Plant Cell Physiol. 44(12): 1350–1358 (2003)

HD-Zip III Homeobox Genes that Include a Novel Member, *ZeHB-13* (*Zinnia*)/ *ATHB-15* (*Arabidopsis*), are Involved in Procambium and Xylem Cell Differentiation

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HD-Zip III homeobox genes are known to be essential transcriptional factors for vascular development. To further understand the relation of HD-Zip III genes in vascular differentiation, we isolated a new member of the HD-Zip III genes, *ZeHB-13*, as a *Zinnia* homolog of *ATHB-15*, and then characterized the expression profile using a *Zinnia* xylogenic cell culture and *Zinnia* plants. We compared the accumulation pattern of transcripts for *ZeHB-13* and other HD-Zip III genes and suggested that the expression of *ZeHB-13* was restricted to the procambium and was not severely suppressed by brassinazole, an inhibitor of brassinosteroid biosynthesis, unlike other HD-Zip III genes. We also characterized its *Arabidopsis* counterpart, *ATHB-15*. A histochemical promoter analysis using *ATHB-15::GUS* transgenic *Arabidopsis* plants indicated that *ATHB-15* was active specifically in the procambium. These results strongly suggest that ZeHB-13/ATHB-15 is a pivotal transcriptional regulator responsible for early vascular development. Based on these results, we will discuss the regulation of xylem development in light of the functions of HD-Zip III members and brassinosteroids.

Keywords: Brassinosteroids — Homeobox gene — Procambium — Vascular development — Xylem differentiation.

Abbreviations: BA, 6-benzyladenine; Bz, brassinazole; DIG, digoxigenin; HD-Zip III, class III of the homeobox leucine-zipper protein; NAA, 1-naphthalenacetic acid; TE, tracheary element; Uni, uniconazole.

The nucleotide sequence reported in this paper has been submitted to the National Institute of Genetics DDBJ database (URL: http://www.ddbj.nig.ac.jp) under accession number AB109562.

Introduction

The vascular bundle is an essential tissue for land plants because it transports not only water and nutrients, but also signaling molecules such as auxin and cytokinin throughout the plant body. It consists of three parts, the xylem, phloem, and procambium/cambium, which are highly organized within the bundle. However, the molecular mechanism that controls vascular development, especially at the early stage of xylem and phloem differentiation from the procambium/cambium, remains unclear. To understand the molecular mechanism that regulates vascular development, we need to elucidate which key transcriptional regulators direct intravascular pattern formation. Recent progress of *Arabidopsis* mutant analyses indicated that class III of the homeobox leucine-zipper protein (HD-Zip III) might be a key transcriptional regulator of vascular development (Baima et al. 2001, McConnell et al. 2001, Zhong and Ye 1999). In *Arabidopsis*, the HD-Zip III includes five members, ATHB-8, ATHB-9, ATHB-14, ATHB-15, and IFL1/REV (Baima et al. 2001). Two gain-of-function mutants that exhibit adaxialized leaves, *phabulosa* and *phavoluta*, are caused by mutations in the *ATHB-14* and *ATHB-9* genes, respectively (McConnell et al. 2001). In *phabulosa*, the xylem surrounds the phloem in leaf veins, although the xylem and phloem are arranged collaterally in plant wild types (McConnell and Barton 1998). Indeed, transcripts of *ATHB-9* and *ATHB-14* are known to accumulate in vascular regions (McConnell et al. 2001). Mutations of the *IFL1/REV* gene are highly pleiotropic, and the most obvious phenotype has an absence of interfascicular fibers in the inflorescence stems (Talbert et al. 1995, Zhong and Ye 1999). In *ifl1* mutants, xylem development is also affected and the number of tracheary elements is reduced. Accumulation of the *ATHB-8* transcript is restricted to the procambium region (Baima et al. 1995), and *ATHB-8* over-expressing transgenic plants exhibit a promotion of xylem formation (Baima et al. 2001). To sum up, four of the five members of HD-Zip III show a close relationship in vascular development. The last HD-Zip III member, *ATHB-15*, has yet to be studied, but might also be expected to have a role in vascular differentiation.

However, such information is limited to *Arabidopsis* plants, and the general roles for HD-Zip III genes in vascular development are still unknown. Therefore, we tried to study HD-Zip III genes using another plant system, an in vitro *Zinnia* culture system. In this system, about half of the isolated mesophyll cells differentiate synchronously into tracheary elements in medium that includes auxin and cytokinin (Fukuda and Komamine 1980). As a result, this system has been used successfully for analyzing xylem differentiation (Demura et al. 2002, Ito and Fukuda 2002, Nakanomyo et al. 2002). We previously isolated three HD-Zip III genes from *Zinnia*, *ZeHB-10* (an *ATHB-8* homolog), *-11* (an *IFL1/REV* homolog) and *-12*

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(an *IFL1/REV* homolog), and showed that all three transcripts accumulated preferentially in xylem cells (Ohashi-Ito et al. 2002). Interestingly, the accumulation of transcripts for *ZeHB-10*, *-11* and *-12* was induced in cultured *Zinnia* cells by brassinosteroids. Because endogenously biosynthesized brassinosteroids are known to initiate the final step of xylem differentiation in *Zinnia* cell culture (Yamamoto et al. 1997, Yamamoto et al. 2001), brassinosteroids may function in the initiation via *ZeHB-10*, *-11* and *-12* gene expression. This finding is consistent with the fact that in brassinosteroid-deficient mutants of *Arabidopsis*, the xylem region, but not the procambium region, is reduced (Choe et al. 1999, Szekeres et al. 1996) and brassinazole, a specific inhibitor of brassinosteroid biosynthesis, suppresses xylem formation but not procambium and phloem formation (Nagata et al. 2001). Although *ATHB-8* is reported to be expressed specifically in procambium region (Baima et al. 1995), *ATHB-8* may be expressed more preferentially in xylem precursor cells than in procambial cells because only xylem formation but not phloem formation is promoted in *ATHB-8*-overproducing *Arabidopsis* plants (Baima et al. 2001). This is consistent with our finding that the *Zinnia* homolog of *ATHB-8*, *ZeHB-10*, is expressed intensively in xylem precursor cells rather than in procambial cells (Ohashi-Ito et al. 2002). Therefore, there may be a procambium-specific transcriptional regulator that is not affected by brassinosteroids. A likely candidate is the last *Arabidopsis* HD-Zip III member, *ATHB-15*, which is most similar to *ATHB-8*.

