Class III HD-ZIPs govern vascular cell fate: An HD view on patterning and differentiation

Running title: HD-ZIP III transcription factors and vascular development

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Date of submission: 4th August, 2016

Number of Tables and Figures: 7

Word count: 8153

Highlight

Multiple aspects of plant vascular development are controlled by HD-ZIP III transcription factors. This review highlights factors that control, and are controlled by HD-ZIP III's to coordinate vascular morphogenesis.

Abstract

Plant vasculature is required for the transport of water and solutes throughout the plant body. It is constituted of xylem, specialised for transport of water, and phloem, that transports photosynthates. These two differentiated tissues are specified early in development and arise from divisions in the procambium, which is the vascular meristem during primary growth. During secondary growth, the xylem and phloem are further expanded via differentiation of cells derived from divisions in the cambium. Almost all of the developmental fate decisions in this process, including vascular specification, patterning and differentiation are regulated by transcription factors belonging to the class III homeodomain-leucine zipper (HD-ZIP III) family. This review draws together the literature describing the roles that these genes play in vascular development, looking at how HD-ZIP III's are regulated, and how they in turn influence other regulators of vascular development. Themes covered vary, from interactions between HD-ZIP III's and auxin, cytokinin, and brassinosteroids, to the requirement for exquisite spatial and temporal regulation of HD-ZIP III expression through microRNA mediated post transcriptional regulation, and interactions with other transcription factors. The literature described places the HD-ZIP III family at the centre of a complex network required for initiating and maintaining plant vascular tissues.

Key words

auxin, (pro)cambium, cytokinin, HD-ZIP III, miR165/166, root, shoot, transcription factors, vascular development, xylem

Introduction

Homeodomain transcription factors have been synonymous with regulation of development since their identification in patterning of the fly more than 30 years ago. In plants, members of the class III homeodomain-leucine zipper (HD-ZIP III) transcription factor family are an excellent example of the incredibly broad range of developmental processes that HD transcription factors regulate. HD-ZIP III's act from cradle to grave, with roles in patterning of the embryo, meristem maintenance, leaf development, inflorescence architecture, ovule development, growth response to environmental signals, and senescence. Characterization of mutations in *REVOLUTA*, one of five *HD-ZIP III* genes present in the model plant *Arabidopsis thaliana*, represents the first description of the consequences of loss of HD-ZIP III function (Talbert et al., 1995). While this paper is notable for its description of the pleiotropic defects present in *HD-ZIP III* mutants, clues begin to emerge as to their importance in controlling vascular development. In particular, Talbert *et al* (1995) noted that there were changes to the numbers of xylem and phloem cells in *rev* mutants compared to wild type plants, accompanied by changes to fibre differentiation. In subsequent years our understanding of the role of *REV*, which is also known as *INTERFASCICULAR FIBRELESS1* or *AMPHIVASAL VASCULAR BUNDLES1*, and the other members of the HD-ZIP III family in Arabidopsis, *Arabidopsis thaliana HOMEOBOX8* (*ATHB8*), *PHABULOSA* (*PHB*)/*ATHB14*, *PHAVOLUTA* (*PHV*)/*ATHB9*, and *CORONA* (*CNA*)/*INCURVATA4*/*ATHB15*, has been considerably elaborated in multiple aspects of vascular development. In this review we will describe in detail these roles in vascular patterning and xylem differentiation in both the shoot and root.

1. Radial patterning of vascular tissues in the shoot

Vascular tissue specification and differentiation occurs in the wider developmental context of organs such as the leaf, stem or root. Several *HD-ZIP III* mutants were initially identified in screens aimed at identifying regulators of leaf development, and these mutants also demonstrated vascular defects (McConnell and Barton, 1998; McConnell et al., 2001). Leaves are initiated at the flanks of the shoot apical meristem. They develop a specialised upper (adaxial or dorsal) side specialised for light capture, and a lower (abaxial or ventral) side specialised for gas exchange. The vascular strands are typically positioned where the adaxial and abaxial domains meet. Xylem is present in the adaxial position and phloem is positioned abaxially. The question of how these specific patterns arise in the leaf was addressed in early experiments, where the initiating leaf primordium was surgically separated

from the apical meristem from which it arose. The aim of these experiments was to determine if all information required for normal leaf formation is present within the initiating primordium or if leaf patterning requires communication with the meristem (Sussex, 1954). These experiments are pertinent to understanding vascular development within the shoot as they also represent some of the first observations of changes in vascular patterns. In a series of elegant papers in the 1950's, Sussex demonstrated that radially symmetric leaves were the consequence of surgically separating initiating primordia from the apical meristem in potato, arguing that a mobile signal emanating from the apical meristem must be involved in leaf patterning. Within these radially symmetric leaves, the vascular tissues were also clearly perturbed (Sussex, 1955). Subsequently, results in similar experiments using willowherb (*Epilobium*) also resulted in radialised leaves that lacked xylem-phloem asymmetry (Snow and Snow, 1959)(Figure 1A-C).

The first paper to place the observations of asymmetry-loss in a genetic context made use of the snapdragon (*Antirrhinum*) *phantastica* (*phan*) mutants, which had radialised leaves similar to those observed in the surgical experiments (Figure 1D-E). *Phan* was described as a "dorsalising factor", i.e. a gene that specifies the upper (and therefore xylem) side of the leaf (Waites and Hudson, 1995). *Phan* encodes a myb transcription factor (Waites et al., 1998), and its Arabidopsis orthologue *ASYMMETRIC LEAVES 1* (*AS1*) (Byrne et al., 2000) was subsequently shown to act as a positive regulator of the expression of *PHB*, *PHV* and *REV* (Fu et al., 2007)(Figure 2). Such observations were consistent with phenotypes of dominant gain-of-function *phb-1d* (Figure 1F-I) and *phv-1d* alleles, which had earlier been described as having amphivasal vascular bundles with xylem surrounding phloem, i.e. xylem present in both adaxial and abaxial positions and therefore gain of adaxial identity (McConnell and Barton, 1998; McConnell et al., 2001). This phenotype is opposite to that observed in loss-of-function *phan*, which has amphicribal bundles where phloem surrounds xylem (thus demonstrating a loss of adaxial identity) (Waites and Hudson, 1995). Cloning of the gain-of-function *phb* and *phv* alleles enabled comparisons of sequences with previously described genes. Similarities were found with *ATHB8*, an early marker of vascularisation (Baima et al., 1995), and with *REV*. While dominant *phb-1d* and *phv-1d* alleles demonstrated the most dramatic loss of asymmetry due to the presence of xylem in positions where phloem might be expected to form (Figure 1I), loss of function alleles demonstrated only subtle, if any, aberrations as single mutants. However, multiple *HD-ZIP III* knockouts resulted in phenotypes converse to those observed in the dominant alleles, i.e. phloem present in

positions where xylem forms in wild type (Emery et al., 2003; Prigge et al., 2005). The influence of the five *HD-ZIP III* genes on asymmetry determination is not equal. The phylogenetically relatively closely related *PHB, PHV* and *REV* clearly play predominant roles, but their paralogous couple *ATHB8* and *CNA* may also contribute to the radial patterning process as *ATHB8* over-expression leads to an increase in the formation of xylem tissue (Baima et al., 2001), and the dominant *icu4* alleles of *CNA* display some characteristics of plants with changes to adaxial-abaxial asymmetry (Ochando et al., 2008; Ochando et al., 2006). All five *HD-ZIP III* genes therefore, to a greater or lesser extent promote adaxial (and therefore xylem) identity within the leaf. Both gain-of-function and loss-of-function *HD-ZIP III* mutants also demonstrate radial patterning defects in the stem with dominant alleles characterised by xylem surrounding phloem, and recessive alleles by phloem surrounding xylem (Emery et al., 2003).

2. miRNA-mediated restriction of HD-ZIP III activity domains

Following their initial identification, a mechanistic understanding of the nature of dominant *HD-ZIP III* alleles was a matter of some speculation. Gain-of-function *HD-ZIP III* alleles have mutations that disrupt a steroidogenic acute regulatory protein-related lipid transfer (START) domain thought to be involved in hydrophobic ligand binding. This led to the hypothesis that a change to the regulatory function of the START domain (e.g. changes to putative ligand binding) may have occurred. However, following the discovery of RNA interference and identification of components of the microRNA (miRNA) machinery, it became apparent that mutations in the dominant alleles were also present in the sequence complementary to miRNA's 165 and 166 (miR165/166; Figure 3A), suggesting that HD-ZIP III's are subject to post transcriptional gene silencing (Reinhart et al., 2002; Rhoades et al., 2002). Consistent with this idea, transgenic plants engineered to have silent mutations disrupting the miRNA target site in *PHB* or *REV* without resulting in protein sequence changes displayed gain-of-function phenotypes (Emery et al., 2003; Mallory et al., 2004)(Figure 3B-D). In experiments using wheat germ extract, it was demonstrated that wild type *PHB* and *PHV* mRNA, but not that of the dominant mutants was subject to cleavage, demonstrating that RNAi can negatively regulate *HD-ZIP III* transcript abundance (Mallory et al., 2004; Tang et al., 2003), in line with the increased levels of *PHB* expression detected in both adaxial and abaxial domains of *phb-1d* leaves (McConnell et al., 2001). Furthermore, *HD-ZIP III* mRNA is expressed ectopically in RNAi machinery mutants, such as *argonaute1* (*ago1*) (Kidner and Martienssen, 2005) or *serrate (se*) (Lobbes et al., 2006).

Consistent with a role in asymmetry patterning miR165 and 166 are found on the abaxial side, and in developing phloem of the leaf primordium in both Arabidopsis and maize. The maize *rolled leaf1* (*rld1*) mutant bears a mutation in the miRNA target site of a *REV* homologue (Juarez et al., 2004b), resulting in adaxialisation and overexpression of the *rld1* gene. Thus, vascular patterning of leaves and stems rely on *HD-ZIP III* expression being restricted through miRNA mediated removal of *HD-ZIP III* mRNA from abaxial domains in both eudicots and monocots. Interestingly, in situ hybridization of miR166 localization in the maize leaf primordium revealed a dynamic and graded distribution on the abaxial/phloem side of the leaf, leading Juarez et al. (2004a) to note that it behaved as a movable signal.

