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Interplay of auxin, KANADI and Class III HD-ZIP transcription factors in vascular tissue formation

Michael Ilegems[1], Véronique Douet[1], Marlyse Meylan-Bettex[1], Magalie Uyttewaal[2], Lukas Brand[3], John L. Bowman[3,*] and Pia A. Stieger[1,*]

SUMMARY

Class III HD-ZIP and KANADI gene family members have complementary expression patterns in the vasculature and their gain-of-function and loss-of-function mutants have complementary vascular phenotypes. This suggests that members of the two gene families are involved in the establishment of the spatial arrangement of phloem, cambium and xylem. In this study, we have investigated the role of these two gene families in vascular tissue differentiation, in particular their interactions with the plant hormone auxin. We have analyzed the vasculature of plants that have altered expression levels of Class III HD-ZIP and KANADI transcription factors in provascular cells. Removal of either KANADI or Class III HD-ZIP expression in procambium cells led to a wider distribution of auxin in internal tissues, to an excess of procambium cell recruitment and to increased cambium activity. Ectopic expression of *KANADI1* in provascular cells inhibited procambium cell recruitment due to negative effects of *KANADI1* on expression and polar localization of the auxin efflux-associated protein PIN-FORMED1. Ectopic expression of Class III HD-ZIP genes promoted xylem differentiation. We propose that Class III HD-ZIP and KANADI transcription factors control cambium activity: KANADI proteins by acting on auxin transport, and Class III HD-ZIP proteins by promoting axial cell elongation and xylem differentiation.

KEY WORDS: Vasculature, Auxin, KANADI, Class III HD-ZIP, Cambium, Xylem, *Arabidopsis*

INTRODUCTION

The seed plant vascular system is composed of differentiated phloem and xylem tissues. As in most seed plants, in *Arabidopsis thaliana* vascular bundles are organized in a collateral pattern (see Fig. 1A,B). Vascular differentiation is initiated from defined cells that are recruited within a growing organ to form continuous files known as the procambium, from which the conducting cells of xylem and phloem differentiate. During this process, some procambial cells remain in their undifferentiated state and function as cambium cells to produce secondary vascular tissues (Busse and Evert, 1999a; Busse and Evert, 1999b; Esau, 1965; Sachs, 1981).

The conducting functions of xylem and phloem require perfect cell alignment and tissue continuity, transverse patterning within veins, proper integration within non-vascular tissues, as well as coordinated maturation of different vascular cell types. Physiological and molecular studies have revealed hormonal regulators and transcription factors that may govern the network of regulation in vascular tissue differentiation. The procambial strand location is defined by directional transport of the plant hormone auxin in a self-reinforcing canalization process from source to sink (Sachs, 1981; Sachs, 1991). This model is based on a feedback effect that auxin exerts on the polarity of its transport (Paciorek et al., 2005; Sauer et al., 2006). Indeed, basal localization of the auxin efflux-associated protein PIN-FORMED1 (PIN1) in cell files is the earliest event observed in developing procambium cells (Reinhardt et al., 2003; Scarpella et al., 2004; Scarpella et al., 2006). Along with the spatial restriction of PIN proteins to single cell files, the expression pattern of the auxin response factor, *MONOPTEROS* [*MP*, also known as *AUXIN RESPONSE FACTOR 5* (*ARF5*)], is restricted to procambium and developing xylem cells (Hardke and Berleth, 1998; Wenzel et al., 2007). The discontinuous vasculature formed in *mp* loss-of-function mutants suggests that MP functions in auxin signal transduction during vascular development (Hardke and Berleth, 1998; Mattsson et al., 2003).

Procambium cells differentiate into xylem and phloem, providing the vascular bundles with a specific transverse pattern. This pattern is likely to be formed and maintained by the dorsiventral activities of antagonistic regulators. Two gene families in particular have been shown to play antagonistic roles in dorsiventral patterning in both leaves and vasculature. These gene families include a subclade of the GARP family of transcription factors, the KANADI genes (*KAN1-4*), which are expressed in the phloem, and the Class III Homeodomain-leucine zipper (Class III HD-ZIP) gene family, expressed in procambium, cambium and developing xylem. KANADI loss-of-function mutants develop phloem cells, indicating that KANADI genes are not required for phloem identity; however, ectopic expression of *KAN1* leads to a complete loss of vascular development (Eshed et al., 2001; Kerstetter et al., 2001).

Five Class III HD-ZIP genes are encoded in the *Arabidopsis thaliana* genome [*PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REVOLUTA* (*REV*), *ATHB15* (also known as *CORONA*) and *ATHB8*] (Baima et al., 1995; McConnell et al., 2001; Ohashi-Ito and Fukuda, 2003; Ohashi-Ito et al., 2005; Otsuga et al., 2001; Prigge et al., 2005). Homologs of *REV*, *ATHB15* and *ATHB8* have also been isolated from *Zinnia* (Ohashi-Ito and Fukuda, 2003). Based on their expression patterns in *Zinnia* and *Arabidopsis*, and on the increased production of specific cell types in gain-of-function mutants of the different Class III HD-ZIP genes, individual functions have been proposed for single members of the gene family (Ohashi-Ito and Fukuda, 2003; Ohashi-

[1]Institute of Biology, University of Neuchâtel, Rue Emile Argand 11, 2009 Neuchâtel, Switzerland. [2]Laboratoire de Reproduction et Développement des Plantes, ENS, 46 allée d'Italie, 69364 Lyon Cedex 07, France. [3]School of Biological Sciences, Monash University, Melbourne, Victoria 3800, Australia.

*Authors for correspondence (john.bowman@sci.monash.edu.au; pia.stieger@unine.ch)

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Ito et al., 2005). *ATHB15/ZeHB13* (*Ze*, *Zinnia elegans*) is expressed in procambium cells before the other gene family members and was proposed to regulate procambium formation. The expression of *ATHB8/ZeHB10* coincides with tracheary element precursors and *ATHB8/ZeHB10* gain-of-function alleles have increased numbers of tracheary elements (Ohashi-Ito et al., 2005; Baima et al., 2001). *REV/ZeHB11/ZeHB12* is expressed in procambium and xylem parenchyma cells and *REV/ZeHB12* gain-of-function mutants have an increased production of procambium and/or xylem precursor cells (Emery et al., 2003; Ohashi-Ito et al., 2005; Zhong and Ye, 2004).

Emery et al. proposed KANADI and Class III HD-ZIP transcription factors to be components of a dorsiventral system for patterning in the vasculature (Emery et al., 2003). An amphivasal vasculature (xylem surrounding the phloem) is found in KANADI multiple loss-of-function mutants and in *REV*, *PHB* and *PHV* gain-of-function mutants, whereas multiple Class III HD-ZIP loss-of-function mutants have amphicribal (phloem surrounding the xylem) vascular bundles (Emery et al., 2003; McConnell and Barton, 1998; McConnell et al., 2001; Zhong and Ye, 2004). However, in roots, the collateral arrangement of xylem and phloem is not changed in Class III HD-ZIP and KANADI multiple loss-of-function mutants, indicating that amphicribal and amphivasal vasculature may be the consequence of radialization of lateral organs, which normally have a dorsiventral shape (Hawker and Bowman, 2004).

Several observations suggest an association of KANADI and Class III HD-ZIP genes with auxin. Atypical expression patterns of the auxin efflux-associated protein PIN1 were observed in KANADI and Class III HD-ZIP multiple mutants (Izhaki and Bowman, 2007). Class III HD-ZIP gene expression patterns are similar to auxin distribution patterns (Floyd et al., 2006; Floyd and Bowman, 2006; Heisler et al., 2005) and expression of *ATHB8*, *REV*, *PHV* and *ATHB15* is induced by auxin (Baima et al., 1995; Zhou et al., 2007). *ETTIN* (*ARF3*) and *ARF4* mediate the KANADI abaxial pathway in lateral organ development and *kan1 kan2* phenotypes are strikingly similar to those of *ettin arf4* double mutants (Pekker et al., 2005).

