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Class III Homeodomain Leucine-Zipper Proteins Regulate Xylem Cell Differentiation

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Although it has been suggested that class III homeodomain leucine-zipper proteins (HD-Zip III) are involved in vascular development, details of the function of individual HD-Zip III proteins in vascular differentiation have not been resolved. To understand the function of each HD-Zip III protein in vascular differentiation precisely, we analyzed the in vitro transcriptional activity and in vivo function of *Zinnia* HD-Zip III genes, *ZeHB-10*, *ZeHB-11* and *ZeHB-12,* which show xylem-related expression. Transgenic *Arabidopsis* plants harboring cauliflower mosaic virus 35S-driven *ZeHB-10* and *ZeHB-12* with a mutation in the START domain (mtZeHB-10, mtZeHB-12) showed a higher production of tracheary elements (TEs) and xylem precursor cells, respectively. A systematic analysis with Genechip arrays revealed that overexpression of *mtZeHB-12* rapidly induced various genes, including brassinosteroid-signaling pathway-related genes and genes for transcription factors that are expressed specifically in vascular tissues in situ. Furthermore, *mtZeHB-12* overexpression did not induce TE-specific genes, including genes related to programmed cell death and lignin polymerization, but did induce lignin monomer synthesis-related genes, which are expressed in xylem parenchyma cells. These results suggest that ZeHB-12 is involved in the differentiation of xylem parenchyma cells, but not of TEs.

Keywords: HD-Zip III — Vascular development — Xylem.

Introduction

The vascular bundle, which is essential for conducting water and solutes throughout the plant, consists of three tissues, the xylem, phloem, and procambium/cambium. These are organized in organ-specific and species-specific patterns. Xylem and phloem also contain several kinds of spatially arranged cells, including conductive tube cells, parenchyma cells, and fiber cells. Recent studies on various mutants of vascular patterns revealed the involvement of new players such as sterols (Carland et al. 2002) and vesicle transport (Koizumi et al. 2005) on the continuous formation of the vascular system. However, little is known about the mechanisms of spatial and temporal organization in each vascular cell. In this context, *APL* is an interesting gene, which may switch the fate of phloem and xylem cells (Bonke et al. 2003). Other important genes that may control cell organization within vascular tissues are the class III homeodomain-leucine zipper genes (HD-Zip III). There are five HD-Zip III genes in *Arabidopsis*, *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REVOLUTA* (*REV/IFL1/AVB1*), *ATHB-8* and *ATHB-15*. Gain-of-function alleles of *PHB* and *PHV* produce adaxialized leaves with amphivasal vasculatures in which xylem surrounds phloem (McConnell and Barton 1998, McConnell et al. 2001). Gain-of-function alleles of *REV/AVB1* also exhibit amphivasal vascular bundles in inflorescences (Zhong et al. 1999, Emery et al. 2003, Zhong and Ye 2004). *PHB*, *PHV* and *REV* show overlapping expression in the adaxial domains of lateral organs, in vascular bundles, and in apical meristems (McConnell et al. 2001, Emery et al. 2003, Prigge et al. 2005). Although loss-of-function alleles of *phb* and *phv* did not show any abnormality, a *phb*, *phv* and *rev* triple loss-of-function mutant produced a single abaxialized cotyledon with an amphicribral vasculature, in which phloem surrounds xylem (Emery et al. 2003). These facts indicate that PHB, PHV and REV act redundantly to provide adaxial identity of lateral organs and to regulate tissue pattern formation in vascular bundles. In addition, PHB, PHV and REV may regulate vascular cell differentiation because loss-of-function *ifl1/rev* alleles cause the reduction of interfascicular xylem fibers in inflorescences (Zhong and Ye 1999) and because double mutants of *rev, phb/+* and *rev, phv* enhanced the vascular defects of the *rev* mutant (Prigge et al. 2005). *ATHB-8* and *ATHB-15* are the other members of the HD-Zip III family and are specifically expressed in the procambium region (Baima et al. 1995, Ohashi-Ito and Fukuda 2003). Furthermore, overproduction of ATHB-8 causes an increase in xylem tissues (Baima et al. 2001). These facts indicate that each HD-Zip III gene may have essential roles in vascular tissue formation, and in differentiation of xylem cells and/or procambium cells. Using in situ hybridization of *Zinnia* HD-Zip III genes, we revealed that transcripts for distinctive HD-Zip III genes accumulated in different manners from procambial layers to mature xylem layers, including tracheary elements (TEs). This finding suggests that each HD-Zip III gene plays a role at a

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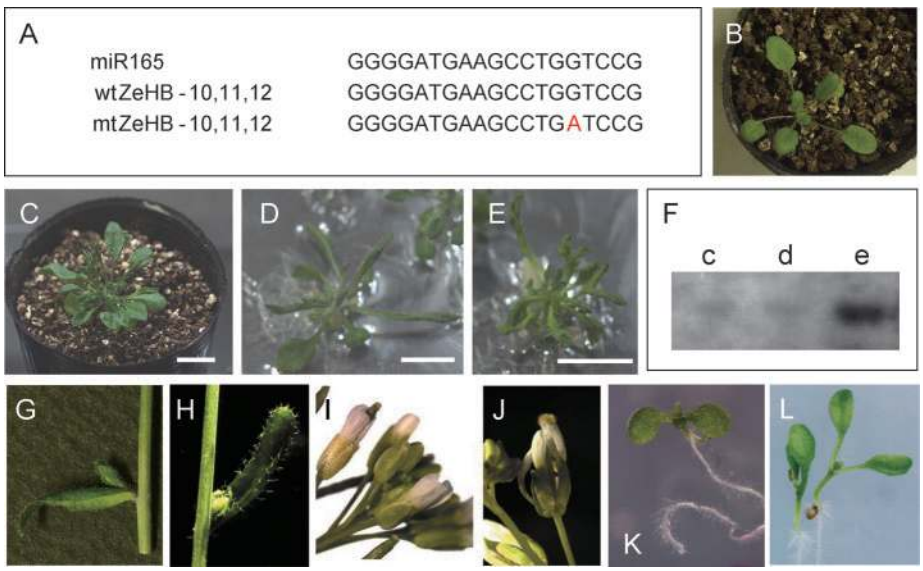

Fig. 1 Phenotypes of transgenic plants that overexpress *mtZeHB-10.* (A) Schematic representation of wild type cDNAs and mutant cDNAs for analysis. A nucleotide substitution was conducted in nucleotide sequences complementary to miR165/166 in the START domain. "wt" and "mt" represent wild type cDNA and mutant cDNA, respectively. (B) A control plant and (C-E) T1 *35S::mtZeHB-10* transgenic plants. Bar: 1 cm. (F) Accumulation of the *ZeHB-10* transcript in transgenic plants. The gel blot was hybridized with DIG-labeled cRNA for *ZeHB-10* as a probe. Lanes c, d, e show levels of the *ZeHB-10* transcript in total RNAs prepared from plants C, D, E, respectively. (G) A cauline leaf of a control plant. (H) A cauline leaf of a *35S::mtZeHB-10* transgenic plant. (I) Flowers of a control plant. (J) Flowers of a *35S::mtZeHB-10* transgenic plant. (K) A control seedling. (L) T3 *35S::mtZeHB-10* transgenic seedlings.

specific stage during xylem development (Ohashi-Ito and Fukuda 2003).