Focussing of miR166 to the abaxial side of the maize leaf is thought to be the result of the action of trans-acting short-interfering RNAs (ta-siRNAs; for review see Chitwood *et al*., 2007). Briefly, in contrast to conventional miRNA directed cleavage which results in the degradation of the target mRNA (e.g. miR165/166 action on *HD-ZIP III* transcripts described above), cleavage of a non-coding *TAS* RNA enables it to become a target for RNA-dependent RNA polymerases. The resulting double stranded RNA is subject to further processing from which 21 bp ta-siRNA's are generated. ta-siRNA's guide cleavage of mRNA targets in a similar manner to miRNA's. ta-siRNA's are derived from miRNA action on non-coding *TAS* transcripts. In Arabidopsis, ta-siRNA's, derived from *TAS3* that has been subjected to cleavage by miR390, negatively regulate *ETTIN* (*ETT*), also known as *AUXIN RESPONSE FACTOR3* (*ARF3*) and *ARF4*, two genes that act redundantly in abaxial leaf identity (Chitwood et al., 2007). In maize, *LEAFBLADELESS1* (*LBL1*) encodes a zinc finger protein required for the generation of ta-siRNA's, and in *lbl1* mutants, the localisation of miR166 is no longer restricted to the abaxial domain of the initiating leaf primordium, but is expressed throughout. *lbl* mutants demonstrate a clear loss of adaxial-abaxial asymmetry (Nogueira et al., 2007), consistent with downstream changes to levels of *HD-ZIP III* transcript (Nogueira et al., 2009). One possibility is that these small RNA's could act non-cell autonomously and thus are candidates for the "Sussex signal", proposed in the early surgical experiments described above that are involved in crosstalk between the shoot apical meristem and initiating leaf primordium (Chitwood et al., 2007).

Disruption of the interactions between miRNA and mRNA target has provided particular insight into the roles that HD-ZIP III's play in vascular tissue formation. HD-ZIP III's are

188 required for vascular tissue in the leaves as over expression of one of the two genes encoding
189 miR165, *MIR165A*, results in leaves that entirely lack vascular tissue (Zhou et al., 2007). An
190 activation tagging line, *jabba-1d* (*jba-1d*) that resulted in increases in expression of
191 *MIR166G*, one of the seven miR166 encoding genes, had concomitant reductions in *PHB*,
192 *PHV* and *CNA* expression. Counter intuitively however, increases in *REV* expression were
193 also observed in this line, leading to the hypothesis that other *HD-ZIP III* genes may repress
194 *REV* (Williams et al., 2005). Interestingly, in high throughput yeast one hybrid (YIH)
195 experiments, PHV was reported to bind to the *REV* promoter (Taylor-Teeples et al., 2015)
196 providing further evidence for such a regulatory relationship. Consistent with perturbation of
197 miRNA - HD-ZIP III homeostasis being required for vascular pattern, *jba-1d* mutants
198 demonstrate changes to vascular organisation. In inflorescence stems, ectopic radially
199 symmetric vascular bundles are present in the centre of the stem that are characterised by
200 xylem surrounding phloem. Collateral bundles in positions similar to those present in wild
201 type also demonstrated changes to morphology (albeit to a lesser degree than those at the
202 centre of the stem) (Williams et al., 2005). A second activation tag mutant, *meristem*
203 *enlargement1* (*men1)*, in which *MIR166A* was overexpressed, demonstrated similar
204 phenotypes (Kim et al., 2005).
205
206 While *HD-ZIP III* mRNA's are negatively regulated by miR165/166, miRNA's are in turn
207 negatively regulated by a member of the AGO family. In contrast to other AGO proteins,
208 which cleave/silence small RNA targets, PINHEAD (PNH; also known as ZWILLE/AGO10)
209 is thought to protect *HD-ZIP III* mRNA from silencing by sequestering miR165/166 (Zhang
210 and Zhang, 2012). Such interactions have mainly been described in the embryo and shoot
211 apical meristem (Zhou et al., 2015; Zhu et al., 2011), but might PNH preform a similar role in
212 the vascular tissue? *PNH* expression in the embryo demonstrates maxima in both the apical
213 meristem expression and in the central provascular cells. Later in development, expression is
214 prominent in the adaxial side of developing leaves, and in the vascular tissue (Lynn et al.,
215 1999; Moussian et al., 1998). *pnh* mutants do not typically demonstrate changes to leaf vein
216 asymmetry, however there is further evidence to suggest that *PNH* could carry out a similar
217 function in vascular tissue. The *pnh* phenotype is enhanced by mutations at the *asymmetric*
218 *leaves2* (*as2*) locus, such that *as2 pnh* leaves demonstrate changes to vascular organisation
219 (Liu et al., 2009). *AS2* encodes a transcription factor that heterodimerises with, and is
220 required for AS1 function (Lin et al., 2003; Semiarti et al., 2001; Xu et al., 2003).
221 Consequently, the *as2 pnh* phenotype may be a combination of a failure to sequester

miRNA's by PNH, and a failure to properly promote *HD-ZIP III* expression by AS2. Furthermore, *PNH* expression, driven from the *ATHB8* promoter is sufficient to restore defects in *pnh* mutants, and while these experiments were used to demonstrate a requirement for focussing *HD-ZIP III* expression in the shoot apical meristem, one could also argue that the *ATHB8::YFP-ZLL* construct used in this analysis could focus *HD-ZIP III* expression in the provascular domain (Tucker et al., 2008). Interestingly, REV has been shown to rapidly promote transcription of *PNH* (Reinhart et al., 2013), perhaps indicating a positive feedback that could have the potential to canalise high REV levels by counteracting miR165/166's (Figure 2).

3. The HD-ZIP III's act in a network of interactions

Mechanisms of post-transcriptional regulation described above are critical in specifying the when and where of HD-ZIP III action. However, interactions between these genes and other factors also determine aspects of vascular development (Figure 2). One group of regulators are members of the LITTLE ZIPPER (ZPR) family of proteins that interact with HD-ZIP III proteins by forming complexes, likely through interactions at the zipper domain, and thereby preventing HD-ZIP III binding to DNA (Husbands et al., 2016; Kim et al., 2008; Wenkel et al., 2007). Overexpression of *ZPR* genes results in vascular defects including cellular proliferation adjacent to veins in the leaf and changes to xylem-phloem asymmetry. Furthermore, expression of *ZPR1* and *ZPR3* is clearly localised to the vascular tissue in the embryo and in developing leaves (Wenkel et al., 2007). In vitro studies suggest that ZPR proteins bind all five members of the HD-ZIP III family, while interactions with PHB and REV have been confirmed *in planta* (Kim et al., 2008).

Genes of the *KANADI* family of GRAS-type transcription factors were shown a number of years ago to act in opposition to HD-ZIP III's in radial patterning. In particular, where HD-ZIP III's specify the adaxial side of the leaf and the xylem side of the vascular tissue, *KAN* genes, of which there are four, specify the abaxial side of the leaf and the phloem side of the vasculature (Emery et al., 2003; Eshed et al., 2004; Kerstetter et al., 2001). Initially, it was not particularly clear how this opposition might occur, despite findings such as negative regulation of *AS2* by KAN1 (Wu et al., 2008). A clearer picture began to emerge in the embryo, where the role of these opposing gene families was shown to control auxin flow (Izhaki and Bowman, 2007). Subsequent studies that focused on the vascular tissue built on these observations, suggesting that *KAN* genes were negative regulators of *PIN-FORMED1*

(*PIN1*) that encodes an auxin efflux carrier (Ilegems et al., 2010). The flow of auxin through preprovascular cells, as directed by PIN1, has been demonstrated to control the process of leaf venation (Scarpella et al., 2006). Strikingly, auxin is thought to induce expression of *HD-ZIP III*'s (Baima et al., 1995; Zhou et al., 2007). *HD ZIP III*'s, in turn promote developmental changes that support canalisation of auxin as plants with reduced levels of HD-ZIP III expression were impaired in cell maturation, demonstrating defects in xylem differentiation and connection of cell files (Ilegems et al., 2010). Interestingly, data is now available that has identified direct, often antagonistic, transcriptional targets of *REV* and *KAN1* (Huang et al., 2014; Reinhart et al., 2013). Of particular note, genes including *ALTERED PHLOEM* (*APL*), required for phloem specification (Bonke et al., 2003), and *CLE41* which encodes a phloem-expressed signal required for vascular proliferation (Etchells et al., 2015; Etchells and Turner, 2010), are negatively regulated by *REV* (Reinhart et al., 2013). In line with the antagonistic interaction between KANADIs and HD-ZIP III's a direct repressive regulation of KAN1 on *PHB* and *ATHB8* was found in one study (Merelo et al., 2013)(Figure 2).

4. Cell-to-cell movement of miR165/166 pattern the root vasculature

A radial section of the Arabidopsis root displays an anatomy with unusually few cells and a diarch xylem arrangement with peripheral protoxylem (with spiral or annular secondary cell wall thickenings) and central metaxylem (with reticulate or pitted walls) (Figure 4A). The xylem axis is flanked by procambium and a phloem pole on either side. The simplicity of the Arabidopsis root vascular anatomy allows for relatively easy detection of aberrant phenotypes. A screen for mutants with vascular defects thus picked up a novel dominant allele of *PHB, phb-7d*, that displayed metaxylem in the place of protoxylem (Carlsbecker et al., 2010) (Figure 4A). Interestingly, it was found that the *short root* (*shr*) and *scarecrow* (*scr*) mutants displayed a very similar vascular phenotype, and SHR had previously been shown to indirectly repress expression of *PHB* and *PHV* (Levesque et al., 2006). Supporting the notion that ectopic *PHB* expression caused the *shr* xylem phenotype, the *shr phb* double mutant had restored protoxylem formation. SHR is produced in the vasculature, but the protein is exported to the endodermal cell layer surrounding the vascular stele, where it activates *SCR* (Helariutta et al., 2000; Nakajima et al., 2001). In the endodermis SHR, together with SCR, activates the transcription of the three genes encoding miR165 and miR166 that are active in roots, *MIR165A*, *MIR166A* and *MIR166B* (Carlsbecker et al., 2010; Miyashima et al., 2011) (Figure 4B).

