

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

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

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24 **Highlight**

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

28

29 **Abstract**

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

46

47 **Key words**

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

50

51

52 **Introduction**

53 Homeodomain transcription factors have been synonymous with regulation of development
54 since their identification in patterning of the fly more than 30 years ago. In plants, members
55 of the class III homeodomain-leucine zipper (HD-ZIP III) transcription factor family are an
56 excellent example of the incredibly broad range of developmental processes that HD
57 transcription factors regulate. HD-ZIP III's act from cradle to grave, with roles in patterning
58 of the embryo, meristem maintenance, leaf development, inflorescence architecture, ovule
59 development, growth response to environmental signals, and senescence. Characterization of
60 mutations in *REVOLUTA*, one of five *HD-ZIP III* genes present in the model plant
61 *Arabidopsis thaliana*, represents the first description of the consequences of loss of HD-ZIP
62 III function (Talbert et al., 1995). While this paper is notable for its description of the
63 pleiotropic defects present in *HD-ZIP III* mutants, clues begin to emerge as to their
64 importance in controlling vascular development. In particular, Talbert *et al* (1995) noted that
65 there were changes to the numbers of xylem and phloem cells in *rev* mutants compared to
66 wild type plants, accompanied by changes to fibre differentiation. In subsequent years our
67 understanding of the role of *REV*, which is also known as *INTERFASCICULAR FIBRELESS1*
68 or *AMPHIVASAL VASCULAR BUNDLES1*, and the other members of the HD-ZIP III family
69 in *Arabidopsis*, *Arabidopsis thaliana HOMEobox8 (ATHB8)*, *PHABULOSA*
70 (*PHB*)/*ATHB14*, *PHAVOLUTA (PHV)/ATHB9*, and *CORONA*
71 (*CNA*)/*INCURVATA4/ATHB15*, has been considerably elaborated in multiple aspects of
72 vascular development. In this review we will describe in detail these roles in vascular
73 patterning and xylem differentiation in both the shoot and root.

74

75 **1. Radial patterning of vascular tissues in the shoot**

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

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

98
99 The first paper to place the observations of asymmetry-loss in a genetic context made use of
100 the snapdragon (*Antirrhinum phantastica* (*phan*) mutants, which had radialised leaves
101 similar to those observed in the surgical experiments (Figure 1D-E). *Phan* was described as a
102 “dorsalising factor”, i.e. a gene that specifies the upper (and therefore xylem) side of the leaf
103 (Waites and Hudson, 1995). *Phan* encodes a myb transcription factor (Waites et al., 1998),
104 and its Arabidopsis orthologue *ASYMMETRIC LEAVES 1* (*ASI*) (Byrne et al., 2000) was
105 subsequently shown to act as a positive regulator of the expression of *PHB*, *PHV* and *REV*
106 (Fu et al., 2007)(Figure 2). Such observations were consistent with phenotypes of dominant
107 gain-of-function *phb-1d* (Figure 1F-I) and *phv-1d* alleles, which had earlier been described as
108 having amphivasal vascular bundles with xylem surrounding phloem, i.e. xylem present in
109 both adaxial and abaxial positions and therefore gain of adaxial identity (McConnell and
110 Barton, 1998; McConnell et al., 2001). This phenotype is opposite to that observed in loss-of-
111 function *phan*, which has amphicribal bundles where phloem surrounds xylem (thus
112 demonstrating a loss of adaxial identity) (Waites and Hudson, 1995). Cloning of the gain-of-
113 function *phb* and *phv* alleles enabled comparisons of sequences with previously described
114 genes. Similarities were found with *ATHB8*, an early marker of vascularisation (Baima et al.,
115 1995), and with *REV*. While dominant *phb-1d* and *phv-1d* alleles demonstrated the most
116 dramatic loss of asymmetry due to the presence of xylem in positions where phloem might be
117 expected to form (Figure 1I), loss of function alleles demonstrated only subtle, if any,
118 aberrations as single mutants. However, multiple *HD-ZIP III* knockouts resulted in
119 phenotypes converse to those observed in the dominant alleles, i.e. phloem present in