In this study, we isolated the *ZeHB-13* gene as a *Zinnia* homolog of *ATHB-15* and characterized its expression profile. We compared the accumulation pattern of transcripts for *ZeHB-13* and other HD-Zip III genes, and suggested that the expression of *ZeHB-13* is restricted to the procambium and is not suppressed severely by brassinazole. Its *Arabidopsis* counterpart, *ATHB-15*, was also found to be expressed specifically in the procambium with *ATHB-15::GUS* transgenic *Arabidopsis* plants. These results strongly suggest that a new member of HD-Zip III, ZeHB-13/ATHB-15, may be an important transcriptional regulator responsible for procambial tissue formation or maintenance.

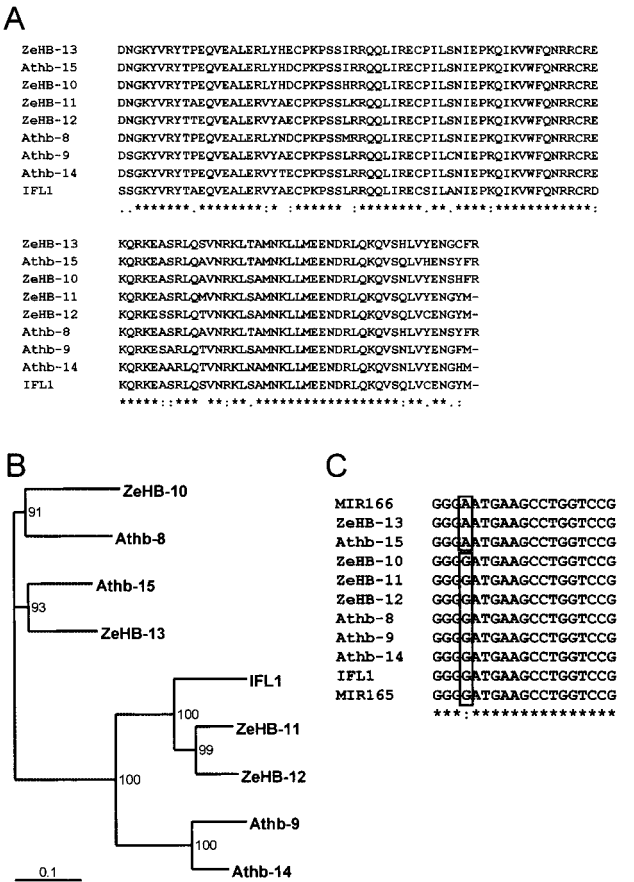

Fig. 1 Sequence analysis of *ZeHB-13*. (A) Amino acid sequence alignment of HD-Zip domains of *Zinnia* ZeHB-13, ZeHB-10, ZeHB-11, and ZeHB-12, and *Arabidopsis* Athb-8, Athb-9, Athb-14, Athb-15, and IFL1 proteins that belong to the HD-Zip III class. * (star) indicates a position that has a single fully conserved residue. : (colon) indicates a position that has a same-property residue. · (dot) indicates a position that has a conserved residue. (B) A phylogenetic tree for *Zinnia* and *Arabidopsis* HD-Zip III proteins. Numbers indicate the bootstrap values. (C) MIR165/166-complementary nucleotide sequences of *Zinnia ZeHB-10*, *-11*, *-12*, and *-13*, *Arabidopsis Athb-8*, *-9*, *-14*, *-15*, and *IFL1*. * (star) indicates a position that has a fully conserved base. : (colon) indicates a position that has a different base between MIR165 and MIR166.

Results

Isolation of cDNA for a new HD-Zip III homeobox gene from Zinnia elegans

An efficient in vitro system for *Zinnia* has been developed that allows for a study of xylem differentiation. Demura et al. (2002) reported many cDNA fragments isolated from *Zinnia* cultured cells differentiating into tracheary elements (TEs). One of these, the No. 3685 fragment, was suggested to be part of the HD-Zip homeobox protein expressed in association with xylem differentiation. We then isolated the full-length cDNA of the No. 3685 fragment from the cDNA library prepared from cells cultured for 48 h in xylogenesis-inducing medium. The full-length cDNA of 2,960 bp, which contained an open reading frame encoding a protein of 838 amino acids, 242 bp of 5′-UTR and 201 bp of 3′-UTR was obtained (DDBJ accession number AB109562). A BLAST search of the protein at NCBI indicated that the predicted protein shares a high sequence identity with HD-Zip III proteins (Fig. 1A). However, it does not completely share sequences with the HD-Zip III proteins we have already analyzed, ZeHB-10, -11 and -12. Therefore, the newly cloned cDNA was designated *ZeHB-13*. Fig. 1B shows the phylogenetic relationship of HD-Zip III proteins between *Zinnia* and *Arabidopsis* and indicates ZeHB-13 is a *Zinnia* homolog of *Arabidopsis* ATHB-15 that has not yet been analyzed. The HD-Zip III proteins share the START domain