Analyses of transcriptional reporters in comparisons with RNA in situ hybridization and translational reporter assays revealed a post transcriptionally restricted activity domain of the *HD-ZIP III* genes, most apparent for *PHB*. Transcriptional reporters for *PHB*, *CNA* and *REV* are active throughout the stele, but mRNA and protein activity domains are focused to the central stele for PHB and CNA, while REV occupies the procambial domain (Carlsbecker et al., 2010; Lee et al., 2006; Miyashima et al., 2011). *ATHB8* displayed transcription and also protein localization specific to the xylem axis. *PHV* had a close to non-detectable activity. The difference between transcriptional and translational reporters supports a miRNA-mediated restriction of *HD-ZIP III* expression domains within the root vasculature. This is particularly evident for *PHB* and genetic analyses showed that ectopic *PHB* activity is primarily responsible for the vascular aberrations of *shr* and *scr*, although *ATHB8* and *CNA* contribute. The post-transcriptionally restricted PHB domain suggested that the miRNA is active primarily in the peripheral stele. Indeed, a miR165-GFP-sensor revealed miR165-activity particularly in these cells. Specifically driving miR165 in ground tissue in *shr* and *scr* restricted the ectopic *PHB* expression to the central stele, and restored the formation of protoxylem (Carlsbecker et al., 2010). Further support for an endodermal-mediated non-cell-autonomous regulation of stele patterning came from an experiment where the *phb-d* phenotype, resulting from driving *PHB* with a mutated miRNA target site under its own promoter, was restored by driving a modified miRNA complementary to the altered *PHB* miRNA-site from an endodermis specific promoter (Miyashima et al., 2011). Hence, miR165/166 derived from the endodermis move several cells away to restrict the mRNA activity domain of the HD-ZIP III TFs (primarily PHB) within the stele, and thereby control vascular patterning (Figure 4B).

The critical role of cell-to-cell trafficking in root vascular patterning was further confirmed by blocking plasmodesmata connections. Gain-of-function alleles of *callose synthase 3* (*cals3-d*) overproduce callose at plasmodesmata hindering macromolecular cell-to-cell passage. This results in a root vascular phenotype similar to that of a *phb-d* or *shr* mutant. In these lines, *PHB* is ectopically active throughout the stele and SHR movement into the endodermis fails (Vatén et al., 2011). Driving a dominant and inducible version of *cals3* by tissue specific promoters further allowed Vatén et al. (2011) to analyse the consequence of blocking plasmodesmata connections between the ground tissue and the stele on miR165 accumulation. In this experiment miR165 and callose synthase was simultaneously induced in the ground tissue of a *shr* mutant. In situ hybridization revealed that miR165 accumulated in

the ground tissue, compared to controls. Thus, these findings demonstrated plasmodesmata mediated cell-to-cell mobility of the miRNA.

Ectopic expression of miR165 throughout the stele results in protoxylem forming in metaxylem positions in the xylem axis. In line with this, plants harbouring mutations in four of the five *HD-ZIP III* genes also display protoxylem throughout the xylem axis, while lower order mutants may display formation of a central metaxylem strand flanked by several protoxylem files (Carlsbecker et al., 2010) (Figure 4A). The quintuple HD-ZIP III mutant does not form xylem at all. These phenotypes, together with that of *phb-d* mutants where metaxylem replace protoxylem, indicate that HD-ZIP III transcription factors determine xylem cell identity in a dose-dependent fashion with high dosage resulting in metaxylem and lower dosage in protoxylem (Carlsbecker et al., 2010). Notably, *phb-d* not only affects xylem cell type formation, but also pericycle cell identity (Miyashima et al., 2011). Thus miR165/166 may form a morphogenetic gradient emanating from the endodermal cell layer, determining stele cell identity.

5. HD-ZIP III activity intersects with auxin and cytokinin signalling for proper xylem patterning

The HD-ZIP III-miRNA gradients in the root is overlayed by balanced auxin and cytokinin signalling domains shown to establish xylem and procambium cell identity, respectively (Bishopp et al., 2011) (Figure 4C). Multiple points of intersection between these two hormones and the HD-ZIP III transcription factors occur during root vascular patterning. Auxin biosynthesis is primarily tryptophan dependent, and consequently requires the enzyme TRYPTOPHAN SYNTHASE. Two alleles (*trp2-12* and *trp2-13*) of the gene encoding the beta subunit (TSB1/TRP2) of this enzyme were identified from a screen for mutants with altered root vascular development. The *trp2* mutants along with other auxin biosynthesis mutants that are defective in down-stream biosynthesis steps, such as the *weak ethylene insensitive 8 tryptophan aminotransferase related 2* (*wei8 tar2*) double mutant or a quintuple *yucca* mutant, displayed defective metaxylem development and protoxylem formation in the metaxylem position, suggesting that auxin biosynthesis is required for mextaxylem formation (Ursache et al., 2014). A similar phenotype was observed in *axr3-3,* which harbours a gain-of-function mutation in *IAA17* that inhibits auxin signalling. The vascular defects in *trp2* were rescued by treatment with L-Trp while treatment with L-Kynurenine (Kyn), which

blocks TAA1/TAR mediated auxin biosynthesis, phenocopied the auxin biosynthesis mutants with the formation of protoxylem in metaxylem position. In line with the similarity of this phenotype to higher order *HD-ZIP III* mutants the expression of *PHB, PHV, CNA* and *ATHB8* was greatly reduced in the *trp2* mutants and upon Kyn treatment of the wild-type. Kyn resistance was brought about by driving *PHB* expression by an auxin non-responsive promoter. Taken together with the partial rescue of the *phb-7d* xylem phenotype by Kyn treatment, this revealed an auxin biosynthesis mediated, HD-ZIP III dependent, vascular development pathway required primarily for metaxylem formation (Ursache et al., 2014).

The interconnection between HD-ZIP III and auxin was previously shown by the auxin inducible characteristic of *ATHB8* (Baima et al., 1995). Studies on vascular patterning in the leaf showed that the accumulation of the DR5 auxin reporter preceded procambium formation, and was closely followed by activation of the auxin response factor *ARF5*/*MONOPTEROS* (*MP*) and *ATHB8* (Mattsson et al., 2003). Donner *et al.* (2009) subsequently demonstrated that *ATHB8* transcription is directly regulated by MP. However, neither in the leaf nor in the root meristem is there a precise correlation between domains of high auxin signalling and transcription domains of the five *HD-ZIP III* genes. Hence, other as of yet unidentified factors likely contribute to their activation and/or restriction. Efforts to identify gene regulatory networks around the *HD-ZIP III* genes may be probed for such candidates (Brady et al., 2011; Taylor-Teeples et al., 2015)(see also section 5 below).

In the postembryonic root meristem, auxin response reporters suggest an auxin sink at the position of the immature xylem axis. The accumulation of auxin is brought about by polar auxin transport, via PIN1 and procambially localized PIN3 and PIN7 mediating lateral auxin transport. Inhibition of polar auxin transport by exogenous supply of *N*-1-naphthylphthalamic acid (NPA) lead to loss of protoxylem strand formation in a dose dependent manner (Bishopp et al., 2011)(Figure 4C). In the protoxylem domain, the auxin maximum activates ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6 (AHP6). AHP6 is an inhibitor of cytokinin signalling and in *ahp6* mutants, the protoxylem strand integrity is affected similar to the wild type root subjected to exogenous cytokinin treatment, and protoxylem become replaced by procambial cells. On the other hand, cytokinin depletion or a block in cytokinin signalling lead to differentiation of all vascular cells as protoxylem (Mähönen et al., 2006). Therefore, inhibition of cytokinin signalling in the xylem axis is necessary for vessel formation and presence of cytokinin signalling in the procambial cells is

required for maintaining them in an undifferentiated state. Interestingly, the *phb-7d* mutant lacks expression of *AHP6* while, in contrast, *athb8 cna phb phv* quadruple mutants demonstrate expansion of the *AHP6* expression domain to the entire xylem axis (Carlsbecker et al., 2010)(Figure 4C). To predict the minimal molecular signalling circuits required for proper radial patterning in the Arabidopsis root Muraro *et al*. generated a mathematical model with which they were able to reconstitute a realistic radial pattern, but only by integrating SHR-miR165/166-PHB with the above described auxin and cytokinin signalling loop (Muraro et al., 2014). In their model, they predicted PHB to act as repressor of *AHP6* expression in the metaxylem domain. In support of this prediction, the expression of *AHP6* rapidly increases upon induction of miR165 (Müller et al., 2016), although it is unknown if this interaction is direct, or occurs via the effect that HD-ZIP III transcription factors have on auxin signalling.

Several observations suggest that levels of HD-ZIP III transcription factors affect auxin signalling: Auxin signalling reporters revealed considerable increases in activity in the *athb8 cna phb phv* mutant compared to the wild type while *phb-7d* mutants displayed severely impaired auxin signalling in the xylem axis not possible to revive by exogenous auxin treatments (Müller et al., 2016). Similarly, up-regulation of miR165 resulted in a wider auxin reporter expression domain, and a number of core auxin signalling genes were increased, along with a down-regulation of primarily *PHB*, *PHV*, and *CNA* (Ilegems et al., 2010; Müller et al., 2016). However, despite being auxin inducible, *MP*, *IAA20*, and *IAA30* were down-regulated upon miR165 induction and PHB was also found to bind the promoters of *MP* and *IAA20* in vivo, suggesting that PHB is required at their promoters for proper activation (Müller et al., 2016). In contrast to most AUX/IAA proteins, IAA20 and IAA30 lack the canonical domain II, recognised by the auxin/TIR receptor complex, and are therefore not degraded even in the presence of high auxin levels. Their interactions with AFRs, however, are not altered, and they may therefore act as ARF scavengers and dampen auxin signalling (Sato and Yamamoto, 2008). The double mutant *iaa20 iaa30* displays formation of extra protoxylem strands suggesting that a balanced auxin response is required for proper root vascular patterning (Müller et al., 2016). Similarities in the phenotypes of a weak *mp* mutant and lines overexpressing *IAA30*, indicate that IAA30 (and IAA20) likely represses the activity of MP. Activation by PHB (and other HD-ZIP III's) of components both promoting and suppressing auxin signalling may balance vascular auxin response and genetic data suggests that this is promoting a stable xylem axis patterning.