In this study, we examined the regulatory functions of Class III HD-ZIP and KANADI transcription factors in procambium formation and xylem and phloem differentiation with a specific focus on their interactions with auxin. In particular, we asked why ectopic *KAN1* expression results in a complete loss of vascular tissue development. We also examined the relationships between Class III HD-ZIP genes and auxin and KANADI expression. We provide strong evidence for a model in which Class III HD-ZIP and KANADI transcription factors control cambium activity: KANADI proteins by acting on auxin transport by inhibiting PIN gene expression, and Class III HD-ZIP proteins by promoting axial cell elongation and xylem differentiation.

MATERIALS AND METHODS

Plant material and growth conditions

Plants were grown in 8 hours light/16 hours dark for 25 days and then transferred to 16 hours light/8 hours dark. Ectopic expression of the different genes was accomplished using the pOp/LhG4 transcription factor system, with promoter>>operator indicating the specific gain-of-function genotype (Moore et al., 1998). For plant genotypes, see Table S1 in the supplementary material. To induce KAN1 protein activity, plants were grown on 0.5×MS plates containing 10 μM dexamethasone 21-acetate (DEX, Sigma), or seedlings were submerged in 10 μM DEX solution for 5 minutes.

RNA extraction, real-time quantitative RT-PCR and semi-quantitative RT-PCR

RNA was extracted from 15-day-old seedlings grown on MS medium using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Purified RNA (1 μg) was treated with DNase RQ1 (Promega, Madison, WI, USA) and reverse transcribed using MMLV reverse transcriptase (Promega) for quantitative PCR and PrimeScript reverse transcriptase (TaKaRa Biotech) for semi-quantitative PCR (see Table S2 in the supplementary material).

Histology, in situ hybridization and immunolabeling

Plant material was fixed, sectioned and hybridized with primary antibodies against PIN1 (AP-20, Santa Cruz Biotechnology) as described (Baluska et al., 2002), or with digoxigenin-labeled riboprobes as described (Vernoux et al., 2000). For plastic sections, plant samples were embedded in Technovit 7100 (Heraeus, Germany), 5 μm sections prepared with a microtome (Leica Microsystems, Germany) and a glass knife and stained with 0.1% Toluidine Blue.

GUS assay and microscopy

Histochemical GUS staining of seedlings was performed as described (Koizumi et al., 2000). After staining, samples were mounted in a 50% glycerol solution or dehydrated and embedded in Technovit 7100. Hypocotyl sections (5 μm) were stained with 0.1% Safranine Orange. For detection of GFP signals, embryos were prepared in 50% glycerol and mounted under a coverslip. Different stages of embryogenesis were obtained by pollinating the stigma and harvesting siliques 3-4 days (transition stage), 5-6 days (heart stage), 7-8 days (torpedo stage) and 10-12 days (mature seedling) after pollination. Hypocotyls were sectioned with a razor blade, stained with FM 4-64 dye at 1-10 μM (Invitrogen, Carlsbad, CA, USA) and suspended in 50% glycerol. Samples were observed using a TCS SP5 DM6000 B confocal microscope (Leica). Images were processed in ImageJ.

RESULTS

KAN1 inhibits procambium activity

Ectopic *KAN1* expression inhibits root and shoot meristem formation as well as vascular tissue differentiation (Eshed et al., 2001; Kerstetter et al., 2001). To better understand how KAN1 negatively affects vascular tissue formation, we expressed *KAN1* in procambium cells using the *ATHB15* promoter. Ectopic expression was accomplished using the pOp/LhG4 transcription factor system (Moore et al., 1998). *ATHB15>>KAN1* seedlings formed no vascular tissue in the hypocotyl (Fig. 1C), shoot (Fig. 1D) or root (Fig. 1E,F), and seedlings had reduced, or lacked, shoot and root apical meristems (Fig. 1D-F) and exhibited reduced shoot and root growth and a variable number of cotyledons that ranged from needle-like to heart-shaped, confirming previous findings (Eshed et al., 2001; Kerstetter et al., 2001).

To determine whether procambium cells and their direct derivates are formed in *ATHB15>>KAN1* plants, we introduced the β-glucuronidase gene (*GUS*) driven by the *ATHB8* (procambium and xylem precursors) and *APL* (phloem precursors) promoters into *ATHB15>>KAN1* plants (Fig. 1E,F). Expression of *ATHB8::GUS* and *APL::GUS* was detected in primary and secondary veins of cotyledons in control plants, but in *ATHB15>>KAN1* seedlings only faint blue staining was observed in a fragmented pattern. These findings suggest that KAN1 interferes with vascular tissue differentiation at the level of procambium formation.

KANADI loss-of-function results in increased cambium activity

Since gain-of-function KANADI alleles result in a loss of cambium activity, we examined vascular anatomy in the hypocotyls of seedlings that lack KANADI function to examine whether cambium activity is increased. In the lower region of wild-type hypocotyls of 15-day-old seedlings, the vasculature is composed of a central xylem plate, two peripheral phloem poles and residual procambium between xylem and phloem. The vasculature is encircled by single layers of pericycle and endodermis (Fig. 1B,G). Below the cotyledonary node and near the point of vasculature bifurcation,

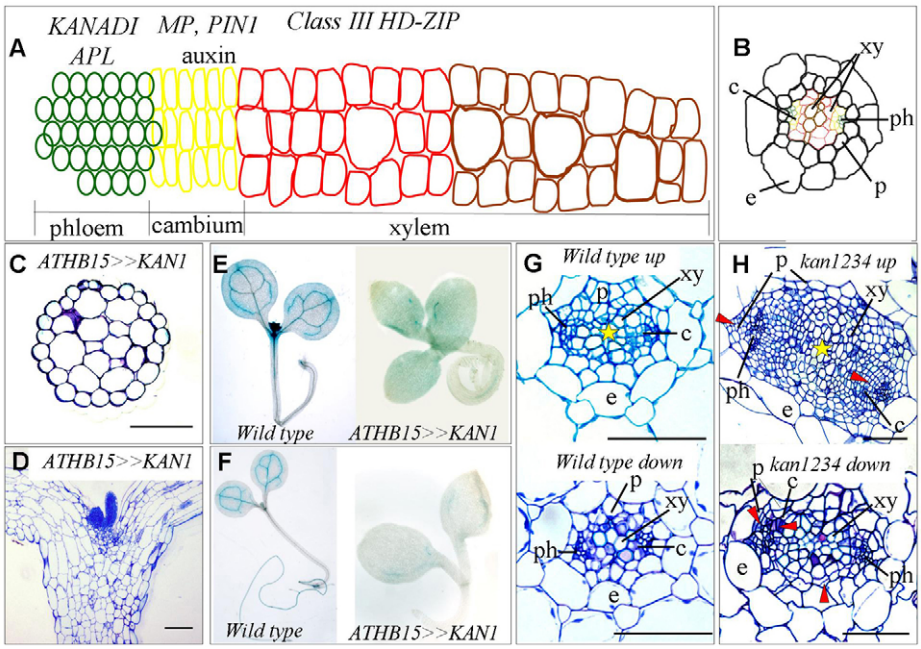

Fig. 1. Effects of altered KANADI gene expression in vascular bundles. (**A**) Schematic of collateral vascular arrangement and the corresponding distribution of factors involved in the regulation of vascular tissue differentiation in *Arabidopsis thaliana*. (**B**) Schematic of vascular arrangement in the hypocotyl. (**C**,**D**) Transverse section through the hypocotyl (C) and longitudinal section through the shoot (D) of an *ATHB15>>KAN1* seedling. (**E**,**F**) Expression of the *ATHB8::GUS* (E) and *APL::GUS* (F) genes in the vasculature of wild-type and *ATHB15>>KAN1* seedlings. (**G**,**H**) Transverse section through the hypocotyl of wild-type (G) and *kan1 kan2 kan3 kan4* (H) seedlings in the lower (down) and the upper (up) part of the hypocotyl. Arrowheads indicate extra cell divisions in the cambium and pericycle; the star indicates the formation of parenchymous cells in the xylem plate; c, cambium; e, endodermis; p, pericycle; ph, phloem; xy, xylem. Scale bars: 100 μm in C,D; 50 μm in G,H.

parenchymous cells interrupt the primary xylem plate (Fig. 1G). In *kan1 kan2 kan3 kan4* hypocotyls, the arrangement of xylem, cambium and phloem was unchanged, but the pericycle was composed of more than one cell layer and the number of cambium cells was increased, leading to precocious secondary growth (Fig. 1H). Extra cell divisions of pericycle and cambium cells were observed in the basal part of the hypocotyl and were more frequent near the cotyledonary node (Fig. 1H, red arrows). The xylem plate was divided by several parenchymous cells, xylem cells were smaller in diameter and fewer cells were lignified as compared with wild type.

