expression, in addition to the positive control exerted by auxin and MP (Abel *et al*., 1995;; Tian *et al*., 2002; Lau *et al*., 2011), we analysed the *BDL* upstream regulatory region. As *BDL* is involved in crucial developmental processes during embryogenesis and later stages of development, we reasoned that the regulation of its expression is probably evolutionarily conserved. Hence, assuming this conserved regulatory mechanism, the *BDL* promoter would be suitable for a phylogenetic shadowing analysis (Boffelli *et al*., 2003; Yamaguchi *et al*., 2013). In order to delineate the relevant conserved regulatory regions within the *BDL* promoter, we compared promoter sequences across different species. We chose to analyse orthologues of *BDL* and its similarly expressed paralogue *IAA13* (Weijers *et al*., 2005) in *Arabidopsis lyrata* and *Brassica rapa*, two Brassicaceae species closely related to *A. thaliana*, and in the more distant species *Populus trichocarpa* belonging to the Salicaceae; the orthologues were identified by

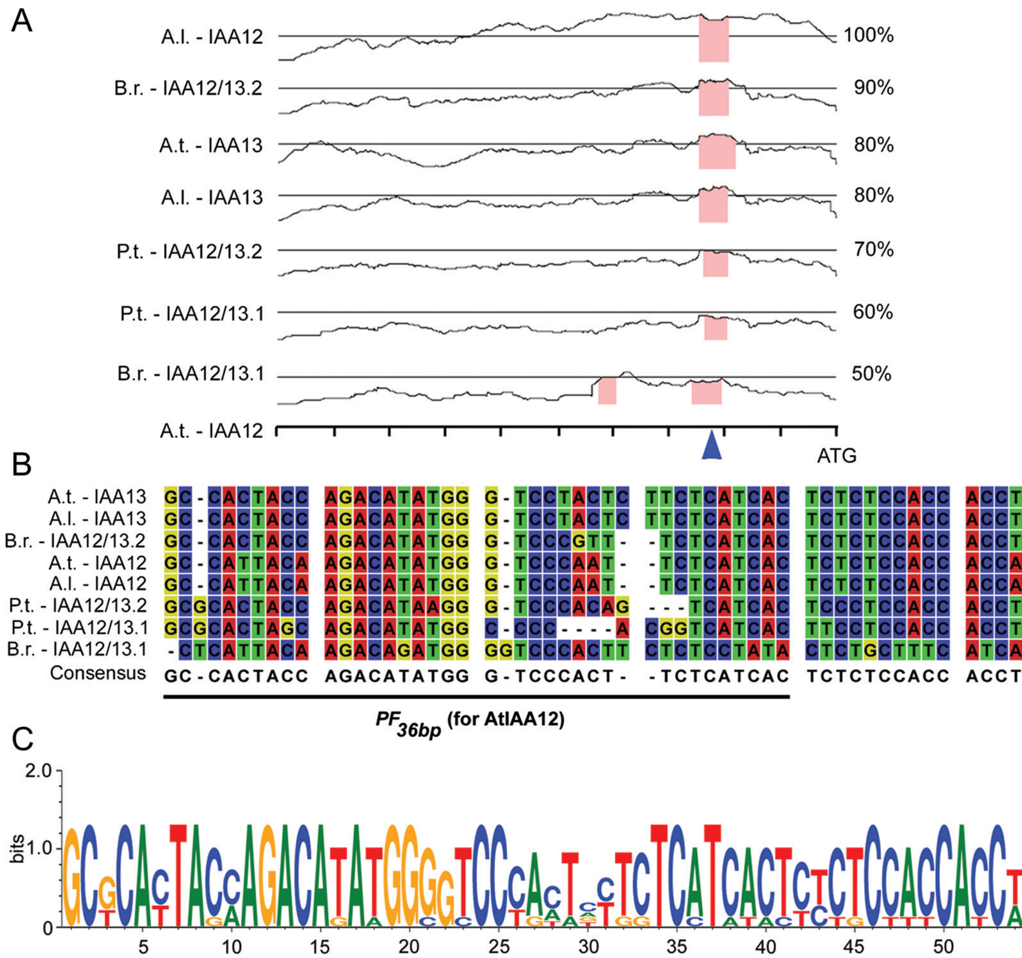

Fig. 1. Identification of a conserved regulatory element in the 5' region of *BDL/IAA12*. (A) mVISTA analysis of 1 kb upstream regulatory regions of the indicated genes with reference to the *AtIAA12* (*BDL*) sequence. Blue arrowhead/pink block, region of conservation. A.l., *Arabidopsis lyrata*; A.t., *Arabidopsis thaliana*; B.r., *Brassica rapa*; P.t., *Populus trichocarpa*. (B) Sequence alignment of a 50 bp stretch of the respective 5' regulatory regions indicated by the blue arrowhead in (A) (corresponding to 245–196 bp upstream of the start codon in *BDL/AtIAA12*). *PF36bp* indicates the fragment used for EMSA (see Fig. 3). (C) Sequence logo of the conserved 50 bp stretch.