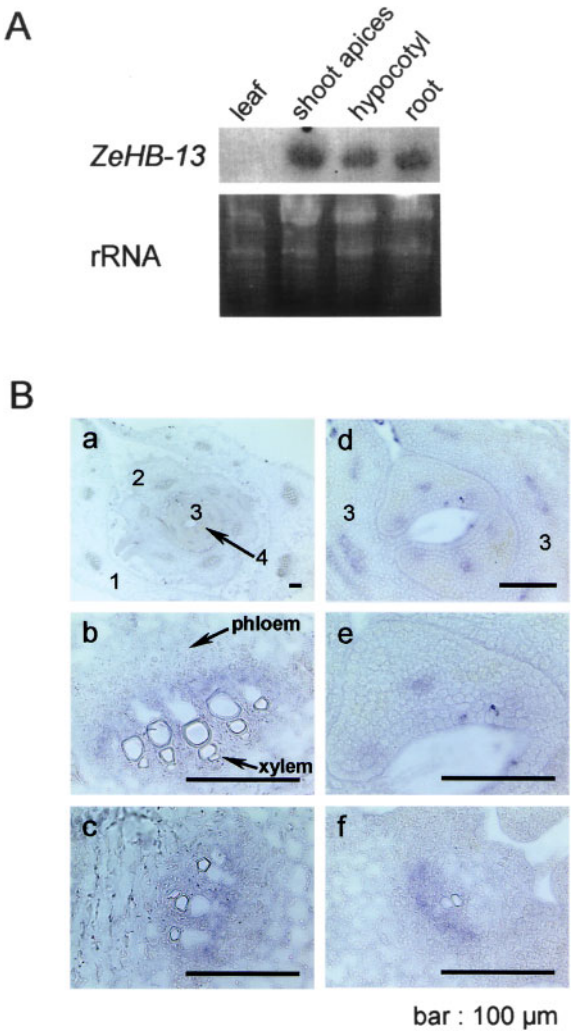

Fig. 2 Accumulation of the *ZeHB-13* transcript in *Zinnia*. (A) Accumulation of the *ZeHB-13* transcript in organs. Total RNA was isolated from the first leaves, shoot apices, hypocotyls, and roots of 14-day-old *Zinnia* seedlings. The gel blot was hybridized with DIG-labeled cRNA for *ZeHB-13* as a probe. Ethidium bromide staining of rRNA is shown below as a marker of equal amounts of applied RNA. (B) Tissue-specific accumulation of the *ZeHB-13* transcript. In situ hybridization of the antisense *ZeHB-13* probe to sections of 14-day-old *Zinnia* seedlings. (a) A cross-section of the shoot apical region hybridized with the antisense *ZeHB-13* probe. In this picture, the numbers 1 to 4 indicate the first to fourth leaves, respectively. (b–e) Magnified images of (a). (b) The main vein of the second leaf. (c) The lateral vein of the second leaf. (d) Third and fourth leaves. (e) The fourth leaf. (f) The main vein of the second leaf hybridized with the antisense probe of *ZeHB-13*.

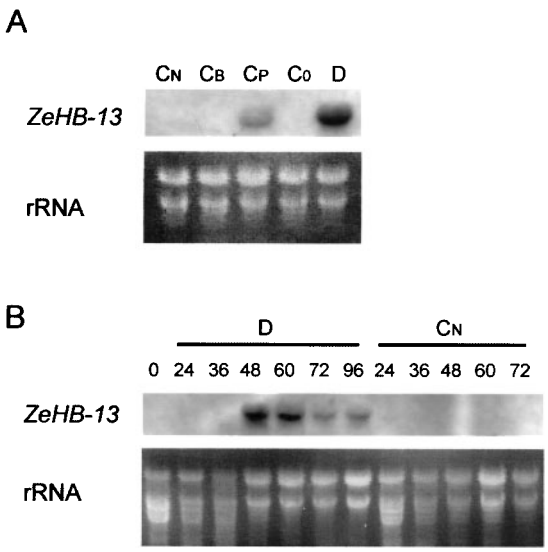

Fig. 3 Accumulation of the *ZeHB-13* transcript in *Zinnia* cells cultured in vitro. (A) Hormonal effects on the level of the *ZeHB-13* transcript. Total RNA was isolated from *Zinnia* cells cultured for 48 h in C_N, C_B, C_P, C_0 and D media containing different concentrations of auxin and cytokinin. The gel blot was hybridized with DIG-labeled cRNA for *ZeHB-13* as a probe. Ethidium bromide staining of rRNA is shown below. (B) Temporal pattern of accumulation of the *ZeHB-13* transcript during in vitro xylem differentiation. Total RNA was extracted from *Zinnia* cells cultured for indicated periods in D and C_N media. TE formation occurred between 48 h and 72 h in cells cultured in D medium, but not at all in cells cultured in C_N medium. The gel blot was hybridized with DIG-labeled cRNA for *ZeHB-13* as a probe. Ethidium bromide-stained gel is shown at the bottom.

(Ponting and Aravind 1999), as well as the homeodomain and leucine zipper domain. Recently, microRNAs MIR165 and MIR166, which are complementary to the START domain of the HD-Zip III proteins, were identified (Reinhart et al. 2002, Rhoades et al. 2002, Tang et al. 2003) and suggested to function as negative regulators of HD-Zip III protein functions (Emery et al. 2003, McConnell et al. 2001). The START domain of *ZeHB-13* and *ATHB-15* was complementary to MIR166, while that of other *Zinnia* and *Arabidopsis* HD-Zip III proteins was complementary to MIR165. These results strongly suggest that ZeHB-13/ATHB-15 is a unique member of HD-Zip III proteins.