Brassinosteroids (BRs) are an important regulator of xylem development (Fukuda 2004). BRs initiate TE differentiation from xylem precursors in cultured *Zinnia* cells (Yamamoto et al. 1997, Yamamoto et al. 2001). A specific inhibitor of BR biosynthesis suppresses xylem differentiation, instead promotes phloem differentiation (Nagata et al. 2001). Recently, we demonstrated that BRs induced the expression of *Zinnia* HD-Zip III genes during xylem differentiation (Ohashi-Ito et al. 2002, Ohashi-Ito and Fukuda 2003). However, HD-Zip III functions in xylem development have not been identified in detail.

In this study, we examined the functions of HD-Zip III proteins in xylem development and transcriptional regulation. Although *Arabidopsis* REV/IFL1/AVB1 has multiple functions in development (Zhong and Ye 1999, Otsuga et al. 2001), *Zinnia* has at least two *REV* homologues, and their functions appear to be separated between vascular development and lateral organ formation. For this reason, the use of *Zinnia* homologues of REV may be more efficient for analyzing the function solely in vascular development. We characterized the functions of HD-Zip III proteins in vascular differentiation using *Zinnia* genes, *ZeHB-10* (which is a homologue of *ATHB-8*) and *ZeHB-11* and *ZeHB-12* (which are homologues of *REV*) with a specific focus on *ZeHB-12*. As a result, we found that ZeHB-12 was involved in the differentiation of xylem parenchyma cells, but not of TEs.

Results

Overexpression of ZeHB-10, ZeHB-11 and ZeHB-12 in Arabidopsis

To investigate the roles of ZeHB-10, ZeHB-11 and ZeHB-12 in vascular differentiation, we tried to overexpress *ZeHB-10*, *ZeHB-11* and *ZeHB-12* in *Arabidopsis* plants. Because it has been reported that a nucleotide substitution in sequences complementary to miR165/166 (*phb/phv* mutations) protect against the rapid degradation of HD-Zip III mRNAs (Tang et al. 2003), we generated mutant cDNAs of *ZeHB-10*, *ZeHB-11* and *ZeHB-12* that contained the same nucleotide substitution in the region complementary to miR165/166 (Fig. 1A). This mutation causes an amino acid change from glycine to aspartic acid. This mutant cDNA was designated "mt" and the original cDNA as "wt". The *wtZeHB-10*, *mtZeHB-10*, *wtZeHB-11*, *mtZeHB-11*, *wtZeHB-12* and *mtZeHB-12* cDNAs were fused to the cauliflower mosaic virus 35S promoter, and introduced into *Arabidopsis* plants to overexpress these genes.

Of 47 independent T1 *35S::mtZeHB-10* lines examined, 20 lines formed rosette leaves that were curled upward toward the adaxial side, in contrast to the flattened rosette leaves of wild-type seedlings (Fig. 1B–E). The most severe phenotype was observed in the line that showed the highest *ZeHB-10* transcript accumulation (Fig. 1F). Although the severe phenotype line did not bolt, other lines bolted and formed upward curling cauline leaves, petals and sepals (Fig. 1G–J). The T3 *35S::mtZeHB-10* transgenic plants exhibited the same phenotypes as

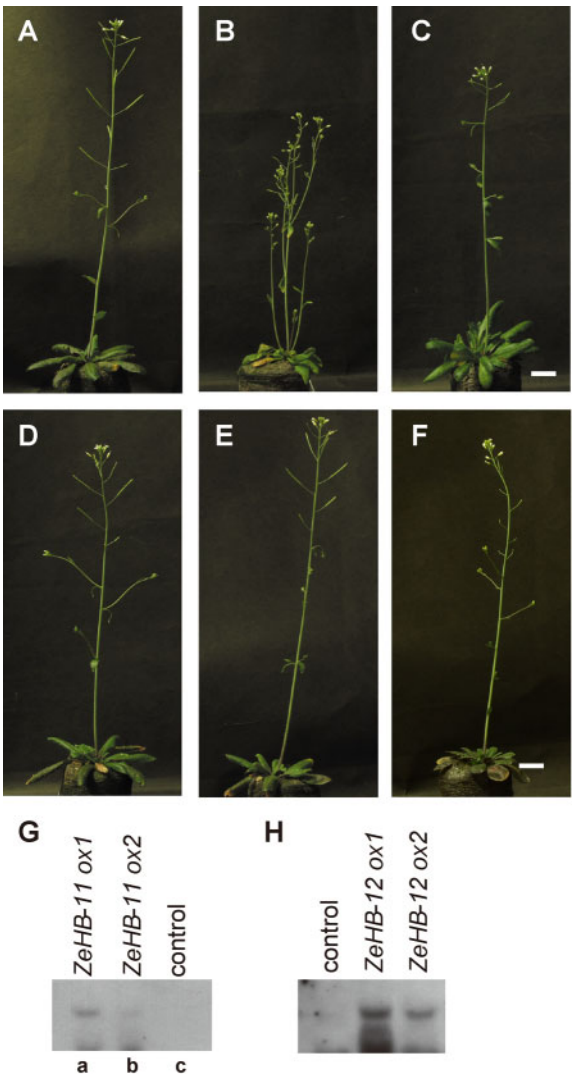

Fig. 2 Phenotypes of the *35S::mtZeHB-11* and *35S::mtZeHB-12* plants. (A) A control plant, (B) a *35S::mtZeHB-11* transgenic plant with many lateral inflorescences, (C) a *35S::mtZeHB-11* transgenic plant resembling a wild type plant. Bar: 2 cm. (D) A control plant, (E, F) *35S::mtZeHB-12* transgenic plants. Bar: 2 cm. (G) Accumulation of the *ZeHB-11* transcript in transgenic plants shown in B and C. The gel blot was hybridized with DIG-labeled cRNA for *ZeHB-11* as a probe. Lanes a and b show levels of the *ZeHB-11* transcript in total RNAs prepared from plants B and C respectively. (H) Accumulation of the *ZeHB-12* transcript in *35S::mtZeHB-12* transgenic plants. The gel blot was hybridized with DIG-labeled cRNA for *ZeHB-12* as a probe.

T1 plants, as shown typically by upward curling blades in cotyledons and young rosette leaves (Fig. 1K, L). These phenotypes were observed in the *mtZeHB-10* plants, but not in the *wtZeHB-10* plants (data not shown). Transgenic plants that overexpressed *wtZeHB-12* and *mtZeHB-12* did not show apparent morphological changes (Fig. 2D–F). In contrast, the *35S::mtZeHB-11* transgenic plants showed an enhanced growth of lateral inflorescences compared with those of control plants (Fig. 2A–C).