database mining and subsequent phylogenetic analyses (Fig. S1 at *JXB* online). In *A. thaliana*, *BDL* is expressed in the apical cell lineage during embryogenesis, and its expression is restricted to the stele in the main root (Hamann *et al*., 2002). A promoter-deletion series for *pBDL::bdl:GUS* revealed that an ~1 kb promoter fragment was sufficient to mimic the *BDL* expression pattern in the root tip (Fig. S2A at *JXB* online). Therefore, we focused our analysis on the 5' region 1 kb upstream of the start codon. Using the mVISTA tool for comparative genomics (Mayor *et al*., 2000; Frazer *et al*., 2004), we identified a conserved 50 bp region in the different 5'-upstream sequences of the homologues from *A. thaliana*, *A. lyrata*, *B. rapa*, and *P. trichocarpa* (Fig. 1A). Sequence alignment of this 50 bp fragment showed its high level of conservation (Fig. 1B, C).

The next step was the functional analysis of this *in silico*-identified conserved region with respect to *BDL* expression in *A. thaliana*. Therefore, we generated specific deletions of the *A. thaliana BDL* promoter. Driving the expression of *bdl:GUS*, a promoter fragment starting 245 bp upstream of the start codon and containing the conserved 50 bp at its 5' end still resulted in normal *BDL* expression and yielded *bdl* mutant phenotypes (*pBDL*$_{245}$*::bdl:GUS*) (Fig. 2A, B). However, by deleting the conserved 50 bp from this promoter fragment, *BDL* expression was reduced and characteristic *bdl* phenotypes were not observed (*pBDL*$_{195}$*::bdl:GUS*) (Fig. 2C, D). Therefore, the highly conserved region that was identified using an *in silico* approach (hereafter referred to as *Promoter Fragment* or *PF*), was also relevant *in planta* for *BDL* expression. To check if *PF* was by itself sufficient for normal *BDL* expression, we fused three copies of this element to *m35S::NLS:3×GFP* (*p3×PF:m35S::NLS:3×GFP*). *p3×PF:m35S::NLS:3×GFP* mimicked the *BDL* expression pattern as visualized by *pBDL::NLS:3×GFP* (Fig. 2E, F and Fig. S2B). However, with neither constructs was it possible to visualize *BDL* expression at the very early stages of embryogenesis. These analyses demonstrated that a fragment of 50 bp is relevant for *BDL* expression and is sufficient to mimic the *BDL* expression pattern, at least in the later stages of embryo development.