KAN1 expressed in preprocambium cells negatively affects *PIN1* expression during embryogenesis

To identify the stage of procambium differentiation that ectopic *KAN1* activity inhibits, we analyzed the expression patterns of *ATHB15*, *PIN1* and *MP* in wild-type and *ATHB15>>KAN1* embryos. We followed the activity domains of the *ATHB15* promoter using a green fluorescent protein (GFP) reporter gene in *ATHB15>>GFP* plants, PIN1 distribution in plants expressing *PIN1::PIN1-GFP*, and *MP* expression by mRNA in situ hybridization. Expression patterns of the three genes in wild-type embryos were detected as described previously (Fig. 2; see Fig. S1A,B in the supplementary material) (Benkova et al., 2003; Friml et al., 2003; Hardke and Berleth, 1998; Ohashi-Ito et al., 2003; Prigge et al., 2005).

In *ATHB15>>GFP* embryos, GFP was detected in the apical regions of the globular embryo, and was resolved to the adaxial regions of the cotyledons and central procambial domain at heart stage (Fig. 2A,C). Ectopic expression of *KAN1* in *ATHB15>>KAN1;GFP* embryos influenced *ATHB15* expression in protoderm and ground tissue cells, but not in procambium precursor cells and the developing shoot apical meristem (SAM) (Fig. 2A-D). In wild-type torpedo-stage and mature embryos, the GFP signal gradually became restricted to procambium cells, whereas the GFP signal decreased in vascular precursors when *KAN1* was ectopically expressed (Fig. 2E-H).

PIN1-GFP expression was altered in *ATHB15>>KAN1* embryos as compared with wild-type embryos. PIN1-GFP was detected in *ATHB15>>KAN1* embryos in the epidermis of upper regions of the embryo at the transition stage, but the convergence points that form in wild-type embryos were absent and, unlike wild type, the GFP signal was not detected in presumptive procambium cells (Fig. 2A,B). When *KAN1* was ectopically expressed, the GFP signal was localized to epidermal cells in the distal regions of developing cotyledons of heart-stage embryos and in a few scattered cells of internal tissue near the cotyledon tips, but not in vascular precursor cells, where expression was evident in wild-type embryos (Fig. 2C,D). In *ATHB15>>KAN1* torpedo-stage embryos, several files of cells in the cotyledons accumulated PIN1-GFP (Fig. 2E,F), and the GFP signal was frequently located at the distal sides of these cells, suggesting auxin transport being directed towards the cotyledon tips (Fig. 2F, arrows). By contrast, in wild-type embryos, PIN1-GFP was detected in a single continuous file of cells in cotyledons and in three cell files of the stele, and was predominantly localized to the basal end of cells (Fig. 2E). In mature *ATHB15>>KAN1* embryos, either no GFP signal was detected, or patches of cells accumulated GFP in a non-polar manner (Fig. 2G,H).

Since PIN1 expression was significantly altered in *ATHB15>>KAN1* embryos, we monitored presumptive auxin distribution using the auxin-inducible synthetic promoter *DR5rev* driving GFP expression. Distribution of *DR5rev::GFP* in *ATHB15>>KAN1* embryos was comparable to the distribution in wild-type embryos until the heart stage. In both genotypes, *DR5rev::GFP* was detected in the hypophysis and in the uppermost suspensor cell, the site of root meristem formation (Fig. 2A-D). In wild-type heart-stage embryos, GFP signals appeared in the tips of the developing cotyledons and in a single cell file of procambium precursor cells. However, in *ATHB15>>KAN1* embryos, the corresponding GFP signal was variable, ranging from normal, to weak, to undetectable in the tips of developing cotyledons and was not narrowed down to a single file of procambium precursor cells. In fully developed wild-type embryos, the GFP signal accumulated in the tips of cotyledons, in provasculature throughout the seedling

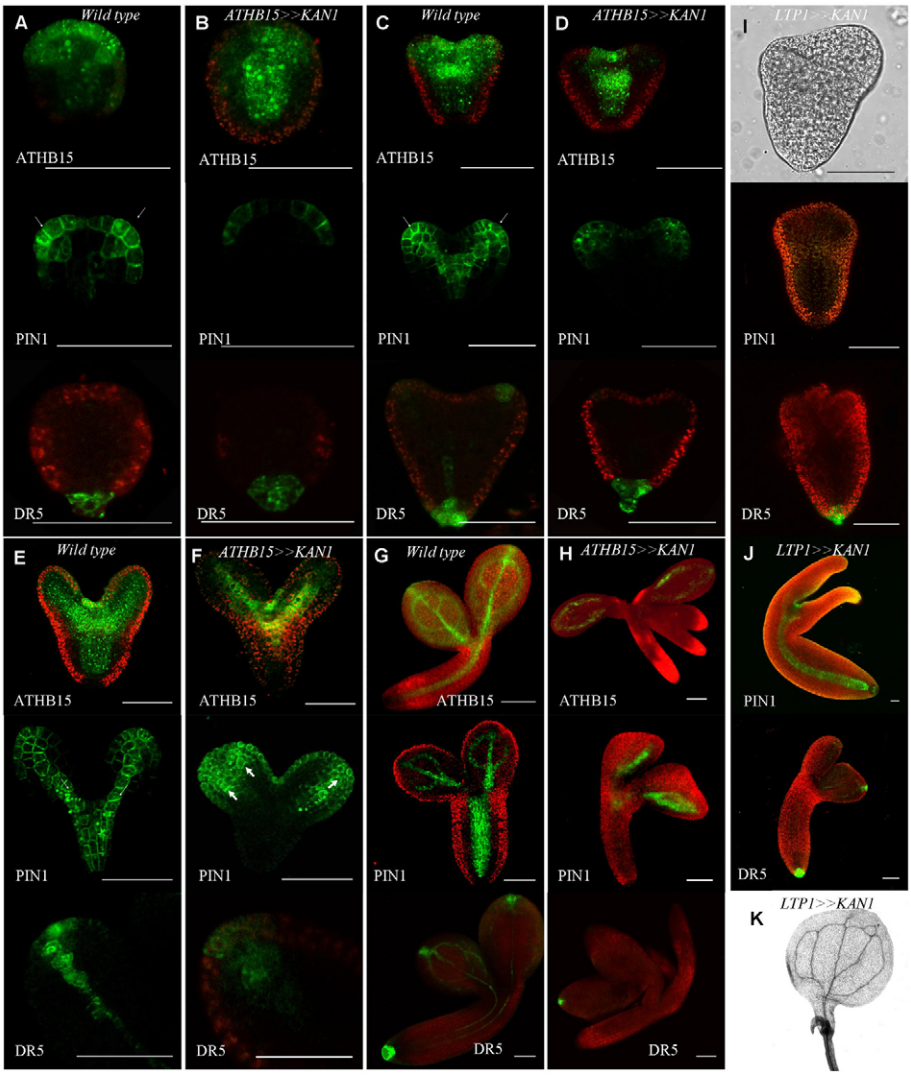

Fig. 2. Effects of ectopic *KAN1* expression on *ATHB15*, *PIN1* and *DR5::GFP* distribution in embryos. (**A-H**) Activity domains of the *ATHB15* promoter, *PIN1::PIN1-GFP* and *DR5rev::GFP* during embryogenesis in wild-type (A,C,E,G) and *ATHB15>>KAN1* (B,D,F,H) embryos. Activity domains of the *ATHB15* promoter are visualized by expressing *ATHB15>>GFP* in wild-type and *ATHB15>>KAN1* embryos. (A,B) Transition stage; (C,D) heart stage; (E,F) torpedo stage; (G,H) mature embryo. Arrows (F, middle) indicate altered local arrangement of PIN1-GFP. (**I,J**) *PIN1::PIN1-GFP* and *DR5rev::GFP* expression at the *LTP1>>KAN1* torpedo stage (I) and in the mature embryo (J). (**K**) Cotyledon vein pattern of a 15-day-old *LTP1>>KAN1* seedling. Scale bars: 50 μm.

and in the root apical meristem (Fig. 2E,G), but was only occasionally detected in the tips of cotyledons and rarely in the root meristem in *ATHB15>>KAN1* seedlings (Fig. 2F,H).