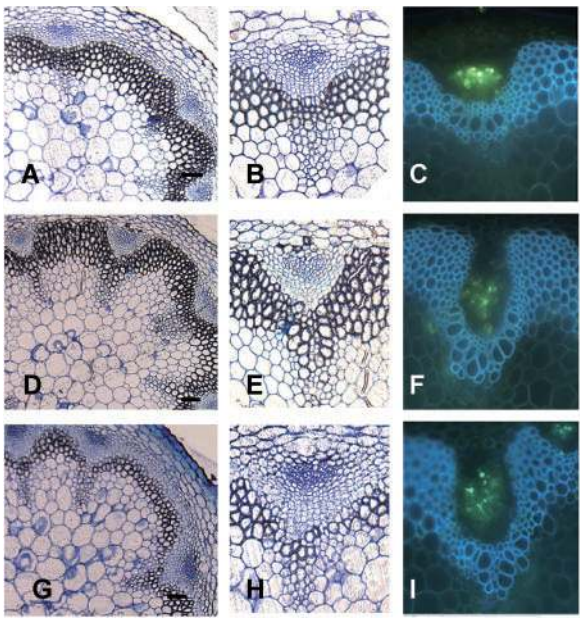

Fig. 3 Histological analysis of the vascular structure in the inflorescence stem of *mtZeHB-10* and *mtZeHB-12* transgenic plants. (A–C) transverse sections of the basal end of the inflorescence stem in control plants transformed with an empty vector. (D–F) transverse sections of the basal end of the inflorescence stem in a *35S::mtZeHB-10* transgenic plant. (G–I) transverse sections of the basal end of the inflorescence stem in a *35S::mtZeHB*-12 transgenic plant. (B, E and H) magnified images of A, D and G, respectively. (C, F, I) vascular bundles stained with aniline blue and viewed under UV light.

Next, the vascular tissue pattern of these transformants was examined at the basal part of the inflorescence. Vascular bundles in the *35S::mtZeHB-10* and *35S::mtZeHB-12* stems showed a weak amphivasal pattern in which xylem nearly surrounded phloem, whereas control stems showed a collateral pattern of vascular bundles (Fig. 3). In addition, the production of TEs of the *35S::mtZeHB-10* stems increased in vascular bundles (Fig. 3D–F), and increased layers of procambium cells and/or xylem precursor cells occurred in the *35S::mtZeHB-12* stems (Fig. 3G–I). These phenotypes were common in the transformants.

Transcriptional activity of ZeHB-10 and ZeHB-12

Although the HD-Zip III proteins are believed to be transcription factors, their transcriptional activities have not been measured. To determine whether these *Zinnia* HD-Zip III proteins are transcriptional activators, each full-length sequence of *wtZeHB-10, mtZeHB-10, wtZeHB-12* and *mtZeHB-12* was fused to the C-terminus of the DNA binding domain of GAL4 (GBD), and introduced into the yeast strain, Y187, which harbored the *GAL1::LacZ* reporter gene.

All constructs could activate the expression of *LacZ* to a level more than 20-fold higher than that of the negative control (Fig. 4). Interestingly, the expression level of *LacZ* by the fusion protein GBD-mtZeHB-12 was about 2-fold higher than

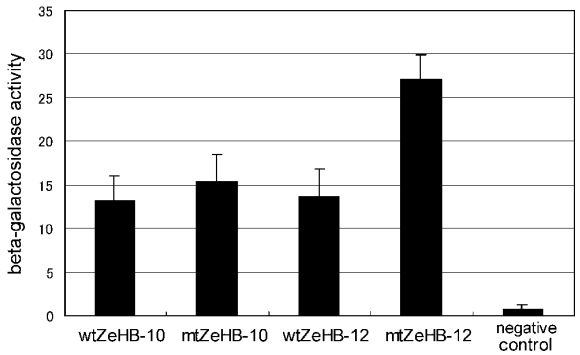

Fig. 4 Transcriptional activity of ZeHB-10 and ZeHB-12. Transcriptional activities of GAL4-BD-fused wtZeHB-10, mtZeHB-10, wtZeHB-12 and mtZeHB-12 proteins in yeast. The columns show the β-galactosidase activity of the effector proteins, which are presented as the measured values in Miller units. The negative control shows transcriptional activity only with GAL4-BD. Error bars represent standard deviations ($n = 3$).

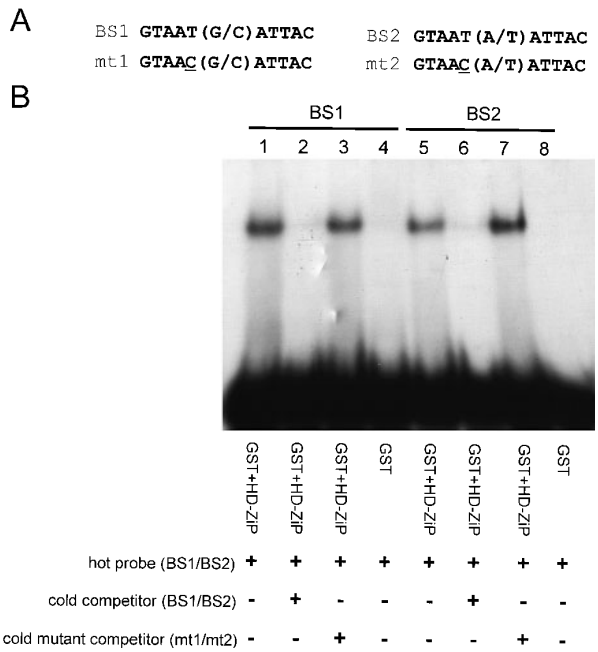

Fig. 5 Sequence-specific binding of ZeHB-12. (A) The binding sequences (BS and BS2) and mutant sequences (mt1and mt2) used in this assay. (B) Gel mobility shift assay using recombinant HD-Zip domain of ZeHB-12. Labeled (hot) oligonucleotides containing BS1 (lanes 1 to 4) and BS2 (lanes 5 to 8) sequences were used as probes. Samples in lanes 2 and 6 contained cold BS1 or BS2 as competitors, respectively. Samples in lanes 3 and 7 contained cold mt1 or mt2 as competitors, respectively. Samples in lanes 4 and 8 contained GST proteins without the HD-Zip domain. (+) indicates present. (–) indicates absent.

that of GBD-wtZeHB-12. These results suggest that ZeHB-10 and ZeHB-12 function as transcriptional activators, and that the mutation in the START domain does not reduce transcriptional activity, but promotes it.

DNA-binding property of the ZeHB-12 protein

The target sequence of the ATHB-9/PHV protein, a member of the *Arabidopsis* HD-Zip III homeobox proteins, has been identified previously as an 11-bp pseudopalindromic sequence (GTAAT(G/C)ATTAC) (Sessa et al. 1998). The same class of HD-Zip families is known to bind similar target sequences (Johannesson et al. 2001). Therefore, we predicted that *Zinnia* HD-Zip III proteins may also interact with the GTAAT(G/C)ATTAC sequence or a similar sequence.

To examine this possibility, an electrophoretic mobility shift assay was performed with ZeHB-12 as an example (Fig. 5). A fusion protein consisting of glutathione *S*-transferase (GST) and the HD-Zip domain of ZeHB-12 was expressed in *Escherichia coli* cells and partially purified. The protein was incubated with the ATHB-9 target sequence (BS1, GTAAT(G/C)ATTAC) or with its related sequence (BS2, GTAAT(A/T)ATTAC), and bound to BS1 and BS2. The binding was completely competed by unlabeled BS1 and BS2, but not competed by their mutant sequences (mt1, mt2) with a substitution of T to C at the fifth nucleotide. These results suggest that ZeHB-12 binds specifically to the sequence GTAATNATTAC.