Thus, several studies show a tight link between HD-ZIP III transcription factors and auxin signalling on many different levels (recently reviewed by Turchi et al., 2015). A direct binding of REV to the promoters of the auxin influx carriers *AUX1*, *LAX2* and *LAX3* was identified (Baima et al., 2014; Huang et al., 2014), and the expression of these genes was significantly altered upon the induction of miR165 in the root and shoot (Baima et al., 2014; Müller et al., 2016). The triple *aux1 lax1 lax2* mutant has aberrant protoxylem formation (El-Showk et al., 2015), and along with previously mentioned results obtained by blocking polar auxin transport, this supports the notion that the activity of both auxin influx and efflux carriers is required to attain sufficient auxin accumulation for proper protoxylem and metaxylem formation. As a consequence of auxin accumulation in the xylem axis a number of downstream genes that play a role in xylem cell specification and differentiation are switched on (see below).

6. A role for HD-ZIP III genes in restricting procambial cell proliferation?

In the embryo, the first vascular cells are initiated in the central globular staged embryo (Scheres et al., 1995). Analyses of expression revealed presence of *REV*, *PHB*, *PHV* and *CNA* expression in apical parts of the embryo from early globular stage, while *ATHB8* appears a little later, at the early heart stage, in the provascular cells where it is later joined by the other family members (Baima et al., 1995; Prigge et al., 2005; Smith and Long, 2010). Thus, although expression of the *HD-ZIP III* genes are initiated early their activity domains are not perfectly overlapping that of the first vascular cells, suggesting that their activity in the procambium follows the initiation of the first vascular cells. A pathway mediated by TARGET OF MONOPTEROS 5 (TMO5) along with its interaction partner LONESOME HIGHWAY (LHW) controls periclinal cell divisions in the embryo essential for the radial vascular axis and also for the maintenance of vascular cell number in the post-embryonic root meristem (De Rybel et al., 2013; Ohashi-Ito et al., 2013) (Figure 4C). Alterations in cell number have been attributed to shifts in the auxin-cytokinin balance as long term treatment with NPA increases the vascular cell number and subsequently the number of xylem poles (Bishopp et al., 2011), while impaired cytokinin signalling results in reduced procambial cell proliferation (Mähönen et al., 2000). *TMO5* and its homolog *TMO5-LIKE1 (T5L1)* express specifically in the xylem axis. As dimers with LHW they directly control the expression of the rate limiting cytokinin biosynthesis genes *LONELY GUY3 (LOG3)* and *LOG4* (De Rybel

et al., 2014; Ohashi-Ito et al., 2014), which would serve to increase cytokinin levels in the xylem axis. However, cytokinin reporters reveal that signalling primarily occurs in the procambium. Potentially, activation of *AHP6* by T5L1/LHW may restrict the effect of cytokinin from the xylem domain (Ohashi-Ito et al., 2014). However, AHP6 is not active in the central metaxylem/PHB-activity domain of the xylem axis. It is possible that PHB contributes by other means to the reduced cytokinin responsiveness of these cells; a recent publication may provide a possible mechanism, as it was found that PHB can prevent the activity of B-type response regulators (B-ARRs) potentially by preventing B-ARR DNA binding, especially under high cytokinin level conditions (Sebastian et al., 2015). The role for PHB and the other HD-ZIP III transcription factors as potential regulators of procambial cell proliferation needs to be substantiated by more research, however, several observations suggest a role for the HD-ZIP III's in regulating procambial cell divisions. The *athb8 cna phb phv* mutant has a significant increase in the number of root procambial cells compared to wild type, resulting in a triarch or tetrarch vascular arrangement. Driving miR165 in the stele also causes a similar increase in the number of vascular cells (Carlsbecker et al., 2010; Ilegems et al., 2010). Conversely, the *phb-d* alleles contain fewer stele cells (Carlsbecker et al., 2010). While there are as yet only clues as to how HD-ZIP III's might ultimately regulate this process, one possibility is that HD-ZIP III's expression in the procambium may be regulated by DOF transcription factors. Seven different DOF genes were found to interact with the promoters of PHB and PHV, and in certain cases a single DOF could act as activator of one HD-ZIP III gene while repressing another (Brady et al., 2011) (Figure 2). DOF-TFs are expressed early in procambium formation in the leaf (Gardiner et al., 2010), and some members of the gene family, act to control vascular cell-division (Guo et al., 2009); see (Le Hir and Bellini, 2013) for review. Complex networks of interactions such as this are present around HD-ZIP III TFs as shown in transcriptional regulatory network analysis for both the stele and xylem (Brady et al., 2011; Taylor-Teeples et al., 2015). The connections in such networks point to interesting regulatory relationships. In the case of DOF regulation of PHB and PHV, further work is required to understand the significance of this interaction.

7. HD-ZIP III regulated differentiation of xylem cells

While the analysis HD-ZIP III function described above looks at changes to vascular patterning and organisation, HD-ZIP III's also function post-patterning, in particular in differentiation of the xylem. Early work on the role of *REV* in xylem differentiation followed

the independent isolation of *REV* loss-of-function alleles by Zhong and Ye (*ifl* alleles of *rev*) (Zhong et al., 1997; Zhong and Ye, 1999) in screens that aimed to identify mutants with xylem defects. Zhong and Ye noted that while vascular bundles in inflorescence stems of *rev* mutants demonstrated few differences when compared to wild type close to the shoot apex, in basal parts of the inflorescence stem, *rev* vascular bundles were characterised by fewer cells (Figure 5). The xylem in inflorescence stem vascular bundles is typically constituted of two cell types that have large secondary cell walls. Xylem vessels transport water, and smaller xylary fibres provide mechanical support. While xylem vessels were present in *rev* mutants, xylary fibres were reduced in weaker alleles, or absent in strong alleles (Zhong and Ye, 1999)(Figure 5). Outside the vascular bundles, a reduction in the number of interfascicular fibres was also observed, and this loss of fibres in *rev* mutants results in large reductions in breaking force (Zhong et al., 1997). It has been suggested that in fibres, the *rev* phenotype is a result of failure to differentiate such that secondary cell wall material is not deposited, rather than a failure in fibre specification (Lev-Yadun et al., 2004). The role that *REV* has in specification of terminal xylem differentiation is influenced by KNOTTED-LIKE HOMEOBOX OF ARABIDOPSIS THALIANA 7 (KNAT7) and BEL1-LIKE HOMEODOMAIN 6 (BLH6) (Figure 6). These homeodomain transcription factors form a heterodimer that binds to the promoter of, and negatively regulates, *REV* expression. Consequently, *knat7 blh6* double mutants demonstrate large increases in *REV* expression that are accompanied by increases in secondary cell wall thickness (Liu et al., 2014). These results support the role of *REV* as a positive regulator of xylem cell wall deposition, specifically in fibres and are consistent with observations that *rev* mutants have reduced secondary walls in fibres. Rather surprisingly, in contrast to fibre walls that are thicker in *knat7 blh6* lines (Liu et al., 2014), vessel secondary walls, and in particular those of *knat7* mutants are thinner than those of wild type counterparts, such that the vessels collapse due to a failure to withstand the negative pressures of water transport (Li et al., 2012). The KNAT7-BLH6-REV interaction consequently does not appear to act in xylem vessels, at least not in the same way that it regulates wall deposition in fibres. One explanation of this phenotype is that KNAT7/BLH6 acts independently from REV in vessel element differentiation.

A number of observations have supported a role for other members of the HD-ZIP III family as having roles in xylem development and differentiation. Analysis of *HD-ZIP III* expression in *Zinnia elegans* leaves found that *REV* homologues, *ZeHB11* and *ZeHB12* demonstrated xylem expression, as did *ATHB8* and *CNA* orthologues (*ZeHB-10* and *ZeHB-13*,

respectively), albeit in an expression domain consistent with these genes having a role in early xylem specification, rather than in deposition of cell wall polymers (Ohashi-Ito and Fukuda, 2003). Such a hypothesis is supported by the observation that constitutive over-expression of *MIR165B*, which results in reductions in *CNA* expression, and likely that of other HD-ZIP III's leads to ectopic deposition of secondary cell wall material in the pith of Arabidopsis stems (Du et al., 2015). Subsequent work, which tested genetic redundancy between *rev* and the other HD-ZIP III transcription factors, showed that *phb* and *phv* were strong enhancers of the *rev* phenotype in the xylem; in extreme cases *rev phb/+* and *rev phv* mutants displayed vascular bundles with remarkably few lignified cells (Prigge et al., 2005). In contrast, lignification of xylem tissue and interfascicular fibres was restored in *athb8 cna rev* triple mutants, i.e. *athb8 cna* suppressed the *rev* phenotype. The idea that *ATHB8* and *CNA* have distinct functions to those of *PHB, PHV* and *REV* is supported by experiments in a *rev* mutant background where expression of HD-ZIP III family members was driven from the *REV* promoter. While *REV::REV, REV::PHB,* and *REV::PHV* constructs rescued the *rev* mutant phenotype. *REV::ATHB8* and *REV::CNA* did not (Prigge et al., 2005). Taken together, these observations suggest that early xylem specification may be controlled by *ATHB8* and *CNA*, while differentiation to mature xylem is repressed by these genes. In contrast, *REV*, *PHV* and *PHB* are positive regulators of the final stages of xylem differentiation.