In contrast to PIN1-GFP and *DR5rev::GFP*, ectopic *KAN1* expression in procambium precursor cells had no influence on *MP* distribution during the early stages of embryogenesis (see Fig. S1A,B in the supplementary material). In torpedo-stage embryos, *MP* was confined to vascular precursor cells in the cotyledons and the stele in both wild-type and *ATHB15>>KAN1* embryos.

In summary, *KAN1* expression in domains of *ATHB15* activity negatively affected *PIN1* expression and distribution in the internal tissues of embryos from the transition stage on. These changes precede the effects of ectopic KAN1 on *ATHB15>>GFP* distribution, which was mostly affected in sub-epidermal peripheral regions and in procambium precursor cells of embryos only after the torpedo stage. Inhibitory effects of ectopic *KAN1* expression on the activity of PIN1 early during embryogenesis suggest that the influence of *KAN1* expression on cambium activity could be mediated by auxin.

KAN1 expression disrupts auxin movement in the epidermal cell layer

Reduced accumulation of *PIN1::PIN1-GFP* in procambium precursor cells during embryogenesis in *ATHB15>>KAN1* plants suggests that KAN1 is altering PIN1 activity and, subsequently, auxin transport. To test this hypothesis and to evaluate whether the effects of KAN1 on PIN1 activity are general, or specific to procambium cells, we ectopically expressed *KAN1* in the epidermal layer of developing embryos using the *LIPID TRANSFER PROTEIN 1* (*LTP1*) promoter and investigated embryo development, *DR5rev::GFP* patterns and *PIN1::PIN1-GFP* distribution (Fig. 2I-K). *LTP1>>KAN1* embryos often had fused cup-shaped cotyledons (Fig. 2I,J), similar to those in embryos in which auxin transport is reduced, such as *pin4 pin7* and *pin1 pin3 pin4* embryos (Friml et al., 2003), *pin4* mutants with ectopic production of auxin (Weijers et al., 2005), and embryos cultured on the auxin transport inhibitors NPA and TIBA (Hadfi et al., 1998; Liu et al., 1993). Moreover, expansion of the cotyledon blade was often increased, leading to rounded cotyledons with an increased number of secondary vein loops and unconnected ends of bifurcated main veins (Fig. 2K), similar to *pin1* mutants (Okada et al., 1991). No *PIN1::PIN1-GFP* was detected in *LTP1>>KAN1* embryos at the torpedo stage (Fig. 2I). In developed seedlings with a weaker phenotype, *PIN1::PIN1-GFP* was detected in the SAM and the provasculature of the hypocotyl and root, but not in the provasculature of cotyledons (Fig. 2J). *DR5rev::GFP* accumulated only in the hypophysis and occasionally in the tips of future cotyledons (Fig. 2I) in torpedo-stage embryos with a strong phenotype, and was faintly detected in provascular strands at the end

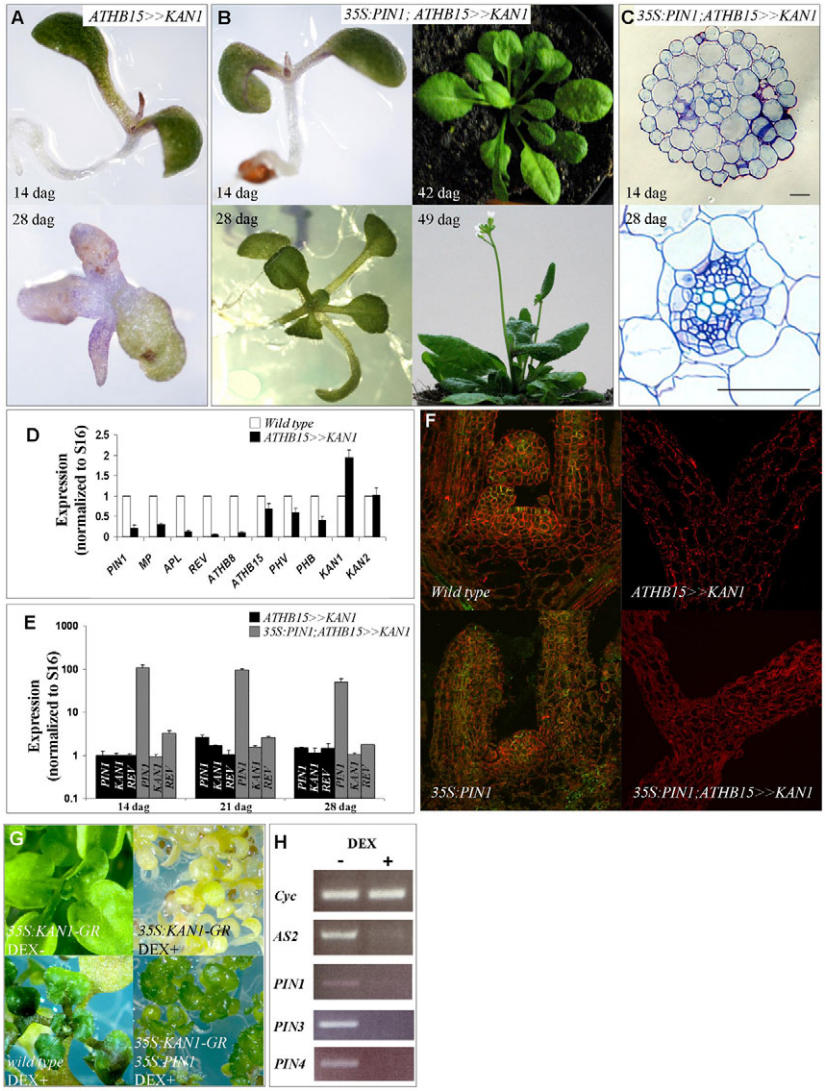

Fig. 3. Complementation of the *ATHB15>>KAN1* and *35S::PIN1* phenotypes by *35S::PIN1* expression. (**A**,**B**) Phenotype of *ATHB15>>KAN1* (A) and *35S::PIN1;ATHB15>>KAN1* (B) plants at different stages of development (dag, days after germination). (**C**) Transverse sections through the hypocotyl of *35S::PIN1;ATHB15>>KAN1* plants. (**D**,**E**) mRNA quantification of genes involved in vascular tissue differentiation by real-time RT-PCR in 15-day-old wild-type (D) and *ATHB15>>KAN1* (E) seedlings. Expression levels were set to 1 in the wild type. (**F**) Localization of immunolabeled PIN1 protein (green) in the shoot apex of 7-day-old seedlings. (**G**) Seeds of *35S::KAN1-GR* were germinated and grown on medium containing 10 μM dexamethasone (DEX+) and the phenotype compared with wild-type (DEX+) and *35S::KAN1-GR;35S::PIN1* (DEX+) seedlings or with wild-type seedlings grown without dexamethasone (DEX–) 30 days after germination. (**H**) Ten-day-old *35S::KAN1-GR* seedlings were incubated in a solution containing 10 μM dexamethasone (DEX+) or in a control solution (DEX–) for 5 minutes and expression levels of the genes indicated determined 80 minutes later by semi-quantitative RT-PCR. Scale bars: 50 μm.

of embryogenesis in plants with a weaker phenotype (Fig. 2J). These results indicate that KAN1 can affect PIN1 activity in general, both in internal tissues and in the epidermis.