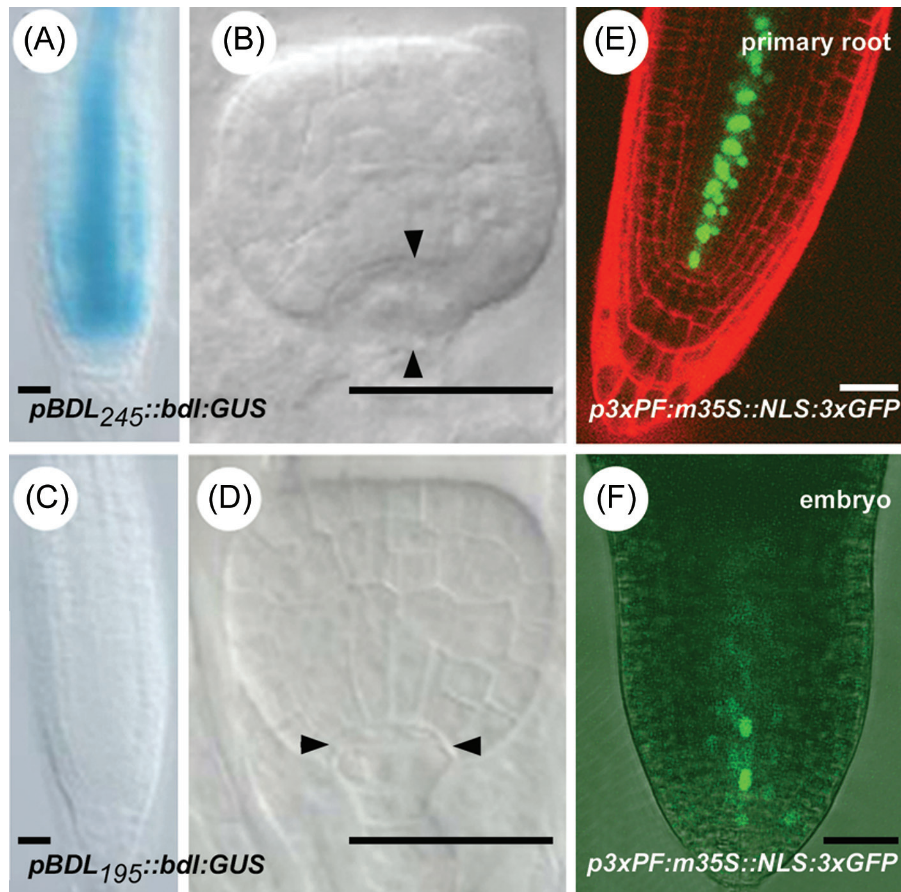

Fig. 2. *pBDL* deletion study. (A) GUS-stained *pBDL245::bdl:GUS* seedling root tip. (B) Hypophysis division defect (vertical instead of horizontal division) in *pBDL245::bdl:GUS* embryos. (C) GUS-stained *pBDL195::bdl:GUS* seedling root tip. (D) Normal hypophysis division in *pBDL195::bdl:GUS* embryos. (E, F) *p3×PF:m35S::NLS:3×GFP* expression in a seedling root counterstained with propidium iodide (E) or in a torpedo-stage embryo (basal part shown) (F). Bars, 25 μm.

HB5 interacts with PF *of the* BDL *promoter*

To identify proteins interacting with *PF*, we performed a yeast one-hybrid screen and isolated several transcription factors as putative *PF* interactors (data not shown). One of these, HB5 (Johannesson *et al.*, 2001, 2003), was chosen for a more detailed analysis because other members of the HD-ZIP family, such as MERISTEM LAYER 1 (ATML1), PROTODERMAL FACTOR 2 (PDF2) and GLABRA 2 (GL2), play important roles in plant development (Ariel *et al.*, 2007).

We confirmed the yeast one-hybrid data by demonstrating that HB5 bound to a 36 bp subfragment of the *PF* element (hereafter referred to as PF_{36bp}) *in vitro* (Fig. 1B and Fig. 3). An EMSA with recombinant HB5 protein revealed a shift of the radiolabelled wild-type probe, and this shift was almost abolished when six mutations were introduced into PF_{36bp} (Fig. 3). Thus, we concluded that HB5 can bind directly to this 36 bp *BDL* promoter fragment.

HB5 accumulates outside the BDL *expression domain*

To assess the biological relevance of this interaction, we monitored *HB5* expression with a *pHB5::HB5:3×GFP* reporter gene. HB5:3×GFP accumulated outside the *BDL* expression domain in the protoderm of the embryo (Fig. 4A, B) and in the epidermis and cortex of the main root tip (Fig. 4C). Thus, the inner cells in which *BDL* is normally expressed in the embryo and in the seedling root (Hamann *et al.*, 2002; Dharmasiri *et al.*, 2005; Weijers *et al.*, 2006) are outside the domain where HB5:3×GFP was detected. This suggested that HB5 is not a positive, but rather is a negative regulator of *BDL* expression.