The roles of *HD-ZIP III* genes in xylem specification and differentiation have been confirmed by experiments that have looked for HD-ZIP III regulators and targets. The phytohormone brassinosteroid (BR) has been implicated as a regulator of wide-ranging aspects of vascular development, both in terms of regulation of number and position of vascular bundles (Caño-Delgado et al., 2010), and xylem differentiation (Cano-Delgado et al., 2004; Yamamoto et al., 2001) (Figure 6). In xylogenic cultures, levels of BR have been shown to dramatically increase at a time point corresponding to entry into the final stages of xylem differentiation. Rapid induction of *REV* homologue transcripts, *ZeHB11* and *ZeHB12*, occurs upon BR treatment. In contrast, expression of the same transcripts is repressed upon treatment with uniconazole, a BR inhibitor (Ohashi-Ito and Fukuda, 2003; Yamamoto et al., 2001; Yamamoto et al., 2007). Intriguingly, the behaviour of the *ATHB8* orthologue *ZeHB10* is similarly regulated, and while *ZeHB13*, a *CNA* orthologue, is not repressed upon perturbation of BR signalling, its expression is also increased upon BR induction (Ohashi-Ito and Fukuda, 2003). This begs the question of how apparently opposing functions of *ATHB8/CNA* and

PHB/*PHV*/*REV* might be reconciled? The answer likely lies in the position that each gene controls within a complex network. *ACAULIS5* (*ACL5*) is a gene encoding a thermospermine synthase, which has been shown to negatively regulate xylem differentiation. *acl5* mutants are characterised by early terminal differentiation of xylem that results in programmed cell death prior to xylem expansion and deposition of a full secondary cell wall (Muñiz et al., 2008). ATHB8 acts together with auxin as a direct positive regulator of *ACL5* which, in turn, slows xylem differentiation, in part by negative regulation of *REV* (Baima et al., 2014). Intriguingly, it was recently found that ACL5 also activates proteins capable of counteracting the cell-proliferation promoting effect of TMO5/LHW (Vera-Sirera et al., 2015) (Figure 4C). ATHB8 together with auxin, therefore, also regulates vascular cell divisions.

Consistent with the idea that ATHB8 is a negative regulator of xylem differentiation, other signalling pathways that act in the procambium to maintain the vascular meristem are thought to positively influence *ATHB8* expression. TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF) and PHLOEM INTERCALATED WITH XYLEM (PXY)/TDIF RECEPTOR (TDR) are a ligand-receptor pair that act both to maintain cell division in, and exclude xylem differentiation from, the procambium. Seedlings grown in liquid media containing TDIF ligand, and plants overexpressing *CLAVATA3/ESR-RELATED 41* (*CLE41*; a gene from which TDIF is derived), demonstrate increases in *ATHB8* expression (Etchells and Turner, 2010; Hirakawa et al., 2008; Ito et al., 2006) (Figure 6), which supports the idea that *ATHB8* acts to slow xylem differentiation.

Genetic analysis has supported a function for *REV*, *PHB* and *PHV* in promoting xylem differentiation as described above. These observations were supported by recent experiments suggesting that *REV* (and *PHV*) both bind to the promoter of the xylem master regulator *VASCULAR-RELATED NAC DOMAIN7* (*VND7*) (Figure 6). In assays where constructs containing the *VND7* promoter controlling expression of a luciferase reporter (*LUC*), were co-bombarded into Arabidopsis leaves with a *35S::REV* construct, a 3-fold increase in promoter activity was observed compared to controls (Endo et al., 2015). Expression of *VND7* has previously been shown to result in adoption of xylem fate (Kubo et al., 2005). This leads to a model whereby adjacent to the procambium, where *ATHB8*/*CNA* show expression maxima, the xylem differentiation process is slowed by positive regulation of *ACL5*. However, expression of *ATHB8/CNA* and consequently *ACL5* expression is lowered further

from the procambium. Therefore *REV* (and possibly *PHB* and *PHV*) would be released from this negative regulation by *ACL5*, enabling promotion of expression of *VND7*.

The HD-ZIP III transcription factors lie at the centre of a network that is required to fine-tune dynamic changes in gene expression throughout vascular development. High throughput YIH screens have recently been used to place *PHB*, *PHV* and *REV* in a network that regulates secondary cell wall deposition. Interactions within this network include both *VND7* and *PHV* binding to the promoter of *REV*, hinting at complex regulatory mechanisms. In particular, VND7 was reported to negatively regulate *REV* expression. REV, in turn, binds to the promoter and negatively regulates the expression of *PHENYLALANINE AMMONIA LYASE4* (*PAL4*) (Taylor-Teeples et al., 2015), a gene involved in lignin biosynthesis (Sewalt et al., 1997). However, as REV has previously been reported to positively regulate expression of *VND7* (Endo et al., 2015), these results suggest that an understanding at cell-type specific resolution is required to understand how these interactions control commitment to xylem differentiation, fibre formation, and deposition of the secondary cell wall.

8. HD-ZIP III regulation of wood formation in trees

In tree species such as poplar, vascular tissue expansion is present in a continuous ring in the stem and is the main driver of secondary growth. It is clear that the HD-ZIP III family have an important role in regulating this process as *REV*, *CNA* and *ATHB8* orthologues, *popREVOLUTA* (*PRE*), *POPCORONA* (*PCN*), and *PtrHB7* are expressed in poplar vascular tissue, and perturbations to the expression of these genes leads to defects in organisation and wood deposition (Du et al., 2011; Robischon et al., 2011; Zhu et al., 2013). While transgenic trees over-expressing a microRNA-resistant form of *PCN* had relatively subtle defects including early onset of secondary growth (Du et al., 2011), phenotypes of miRNA-resistant *PRE* over-expressers demonstrated much more dramatic phenotypes, including areas of xylem present on both sides of the cambium. This is in contrast to wild type poplar (and other woody species), where xylem is strictly restricted to the inner side of the cambium (Robischon et al., 2011). In another experiment, over expression in poplar of the native REV-homologue also resulted in reduction in fibre to vessel ratio and associated changes in many genes relating to cell wall synthesis (Côté et al., 2010). Interestingly, genome wide association studies identify links between the multiple splice variants in the 3' end of the *REV* locus and wood cellulose content in poplar (Porth et al., 2014). One of the striking features of perennial woody plants are the annual rings that form in the wood due to differences in

seasonal growth. In hybrid aspen, miR166 has been shown to be seasonally regulated, with a large peak in expression in the winter months. Elevated winter miR166 coincides with reductions in expression of *PtaHB1*, a *REV* orthologue (Ko et al., 2006), suggesting that seasonal control of *REV*-directed wood development is at least in part via miR166 regulation. It may be interesting to observe the roles that HD-ZIP III's and miRNA's might have in patterning of plants with unusual cambial organisations for example those with included phloem such as *Avicennia* and *Bougainvillea* (Studholme and Philipson, 1966; Zamski, 1979), or plants that develop phloem wedges, such as members of the *Bignonieae* (Pace et al., 2009; Spicer and Groover, 2010). Aside from miRNA mediated regulation of HD-ZIP III's, other regulatory interactions are likely to be conserved across plants with differing growth habits. One such regulatory interaction is that between *PttHB8* (an *ATHB8* orthologue) and poplar *ACL5* (*POPACAULIS5*). *POPACAULIS5* represses *PttHB8* expression, while in contrast *PttHB8* promotes expression of *POPACAULIS5* expression, suggesting that thermospermine levels and *PttHB8* expression are balanced by feedback control (Milhinhos et al., 2013). Conifers and other gymnosperms also display extensive secondary development, and also here HD-ZIP III transcripts are associated with secondary xylem (Côté et al., 2010; Duval et al., 2014). However, in conifers the xylem tissues contain only tracheids, while vessels and fibres are missing. Potentially reflecting this, conifers have relatively few NAC-domain-containing VND-homologues, while this gene family has expanded considerably in angiosperms (Nystedt et al., 2013). A recent study employing *Agrobacterium* mediated transformation of embryonic spruce cells to test for promoter-transcription factor interactions in planta in a semi-high throughput manner found evidence for the regulation of multiple genes regulating secondary cell wall formation by a NAC-domain transcription factor (Duval et al., 2014), including interaction with a homologue to the angiosperm *HD-ZIPIII* genes from *Picea glauca*. However, the NAC-domain transcription factor most closely related to the VNDs, which also displayed expression during secondary growth, did not show interaction with the HD-ZIP promoters tested. Thus, despite the ca 300 million years of separate evolution molecular circuits connecting HD-ZIP III's and NACs may be at least partially conserved. It will be interesting to learn if the HD-ZIP III's are also important for conifer tracheid formation.

9. Perspectives and outlook

While clearly a considerable amount is now known about the roles that HD-ZIP III's play in multiple aspects of vascular development, there are still a number of unanswered questions,

660 in particular pertaining to the apparently very complex loops of regulation these factors act
661 in. Omics based methods such as transcriptome analyses, Chip-seq, together with high
662 throughput interaction screening using YIH, have revealed a complex transcriptional network
663 around these factors. Furthermore, despite the apparent redundancy these five factors display
664 in certain genetic analyses, they act sometimes antagonistically, and the molecular basis for
665 this will likely continue to be revealed by large scale approaches. However, it is conceivable,
666 or even likely, that different cellular, tissue and organ contexts provide opportunities for
667 different positions in molecular networks of the five family members. Therefore,
668 improvements in techniques for cellular and tissue resolution of large scale omics assays, in
669 methods for determining molecular interactions, and in modelling of both networks and
670 development, are promising. To complicate the image further the HD-ZIP III's are, as
671 mentioned, also regulated post-transcriptionally by miRNA providing additional levels of
672 complexity. In addition, HD-ZIP III protein activity is most likely closely regulated as well;
673 the presence of the highly conserved START domain strongly suggests interactions with an
674 as of yet unidentified ligand. Furthermore, the C-terminus is occupied by a conserved
675 domain, the MEHKLA domain, displaying similarity to Per Arnt Sim (PAS)-domains known
676 to sense light, redox or other stimuli (Mukherjee and Burglin, 2006). Thus far its function is
677 not clear: the MEHKLA domain has been shown to be a site for protein-protein interactions
678 (Chandler et al., 2007), alternative folding of this domain regulates REV activity (Magnani
679 and Barton, 2011) and a point mutation in the MEHKLA domain of the *hoc* allele of *CNA*
680 confer high regeneration competence, even in the absence of hormones (Duclercq et al.,
681 2011). Intriguingly, whereas the MEHKLA domain might be redox sensitive, DNA binding
682 of HD-ZIP III's can also be redox regulated (Comelli and Gonzalez, 2007; Xie et al., 2014).
683 Considering that HD-ZIP III transcription factors appear active in the plant vasculature after
684 its development programme is complete, it is tempting to speculate that these factors not only
685 regulate the development of the vascular tissues, but also contribute to the function of the
686 vasculature as an information highway, perhaps by transmitting information from one part of
687 the plant to another.
688
689 The HD-ZIP III-miR165/166 regulon is highly conserved, and found not only in vascular
690 plants but in all land plants, including mosses and liverworts (Floyd and Bowman, 2006;
691 Floyd et al., 2006; Prigge and Clark, 2006). Strikingly, a *HD-ZIP III* from the moss (i.e.
692 prevascular) species *Physcomitrella patens* regulates moss leaf development, including the
693 conducting tissues, and partially suppresses the *Arabidopsis rev* phenotype (Prigge and Clark,