Effects of ectopic *KAN1* expression in cambium cells are rescued by *35S::PIN1*

The distribution patterns of PIN1-GFP in plants with ectopic *KAN1* expression suggest that KAN1 affects the transcriptional regulation of *PIN1* or the localization and activity of the protein. We introduced *35S::PIN1* into *ATHB15>>KAN1* plants to test whether constitutive expression of *PIN1* could rescue the loss of vascular development caused by ectopic expression of *KAN1* in meristematic and internal tissues. Following germination, *ATHB15>>KAN1* seedlings occasionally developed a few needle-like leaves before all growth arrested (Fig. 3A). *35S::PIN1;ATHB15>>KAN1* seedlings were similar to *ATHB15>>KAN1* seedlings 7 days after germination, but 14 days after germination leaves had already started to form regularly and the root had elongated and formed side roots. Rosette leaves were comparable to those of the wild type, although their growth was slower and, in a few cases, the leaf lamina extended irregularly and resembled the leaf shape of *pin1* mutants. The inflorescence stem formed fertile flowers, but the number of seeds in siliques was slightly reduced (Fig. 3B). No vascular traces were detected in alcohol-cleared *35S::PIN1;ATHB15>>KAN1* seedlings 14 days after germination (data not shown), but a few smaller cells surrounded by the endodermis were visible in transverse sections of the hypocotyl (Fig. 3C). Three weeks after germination, the vasculature was visible in alcohol-cleared seedlings in the root, hypocotyl and leaves (data not shown), and 4 weeks after germination the vascular system in the hypocotyl had differentiated in a regular pattern (Fig. 3C).

We also quantified the expression levels of genes involved in early steps of vasculature differentiation in 14-day-old *ATHB15>>KAN1* seedlings (Fig. 3D), and compared the expression levels of selected genes between *ATHB15>>KAN1* and *35S::PIN1;ATHB15>>KAN1* seedlings during a growth period of 28 days (Fig. 3E). Ectopic *KAN1* expression reduced the expression of the Class III HD-ZIP transcription factors *PHB*, *PHV*, *ATHB15* and, especially, *ATHB8* and *REV*. Since ectopic *KAN1* expression is driven by the *ATHB15* promoter, we quantified *KAN1* expression. *KAN1* expression was high in *ATHB15>>KAN1* seedlings compared with controls, whereas expression of *KAN2* was unchanged, indicating that the transgene *pATHB15::LhG4* activated *pOp::KAN1*. Expression of *PIN1*, *MP* and *APL* was also reduced by ectopic *KAN1* expression (Fig. 3D). Expression levels of *PIN1* were highly elevated in *35S::PIN1;ATHB15>>KAN1* seedlings 14 days

after germination and remained high 28 days after germination as compared with *ATHB15>>KAN1* seedlings, whereas *KAN1* expression was comparable in the two genotypes and remained stable over time (Fig. 3E). Expression levels of *REV* in *35S::PIN1;ATHB15>>KAN1* seedlings were comparable to those in wild type (data not shown) and were increased compared with *ATHB15>>KAN1* seedlings 14 and 21 days after germination (Fig. 3E).

The slow onset of rescue in *35S::PIN1;ATHB15>>KAN1* seedlings might be due to reduced activity of the 35S promoter during embryogenesis and the early stages of seedling development. To test this, we visualized the PIN1 protein in the apical part of seedlings 7 days after germination in wild-type, *35S::PIN1*, *ATHB15>>KAN1* and *35S::PIN1;ATHB15>>KAN1* seedlings (Fig. 3F). Localization of PIN1 protein was similar in the meristem and leaf primordia of *35S::PIN1* and wild-type seedlings. In *ATHB15>>KAN1* seedlings, no meristem had formed and no PIN1 protein was detected in the shoot or the cotyledons. Likewise, at this stage in *35S::PIN1;ATHB15>>KAN1* seedlings, no PIN1 protein was detected in the shoot or cotyledons. These results suggest that there is significant post-transcriptional regulation of PIN1 protein accumulation and that the 35S promoter activity is insufficient for phenotypic rescue during early embryogenesis.

To obtain further evidence that ectopic *PIN1* expression can rescue the phenotypic effects of ectopic KAN1, we utilized a line harboring a transgene that results in widespread expression of a hormone-inducible KAN1 protein, *35S::KAN1-GR* (Hawker and Bowman, 2004). When seeds homozygous for the *35S::KAN1-GR* transgene were germinated in the presence of dexamethasone (DEX), both shoot and root meristems were arrested, no leaf primordia were produced, and seedlings died within a couple weeks of germination (Fig. 3G). By contrast, when seeds homozygous for both the *35S::KAN-GR* and *35S::PIN1* transgenes were germinated in the presence of DEX, the shoot meristem was not arrested and seedlings produced many leaves that, although small, were relatively normal (Fig. 3G). In this genotype, mRNA levels of the ectopically expressed transgenes *PIN1* and *KAN1* were increased compared with those in wild-type seedlings (see Fig. S1C in the supplementary material).

That gain-of-function *KAN1* alleles are suppressed by ectopic *PIN1* expression, and loss-of-function KANADI alleles result in ectopic *PIN1* expression (Izhaki and Bowman, 2007), suggest that KAN1 regulates *PIN1* expression. To test this hypothesis, we examined the expression of *PIN1* and related genes in *35S::KAN1-GR* plants treated with DEX. As positive and negative controls, we followed the expression of *ASYMMETRIC LEAVES 2* (Wu et al., 2008) and cyclophilin, respectively. When assayed 80 minutes after DEX treatment, expression of *AS2*, *PIN1*, *PIN3* and *PIN4* was reduced in hormone-treated plants relative to controls, whereas cyclophilin expression was unchanged (Fig. 3H). *PIN2*, *PIN5*, *PIN6* and *PIN8* levels were unaffected and *PIN7* was only moderately reduced, but expression levels were also very low in controls (data not shown). Thus, KAN1 rapidly regulates, either directly or indirectly, the transcription of *PIN1*, *PIN3* and *PIN4* in the tissues analyzed.

Class III HD-ZIP loss-of-function affects polar cell elongation and xylem differentiation

The results obtained above suggest that KAN1 acts on auxin homeostasis and that reduced levels of Class III HD-ZIP expression might be an indirect consequence of a lack of cambium tissue formation. However, antagonistic activities of the two gene families were suggested in the past (Emery et al., 2003). To analyze relationships between the two gene families, we studied the vasculature in plants with reduced levels of Class III HD-ZIP expression in procambium cells. If KANADI transcription factors directly inhibit Class III HD-ZIP expression, the phenotype of Class III HD-ZIP multiple loss-of-function mutants should phenocopy the ectopic expression of KANADI.

Since Class III HD-ZIP genes are post-transcriptionally regulated by *miR165* and *miR166* (Emery et al., 2003; Jones-Rhoades and Bartel, 2004; Jung and Park, 2007; Kim et al., 2005; Mallory et al., 2004; Tang et al., 2003; Williams et al., 2005), and as it has been shown that multiple related genes can be quantitatively regulated by miRNA expression (Alvarez et al., 2006), we made use of *miR165* to reduce the mRNA levels of all Class III HD-ZIP genes in cambium cells. We activated *miR165* expression in the promoter activity domains of *REV* and *ATHB15* (Fig. 4A-E). Although bilateral symmetry was maintained in *ATHB15>>miR165* seedlings, they exhibited features typical of abaxialization, as found in Class III HD-ZIP multiple loss-of-function mutants (Fig. 4A). Cotyledons and leaves curled downwards, expansion of leaf lamina was inhibited, and petals were radialized, indicating that several Class III HD-ZIP genes were reduced (Prigge et al., 2005). In addition, plant stature was dwarfed, growth was reduced and flowering was retarded. Expression of *REV>>miR165* had a similar effect on plant development (data not shown). In contrast to *ATHB15>>KAN1* plants, in *ATHB15>>miR165* and *REV>>miR165* seedlings, a vascular system developed, but xylem elements in leaves and stems were partially disconnected (Fig. 4B), and expression of the procambium and protoxylem marker *ATHB8::GUS* was often lacking in parts of the vasculature of *ATHB15>>miR165* plants (Fig. 4C). In the hypocotyl, the size of the stele and cell number within the stele were increased (Fig. 4E). Most vascular cells displayed characteristics of cambium or parenchyma cells. Tracheary element differentiation was inhibited and only a few vessel elements differentiated at random locations within the stele (Fig. 4E, stars).