Expression of bdl *in the epidermis impairs cotyledon development*

Auxin response in the (globular) embryo is important for cotyledon initiation and development (Hamann *et al.*, 1999; Benková *et al.*, 2003; Ploense *et al.*, 2009). Therefore, it could be significant that *BDL* expression gets restricted to the inner cells of the embryo (Hamann *et al.*, 2002), which coincides with the detectable onset of *HB5* expression in protodermal cells at the globular stage during embryogenesis (Fig. 4A). Because in the *hb5-1* knockout mutant (Johannesson *et al.*, 2003) cotyledon formation and embryogenesis in general are not obviously impaired, HB5 is either not the only factor involved in excluding *BDL* expression from the protoderm and/or the

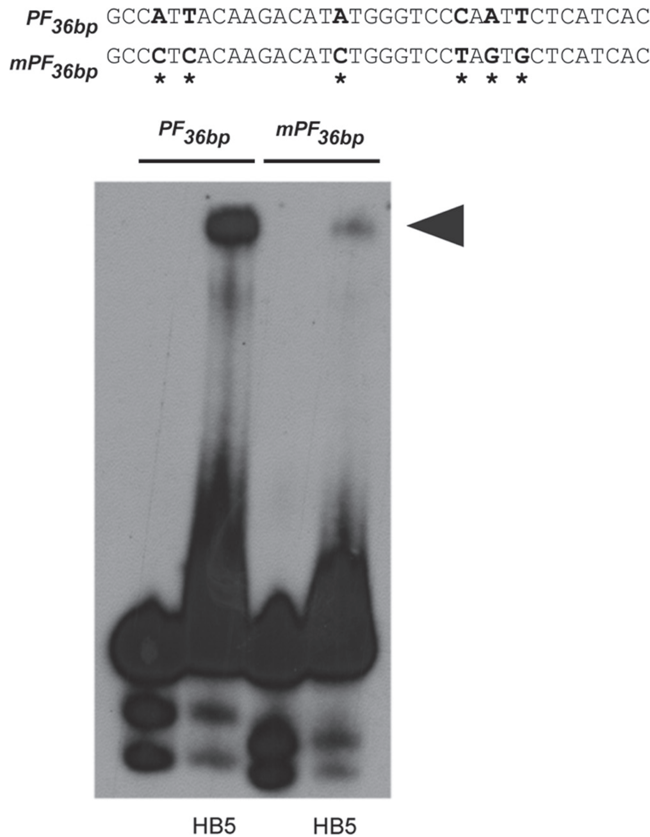

Fig. 3. Direct binding of HB5 to a *BDL* promoter fragment (see Fig. 1). Results of an EMSA with *PF36bp* and *mPF36bp* in the absence or presence of HB5. Mutations are indicated by asterisks and the shifted band by an arrowhead.

effect of possible misexpression of *BDL* in the *hb5-1* mutant is not strong enough to affect cotyledon development. The latter is in agreement with the absence of obvious phenotypes when altering the levels of wild type *AUX/IAA*s—despite higher *BDL* transcript levels, BDL protein levels might actually not increase due to auxin-facilitated BDL degradation.

To circumvent these problems, we expressed stabilized BDL (bdl), which is not prone to auxin-facilitated degradation, ectopically in the developing epidermis, using the protoderm-specific driver line pLTP1 for transactivation of *bdl* (*pLTP1>>bdl*) (Baroux *et al*., 2001; Weijers *et al*., 2006). This resulted in mild effects on cotyledon development in F1 *LTP1>>bdl* seedlings (*n*= 22). For example, seedlings with no cotyledons (1%), with only one fully developed cotyledon (16%), or with cotyledons of different size (9%) were observed, in contrast to the wild-type control (Fig. 5A–C). These defects had their origin in embryogenesis, with embryos not developing two equal-sized cotyledons or appearing cup-shaped, which contrasted to wild-type embryos with two normally developing cotyledons (Fig. 5D–F). These results suggested that HB5 might contribute to the repression of *BDL* expression in the protodermal layer.

Ectopic expression of HB5 *rescues the* bdl *rootless phenotype*

To assess the repressive effect of HB5 on *BDL* expression when expressing *HB5* in the normal *BDL* expression domain, we used a transgenic line expressing stabilized BDL (bdl) from its endogenous promoter (*pBDL::bdl:GUS*). The *pBDL::bdl:GUS* line resembles the originally identified *bdl* line and gives rise to ~29% rootless seedlings (Dharmasiri *et al*., 2005) (Fig. 6A, red bars). *HB5* was ectopically expressed in this *pBDL::bdl:GUS* line via the strong embryo promoter *pRPS5A* (Weijers *et al*., 2001) (*pRPS5A::HB5*) to determine if the rootless seedling phenotype resulting from the non-degradation of bdl could be suppressed. Indeed, ectopic expression of *HB5* in the *pBDL::bdl:GUS* transgenic background reduced the proportion of characteristic *bdl* rootless seedlings in multiple independent transgenic lines variably to a minimum of 9% (Fig. 6A). We observed rescued plants that were homozygous for *pBDL::bdl:GUS* (Fig. 6B, C). One T_1 plant carrying two *pRPS5A::HB5* transgenes was homozygous for *pBDL::bdl:GUS* and segregated only about 35% rootless seedlings (Fig. 6A, blue bar). Taken together, these data