Accumulation of the ZeHB-13 transcript in Zinnia plants

To characterize the expression of *ZeHB-13* in plants, we performed an RNA gel blot analysis for organs of 14-day-old *Zinnia* seedlings. The transcript for *ZeHB-13* was below the detectable level in mature leaves, but abundant in shoot apices, hypocotyls, and roots (Fig. 2A). Localization of the transcript for *ZeHB-13* was investigated by in situ hybridization in shoot apical regions of 14-day-old *Zinnia* seedlings and found to accumulate in vascular regions (Fig. 2B-a). In vascular regions, the signal of *ZeHB-13* was localized preferentially in procambium cells that existed between the xylem and phloem cells, in main veins and the lateral veins of second leaves (Fig. 2B-b, -c, -f). At the fourth leaves, where vascular tissues had not yet fully developed and visible xylem cells were not present, the transcript for *ZeHB-13* accumulated in cells located at future vascular regions (Fig. 2B-e). In the third leaves, the signal of *ZeHB-13* extended as a line that consisted

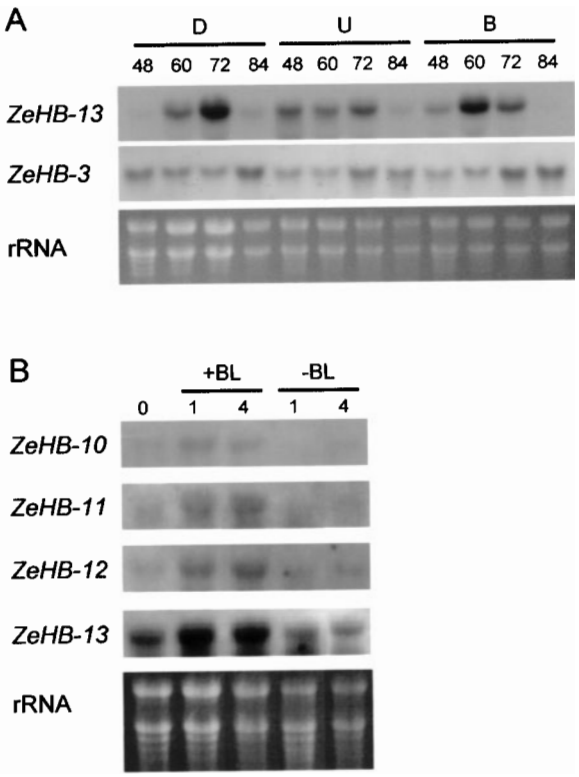

Fig. 4 Effect of brassinosteroids on accumulation of transcripts for *ZeHB-13*, *-10*, *-11*, *-12*, and *-3*. (A) Total RNA samples, which were prepared from *Zinnia* cells cultured for indicated periods in the absence of (D) or the presence of 5 μM uniconazole (U), or 5 μM uniconazole plus 10 nM brassinolide (B), were used for gel blot analysis. Hybridization was performed with DIG-labeled cRNA for each gene as a probe. Ethidium bromide staining of rRNA is shown at the bottom. (B) Total RNA was prepared from *Zinnia* cells cultured for 48 h in the presence of 5 μM brassinazole (0) and cells further cultured for indicated hours with (+ BL) or without (– BL) brassinolide. Gel blots were hybridized with DIG-labeled cRNA for *ZeHB-10*, *-11*, *-12*, and *-13* as probes. Ethidium bromide staining of rRNA is shown at the bottom.

of one or two cell layer(s), which corresponded to procambium cells (Fig. 2B-d). These results show that the *ZeHB-13* transcript accumulates preferentially in procambium cells throughout vascular development.

Accumulation of the ZeHB-13 transcript in a Zinnia cell culture

We investigated the accumulation pattern of the *ZeHB-13* transcript in *Zinnia* cells cultured for 48 h in the presence or absence of auxin and/or cytokinin (Fig. 3A). The transcript for *ZeHB-13* was abundant in cells cultured in xylogenesis-inducing medium (D), but did not accumulate in cells cultured in C_N, C_B, and C_0 media in which cells do not differentiate into TEs. The *ZeHB-13* transcript accumulated at a low level when cultured in C_P medium, where a small population of cells differentiated into TEs. To reveal the time course of accumulation of the *ZeHB-13* transcript during xylem differentiation, we performed an RNA gel blot analysis for cells cultured for various periods in D and C_N media. In C_N-cultured cells, accumulation of the *ZeHB-13* transcript was not detected throughout the culture period. In D-cultured cells, the transcript for *ZeHB-13* began to accumulate after 48 h in culture, and then decreased gradually (Fig. 3B). These results suggest that the *ZeHB-13* transcript accumulates in a xylogenesis-specific manner.

Effect of brassinosteroids on transcript levels of Zinnia HD-Zip III genes

It was revealed that brassinosteroid is essential for TE differentiation (Iwasaki and Shibaoka 1991, Yamamoto et al. 1997). We previously showed that the accumulation of transcripts for *ZeHB-10*, *-11* and *-12* was almost completely suppressed by uniconazole, an inhibitor of brassinosteroid biosynthesis, and such suppression was reversed by the addition of brassinolide, a biologically active brassinosteroid (Ohashi-Ito et al. 2002). To investigate whether the accumulation of the *ZeHB-13* transcript was regulated by brassinosteroid or not, we performed an RNA gel blot analysis for cells cultured in D-medium containing uniconazole (U), uniconazole and brassinolide (B), and none of them as a control (D). In cells cultured in U-medium, accumulation of the *ZeHB-13* transcript was slightly suppressed when compared to that in the D-medium. Such suppression was reversed with the addition of brassinolide (Fig. 4A). These results indicate that brassinosteroids are not needed to induce the accumulation of the *ZeHB-13* transcript, but can promote the accumulation of the *ZeHB-13* transcript. In contrast, the accumulation pattern of the transcript for *ZeHB-3*, which is a HD-Zip I gene and a marker of immature phloem (Nishitani et al. 2001), was not significantly affected by uniconazole or by brassinolide.