Expression of ZeHB-12 under the control of an inducible promoter

The analysis of transcriptional activity and the DNA binding activity of ZeHB-12 revealed that ZeHB-12 is a transcriptional activator bound to specific sequences. Therefore, we next examined the transcriptional network regulated by ZeHB-12 by generating transgenic *Arabidopsis* plants with pTA7002-mtZeHB-12, in which the *mtZeHB-12* transcript can be transiently overexpressed by dexamethasone (DEX) (Aoyama and Chua 1997). T3 seedlings of these transgenic plants were treated with 50 μM DEX for 3 h. One line, pTA7002-mtZeHB-12–1, which had the highest inducibility of *ZeHB-12* mRNA, was selected by RNA gel blot analysis and used for further analysis (data not shown).

Identification of genes regulated by ZeHB-12

To identify downstream target genes of ZeHB-12, a transcriptome analysis using microarrays was performed. The approach was based on the detection of genes whose mRNA levels were affected after induction of *mtZeHB-12* mRNA. The pTA7002-mtZeHB-12–1 transgenic plants were treated with DEX (50 μM) for 3 h and 6 h. As controls, pTA7002-mtZeHB12–1 plants without the DEX treatment and pTA7002-luciferase (LUC) plants harboring the inducible LUC transgene instead of *ZeHB-12* both with and without DEX were used. DEX induced *mtZeHB-12* mRNA by 0.5 h and increased it at least until 6 h in the pTA7002-mtZeHB-12–1 plants (data not shown). Therefore, to investigate genes regulated by ZeHB-12, we prepared total RNAs from the plants treated for

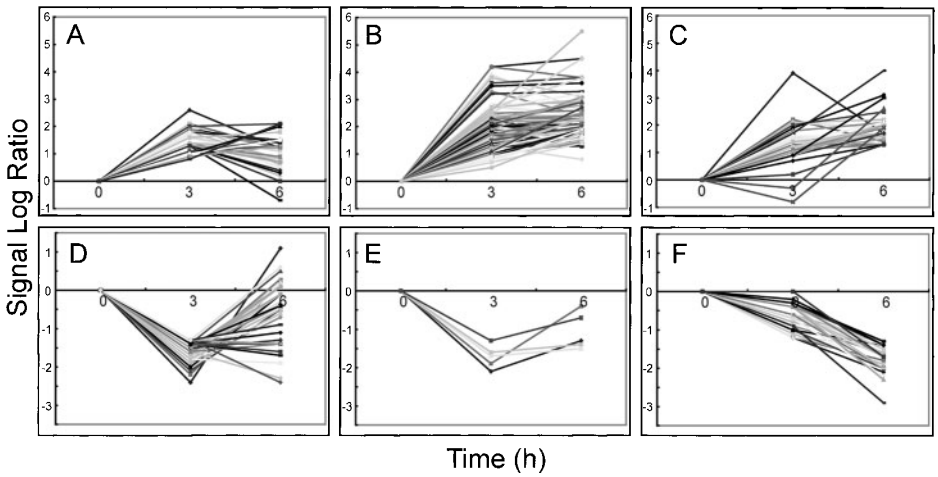

Fig. 6 Hierarchical clustering of genes whose levels of expression were changed. *Arabidopsis* plants harboring the pTA7002-mtZeHB-12 were treated with DEX for 3h and 6 h. Total RNA prepared from the plants was subjected to a 23K genechip. On the basis of expression patterns, 205 genes with a greater than 2.5-fold change after induction of *ZeHB-12* expression were divided into six groups. The y axis indicates signal $\log_2$ ratio.

3 h and 6 h with and without DEX and subjected them to the 23K Genechip.

Of 22,500 genes on the Genechip, 205 genes (0.91%) were specifically up- or down-regulated (>2.5-fold) in pTA7002-mtZeHB-12–1 plants by the DEX treatment but not in pTA7002-LUC plants. The up- and down-regulated genes were divided into six groups by their expression patterns (Fig. 6A–F, Supplementary Table 1). Groups A–C included 136 genes that were up-regulated (the "up-regulated category"). Groups D–F included 69 genes that were down-regulated (the "down-regulated category"). This result is consistent with the fact that ZeHB-12 is a transcriptional regulator, which functions by promoting the transcription of target genes.

Xylem differentiation-related genes—First, we examined whether genes encoding proteins that may function in xylem differentiation were included among genes up- or down-regulated by the overexpression of *mtZeHB-12* (Fig. 7A, B). Accumulation of transcripts for the developing TE-specific cysteine proteinases, *XCP1* and *XCP2*, which function in the cell death process of TE differentiation, was not changed by *mtZeHB-12* overexpression (Zhao et al. 2000, Funk et al. 2002). *BFN1*, a homologue of *Zinnia ZEN1*, encodes a bifunctional nuclease that also has a role in the cell death process and is expressed specifically in developing TE cells (Ito and Fukuda 2002, N. Saito and H. Fukuda, unpublished results). Expression of *BFN1* was not affected by induction of the *mtZeHB-12* transcript either.