The phenotype of *ATHB15>>miR165* and *REV>>miR165* plants suggests that Class III HD-ZIP proteins are reduced. In 14-day-old seedlings, *PHB* and *PHV* mRNAs were reduced in both genotypes, whereas *ATHB15* was only reduced in *ATHB15>>miR165* seedlings, and, contrary to our expectations, mRNAs of *REV* and, to a minor extent, of *ATHB8*, were increased in *ATHB15>>miR165* and *REV>>miR165* plants (Fig. 4D). To evaluate whether the accumulation of cambium and parenchyma cells in the hypocotyl of *ATHB15>>miR165* and *REV>>miR165* plants was due to elevated expression levels of *REV*, we analyzed the effect of *ATHB15>>miR165* expression in *rev-9* loss-of-function mutants (Fig. 4E, *ATHB15>>miR165;rev-9*). Comparable to *ATHB15>>miR165* and *REV>>miR165* seedlings, cells in the stele of these plants showed characteristics of either cambium or parenchyma cells and very few cells differentiated into tracheary elements at random positions. In *rev-9* single and *rev-9 phb-6 phv-5* triple mutants, tracheary elements formed in the center of the stele, but in reduced numbers compared with wild-type plants (Fig. 4F). Therefore, we conclude that the vascular phenotype of *ATHB15>>miR165* and *REV>>miR165* seedlings was caused by reduced levels of several Class III HD-ZIP transcription factors in the vasculature.

To examine the effects of gain-of-function Class III HD-ZIP alleles on vascular development, we used *rev-10d*, as well as *ATHB15>>ATHB8-δmiR* plants, both of which contain a one-nucleotide substitution in the region complementary to *miR165/166*.

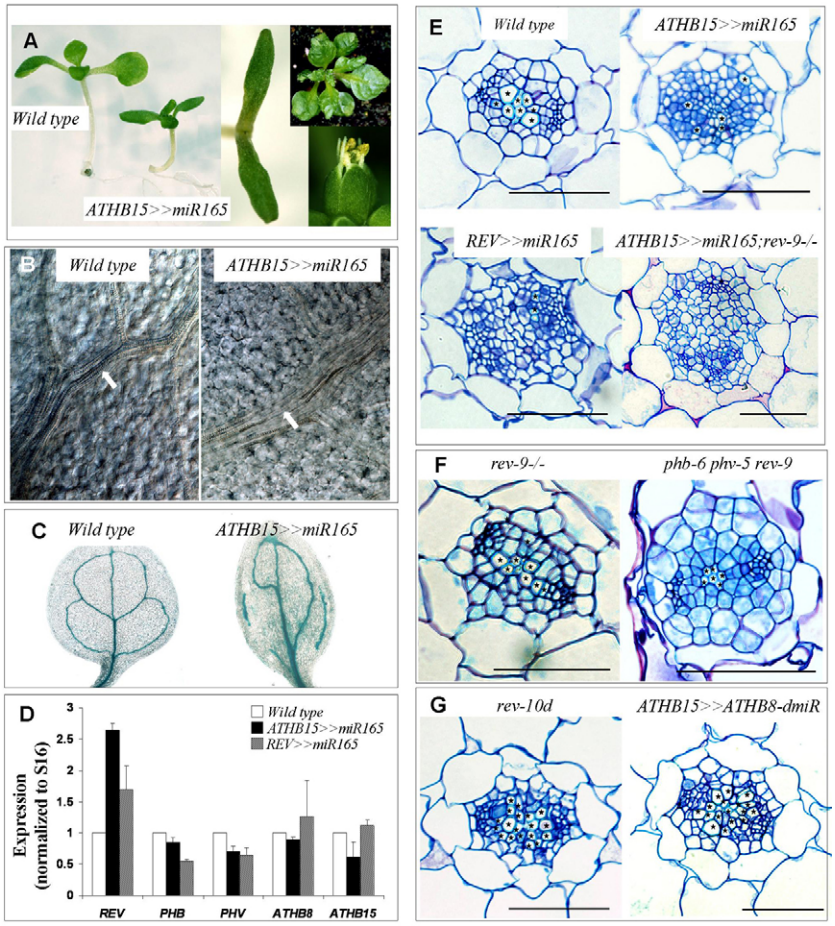

Fig. 4. Effects of altered Class III HD-ZIP expression in vascular bundles. (**A**) Phenotype of *ATHB15>>miR165* seedlings at different stages of development. (**B**,**C**) Disconnected xylem strands in leaves of *ATHB15>>miR165* plants (B, arrows) and expression of the *ATHB8::GUS* gene in cotyledons (C). (**D**) mRNA quantification of genes involved in vascular tissue differentiation by real-time RT-PCR in 15-day-old seedlings. Expression levels were set to 1 in wild type. (**E**) Transverse sections through hypocotyls of wild-type, *ATHB15>>miR165*, *REV>>miR165* and *ATHB15>>miR165;rev-9* seedlings 15 days after germination. (**F**) Transverse sections through hypocotyls of *rev-9* and *rev-9 phb-6 phv-5* mutants 15 days after germination. (**G**) Transverse sections through the hypocotyl of *rev-10d* and *ATHB15>>ATHB8δmiR* seedlings 15 days after germination. Stars indicate tracheary elements. Scale bars: 50 μm.

Expression of the microRNA-resistant form of either *REV* or *ATHB8* had minor effects on vasculature development in the hypocotyl, in that the stele contained a slightly increased number of differentiated xylem cells (Fig. 4G).

In summary, cambium cells were formed in plants with reduced Class III HD-ZIP levels, but coordinated cell expansion and cell maturation were impaired and xylem differentiation and proper connection of cell files to form vessels did not occur. These results suggest that KAN1 and Class III HD-ZIP actions are not directly linked, but rather indicates a role for Class III HD-ZIP genes in maintaining a balance between cambium and differentiating xylem cells and in vascular continuity and orientation of cell elongation.

Auxin accumulates in broader patterns in the vasculature of *ATHB15>>miR165* plants

Expression patterns of Class III HD-ZIP genes correlate with auxin distribution in the shoot meristem and in meristematic tissues of the vasculature (Emery et al., 2003; McConnell et al., 2001; Otsuga et al., 2001; Prigge et al., 2005) (Fig. 2). A common task of Class III HD-ZIP genes and auxin may be the regulation of polar cell elongation during xylem differentiation, and defects in polar cell expansion and xylem maturation in Class III HD-ZIP loss-of-function mutants might be related to changes in auxin movement and/or signaling.

To evaluate correlations of Class III HD-ZIP gene expression and auxin during vascular tissue differentiation, we analyzed the distribution of *DR5rev::GFP* in embryos and hypocotyls of *ATHB15>>miR165* plants (Fig. 5A,B). *DR5rev::GFP* signal was first detected in procambium cells in heart-stage embryos and was confined to two strands in the root and hypocotyl of mature wild-type embryos (Fig. 5A, Fig. 2). In 15-day-old wild-type seedlings, *DR5rev::GFP* accumulated in pericycle, cambium and vessel mother cells (Fig. 5B). In embryos of *ATHB15>>miR165* plants, *DR5rev::GFP* accumulated in a much more widespread pattern in the root and hypocotyl and was not bundled in single strands (Fig. 5A). In the hypocotyl of *ATHB15>>miR165* seedlings 15 days after germination, *DR5rev::GFP* accumulated throughout the stele in a more widespread pattern than in wild-type plants (Fig. 5B).