To determine earlier effects of brassinosteroids on the accumulation of transcripts for HD-Zip III genes, we followed the changes in levels of *ZeHB-10*, *-11*, *-12* and *-13* transcripts. This was undertaken after the addition of brassinosteroids to cells cultured in D-medium that included brassinazole, a specific inhibitor of brassinosteroid biosynthesis (Sekimata et al. 2001), for 48 h from the start of culture (Fig. 4B). Transcripts of *ZeHB-10*, *-11* and *-12* accumulated only slightly in the presence of brassinazole (48 h) and appeared within 1 h after the addition of brassinolide. Similarly, the level of the *ZeHB-13* transcript increased within 1 h after the brassinolide-treatment, although its substantial level accumulated even in the presence of brassinazole. These results indicate that HD-Zip III genes are early targets of brassinosteroid signaling.

The accumulation of transcripts for HD-Zip III genes in specific holes

To understand the spatial regulation of the expression of HD-Zip III genes in detail, we performed in situ hybridization using serial sections of shoot apices of 14-day-old *Zinnia* seedlings (Fig. 5). The signal of the *ZeHB-3* transcript was restricted to immature phloem cells near the sieve element cells

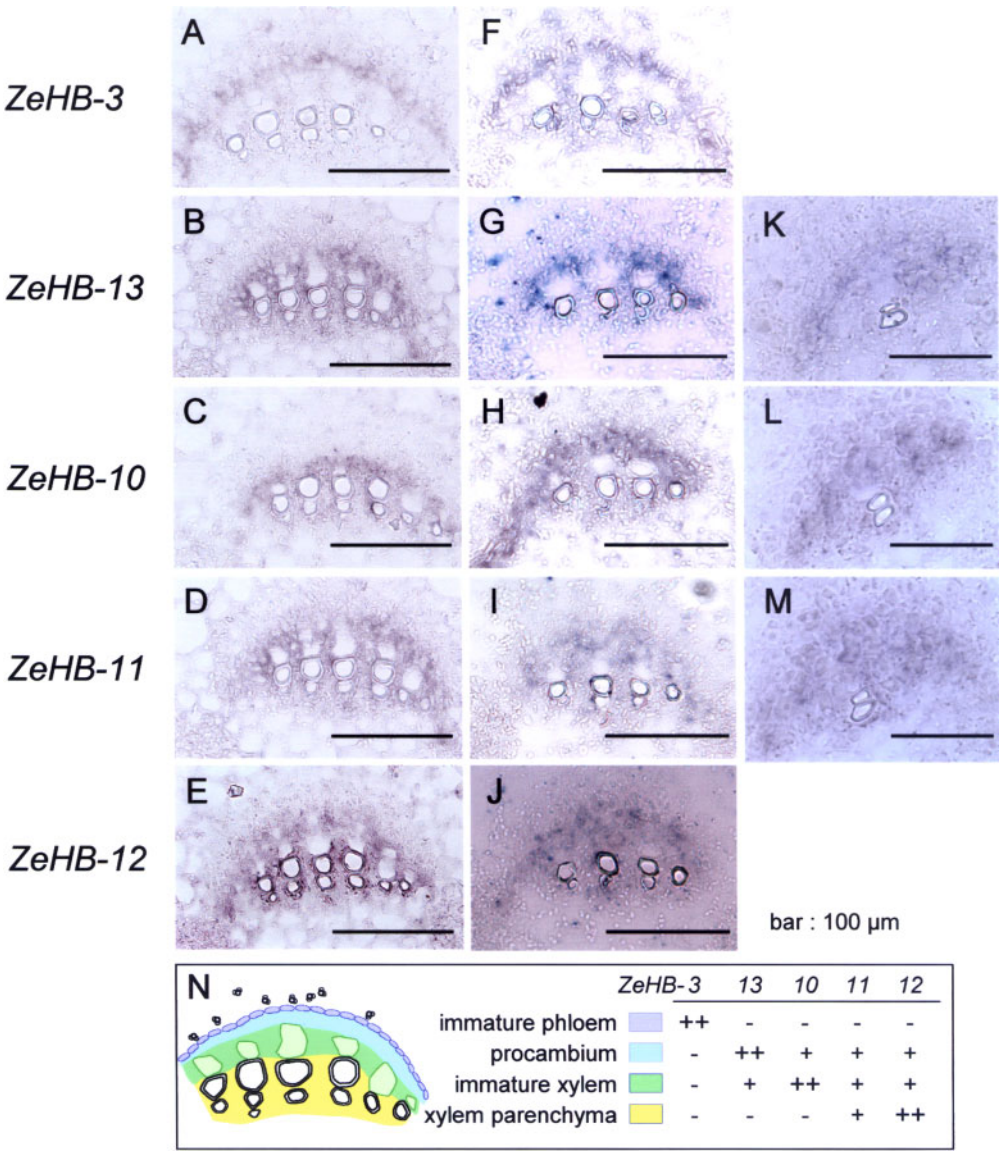

Fig. 5 Accumulation pattern of *ZeHB-3*, *-10*, *-11*, *-12*, and *-13* transcripts in vascular tissues. In situ hybridization with antisense probes for *ZeHB-3*, *-10*, *-11*, *-12*, and *-13* was performed in serial cross-sections of shoot apices of 14-day-old *Zinnia* seedlings. (A–E) The main vein of the second leaf hybridized with antisense sequences for *ZeHB-3*, *-10*, *-11*, *-12*, and *-13*, respectively. (F–J) The main vein of the second leaf hybridized with *ZeHB-3*, *-10*, *-11*, *-12*, and *-13* probes, respectively. (K–M) The main vein of the third leaf hybridized with *ZeHB-10*, *-11* and *-13* probes, respectively. (N) Schematic diagram of accumulation patterns of *ZeHB-3*, *-10*, *-11*, *-12*, and *-13* transcripts in vascular tissues. Purple, light blue, green, and yellow indicate immature phloem cells, procambium cells, immature xylem cells and developing TEs, and parenchyma cells, respectively. ++ indicates a strong signal. + indicates the signal exists. – indicates no signal.

as expected (Fig. 5A, F). The *ZeHB-13* transcript was detected preferentially in cells just below the cells that accumulated the *ZeHB-3* transcript (Fig. 5B, G, K). The *ZeHB-10* transcript accumulated in cells next to the TEs on the upper side and in cells that were progressing to become TEs (Fig. 5C, H, L). Compared to the accumulation of the *ZeHB-13* transcript, the *ZeHB-10* transcript signal was more restricted to the xylem side. The accumulation pattern of *ZeHB-11* and *-12* transcripts was very similar. These signals were observed in the *ZeHB-13* and *-10* transcript-accumulated cells and xylem parenchyma cells that existed between the TEs (Fig. 5D, E, I, J, M). The only difference was that the *ZeHB-12* transcript signal was stronger than the *ZeHB-11* transcript signal in xylem parenchyma cells.