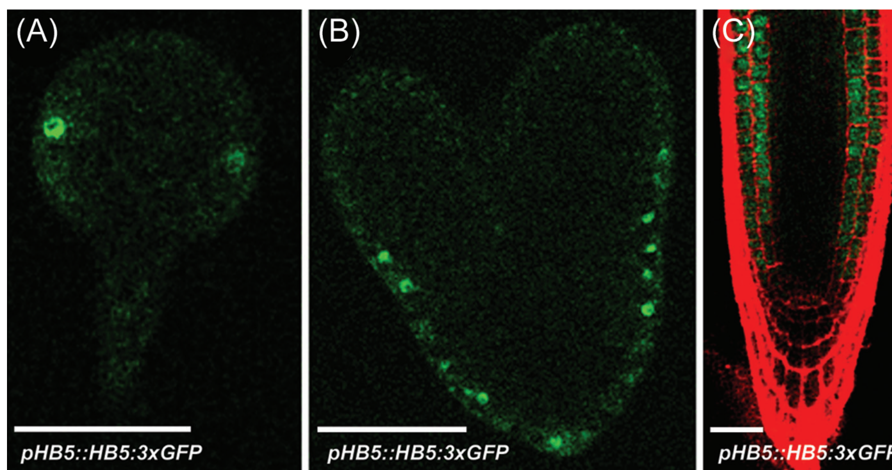

Fig. 4. *HB5* expression. (A–C) *pHB5::HB5:3×GFP* expression (green) in a globular-stage embryo (A), heart-stage embryo (B), and seedling root counterstained with propidium iodide (red) (C). Bars, 50 µm.

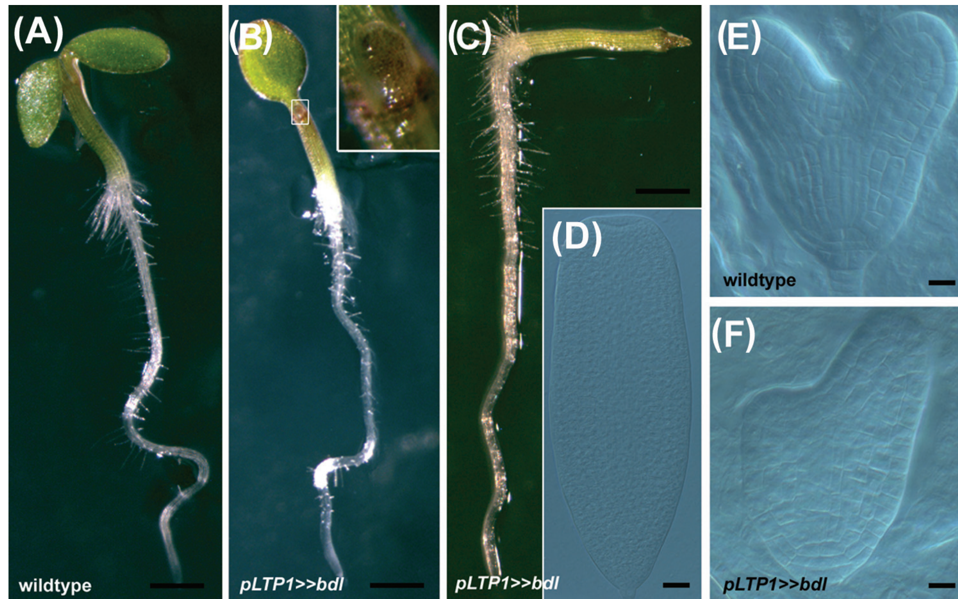

Fig. 5. Transactivation of *bdl* by *pLTP1* results in cotyledon defects. (A–C) Seedlings with cotyledon defects caused by protoderm-specific transactivation of *bdl* expression (B, C) compared with the wild-type (A). Bar, 1 mm. (D–F) Cotyledon development is already impaired in embryogenesis in *pLTP1*>>*bdl* (compare panels D and F with panel E). Bars, 10 μm.

suggested that, *in planta*, HB5 plays a negative regulatory role in controlling the expression of *BDL*.

