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).
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 (Ploene 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; Züñiga-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 (Himmelbacher 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 HOMEBOX PROTEIN 5 (HB5)/ARABIDOPSIS THALIANA HOMEBOX 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 has\(\_\)5 as well as transgenic lines pBDL::bdll::GUS, pBDL::NLS::3×GFP, LTP::LhG4, and UAS::bdll 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).
**Data mining**

Protein and genomic sequence information used in this study were retrieved from the Arabidopsis Information Resource (http://www.arabidopsis.org), Joint Genome Institute (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: 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 parologue 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
De Smet et al. 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 (pBDL245::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 (pBDL195::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. 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; Pt., 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.
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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 36bp 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 36bp BDL promoter fragment.

HB5 accumulates outside the BDL expression domain

To assess the biological relevance of this interaction, we monitored HB5 expression with a $pHB5::HB5:3\timesGFP$ reporter gene. HB5:3GFP 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 HB5:3GFP 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; Ploence 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. 2. pBDL deletion study. (A) GUS-stained $pBDL_{245}:\text{bdl}:\text{GUS}$ seedling root tip. (B) Hypophysis division defect (vertical instead of horizontal division) in $pBDL_{245}:\text{bdl}:\text{GUS}$ embryos. (C) GUS-stained $pBDL_{195}:\text{bdl}:\text{GUS}$ seedling root tip. (D) Normal hypophysis division in $pBDL_{195}:\text{bdl}:\text{GUS}$ embryos. (E, F) $p3\times\text{PF}:m35S::\text{NLS}:3\times\text{GFP}$ expression in a seedling root counterstained with propidium iodide (E) or in a torpedo-stage embryo (basal part shown) (F). Bars, 25 $\mu$m.
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.

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 transgenic background 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 T1 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. 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.

**Fig. 4.** HB5 expression. (A–C) pH5::HB5:3xGFP expression (green) in a globular-stage embryo (A), heart-stage embryo (B), and seedling root counterstained with propidium iodide (red) (C). Bars, 50 μm.
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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×PF36bp:m35S::LUC (Fig. 7A, B), where copies of these PFs

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**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.

**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 (T2 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.
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/IAAs 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 

\[ \text{P}_{3000}\text{g} \] 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) \( pBDL_{3000g}::bdl:GUS \) expression in the seedling root tip. (B) \( pBDL::NLS:3\timesGFP \) expression in a torpedo-stage embryo.

**Supplementary Fig. S3.** (A) Empty vector (\( pJIT60 \)) does not significantly repress \( 3\timesPF::LUC \) 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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