Expression pattern of Arabidopsis ATHB-15 promoter in seedlings

To compare with the expression pattern of *ZeHB-13*, we examined the expression pattern of *ATHB-15*, which is the *Arabidopsis* gene most similar to *ZeHB-13*. The GUS reporter gene

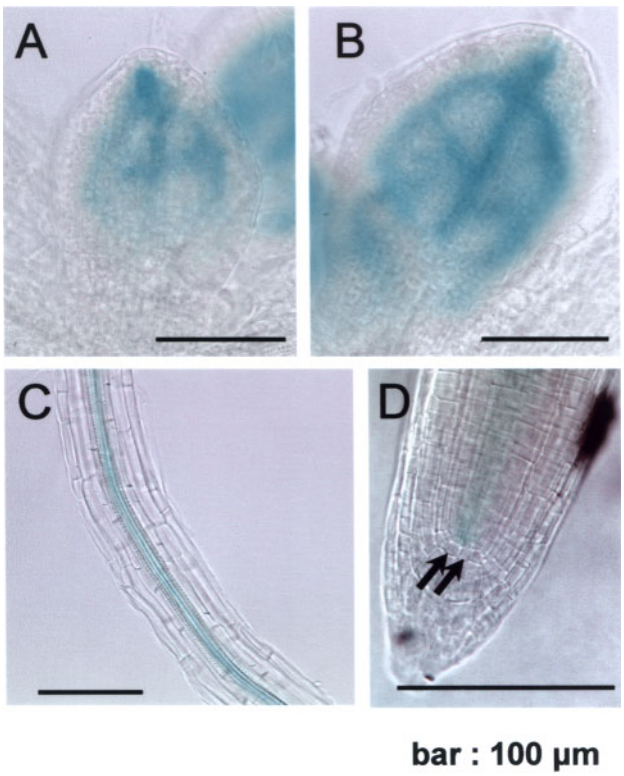

Fig. 6 *GUS* expression pattern in *Arabidopsis* seedlings harboring *Athb-15 promoter::GUS*. (A) The second leaf of 4-day-old seedlings, (B) the first leaf of 4-day-old seedlings, (C) the root of 6-day-old seedlings, and (D) the primary root tip of 6-day-old seedlings. Arrows indicate the quiescent centers.

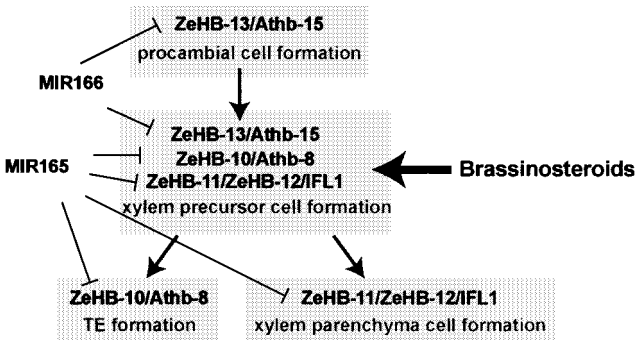

Fig. 7 Model of how HD-Zip III genes direct the radial pattern of xylem. An unknown vascular cell-initiating signal induces the expression of *ZeHB-13/Athb-15*, which functions in the formation or maintenance of procambial cells. Procambial cells produce brassinosteroids, which promote the expression of *ZeHB-13/Athb-15* and form a positive feedback loop, and at the same time induce the expression of *ZeHB-10/Athb-8* and *ZeHB-11/ZeHB-12/IFL1*. Cells expressing *ZeHB-10/Athb-8* differentiate into TEs, while cells expressing *ZeHB-11/ZeHB-12/IFL1* become xylem parenchyma cells. MIR166 functions as a negative regulator of *ZeHB-13/Athb-15* and restricts the distribution of its transcript within the procambium. In contrast, MIR165 limits the distribution of the other HD-Zip III transcripts in xylem tissues.

under the control of the 2.2 kb *ATHB-15* promoter was introduced into *Arabidopsis* plants by *Agrobacterium*-mediated transformation. Transformants grown for 4 or 6 d on standard Murashige and Skoog medium plates were histologically stained to analyze the GUS expression. *ATHB-15::GUS* expression was clearly seen in young leaves and roots (Fig. 6). In young leaves, in which leaf veins had yet to appear, GUS expression was restricted to future leaf veins (Fig. 6A, B). In primary roots, GUS expression was found in the vascular tissue, especially in cells located between two protoxylems (Fig. 6C). In root tips, GUS expression was restricted to vascular cell files that started from cells next to quiescent center cells (Fig. 6D). These results clearly indicate that *ATHB-15* is expressed in a procambium-cell-specific manner from a very early stage of vascular differentiation.