2006; Yip et al., 2016). In early vascular plants, lycophytes and ferns, *HD-ZIP III*'s are associated with leaf development and procambium (Floyd and Bowman, 2006; Vasco et al., 2016). It is conceivable that the HD-ZIP III-miR165/166 regulon evolved from an ancestral function in leaf patterning and growth to also govern vascular differentiation with secondary cell walls. Analyses of the molecular networks in which the moss and liverwort *HD-ZIP III* homologues act will likely contribute not only to our understanding of vascular plant evolution, but perhaps also to the function of the famous five in the complex processes of patterning and differentiation of vascular tissues in Arabidopsis, and other vascular plants.

Acknowledgements

This work was supported by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS; 2013-953) to A.C., and an EU-FP7 Marie Sklodowska-Curie fellowship to J.P.E.

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Figure legends

Figure 1. Vascular tissue formation within radialised leaves.
Separation of incipient leaf primordium (I_1) from apical meristem by cut '*x*' **(A)** leads to loss of adaxial-abaxial leaf asymmetry **(B)** and amphicribal vascular tissue **(C)** with phloem surrounding xylem in *Epilobium*. Cut '*y*' **(A)** represents the separation between meristem and initiating leaf performed by Sussex (1955) with similar results. P_1, P_2 and P_3 denote leaf primordia formed by the meristem prior to the cut. *phan* mutant from *Antirrhinum* **(E)** with radialised vascular tissue similar to that described in **(C)**, compared with that of a wild-type *Antirrhinum* leaf which demonstrates adaxial-abaxial asymmetry **(D)**. Phenotype of *phb-1d* mutant with radially symmetric trumpet-shaped leaves **(G, I)** with amphivasal vascular tissue compared to wild type plants **(F, H)**, in which xylem is restricted to the adaxial domain and phloem to the abaxial. **(H, I)** Toluidine blue stained cross sections of leaf petioles. Scale bars are 50 μm **(D)**, 5 mm **(F, G)** and 20 μm **(H, I)**. x, p, pa and ve are xylem, phloem, parenchyma, and ventral epidermis, respectively. **(A-C)** Reproduced from Snow & Snow (1959), with permission. **(D-E)** Reproduced from Waites & Hudson (1995), with permission. **(F-I)** Reproduced from McConnell & Barton (1995), with permission.

Figure 2. HD-ZIP III transcription factors in the formation of leaf vasculature.
HD-ZIP III members lie at the core of a signalling network that patterns and determines xylem identity in the adaxial domain of the leaf. The cartoon shows a cross section through a leaf vascular strand, with the network overlayed. The activity domains of the various factors are approximately indicated. Black arrows indicate positive and red blocked arrows negative interactions.

Figure 3. Dominant *HD-ZIP III* alleles discussed in this review.
(A) HD-ZIP III domain structure, with miRNA complementary site marked. Protein (upper) and nucleotide (lower) sequences from the different *HD-ZIP III* alleles are shown below. **(B-D)** Toluidine blue stained cross sections of vascular bundles from the inflorescence stems of wild type **(B)** which has xylem to the centre of the stem and phloem towards the outside, compared to that of *rev-10d* **(C)** where xylem surrounds the phloem. In plants expressing a version of *REV* harbouring silent point mutations in the miRNA target site (**D**; *rev-δmiRNA*) some vascular bundles (lower right in **D**) demonstrate similar phenotypes to *rev-10d* **(C)**,

with xylem surrounding phloem. ph is phloem, xy is xylem, arrowheads point to xylem cells. **(B-D)** Reproduced from Emery et al. (2003) with permission.

Figure 4. Root vascular patterning is mediated by cell-to-cell movement of miR165/166 and interactions with auxin and cytokinin signalling.

(A) Levels of HD-ZIP III transcription factors determine xylem cell type: In wild type (WT), central image, protoxylem (yellow arrowhead) form at the periphery of the xylem axis, and metaxylem (blue arrowhead) at the centre. In *phb-7d,* left image, ectopic metaxylem form in peripheral positions, while in the *athb8 cna phb phv* mutant all xylem differentiate as protoxylem. The confocal images display lignified xylem cells stained with basic fuchsin. **(B)** HD-ZIP III (primarily PHB) activity is focused to the central, metaxylem, domain of the stele, through SHR and SCR mediated production of miR165/166 in the endodermis and their subsequent movement into the stele. Solid arrow indicate direct activation, dashed arrow indicate cell-to-cell molecular movement. **(C) C**artoon displaying a cross section of the central part of the Arabidopsis root, a few cells shootward of the vascular stem cells within the root apical meristem. The endodermis (pink) surrounds the stele with its pericycle (green), procambium (grey) and central xylem axis with protoxylem (orange) and metaxylem (blue) precursor cells. Overlayed is a network of interactions between the HD-ZIP III transcription factors and auxin and cytokinin signalling at multiple levels, as described in the text. The activity domains of the various factors are approximately indicated. Black arrows indicate positive and red blocked arrows negative interactions.

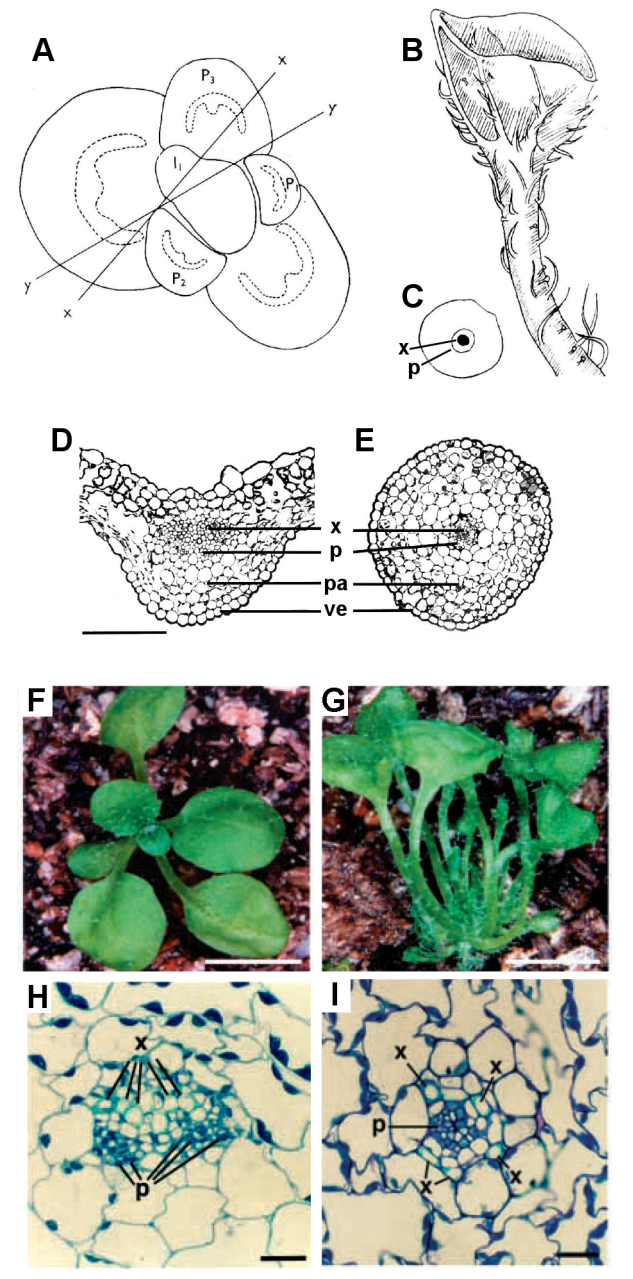
Figure 5. Xylem phenotypes of *rev-6* mutants in inflorescence stem.

Transverse sections through inflorescence stem tissue of 5 weeks old wild type (WT) plants **(A)** and *rev-6* **(B)**. Phloroglucinol stains lignin, and is reduced in hand sections of *rev-6* compared to wild type (pink staining on left hand side panels). Toluidine blue stained sections with close-ups of the vascular bundles (right hand side panels). Xylem fibres that lack secondary cell walls are present in *rev* (**B**; arrowheads), but all fibres in wild type **(A)** have thick secondary cell walls. X indicates xylem, ph indicates phloem. Scale bars are 50 μm.

Figure 6. HD-ZIP III regulation of xylem specification and differentiation in stem.

Co-action of the HD-ZIP III and hormonal (BR and auxin) signalling networks ensures maintenance of a balance between the procambial domain and the differentiating xylem

domain. The cartoon displays a cross section of a vascular bundle of the stem. Black arrows indicate positive and red blocked arrows negative interactions.