We analyzed the influence of *miR165* expression in procambium cells on *ATHB15* promoter activity by following GFP in *ATHB15>>miR165;GFP* plants. The GFP signal accumulated in *ATHB15>>miR165* embryos in a similar pattern as in *ATHB15>>GFP* control embryos until torpedo stage (data not shown), but at embryo maturity, an enhanced and more broadly distributed signal of GFP was observed in *ATHB15>>miR165* plants (Fig. 5C). In the hypocotyl, *ATHB15>>GFP* accumulated in pericycle, cambium and vessel mother cells in control plants and in patches throughout the stele in *ATHB15>>miR165* plants (Fig. 5D). The procambium marker *ATHB8::GUS* accumulated in the pericycle, procambium and developing tracheary elements, but was absent from the phloem and fully differentiated xylem in wild-type hypocotyls. By contrast, *ATHB8::GUS* was strong in all stelar cells, with the exception of phloem cells and a few cells that had initiated secondary cell wall formation in *ATHB15>>miR165* hypocotyls (Fig. 5E). These results indicate that reduction of Class III HD-ZIP genes within the *ATHB15* expression domain during embryogenesis

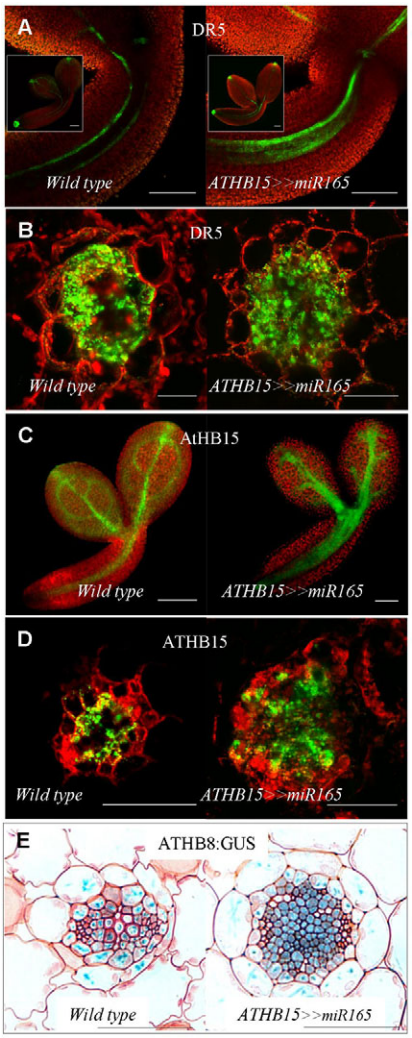

Fig. 5. Expression patterns of *DR5rev::GUS*, *ATHB15>>GFP* and *ATHB8::GUS* in *ATHB15>>miR165* plants. (**A-D**) Expression patterns of *DR5rev::GFP* (A,B) and *ATHB15>>GFP* (C,D) were analyzed in mature embryos (A,C) and in hypocotyls 15 days after germination (B,D) of wild-type (left) and *ATHB15>>miR165* (right) plants. Insets in A show entire embryo. (**E**) Distribution of *ATHB8::GUS* in vascular cells of the hypocotyl of wild-type and *ATHB15>>miR165* seedlings was analyzed 15 days after germination. Scale bars: 50 μm.

inhibits the restriction of auxin to single cell strands and favors the formation of cell tissues in which the expression of *ATHB8* and *ATHB15* is stimulated.

In summary, *ATHB15>>miR165* plants formed an excess of procambium cells in which *DR5rev::GFP* and Class III HD-ZIP transcripts accumulated. In *ATHB15>>KAN1* plants, procambium cell formation and accumulation of *DR5rev::GFP*, as well as Class III HD-ZIP expression, were inhibited. This suggests that auxin-containing procambium cells stimulate Class III HD-ZIP expression and that KAN1 indirectly acts on Class III HD-ZIP activity through its negative action on auxin transport and procambium cell formation.

DISCUSSION

A key role for Class III HD-ZIP and KANADI transcription factors in transverse patterning of vascular tissues is inferred from complementary vascular phenotypes of mutants of the two families. We show that KANADI and Class III HD-ZIP genes act on auxin distribution during procambium formation. Class III HD-ZIP genes influence auxin distribution by promoting axial cell elongation, meristematic activity and tracheary element differentiation, whereas KAN1 indirectly influences cambium activity by regulating auxin movement. We suggest that amphivasal vascular phenotypes in KANADI and Class III HD-ZIP mutants are due to an expansion in the domain in which auxin is present (see Fig. S2 in the supplementary material).

KANADI factors negatively affect PIN activity

We have shown that ectopic expression of *KAN1* in presumptive procambium cells reduces gene expression and alters the local arrangement of PIN1 in procambium precursor cells (Figs 2, 3), and that in *kan1 kan2 kan3 kan4* loss-of-function mutants, cambium and pericycle cell divisions are increased (Fig. 1). These observations imply a negative action of KAN1 on procambium cell formation and division, either due to a general effect of *KAN1* expression on meristematic cell activity, or to an influence on the distribution of auxin. Several lines of evidence suggest that KAN1 activity impinges on the distribution of auxin. Ectopic expression of *KAN1* in the epidermis reduced PIN1-GFP accumulation not only in the epidermis, but also in internal tissues (Fig. 2). The phenotype of *LTP1>>KAN1* embryos and seedlings is similar to the phenotype of single and multiple loss-of-function mutants of PIN proteins, suggesting that auxin transport is severely inhibited in these mutants. The lack of *DR5rev::GFP* signal in internal tissues can be interpreted as a consequence of reduced auxin flow through the epidermis of the plant body. Finally, the observation that constitutive expression of *PIN1* in seedlings with ectopic *KAN1* expression compensates developmental defects after embryogenesis (Fig. 3) indicates that the primary cause of loss of vascular tissues in plants ectopically expressing *KAN1* is a loss of PIN activity. That PIN gene expression is reduced within 80 minutes of induction of KAN1 activity suggests that regulation is at the level of transcription.

Class III HD-ZIP genes are essential for proper cell elongation and xylem maturation

Class III HD-ZIP proteins may be seen as differentiation-promoting factors that temporally and spatially coordinate procambium formation and differentiation of xylem cells in vascular tissues. Ohashi-Ito and Fukuda proposed that a positive-feedback loop between procambium cells and *ATHB15/ZeHB13* expression maintains procambium cell formation and that procambium cells induce the expression of *ATHB8/ZeHB10* and *REV/ZeHB11/ZeHB12*, which initiate the formation of xylem and parenchyma precursors (Ohashi-Ito and Fukuda, 2003). We show that ectopic expression of *ATHB8* and *REV* results in increased tracheary element formation in the hypocotyl. Furthermore, plants with ectopic *miR165* expression in provascular cells have defects in maintaining the balance between procambium cell proliferation and tracheary element differentiation, as well as in axial cell elongation and connection (Fig. 4). In addition, a broader *DR5rev::GFP* distribution in the vasculature (Fig. 5) suggests that auxin is not canalized in single cell files. Alterations in polar auxin transport and defects in interfascicular fiber differentiation have been observed previously in the *ifl=rev* mutant (Zhong and Ye, 2001). We propose that Class III HD-ZIP genes are involved in auxin canalization by promoting axial cell elongation, connection of procambium cells and tracheary element differentiation.

Interaction of auxin and Class III HD-ZIP transcription factors

PIN1 is accompanied by *MP* expression in the ground tissue of leaf primordia, with PIN1 expression gradually refined to single cell files after induction of *ATHB8* expression and formation of a procambium strand, suggesting a regulatory feedback loop between auxin, PIN1, MP and ATHB8 (Hardke and Berleth, 1998; Scarpella et al., 2006; Wenzel et al., 2007). During embryogenesis, *PIN1*, *MP* and Class III HD-ZIP expression overlap. Subsequently, partial separation of Class III HD-ZIP and *PIN1* expression domains occurs in mature embryos and coincides with xylem and phloem precursor formation (Figs 2, 5; see Fig. S1 in the supplementary material). In *ATHB15>>KAN1* embryos, changes in *PIN1* expression preceded changes in *ATHB15* and *MP*, suggesting that reductions in *ATHB15* and *MP* expression were due to loss of auxin movement resulting in a loss of procambium cell formation. In embryos with ectopic *miR165* expression in provascular cells, the restriction of *DR5rev::GFP* to two strands was inhibited and the promoter activity domains of Class III HD-ZIP genes were enlarged, suggesting a positive feedback between auxin flow and Class III HD-ZIP expression (Fig. 5). This provides an explanation for the increased expression of some Class III HD-ZIP genes when *miR165* is expressed in the *ATHB15* expression domain. In addition, tracheary element differentiation was reduced, suggesting that it might be initiated at the time of Class III HD-ZIP restriction to central parts of the developing stele.