Discussion

ZeHB-13/ATHB-15 functions in the procambium

In this study, we characterized a new class member of the HD-Zip III gene family, *Zinnia ZeHB-13*, and *Arabidopsis ATHB-15*. The two genes shared not only sequences of the homeo domain and the leucine zipper domain, but also a sequence within the START domain that is complementary to microRNA, MIR166. On the other hand, the other members of the HD-Zip III of *Arabidopsis* and *Zinnia* shared a sequence in the START domain complementary to MIR165. The *ZeHB-13* transcript accumulated preferentially in procambial cells in planta (Fig. 2B, 5) and in procambium-like cells in *Zinnia* culture (Fig. 3). Similarly, *ATHB-15* promoter expression was restricted to procambial cells in leaves and roots (Fig. 6). These results strongly suggest that *ZeHB-13/ATHB-15* is a unique member of the HD-Zip III family and is involved in procambial function. In situ hybridization and gel blot analysis of RNA from cultured cells indicated that *ZeHB-13/ATHB-15* is the earliest expressed gene during vascular development among *ZeHB-13/ATHB-15*, *ZeHB-10/ATHB-8*, and *ZeHB-11/ZeHB-12/IFL1* (Fig. 3, 5). Although *ATHB-8* is known as one of the earliest vascular markers expressed in the procambium (Baima et al. 1995, Kang and Dengler 2002), our previous and present results indicate that *ATHB-8/ZeHB-10* is rather expressed in xylem precursor cells (Fig. 5, Ohashi-Ito et al. 2002).

ZeHB-13 expression is regulated by an unknown factor and brassinosteroids

Brassinosteroid-deficient mutants with defects in different enzymes in the brassinosteroid biosynthetic pathway show a typical phenotype that includes dwarf, altered photomorphogenesis, and an abnormal vascular pattern in which phloem increases and xylem decreases (Choe et al. 1999, Jang et al. 2000, Szekeres et al. 1996). In cultured *Zinnia* cells, uniconazole inhibited differentiation of TEs from procambial cells, and brassinolide overcame its inhibition (Iwasaki and Shibaoka 1991, Yamamoto et al. 1997). These findings strongly suggest

that endogenously biosynthesized brassinosteroids only promote xylem formation. We indicated that inhibitors of brassinosteroid biosynthesis completely suppress the accumulation of transcripts for *ZeHB-10*, *-11*, and *-12* (Ohashi-Ito et al. 2002), and that brassinosteroids immediately induce the accumulation of transcripts in developing xylem cells, but not the accumulation of the *ZeHB-3* transcript in immature phloem cells (Fig. 4). This result not only supports the specific involvement of brassinosteroids in xylem formation, but also suggests that brassinosteroids may be initiators of *ZeHB-10*, *-11*, and *-12* expression. On the other hand, inhibitors of brassinosteroid biosynthesis did not completely suppress the accumulation of the *ZeHB-13* transcript, which indicates the presence of a factor other than brassinosteroids that initiates *ZeHB-13* expression. Interestingly, however, the expression of *ZeHB-13* was promoted by brassinosteroids (Fig. 4). Because brassinosteroids seem to be produced in procambial cells (Yamamoto et al. 2001), these findings may imply the presence of a feedback promotion in which ZeHB-13 functions in the differentiation of procambial cells, producing brassinosteroids, which in turn promote *ZeHB-13* expression. Another possibility is that *ZeHB-13* may be expressed and function in both procambial and xylem precursor cells, and only the latter cells may possess brassinosteroid perception machinery. Further analysis is required to elucidate the relationship between brassinosteroids and *ZeHB-13* expression.

HD-Zip III genes may be involved in radial pattern formation of xylem tissues

Individual species of vascular plants have unique radial patterns of vascular tissues. Even in a plant species, distinctive intravascular organization is formed in different organs such as roots, stems, and leaves. Procambial/cambial cells produce the radial pattern of xylem and phloem tissues. Towards the inside, procambial/cambial cells differentiate primarily into xylem precursor cells, which in turn differentiate into TE precursor cells or xylem parenchyma precursor cells, and then into TEs or xylem parenchyma cells. Based on our findings shown here, we present a working hypothesis for the radial pattern formation of xylem tissues (Fig. 7). An unknown vascular cell-initiating signal induces the expression of *ZeHB-13/ATHB-15*, which functions in the formation or maintenance of procambial cells. The possible candidates for vascular cell-initiating signals may be a combination of auxin and cytokinin, because the combination is a prerequisite for *ZeHB-13* mRNA accumulation in *Zinnia* xylogenic culture (Fig. 3). In *woodenleg* mutants defective in a cytokinin receptor, the number of procambial cells in embryos is reduced and the vascular system in emerging roots is composed only of xylem (Scheres et al. 1995, Mähönen et al. 2000), which suggests that cytokinin function is necessary for the initiation and maintenance of procambial activity. It is well known that auxin is also necessary for inducing vascular cells including procambial cells (Fukuda 1997). Genes involved in auxin signaling such as *Zinnia* homologues of *ARF5/MONOPTEROS*, *IAA8/IAA9*, and *AUX1* are expressed before *ZeHB-13* expression in *Zinnia* xylogenic culture (Demura et al. 2002). Taken together with the recent finding that the expression of *ATHB-8* is promoted via ARF5/MONOPTEROS activity by auxin (Mattsson et al. 2003), it is plausible that the establishment of new intracellular auxin signaling systems occurs in differentiation of procambial cells. Procambial cells produce brassinosteroids in the presence of auxin and cytokinin (Yamamoto et al. 2001). Brassinosteroids promote the expression of *ZeHB-13/ATHB-15* and form a positive feedback loop, and at the same time induce the expression of *ZeHB-10/ATHB-8*, *ZeHB-11/IFL1*, and *ZeHB-12/IFL1*. However, auxin in addition to brassinosteroids may also be necessary for inducing these genes, because the differentiation from procambial cells to TEs in *Zinnia* culture requires the presence of both auxin and brassinosteroids (Iwasaki and Shibaoka 1991). We have revealed that multiple HD-Zip III transcripts accumulate in a different xylem cell type in a distinctive combination (Fig. 5) and that HD-Zip III proteins can make heterodimers as well as homodimers (Ohashi-Ito et al. 2002). Therefore, it is possible that a specific combination of heterodimers and homodimers of HD-Zip III proteins functions in specifying some xylem cell types. The combination of *ZeHB-10/ATHB-8*, *ZeHB-11/IFL1*, and *ZeHB-12/IFL1* (or plus *ZeHB-13/ATHB-15*) may initiate the formation of xylem precursor cells. Cells expressing *ZeHB-10/ATHB-8* may differentiate into TEs, while cells expressing *ZeHB-11/IFL1*, and *ZeHB-12/IFL1* may become xylem parenchyma cells. MicroRNAs MIR165 and MIR166 may also function in the specific distribution of these transcripts in vascular tissues (Reinhart et al. 2002, Rhoades et al. 2002, Tang et al. 2003). MIR166 may function as a negative regulator of *ZeHB-13/ATHB-15* and restrict the distribution of its transcript within the procambium. In contrast, MIR165 may limit the distribution of the other HD-Zip III transcripts in xylem tissues.