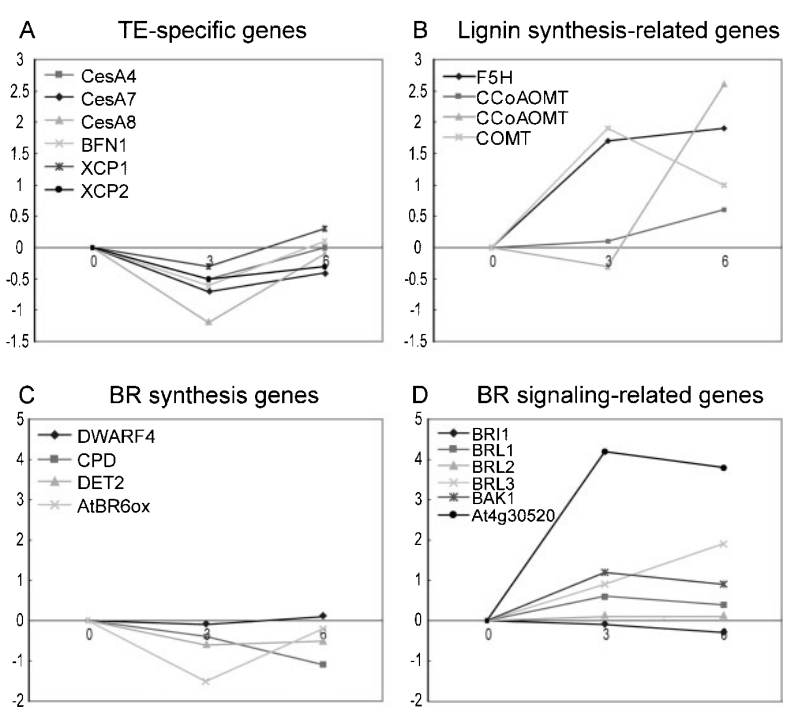

Fig. 7 Expression patterns of xylogenesis-related genes and brassinosteroid-related genes during *ZeHB-12* overexpression. (A) TE-specific expressing genes. *CesA4*: AT5G44030, *CesA7*: AT5G17420, *CesA8*: AT4G18780, *BFN1*: AT1G11190, *XCP1*: AT4G35350, *XCP2*: AT1G20850. (B) lignin monomer synthesis-related genes. *F5H* (*ferulate 5-hydroxylase*): AT4G36220, *CCoAOMT* (*caffeoyl CoA O-methyltransferase*): AT4G34050, AT3G61990, *COMT* (*caffeic acid/5-hydroxyferulic acid O-methyltransferase*): AT1G21130. (C) brassinosteoid biosynthesis-related genes. *DWARF4*: AT3G50660, *CPD*: AT5G05690, *DET2*: AT2G38050, *AtBR6ox*: AT5G38970. (D) brassinosteroid signaling-related genes. *BRI1*: At4g39400, *BRL1*: AT1G55610, *BRL2*: AT2G01950, *BRL3*: At3g13380, *BAK1*: At4g33430. The y axis indicates signal log2 ratio (SLR).

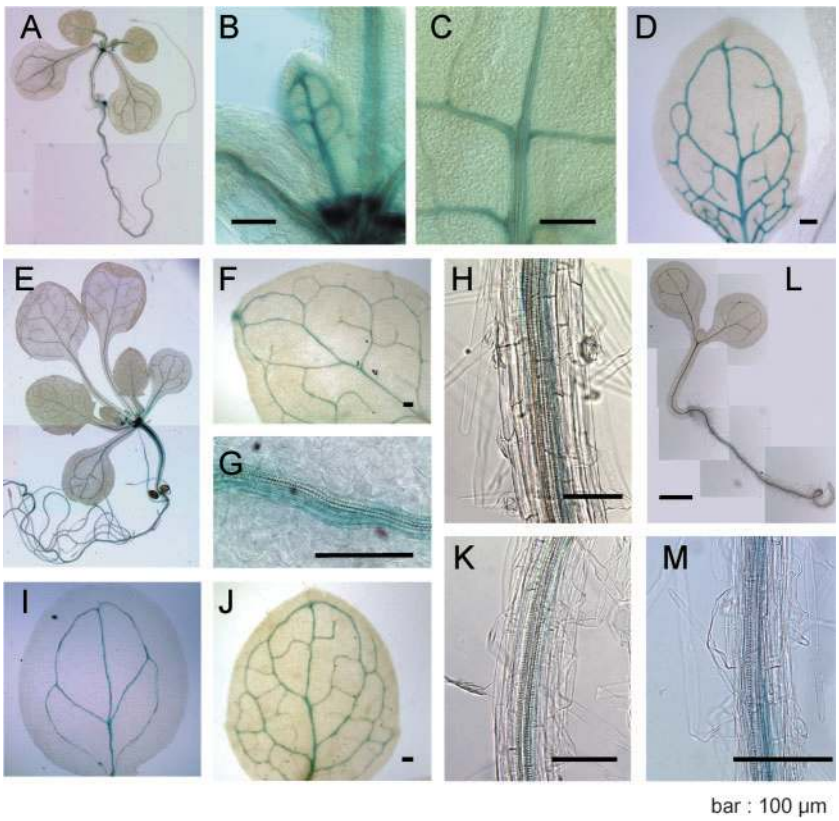

Fig. 8 GUS expression patterns in *Arabidopsis* seedlings. (A–D) The At4g30520 promoter:: GUS expression pattern. The GUS expression in a whole seedling (A) and rosette leaves (B–D). (E–H) The At4g31800 (*WRKY18*) promoter:: GUS expression pattern. The GUS expression in a whole seedling (E), rosette leaves (F, G) and a root (H). (I–K) The At4g36710 promoter::GUS expression pattern. The GUS expression in a cotyledon (I), a rosette leaf (J) and a root (K). (L, M) The At3g10500 promoter::GUS expression pattern. The GUS expression in a whole seedling (L) and a root (M).

Six cellulose synthase catalytic subunits, CesA1, CesA3, CesA4, CesA6, CesA7 and CesA8, are known to function in TE cells. In particular, genes for *CesA4*, *CesA7* and *CesA8* are expressed specifically in developing TEs (Demura et al. 2002, Gardiner et al. 2003). Expression of all *CesA* genes was not affected by mtZeHB-12 (Fig. 7A).

Lignin formation occurs specifically in developing TEs through the polymerization of lignin monomers by laccases and peroxidases (Blee et al. 2003, Boerjan et al. 2003). In the Genechip, 15 genes that were annotated as laccase were located. Their expression was not affected by ZeHB-12, although many laccase genes are known specifically to be up-regulated in association with TE differentiation in *Zinnia* (Demura et al. 2002). These results showed that ZeHB-12 did not regulate the expression of TE-specific genes related to programmed cell death and secondary cell wall formation. In contrast, expression of four genes that encode enzymes related to the synthesis of lignin monomers was up-regulated by *mtZeHB-12* induction. These enzymes were a ferulate-5-hydroxylase, two caffeoyl-CoA *O*-methyltransferases, and a caffeic acid 5-*O*-methyltransferase (Hertzberg et al. 2001, Fig. 7B). It has been suggested that lignin monomers are synthesized in both TEs and xylem parenchyma cells, although lignin polymerization occurs specifically in developing TEs (Hosokawa et al. 2001). Therefore, these results suggest that ZeHB-12 may promote gene expression in relation with the differentiation of xylem parenchyma cells or immature xylem cells, but not of TEs.

Plant hormone-related genes—Although changes in the expression of genes related to signaling and biosynthesis of auxin, cytokinin, ethylene, gibberellin, abscisic acid and jasmonate acid were examined, few genes were affected by *mtZeHB-12* induction.

Next, we investigated BR-related genes intensively because BR is necessary for differentiation of xylem and up-regulates *ZeHB-12* expression (Yamamoto et al. 1997, Ohashi-Ito et al. 2002). Expression of genes encoding enzymes that are associated with BR synthesis such as DET2 (3-oxo-5-alpha-steroid 4-dehydrogenase), DWF4 (steroid 22-alpha-hydroxylase), CPD (cytochrome P450 90A1) and AtBR6ox (6-deoxycastasterone C6-oxidase) was not significantly affected (Bishop and Koncz 2002, Fig. 7C).

BR signaling is transduced inside the cell by transmembrane receptors that may involve two types of leucine-rich repeat (LRR) receptor-like kinases (RLKs), BRASSINOSTEROID INSENSITIVE 1 (BRI1) and BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1). BRI1 interacts with BAK1, and they bind BR either directly or indirectly (Nam and Li 2002). There are three BRI1-like proteins, BRL1, BRL2 and BRL3, in *Arabidopsis*, two of which (BRL1 and BRL3) bind BR (Yin et al. 2002). Of them, the expression of *BRL3* gradually increased after induction of the *mtZeHB-12* transcript (Fig. 7D). Of the BAK1-type LRR-RLK family, one gene (253620_at, At4g30520) was up-regulated (Fig. 7D).