120 positions where xylem forms in wild type (Emery et al., 2003; Prigge et al., 2005). The
121 influence of the five *HD-ZIP III* genes on asymmetry determination is not equal. The
122 phylogenetically relatively closely related *PHB*, *PHV* and *REV* clearly play predominant
123 roles, but their paralogous couple *ATHB8* and *CNA* may also contribute to the radial
124 patterning process as *ATHB8* over-expression leads to an increase in the formation of xylem
125 tissue (Baima et al., 2001), and the dominant *icu4* alleles of *CNA* display some characteristics
126 of plants with changes to adaxial-abaxial asymmetry (Ochando et al., 2008; Ochando et al.,
127 2006). All five *HD-ZIP III* genes therefore, to a greater or lesser extent promote adaxial (and
128 therefore xylem) identity within the leaf. Both gain-of-function and loss-of-function *HD-ZIP*
129 *III* mutants also demonstrate radial patterning defects in the stem with dominant alleles
130 characterised by xylem surrounding phloem, and recessive alleles by phloem surrounding
131 xylem (Emery et al., 2003).

132

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

134 Following their initial identification, a mechanistic understanding of the nature of dominant
135 *HD-ZIP III* alleles was a matter of some speculation. Gain-of-function *HD-ZIP III* alleles
136 have mutations that disrupt a steroidogenic acute regulatory protein-related lipid transfer
137 (START) domain thought to be involved in hydrophobic ligand binding. This led to the
138 hypothesis that a change to the regulatory function of the START domain (e.g. changes to
139 putative ligand binding) may have occurred. However, following the discovery of RNA
140 interference and identification of components of the microRNA (miRNA) machinery, it
141 became apparent that mutations in the dominant alleles were also present in the sequence
142 complementary to miRNA's 165 and 166 (miR165/166; Figure 3A), suggesting that *HD-ZIP*
143 *III*'s are subject to post transcriptional gene silencing (Reinhart et al., 2002; Rhoades et al.,
144 2002). Consistent with this idea, transgenic plants engineered to have silent mutations
145 disrupting the miRNA target site in *PHB* or *REV* without resulting in protein sequence
146 changes displayed gain-of-function phenotypes (Emery et al., 2003; Mallory et al.,
147 2004)(Figure 3B-D). In experiments using wheat germ extract, it was demonstrated that wild
148 type *PHB* and *PHV* mRNA, but not that of the dominant mutants was subject to cleavage,
149 demonstrating that RNAi can negatively regulate *HD-ZIP III* transcript abundance (Mallory
150 et al., 2004; Tang et al., 2003), in line with the increased levels of *PHB* expression detected in
151 both adaxial and abaxial domains of *phb-1d* leaves (McConnell et al., 2001). Furthermore,
152 *HD-ZIP III* mRNA is expressed ectopically in RNAi machinery mutants, such as *argonaute1*
153 (*ago1*) (Kidner and Martienssen, 2005) or *serrate (se)* (Lobbes et al., 2006).

154

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

164

165 Focussing of miR166 to the abaxial side of the maize leaf is thought to be the result of the
166 action of trans-acting short-interfering RNAs (ta-siRNAs; for review see Chitwood *et al.*,
167 2007). Briefly, in contrast to conventional miRNA directed cleavage which results in the
168 degradation of the target mRNA (e.g. miR165/166 action on *HD-ZIP III* transcripts described
169 above), cleavage of a non-coding *TAS* RNA enables it to become a target for RNA-dependent
170 RNA polymerases. The resulting double stranded RNA is subject to further processing from
171 which 21 bp ta-siRNA's are generated. ta-siRNA's guide cleavage of mRNA targets in a
172 similar manner to miRNA's. ta-siRNA's are derived from miRNA action on non-coding *TAS*
173 transcripts. In Arabidopsis, ta-siRNA's, derived from *TAS3* that has been subjected to
174 cleavage by miR390, negatively regulate *ETTIN (ETT)*, also known as *AUXIN RESPONSE*
175 *FACTOR3 (ARF3)* and *ARF4*, two genes that act redundantly in abaxial leaf identity
176 (Chitwood et al., 2007). In maize, *LEAFBLADELESS1 (LBL1)* encodes a zinc finger protein
177 required for the generation of ta-siRNA's, and in *lbl1* mutants, the localisation of miR166 is
178 no longer restricted to the abaxial domain of the initiating leaf primordium, but is expressed
179 throughout. *lbl* mutants demonstrate a clear loss of adaxial-abaxial asymmetry (Nogueira et
180 al., 2007), consistent with downstream changes to levels of *HD-ZIP III* transcript (Nogueira
181 et al., 2009). One possibility is that these small RNA's could act non-cell autonomously and
182 thus are candidates for the "Sussex signal", proposed in the early surgical experiments
183 described above that are involved in crosstalk between the shoot apical meristem and
184 initiating leaf primordium (Chitwood et al., 2007).