A
x
y
P3
I1
P1
P2
y
x
B
C
x
p
D
E
x
p
pa
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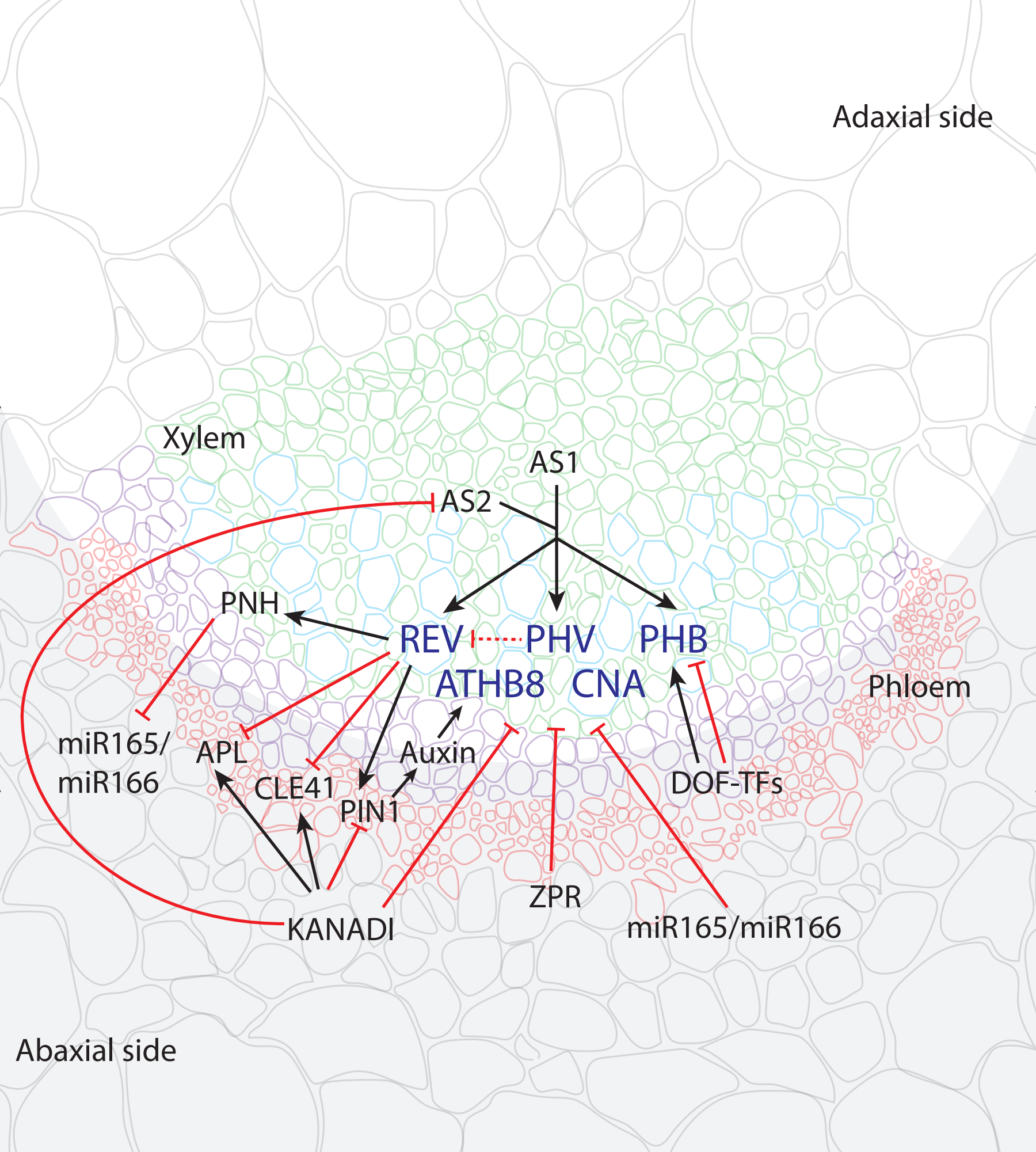
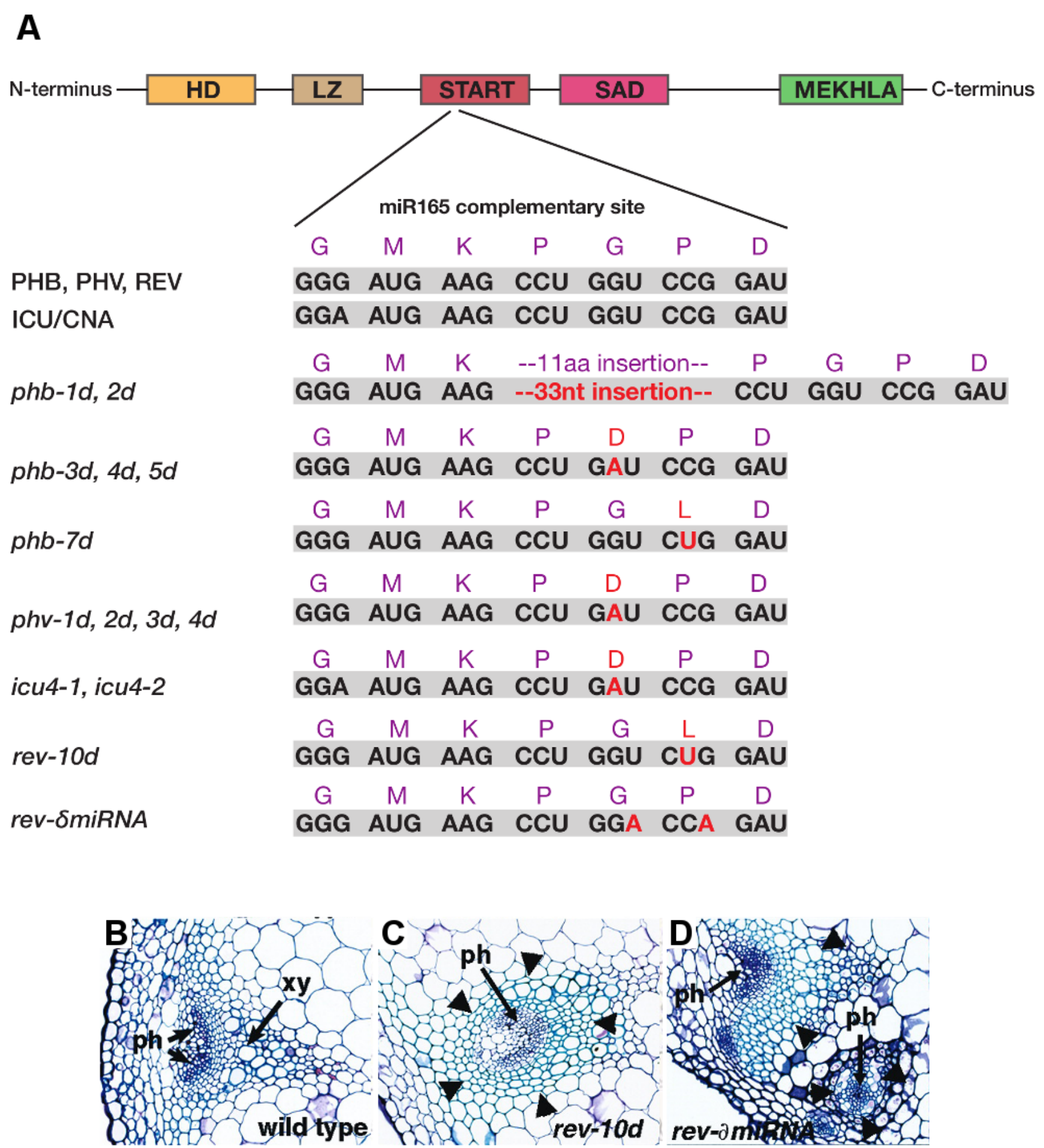
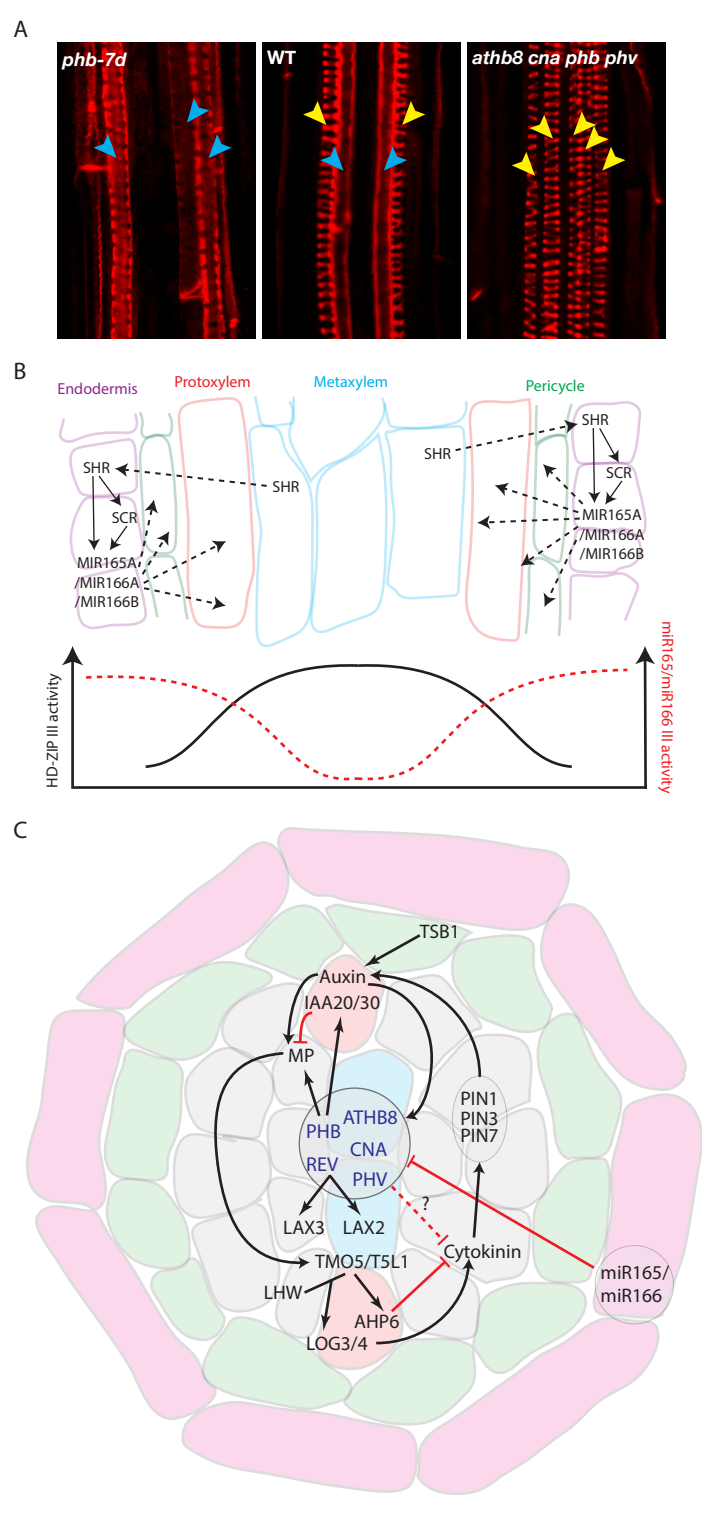
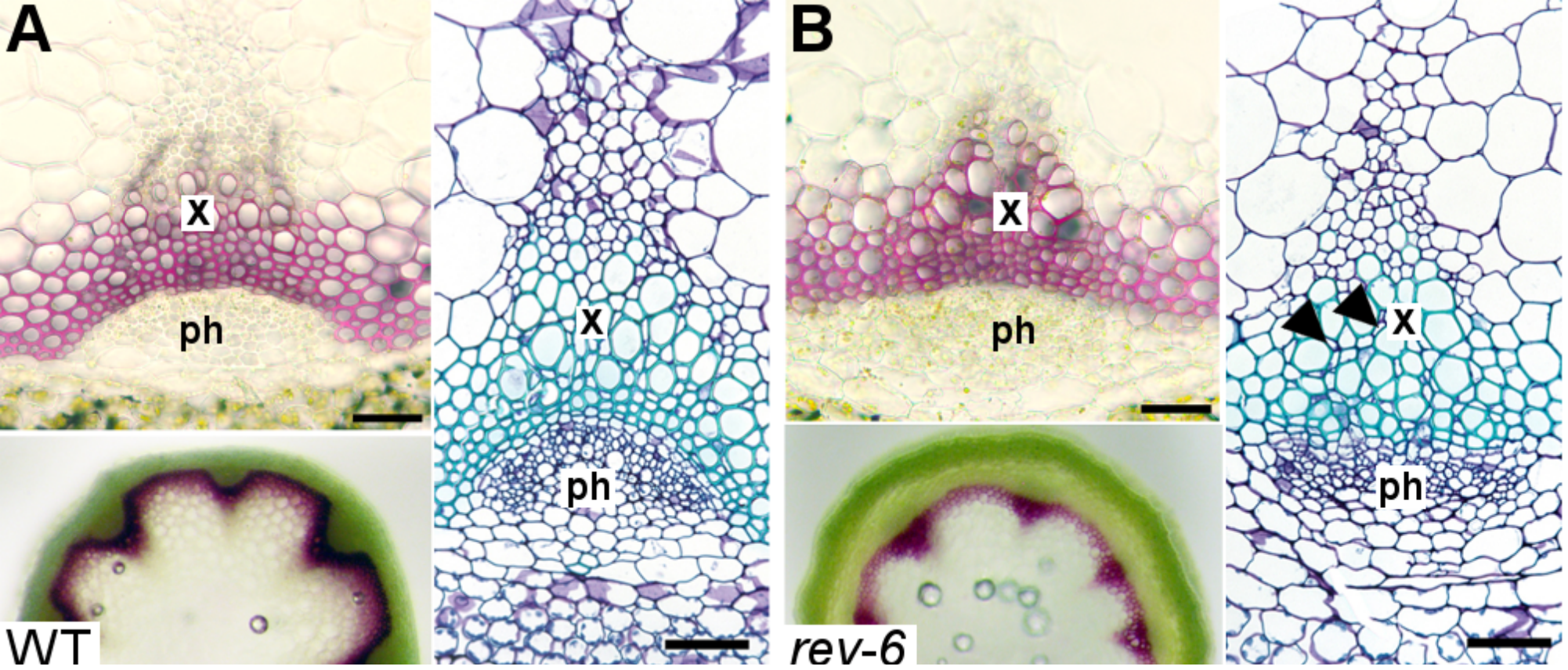
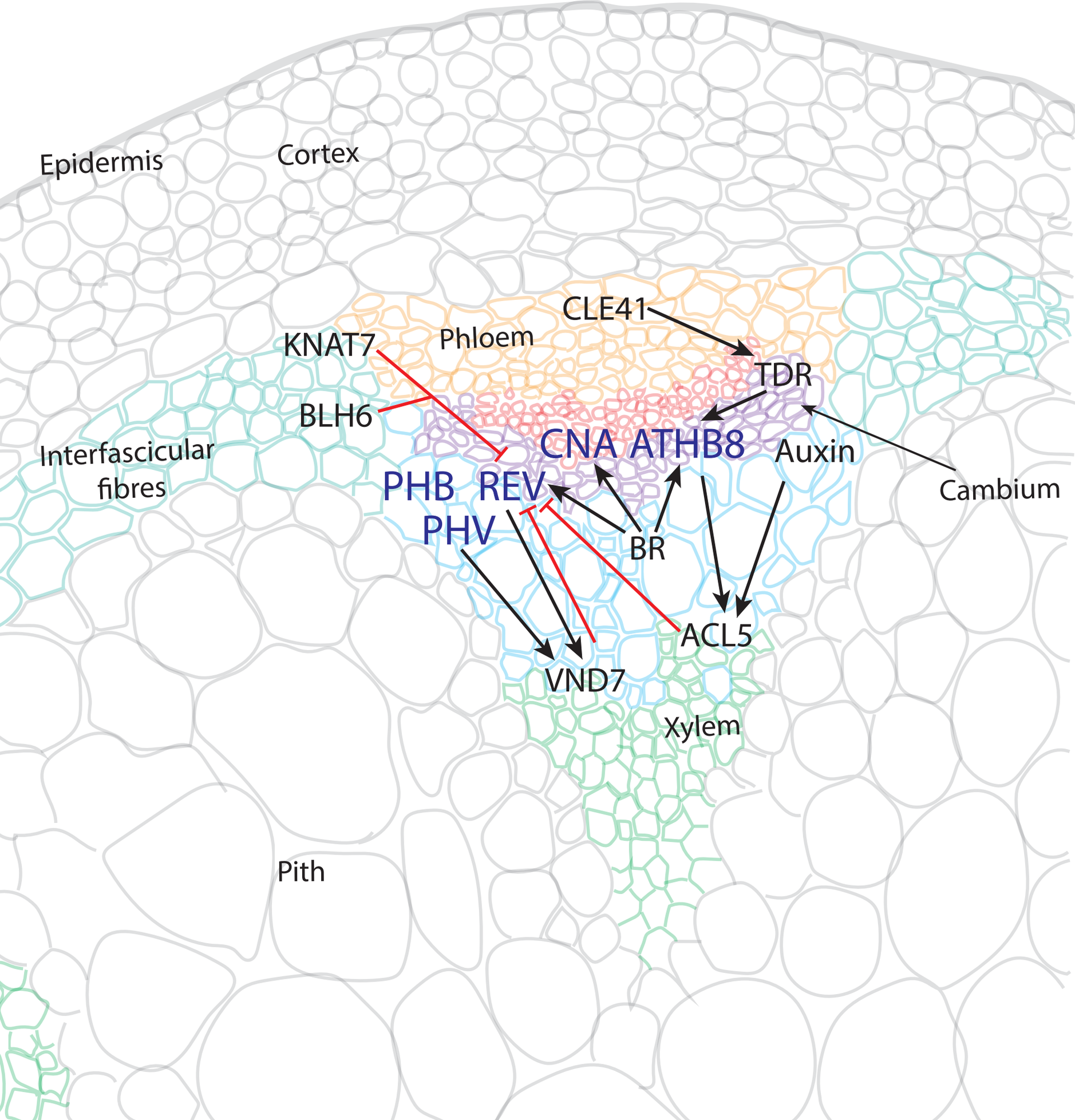
F
G
H
x
p
I
x
x
p
x
x