In SAMs, it has been shown that the AP2-domain proteins DORNROESCHEN (DRN) and DRN-like (DRNL) control SAM development by promoting differentiation in the peripheral zone (Kirch et al., 2003), and by acting negatively upon auxin response factors and influencing auxin transport during embryogenesis (Chandler et al., 2007; Nag et al., 2007). Class III HD-ZIP proteins interact with DRN and DRNL (Chandler et al., 2007). It is therefore tempting to speculate that Class III HD-ZIP proteins, in concert with DRN/DRNL, balance cell proliferation and differentiation in meristematic tissues via interactions with auxin signaling and perception. A feedback mechanism between PIN proteins and the auxin-inducible AP2-domain transcription factor PLETHORA (PLT) regulates root meristem patterning by focusing the auxin maximum, which in turn restricts the expression domain of PLT genes (Blilou et al., 2005). A similar regulatory mechanism between auxin, PIN proteins and Class III HD-ZIP transcription factors might restrict auxin transport domains, as well as Class III HD-ZIP expression domains, in the differentiating vasculature.

The formation of amphivasal and amphicribal vasculature in KANADI and Class III HD-ZIP mutants

Earlier studies have shown that KANADI multiple loss-of-function mutants develop cambium and tracheary elements also on the abaxial side of phloem cells (Emery et al., 2003; Eshed et al., 2004; Izhaki and Bowman, 2007). Amphivasal vasculature is also observed in the inflorescence stems of *REV*, *PHB* and *PHV* gain-of-function mutants (McConnell and Barton, 1998; McConnell et al., 2001; Emery et al., 2003; Zhong and Ye, 2004). We interpret the ectopic xylem and fiber differentiation in amphivasal vascular bundles of KANADI loss-of-function mutants as being caused by the canalization of auxin in ectopic positions due to a lack of inhibition of PIN1 activity at these positions. Likewise, in Class III HD-ZIP gain-of-function alleles, ectopic Class III HD-ZIP activity leads to ectopic activation of meristematic cells and hence to ectopic xylem and fiber differentiation. By contrast, the amphicribal vascular arrangement in *phb-6 rev-6 phv-5* mutants results from a reduction of tracheary element formation due to a reduction of Class III HD-ZIPs. This interpretation is supported by the observations that fewer tracheary elements are formed in the *rev-9* single mutant and that ectopic expression of *miR165* mostly inhibited tracheary element formation.

Conclusions

Polar auxin flow is essential for cell divisions and cell alignment to form procambium cells, and our results suggest that KANADI and Class III HD-ZIP genes have a role in the canalization of auxin flow: KANADI genes function to restrict flow by inhibiting PIN activity, whereas Class III HD-ZIP genes function to promote axial cell elongation and tracheary element differentiation and hence canalize auxin flow. Thus, the mutual antagonism between KANADI and Class III HD-ZIP activities is not mediated directly, but through effects on the canalization of auxin flow.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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Table S1. Plant genotypes used for this study

Plant genotype	Description/reference
pATHB15::LhG4	Five thousand bp of the 5′ upstream sequence of *ATHB15* were amplified by PCR and cloned in front of the *LhG4* sequence
10Op::ATHB8δmiR	A non-silent single-point mutation (G to D) was introduced in the *miR165/166* complementary site of an *ATHB8* cDNA and the cDNA was cloned behind the *10Op* sequence
pLTP1::LhG4	Moore et al., 2006
pREV::LhG4	Moore et al., 2006
6Op::KAN1	Eshed et al., 2001
10Op::miR165	Alvarez et al., 2005
10Op::ATHB8δmiR	Alvarez et al., 2005
10Op::GFP	Alvarez et al., 2005
APL::GUS	Bonke et al., 2003
ATHB8::GUS	Baima et al., 1995
PIN1::GFP	Benkova et al., 2003
35S::PIN1	Benkova et al., 2003
DR5rev::GFP	Friml et al., 2003
35S::KAN1-GR	Hawker and Bowman, 2004
rev-9	Emery et al., 2003
rev-10d	Emery et al., 2003
phb-6 phv-5 rev-9	Emery et al., 2003
kan1-2 kan2-1 kan3-1 kan4-3	Izhaki and Bowman, 2007

Plants homozygous for the transactivation driver lines *pATHB15::LhG4, pLTP1::LhG4* and *pREV::LhG4* were crossed to plants homozygous for the various reporter constructs *6Op::KAN1*, *10Op::miR165, 10Op::ATHB8δmiR* and *10Op::GFP*. *pATHB15::LhG4* and *10Op::miR165* were crossed to *rev-9* mutants and segregating plants homozygous for *pATHB15::LhG4* and *rev-9* were crossed with segregating plants homozygous for *10Op::miR165* and *rev-9*. *APL::GUS* and *ATHB8::GUS* plants were crossed with the *pATHB15::LhG4* driver line and plants homozygous for *pATHB15::LhG4* and either *APL::GUS* or *ATHB8::GUS* were crossed with homozygous reporter lines. Similarly, *PIN1::GFP, 35S::PIN1* and *DR5rev::GFP* were introduced into the *pATHB15::LhG4* and the *pOP::KAN1* lines. *35S::KAN1-GR* plants were crossed with *35S::PIN1* plants and F3 plants homozygous for both gene constructs were selected.

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Table S2. Primer sequences for quantitative and semi-quantitative PCR and cloning of *ATHB15::LhG4* and *10OpTATA::ATHB8-δmiR*

Primer	Sequence (5′ to 3′) or source
S16	QT00833819 (Qiagen)
KAN1	QT00830802 (Qiagen)
KAN2	GCAGCTTCGTCAGGACAATC and TCTCCGGAAGAATTGGTCCA
REV	CGAATAGTCCTGCTGGATTG and GATCTCTGCAATCTTCATAG
PHB	QT00716583 (Qiagen)
PHV	QT00870877 (Qiagen)
AtHB15	AGTCCTGCAGGACTTTTGTC and CAATCTCTGCAACCCTTGTA
AtHB8	AAGCAGAGGAAACTCAAG and AAATGTGAATACCGGAGAT
PIN1	QT00892654 (Qiagen)
APL	QT00744926 (Qiagen)
MP	QT00861847 (Qiagen)
Cyclophilin	TCTTCCTCTTCGGAGCCATA and AAGCTGGGAATGATTCGATG
AS2	ATGGCATCTTCTTCAACAAAC and TCAAGACGGATCAACAGTAC
PIN1	ATGATTACGGCGGCGGACTTC and TCATAGACCCAAGAGAATGTAGTAGAG
PIN3	CAAGATGATCTCATGGCACG and GCTTATAACCCGAGTAGAATG
KAN1-GR	GGAGAAGAAGAAATGGGC and CTTTGCCCATTTCACTGC
PIN4	GGAAAAAATGATTACGTGGC and GGGACCAATTCAAAGGCC
AtHB15 promoter	GGCGCGCCATAGAGAGCAAATGTGATGGTG and GGGGTACCTACTCCTCAGCAAAACTCTT
ATHB8-δmiR	ACCTCGAGATGGGAGGAGGAAGCAATA and GCGGTACCCATATAAAAGACCAGTTGA

Either the QuantiTect Primer Assay (Qiagen) or oligonucleotide primers (Microsynth, Balgach, Sg, Switzerland) were used at a concentration of 0.5 μM. Real-time quantitative RT-PCR was performed with an iCycler iQ Real-Time PCR Detection System (BioRad, Hercules, CA, USA), using Absolute SYBR Green Fluorescein, which binds non-specifically to double-stranded DNA (ABgene, Epsom, Surrey, UK). The ribosomal *S16* gene was used as a standard control. Reactions were performed in triplicate in 20 μl using 100 ng cDNA and 0.4 mM $MgCl_2$ per reaction. Negative controls were performed in duplicate. Thermal cycling parameters were 95°C for 15 minutes, followed by 45 cycles at 95°C for 30 seconds, 59.7°C for 30 seconds and 72°C for 45 seconds. RT-PCR specificity was tested with melting-curve analysis and electrophoresis. Experiments were repeated three times. All calculations were performed with the Gene Expression Macro 1.1 (Bio-Rad) macros for Excel (Microsoft, Redmond, WA, USA). Semi-quantitative real-time PCR on 1 μl cDNA was performed using ExTaq (TaKaRa Biotech).