Materials and Methods

Plant material

Seeds of *Zinnia elegans* (cv. Canary Bird) were purchased from Takii Shubyo (Kyoto, Japan). *Zinnia* seedlings were grown on vermiculite at 25°C under a daily 14 h light period. The first leaves of 14-day-old seedlings were used for the isolation of mesophyll cells in the suspension culture.

Cloning of full-length cDNA of ZeHB-13

The cDNA fragment of Z3685 (Demura et al. 2002) was labeled by radioisotope according to the manufacturer's instructions (Promega, Madison, WI, U.S.A.). Using this probe, we performed screening of the cDNA phage library produced from *Zinnia* xylogenic culture cells. The cDNA insert was cloned into the phagemid vector pBluescript SK by in vivo excision according to the manufacturer's instructions (Stratagene, La Jolla, CA, U.S.A.).

Construction of a phylogenetic tree

The CLUSTAL W program on the DNA Data Bank of Japan was used for alignment and tree calculation. The phylogenetic tree was displayed using Tree View software from DDBJ.

RNA gel blot analysis

cDNAs cloned into the pBluescript SK vector were amplified by PCR using T3 and T7 primers. Digoxigenin-labeled antisense RNA probes were synthesized from these PCR products using a DIG RNA labeling kit (Roche, Basel, Switzerland). Total RNA was isolated according to Ozeki et al. (1990). 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).

In situ hybridization

Fourteen-day-old seedlings were used for in situ hybridization. Eight-micrometer serial sections from shoot apical regions were prepared according to Demura and Fukuda (1994). Hybridization was performed according to Demura and Fukuda (1994) with some modifications. The hybridization solution, containing 50% formamide, 250 mM NaCl, 125 mM Na_2HPO_4, 1 mM EDTA, 7% SDS, and 10% PEG6000, and a DIG-labeled RNA probe were layered onto slides to which sections had been fixed. The slides were incubated at 50°C for 16 h and soaked in 4× SSC at 50°C for 10 min. Any non-hybridized probe was removed electrophoretically at 3 V cm^{-1} for 1 h in TAE buffer (0.04 M Tris, 0.04 M CH_3COOH, 1 mM EDTA, pH 8.0). The hybridization signal was detected colorimetrically using an anti-DIG Fab fragment conjugated to alkaline phosphatase.

Cell suspension culture

Mesophyll cells were isolated mechanically in a culture medium with a blender by the maceration of surface-sterilized first leaves of 14-day-old *Zinnia* seedlings according to the procedure of Sugiyama and Fukuda (1995). Isolated mesophyll cells were cultured in a modified version of Fukuda and Komamine's medium (Fukuda and Komamine 1980) in the dark at 27°C, while being rotated at 10 rpm on a revolving drum. The culture medium for the induction of TE differentiation (D medium) contained 0.1 mg $liter^{-1}$ 1-naphthalenacetic acid (NAA) and 0.2 mg $liter^{-1}$ 6-benzyladenine (BA). For control cultures, in which few or no TEs were differentiated, C_P medium containing 0.1 mg $liter^{-1}$ NAA and 0.001 mg $liter^{-1}$ BA, C_N medium containing only 0.1 mg $liter^{-1}$ NAA, C_B medium containing only 0.2 mg $liter^{-1}$ BA, and C_0 medium that did not contain any phytohormone were used instead of the D medium. To study the relationship between gene expression and the brassinosteroid, cells were cultured in the presence of 5 μM uniconazole (Uni), 5 μM brassinazole (Bz, a gift from Prof. S. Yoshida, Sekimata et al. 2001), or 5 μM Uni plus 10 nM brassinolide (BL), or 5 μM Bz plus 10 nM BL. In this experiment, all cultures contained a final concentration of 0.5% DMSO as a solvent of agents that did not affect xylem differentiation (Fukuda and Komamine 1981).

ATHB-15 promoter analysis

The 2.2 kb upstream sequence of the ATG translation start of *ATHB-15* was amplified from *Arabidopsis* (Columbia) and fused to the GUS gene to produce an *ATHB-15* promoter::GUS fusion construct. *Arabidopsis* flowering plants were transformed using the floral dip method (Clough and Bent 1998) and transformed plants carrying the construct were screened by resistance to bialaphos. 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).

Acknowledgments

We are grateful to Dr Taku Demura (PSC, RIKEN, Yokohama, Japan) for providing the cDNA fragment of Z3685. We also thank Dr Shigeo Yoshida (PSC, RIKEN, Wako, Japan) for supplying the brassinazole. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (14036205), from the Japan Society for the Promotion of Science (1570018), and from the Ministry of Agriculture, Forestry and Fisheries ("Gene discovery and elucidation of functions of useful genes in rice genome by gene-expression monitoring system") to H.F.

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(Received August 26, 2003; Accepted September 24, 2003)