HB5 represses BDL *expression in protoplasts*

To further support the results on the negative effect of HB5 on *BDL* expression, we investigated this relationship quantitatively, using a well-established luciferase reporter system in protoplasts (Lau *et al*., 2011; Niu and Sheen, 2012). To examine HB5 for *BDL*-repressing activity, we made use of the auxin inducibility of *BDL* expression (Abel *et al*., 1995; Tian *et al*., 2002; Lau *et al*., 2011). Auxin inducibility of *BDL* could be mimicked in protoplasts by *p3×PF:m35S::LUC* and *p4×*PF_{36bp}*:m35S::LUC* (Fig. 7A, B), where copies of these *PF*s

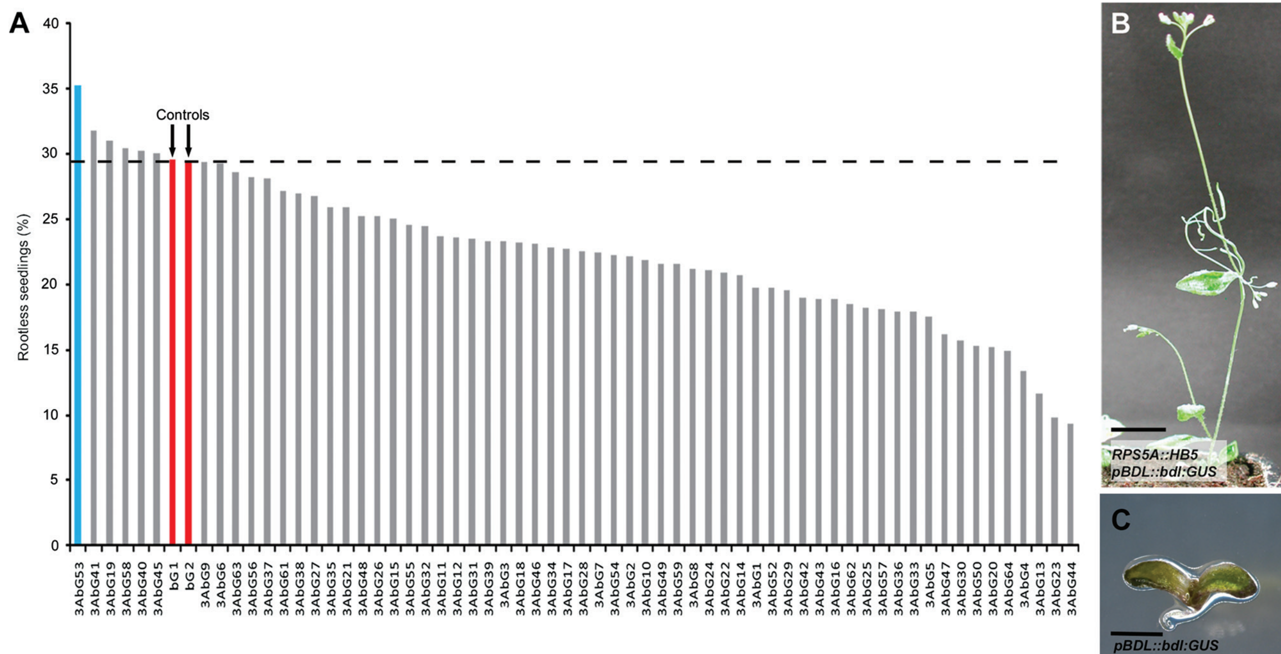

Fig. 6. Rescue of the *bdl* rootless phenotype by *HB5* overexpression. (A) Segregation analysis of independent *pRPS5A::HB5* transgenic lines in the *pBDL::bdl:GUS* background (T_2 seedlings were counted). Bars for the *pBDL::bdl:GUS* controls are shown in red and the bar for the homozygous *pBDL::bdl:GUS* line with two *pRPS5A::HB5* transgenes in blue. (B) Five-week-old plant homozygous for *pBDL::bdl:GUS* 'rescued' by *pRPS5A::HB5*. Bar, 1 cm. (C) One-week-old rootless *pBDL::bdl:GUS* plant. Bar, 1 mm.