Table 1 Putative ZeHB-12 binding sequences in upstream regions of genes from groups A, B, D and E

Group A and B 13/50			
AGI No.	Probe set ID	Annotation	binding sequences*
At3g29770	256564_at	alpha/beta hydrolase, putative	ttaatgattat (1500)
At3g10500	258921_at	NAC-like protein	ataataattat (1550)
At2g19440	267335_s_at	putative beta-1,3-glucanase	gtaattattat (1800), ttaattattag (940)
At3g13380	256981_at	brassinosteroid receptor like kinase 3	ttaataattag (140), ataatgattat (110)
At1g61810	264433_at	beta-glucosidase, putative	ttaattattaa (670)
At1g24140	264866_at	putative metalloproteinase	ttaataattat (1100), ataattattaa (1200)
At1g14460	261471_at	hypothetical protein similarity to DNA polymerase III	ttaatcattaa (950)
At1g05790	261312_at	putative NPK1-related protein kinase 2	ataataattaa (310)
At4g36010	253104_at	thaumatin-like protein	gtaataattaa (610)
At2g47260	260528_at	putative WRKY-type DNA binding protein	ttaattattaa (480), ttaattattaa (1740)
At1g23730	265170_at	putative carbonic anhydrase	ctaattattac (1430), ctaataattaa (1840)
At4g36710	*246230_at*	SCARECROW-like protein	ttaatcattaa (1630)
At1g14210	262657_at	ribonuclease contains similarity to RNase	gtaatcattaa （180)
Group D and E 6/50			
AGI No.	Probe set ID	Annotation	binding sequences
At5g25190	246932_at	ethylene-responsive element – like protein	tattaattattaa (1200)
At5g23010	249866_at	2-isopropylmalate synthase-like protein	ttaattattat (1180)
At3g50440	252168_at	putative protein pir7a protein	gtaattattat (160)
At4g19530	254553_at	TMV resistance protein N – like protein	ttaattattaa (1080)
At4g04330	255331_at	hypothetical protein	ctaataattaa (1700)
At2g18300	265342_at	hypothetical protein predicted by genscan	ttaattattaa (1400)

One hundred genes randomly selected from the groups A, B, D and E were examined for the presence of the TAATNATTA-like sequences with 2.0 kb region upstream of ATG. * Left sequences show the TAATNATTA DNA sequences located within the 2.0 kb upstream region. Numbers in parentheses indicate the rough positions of TAATNATTA from the point of transcriptional start.

To determine whether the At4g30520 gene is expressed in vascular cells, a fusion gene composed of the At4g30520 promoter region and GUS gene was introduced into *Arabidopsis*. In seedlings harboring the At4g30520 promoter::GUS transgene, expression was observed strongly in the vascular bundles of shoots (Fig. 8A–D). GUS expression was also detected in both young and developed leaves. In young leaves, GUS activity was observed in vascular bundles that had not yet developed (Fig. 8B–D). *BRL3* is specifically expressed in vascular bundles, although *BRI1* is ubiquitously expressed in plants (Friedrichsen et al. 2000, Cano-Delgado et al. 2004). These results showed that some BR signaling-related genes were up-regulated by ZeHB-12, although BR biosynthesis-related genes were not.

Transcription factors—The up-regulated category contained 17 genes encoding transcription factors. These transcription factors belong to various families such as myb, zinc finger, GRAS, bZIP, WRKY and AP2 families. To determine whether these genes are expressed in association with vascular differentiation, we randomly picked three genes and examined their expression patterns.

The 253485_at gene is identical to *WRKY18* (At4g31800), which has been reported as a transcription factor with a WRKY zinc-finger motif, and is induced in response to pathogens (Dong et al. 2003). The *WRKY18* promoter::GUS reporter was introduced into *Arabidopsis* plants and a signal detected in vascular bundles throughout the plant, but not in very young leaves (Fig. 8E–H). This result suggested that WRKY18 was involved not only in pathogen response, but also in vascular development. The 258921_at gene (At3g10500) encodes a NAC-like protein and is a member of the GRAS family. GUS expression driven by the At3g10500 gene promoter was observed strongly in the central region of roots including vascular tissues (Fig. 8L, M). The 246230_at gene (At4g36710) also encodes a member of the GRAS family, SCARECROW-like protein. At4g36710 gene expression was specifically observed in vascular bundles throughout the plant using At4g36710 promoter GUS fusions (Fig. 8I–K).

We described above that ZeHB-12 binds the TAATNATTA sequence. Therefore, we searched this sequence in the 2.0 kb upstream region of the three genes. The At3g10500 and At4g36710 genes have the sequences in their promoters, suggesting the possibility that these genes are regulated directly by ZeHB-12. Of 50 genes randomly selected from the up-regulated category, 13 have TAATNATTA-like sequences (Table

1). Nevertheless, six of 50 genes selected from the down-regulated category also have TAATNATTA-like sequences.

Discussion

Roles of HD-Zip III genes in vascular differentiation

Although it has been suggested that HD-Zip III homeobox genes are involved in vascular development, details of individual HD-Zip III functions in vascular differentiation have not been resolved. We previously characterized the expression patterns of HD-Zip III genes in vascular differentiation using *Zinnia* in vitro culture and plants (Ohashi-Ito et al. 2002, Ohashi-Ito and Fukuda 2003). Therefore, to precisely determine each HD-Zip III function in vascular differentiation, we analyzed in vitro transcriptional activity and the in vivo function of *Zinnia* HD-Zip III genes.

Based on expression data and data from this study, we could suggest their functions in vascular differentiation. *ZeHB-10* transcript accumulation was observed preferentially in xylem precursor cells (TE precursor cells) in developing vascular bundles and transiently before secondary cell wall formation and cell death in xylogenic culture. The *mtZeHB-10* overexpressing transgenic plants showed a higher production of TE cells in the vascular bundles (Fig. 3). These results indicate that ZeHB-10 might function in promoting differentiation into TEs within xylem precursor cells rather than procambium cells, although ATHB-8, the *Arabidopsis* ortholog is believed to play a role in procambial cells based on its expression pattern (Baima et al. 1995). These results are consistent with the fact that ectopic expression of *ATHB-8* increases the amount of xylem tissue (Baima et al. 2001).

Arabidopsis HD-Zip III genes are divided into two clades according to the phylogenetic tree. It has been suggested that the first group, PHB, PHV and REV/IFL1, has a role in the establishment of adaxial fate, whereas the second group, ATHB-8 and ATHB-15, appears to function only in vascular development (Emery et al. 2003). However, upward-curling leaves were observed in the plants overexpressing *mtZeHB-10*. The plants overexpressing *ATHB-8* also had slightly upward-curved leaves (Baima et al. 2001). These phenotypes indicate that the overexpressed gene is involved in the establishment of adaxial identity because plants weakly overexpressing *PHB* showed such a phenotype. In addition, *ZeHB-10* transcripts accumulated in the adaxial domain of very young leaves (data not shown). Thus, ZeHB-10 and ATHB-8 might also have a role in adaxial establishment.