Adaxial side
Xylem
AS1
AS2
PNH
REV
PHV
PHB
ATHB8
CNA
Phloem
miR165/
miR166
APL
CLE41
PIN1
Auxin
DOF-TFs
ZPR
KANADI
miR165/miR166
Abaxial side


A
N-terminus
HD
LZ
START
SAD
MEKHLA
C-terminus
miR165 complementary site
G M K P G P D
PHB, PHV, REV
GGG AUG AAG CCU GGU CCG GAU
ICU/CNA
GGA AUG AAG CCU GGU CCG GAU
G M K --11aa insertion-- P G P D
phb-1d, 2d
GGG AUG AAG --33nt insertion-- CCU GGU CCG GAU
G M K P D P D
phb-3d, 4d, 5d
GGG AUG AAG CCU GAU CCG GAU
G M K P G L D
phb-7d
GGG AUG AAG CCU GGU CUG GAU
G M K P D P D
phv-1d, 2d, 3d, 4d
GGG AUG AAG CCU GAU CCG GAU
G M K P D P D
icu4-1, icu4-2
GGA AUG AAG CCU GAU CCG GAU
G M K P G L D
rev-10d
GGG AUG AAG CCU GGU CUG GAU
G M K P G P D
rev-δmiRNA
GGG AUG AAG CCU GGA CCA GAU
B
xy
ph
wild type
C
ph
rev-10d
D
ph
ph
rev-∂miRNA


A
phb-7d
WT
athb8 cna phb phv
B
Endodermis
Protoxylem
Metaxylem
Pericycle
SHR
SCR
MIR165A
/MIR166A
/MIR166B
HD-ZIP III activity
miR165/miR166 III activity
C
TSB1
Auxin
IAA20/30
MP
PHB
ATHB8
CNA
REV
PHV
PIN1
PIN3
PIN7
?
LAX3
LAX2
Cytokinin
TMO5/T5L1
LHW
AHP6
LOG3/4
miR165/
miR166


A
x
ph
x
ph
B
x
ph
x
ph
WT
rev-6


Epidermis
Cortex
Phloem
KNAT7
BLH6
CLE41
TDR
CNA ATHB8
Auxin
Cambium
Interfascicular
fibres
PHB REV
PHV
BR
ACL5
VND7
Xylem
Pith