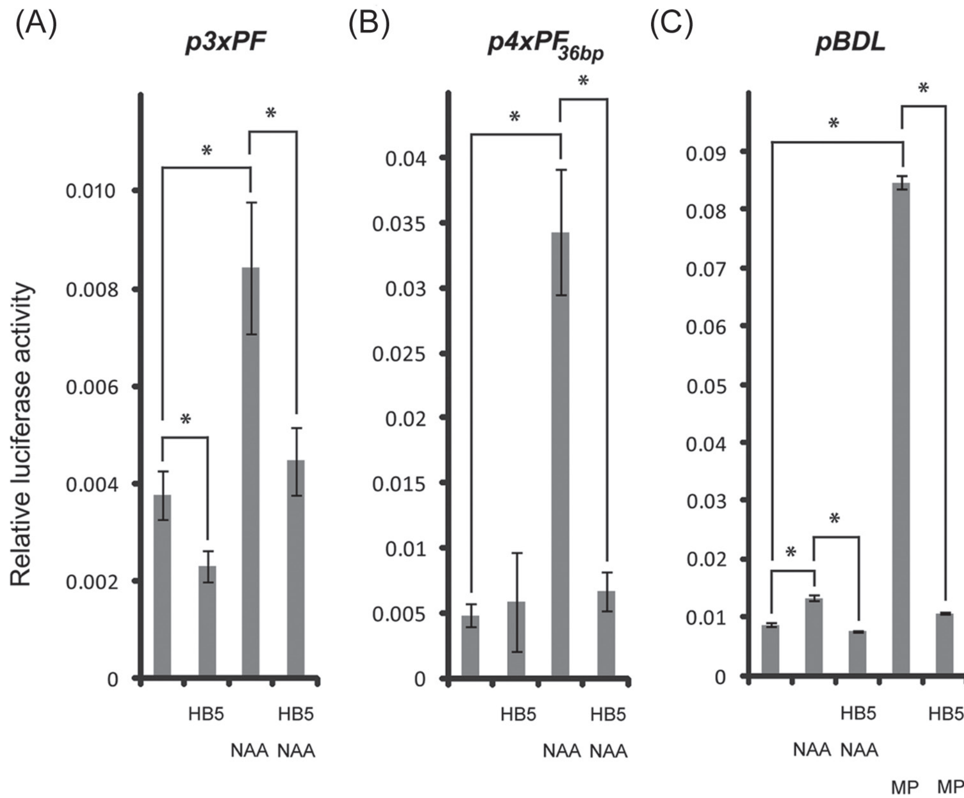

Fig. 7. Effect of HB5 on the expression of different *pBDL* reporter constructs. (A–C) Transient activity assays using *p3×PF::LUC* (A), *p4×PF36bp::LUC* (B), or *pBDL::LUC* (C) as reporter constructs in the presence or absence of HB5, MP, and 1-naphthaleneacetic acid (NAA), as indicated in the respective panels. Values represent mean ±standard error. Statistically significant differences (P <0.05) are indicated by asterisk (Student's t-test; for statistical details see Table S1).

were fused in tandem to a minimal cauliflower mosaic virus 35S promoter to drive expression of firefly *LUCIFERASE* (*LUC*). This auxin-mediated induction was repressed by HB5 (Fig. 7A, B). To rule out non-specific *trans*-effects of the *HB5* effector construct, we also co-transfected the empty effector vector, which had no comparable effects (Fig. S3A at *JXB* online). Furthermore, HB5 repressed auxin-induced expression of the full-length *BDL* promoter as well as its recently shown stronger induction by MP (Lau *et al.*, 2011) (Fig. 7C). Taken together, these results demonstrated that HB5 functions as a negative regulator of *BDL* expression *in vivo*.

To get an idea of whether HBs other than HB5 might negatively regulate *BDL* expression, we analysed whether its close homologue, HB6 (Henriksson *et al.*, 2005; Ariel *et al.*, 2007), would also be able to repress auxin- or MP-mediated induction of *BDL* expression. Indeed, in transient activity assays, HB6 repressed the induction of *pBDL::LUC* by auxin or MP essentially as efficiently as HB5 (Fig. S3B). Given that *in silico* analyses using CORNET (De Bodt *et al.*, 2012) suggested co-expression between HB5 and HB6 (data not shown), there is probably functional redundancy among HB5-related transcription factors regulating *BDL* expression.