The *IFL1/REV* gene is an *Arabidopsis* homologue of *ZeHB-12* and *ZeHB-11*. IFL1/REV has multiple functions, including xylem differentiation, lateral meristem formation, and the establishment of lateral organ polarity (Zhong and Ye 1999, Otsuga et al. 2001, Emery et al. 2003). Because *Zinnia* has at least two IFL1/REV homologues, we considered that IFL1/REV functions might be split between the two. Plants overexpressing *mtZeHB-11* made many lateral inflorescences, whereas plants overexpressing *ZeHB-12* did not show such a phenotype. Instead, they showed phenotypes in vasculatures. This overexpression experiment suggested that ZeHB-11 may function in the formation of lateral meristems, and that ZeHB-12 may have a role in xylem development. Therefore, to investigate the mechanism of gene regulation by IFL1/REV that leads to xylem differentiation, the use of ZeHB-12 must be more effective. Histochemical analyses of plants overexpressing *mtZeHB-12* revealed an increase in the layers of procambium cells and/or xylem precursor cells, but not of TEs (Fig. 3). This result implies that ZeHB-12 may promote the production of cells toward xylem, but not toward TEs. In *Zinnia* xylogenic culture, accumulation of the *ZeHB-12* transcript started at 48 h and continued to 96 h, when TE differentiation had almost finished and the TEs died. This suggests that *ZeHB-12* is expressed from an early stage of xylem development and in cells other than TEs. It has been suggested that, at 96 h of culture, xylem parenchyma-like cells are differentiated (Shinohara et al. 2000, Ito et al. 2004). In addition, the *ZeHB-12* transcript accumulated preferentially in xylem parenchyma cells in situ (Ohashi-Ito and Fukuda 2003). These results strongly suggest that ZeHB-12 may have roles in the differentiation of xylem precursors and xylem parenchyma cells. This idea is supported by our microarray data, which showed that *mtZeHB-12* overexpression did not induce TE-specific genes, including genes related to programmed cell death and lignin polymerization, but did induce lignin monomer synthesis-related genes. This is because xylem parenchyma cells produce enzymes for the biosynthesis of lignin monomers, but not enzymes to polymerize them (Hosokawa et al. 2001, Ito et al. 2004).

Genes regulated by ZeHB-12

A systematic analysis with Genechip arrays revealed that overexpression of *mtZeHB-12* rapidly promoted or suppressed 205 genes that encode various proteins, including transcription factors and signaling molecules, although they might include genes regulated by ZeHB-12 indirectly because not all genes have the ZeHB-12 binding sequence. Indeed, some of these transcription factors were expressed specifically in vascular tissues in situ. We have indicated that BRs play a pivotal role in the progression of xylem differentiation (Yamamoto et al. 1997, Yamamoto et al. 2001), and in the expression of HD-Zip III genes, including *ZeHB-12* (Ohashi-Ito et al. 2002, Ohashi-Ito and Fukuda 2003). Therefore, expression profiles of BR-related genes were examined.

Interestingly, two BR-signaling pathway-related genes, *BRL3* (a BRI1 like LRR-RLK) and At4g30520 (a BAK like LRR-RLK), were up-regulated rapidly by the overproduction of mtZeHB-12, although the other BR-signaling-related genes and all the BR-biosynthesis-related genes identified on microarrays were not affected. BRL3 is known to appear in a vascular bundle-specific manner (Cano-Delgado et al. 2004). We also found that At4g30520 was expressed specifically in vascu-

lar tissues. Our microarray data with in vitro *Arabidopsis* xylogenic culture also indicated that *BRL3* and At4g30520 are both expressed preferentially in xylem differentiation (M. Kubo, unpublished results).

BRI1 and BAK1 are known to interact with each other and to transduce BR signals. BRL3 plays a role similar to that of BRI1 (Cano-Delgado et al. 2004). Therefore, BRL3 and At4g30520 may have a specific role as a xylem-specific BR signaling complex downstream of ZeHB-12, while BRI1, which is expressed ubiquitously, may have a general role in BR signaling. These findings provide a new insight into the complex gene regulation underlying xylem development governed by BR.

MicroRNAs involved in the regulation of HD-Zip III genes

MicroRNAs (miRNAs) have essential roles in plant development (Aukerman and Sakai 2003, Palatnik et al. 2003). Target mRNAs are cleaved in positions complementary to miRNAs (Llave et al. 2002, Tang et al. 2003, Mallory et al. 2004). miRNAs also act through specific translational inhibition (Aukerman and Sakai 2003) and DNA methylation (Bao et al. 2004). In any case, plant miRNAs negatively regulate functions of the target genes. HD-Zip III genes include sequences that are complementary to miRNAs, miR165/166, in the START domain. The overexpression of *mtZeHB-10*, which has a nucleotide substitution in the region complementary to miR165/166, induced altered shoots with upward curling leaves in transgenic *Arabidopsis* plants. However, overexpression of wild type *ZeHB-10* did not induce substantial abnormal phenotypes. The enhanced effect from the mutation in the START domain was also observed in transgenic *Arabidopsi*s plants with *ZeHB-11*, *ZeHB-12* and *ZeHB-13* (K. Ohashi-Ito, unpublished results). Thus, even in a heterogous system, miRNA appears to function.

These results strongly suggest that miR165/166 generally plays a role in developmental processes governed by HD-Zip III genes. Emery et al. (2003) revealed that the substitution of nucleotides of the miR165/166 binding site without amino acid changes in the START domain of REV/IFL1 caused the gain-of-function type of phenotype. This suggests that the nucleotide sequence in the START domain is essential as the miR165/166 binding site. Similarly, Mallory et al. (2004) indicated that disrupted miRNA pairing without changes in the protein sequence in the *PHB* gene caused the *phb-1d*-like gain-of-function defects. However, amino acid changes in the START domain might cause additional alterations to protein function, because the mutation in the START domain with one amino acid change causes an alteration in the *rev-5* allele (Otsuga et al. 2001). Our finding that mtZeHB-12 proteins with an amino acid change in the START domain had a greater transcriptional activity than that of the wild type in yeast is consistent with the role of the START domain as a peptide sequence. Indeed, the START domain is known as a sequence to which lipids or sterols bind to regulate protein function (Tsujishita and Hurley 2000). Therefore, further analysis is required to identify a ligand that binds to the START domain.

Materials and Methods

Site-Directed mutagenesis

The mutant *ZeHB-10*, *ZeHB-11* and *ZeHB-12* cDNA were generated from these original cDNA clones using the Quikchange Site-Directed mutagenesis kit (Stratagene, La Jolla, CA, USA) with primers of sequence 5′-CCTGGGATGAAGCCTGATCCGGATTCCATAGG-3′ (*ZeHB-10*), 5′-CCTGGGATGAAGCCTGATCCGGATTCGGTTGGG-3′ (*ZeHB-11*), 5′-CCTGGGATGAAGCCTGATCCGGATTCAGTTGGG-3′ (*ZeHB-12*) and its exact complement according to the manufacturer's instructions.