Discussion

Auxin plays a major role in plant development. Auxin response relies on AUX/IAA degradation to release ARFs from inhibition (De Smet and Jürgens, 2007; Lau *et al.*, 2008; Vanneste and Friml, 2009). Next to their auxin-mediated degradation, *AUX/IAA*s exhibit distinct expression patterns (Fukaki *et al.*, 2002; Hamann *et al.*, 2002; Vanneste *et al.*, 2005; Ploense *et al.*, 2009; De Rybel *et al.*, 2010). While the spatio-temporal control of *AUX/IAA* expression is likely to be relevant for the proper execution of developmental processes, very little is known about their transcriptional regulation. To address this problem, we focused on *BDL*, an *AUX/IAA* involved in embryonic and post-embryonic processes (Hamann *et al.*, 1999; Hamann *et al.*, 2002; De Smet *et al.*, 2010). Previously, we showed that MP activates the expression of its AUX/IAA inhibitor BDL, with auxin being able to act as a threshold-specific trigger by promoting the degradation of the inhibitor (Lau *et al.*, 2011). Here, we explored whether HB5 might be an additional transcriptional regulator contributing to the control of *BDL* expression.

Many animal and plant homeodomain proteins play a critical role in diverse developmental processes, including pattern formation and specification of cell fates of many tissues (Gehring *et al.*, 1994; Hake *et al.*, 2004; Ariel *et al.*, 2007). HB5 has been described as a potential regulator of abscisic acid (ABA) responsiveness, but has not been implicated in auxin response (Johannesson *et al.*, 2003). HD-ZIPs function as transcriptional regulators that are characterized by an evolutionarily conserved HD responsible for DNA binding and a leucine zipper motif adjacent to the HD, which facilitates

homo- and heterodimerization of these transcriptional regulators (Gehring *et al.*, 1994; Johannesson *et al.*, 2001; Ariel *et al.*, 2007). Members of the HD-ZIP I and II families form homo- and heterodimers exclusively with other members of their own family as a prerequisite to DNA binding, and target similar *cis* elements under *in vitro* conditions (Harris *et al.*, 2011). For example, HB16 regulates leaf development and flowering time, and has been demonstrated to heterodimerize with HB5 *in vitro* (Johannesson *et al.*, 2001; Wang *et al.*, 2003). *In vitro* DNA-binding assays have shown that HB5 preferentially interacts with the pseudopalindromic binding site CAATNATTG (Johannesson *et al.*, 2001). At least half of such a site is present in the *BDL* promoter, namely in the PF_{36bp} element to which HB5 binds *in vitro*. This supported the view that HB5 interacts with the *BDL* promoter but did not reveal the regulatory effect of HB5.

HD-ZIPs can act as positive and negative regulators (Harris *et al.*, 2011). HD-ZIP Is, including HB5, have been described to be able to induce transcription (Henriksson *et al.*, 2005); HB7 and HB12, also members of HD-ZIP Is, have been reported to act as both transcriptional activators and repressors (Valdés *et al.*, 2012); and HB2 has been described to negatively regulate gene expression (Steindler *et al.*, 1999; Ohgishi *et al.*, 2001). Within the clade containing HB5, the ABA-inducible *HB6* positively regulates gene expression in protoplasts, and overall represents a negative regulator of the ABA signalling pathway downstream of ABI1 (Himmelbach *et al.*, 2002). Here, we demonstrated that HB5 acts as a negative regulator of *BDL* expression, and thus might contribute to the exclusion of BDL from the epidermis and cortex. The transcriptional regulation of *BDL* by HB5, HB6 and potentially other HD-ZIPs, might thus represent another means of auxin-response control—in addition to the auxin-inducible degradation of BDL.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Phylogenetic relationship of IAA12 and IAA13 homologues and AtIAA3, AtIAA9, AtIAA10, AtIAA11 and AtIAA14.

Supplementary Fig. S2. (A) $pBDL_{921bp}$*::bdl:GUS* expression in the seedling root tip. (B) *pBDL::NLS:3×GFP* expression in a torpedo-stage embryo.

Supplementary Fig. S3. (A) Empty vector (*pJIT60*) does not significantly repress *p3×PF::LUC* expression. (B) HB6 represses auxin- or MP-induced expression of *pBDL::LUC*.

Supplementary Table S1. Statistical details.

Supplementary List S1. Sequences used for the phylogenetic and the VISTA analysis.

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