Arabidopsis transformants

The DNA fragments corresponding to protein coding region of wtZeHB-10 and mtZeHB-10, wtZeHB-11, mtZeHB-11, wtZeHB-12 and mtZeHB-12 were obtained by PCR amplification with primers located at the 5′ and 3′ ends of these genes that contained restriction enzyme sites. These fragments were digested by restriction enzymes and inserted into a binary vector pBI121. A plasmid pTA7002 (Aoyama and Chua 1997) was used for construction of two component glucocorticoid-inducible system. The *ZeHB-12* coding region including the nucleotide substitution (G 581 A) was obtained by PCR amplification with forward and reverse primers containing a *Xho* I site and a *Spe* I site, respectively. The PCR product and pTA7002 vector were digested with *Xho* I and *Spe* I. The PCR fragment was inserted into the pTA7002 vector to give rise to pTA7002-mtZeHB-12. For promoter analysis, the 2.2 kb upstream sequences from the ATG translation start of selected genes were amplified from *Arabidopsis* (Columbia) genomic DNA by PCR and cloned into pENTR/D-TOPO vector according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Then, the promoter sequence was transferred by site-specific recombination to the binary vector harboring the GUS reporter gene using GATEWAY system (Invitrogen). These vectors were introduced into *Agrobacterium tumefaciens* strain C58C1 and then into *Arabidopsis thaliana* (ecotype Columbia) using the floral dip method (Clough and Bent 1998). Transformed plants carrying these transgenes were screened by resistance to hygromycin or bialaphos. The detailed morphological analysis of transgenic plants was done on T3 generation of homozygotes. The vectors for promoter analysis were introduced into *Agrobacterium* strain GV3101::pMP90 and then into *Arabidopsis* using the same methods.

RNA gel blot analysis

Total RNA was extracted by using Concert Plant RNA Reagent (Invitrogen). Total RNA (10 μg) was separated electrophoretically on a 1.2% agarose gel and transferred to a positively charged nylon membrane (Roche) and hybridized with a digoxigenin (DIG)-labeled antisense RNA probe under highly stringent conditions. Hybridization signals were visualized immunologically with an anti-DIG Fab fragment conjugated to alkaline phosphatase according to the manufacturer's instructions (Roche).

Histological analysis

Cross sections of 1 μm thickness were made from tissues that had been fixed in FAA (formaldehyde: acetic acid: 70% ethanol; 1 : 1 : 18, by vol.), dehydrated in a graded ethanol series, and embedded in the Technovit 7100 resin (Heraeus Kulzer, Germany). These sections were stained with toluidine blue and observed under a light microscope. Hand-cut sections of the base of stems were stained with 0.05%

Aniline Blue (0.67 M phosphate buffer, pH 8.0) and observed using a UV microscope. For GUS detection, samples were incubated for 16 h at 37°C in GUS staining buffer (100 mM phosphate buffer, pH 7, 0.1% Triton X-100, 0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-glucuronic acid, 5 mM FeCN, and 10 mM EDTA) and rinsed in 70% ethanol. The samples were cleared in chloral hydrate:water:glycerol (8 : 2 : 1, by vol.), and then observed under a microscope (Olympus, Tokyo, Japan).

Gel mobility shift assay

The DNA fragments corresponding to the HD-Zip domains of ZeHB-12 were obtained by PCR amplification. The PCR fragments were cloned into a glutathione S-transferase (GST) expression vector, pGEX6P-1 vector (Amersham Biosciences). The vector was transformed into *Esherichia coli* BL21 strain, and transformed cells were cultured at 37°C and induced by the addition of isopropyl β-D-thiogalactopyranoside to be a final concentration of 0.1 mM. Nucleotide sequences of double-stranded oligonucleotides, BS1 (5′-GAGTAAGTAATGATTACTTTCTC-3′), BS2 (5′- GAGTAAGTAATAATTACTTTCTC-3′) and mtBS (5′-GAGTAAGTAAC- GATTACTTTCTC-3′) were annealed and labeled using α-32P-dCTP and the Klenow frnagment of DNA polymerase I. Five fmol μl^{-1} of the labeled DNA was incubated with 0.1 μg μl^{-1} of a sonicated *E. coli* extract containing GST-ZeHB-12 fusion protein or GST as a control, 0.1 μg μl^{-1} poly (dI-dG) and DNA competitors (500-fold molar excess) in a binding buffer (20 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 1 mM EDTA, 50 mM KCl, and 10% glycerol) at 4°C for 30 min. The reacted samples were loaded onto 4% polyacrylamide gels containing 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 190 mM Glycine.

Transcriptional activation experiment in yeast

The DNA fragments corresponding to ZeHB-10 and ZeHB-12 proteins were obtained by PCR amplification. The PCR fragments were inserted into yeast expression vector pGBKT7 (Clontech, Palo Alto, CA, USA), which expresses proteins fused to amino acids 1–147 of the GAL4 DNA binding domain. In this experiment, the yeast strain Y187 was used. ONPG (o-nitrophenyl β-D-galactopyranoside) was used as a substrate. Yeast transformation and β-galactosidase assays were done according to the manufacturer's instructions.

Chemical treatment of Arabidopsis plants

The pTA7002-mtZeHB-12 transgenic plants and control pTA7002-LUC transgenic plants were grown on agar plates for 18 d and transferred together with the agar onto plastic meshes with 2–3 mm high spacers attached to the lower side. They were placed in plastic container with just enough water. For chemical treatment, the water was replaced with a solution containing 50 μM DEX or with a solution containing same volume of ethanol as DEX solvent. The treatments were continued for 3 h and 6 h and then the treated plants were harvested.

DNA microarray analysis

Total RNA was extracted with Concert Plant RNA Reagent (Invitrogen) and purified with RNeasy columns according to the manufacturer's instructions (QIAGEN, Chatsworth, CA). Double-stranded cDNA was synthesized from the poly (A^+) mRNA present in the total RNA (10 μg) using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitorogen) with an oligo $(dT)_{24}$ primer containing a T7 polymerase promoter site. The synthesized double-stranded cDNA was used as a template to generate biotin-labeled cRNA from an in vitro transcription, using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Inc., Farmingdale, NY). Biotin-labeled cRNA was purified with the use of the RNeasy columns (Qiagen). Twenty micrograms of the resulting biotin-labeled cRNA was fragmented according to the manufacturer's instructions (Affymetrix). Fifteen micrograms of the fragmented cRNA was hybridized at 45°C with rotation for 16 h to probe sets present on an Affymetrix ATH1 arrays. The arrays were washed and then stained (streptavidin-phycoerythrin) in an Affymetrix fluidics station following the manufacturer's protocol. The arrays were scanned by a confocal microscope scanner (HP Genome Array Scanner, Affymetrix) at a wavelength of 570 nm.

Analysis of absolute and differential gene expression was performed with the GeneChip software, Microarray Suite (version 5.0, Affymetrix). Each gene was compared between the samples without DEX treatment and the samples with DEX treatment. Genes that were up- or down-regulated as reflected by a more than 2.5-fold difference in pTA7002-mtZeHB-12 were identified. However, genes that were up- or down-regulated more than 1.5-fold difference in pTA7002-LUC were excluded. Furthermore, genes that had less than 50 signal intensity in all samples were also excluded. These experiments were performed twice.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oupjournals.org.

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