185

186 Disruption of the interactions between miRNA and mRNA target has provided particular
187 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-Teeple 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

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

231

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

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

245

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

256 (*PIN1*) that encodes an auxin efflux carrier (Ilegems et al., 2010). The flow of auxin through
257 preprovascular cells, as directed by PIN1, has been demonstrated to control the process of
258 leaf venation (Scarpella et al., 2006). Strikingly, auxin is thought to induce expression of *HD-*
259 *ZIP III*'s (Baima et al., 1995; Zhou et al., 2007). *HD ZIP III*'s, in turn promote developmental
260 changes that support canalisation of auxin as plants with reduced levels of HD-ZIP III
261 expression were impaired in cell maturation, demonstrating defects in xylem differentiation
262 and connection of cell files (Ilegems et al., 2010). Interestingly, data is now available that has
263 identified direct, often antagonistic, transcriptional targets of *REV* and *KAN1* (Huang et al.,
264 2014; Reinhart et al., 2013). Of particular note, genes including *ALTERED PHLOEM (APL)*,
265 required for phloem specification (Bonke et al., 2003), and *CLE41* which encodes a phloem-
266 expressed signal required for vascular proliferation (Etchells et al., 2015; Etchells and Turner,
267 2010), are negatively regulated by *REV* (Reinhart et al., 2013). In line with the antagonistic
268 interaction between KANADIs and HD-ZIP III's a direct repressive regulation of KAN1 on
269 *PHB* and *ATHB8* was found in one study (Merelo et al., 2013)(Figure 2).

270

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

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

289

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

313

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

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

326

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

339

340 **5. HD-ZIP III activity intersects with auxin and cytokinin signalling for proper xylem** 341 **patterning**

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

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

365

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

377

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

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

403

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

425

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

438

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

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

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

486

487 **7. HD-ZIP III regulated differentiation of xylem cells**

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

491 the independent isolation of *REV* loss-of-function alleles by Zhong and Ye (*ifl* alleles of *rev*)
492 (Zhong et al., 1997; Zhong and Ye, 1999) in screens that aimed to identify mutants with
493 xylem defects. Zhong and Ye noted that while vascular bundles in inflorescence stems of *rev*
494 mutants demonstrated few differences when compared to wild type close to the shoot apex, in
495 basal parts of the inflorescence stem, *rev* vascular bundles were characterised by fewer cells
496 (Figure 5). The xylem in inflorescence stem vascular bundles is typically constituted of two
497 cell types that have large secondary cell walls. Xylem vessels transport water, and smaller
498 xylary fibres provide mechanical support. While xylem vessels were present in *rev* mutants,
499 xylary fibres were reduced in weaker alleles, or absent in strong alleles (Zhong and Ye,
500 1999)(Figure 5). Outside the vascular bundles, a reduction in the number of interfascicular
501 fibres was also observed, and this loss of fibres in *rev* mutants results in large reductions in
502 breaking force (Zhong et al., 1997). It has been suggested that in fibres, the *rev* phenotype is
503 a result of failure to differentiate such that secondary cell wall material is not deposited,
504 rather than a failure in fibre specification (Lev-Yadun et al., 2004). The role that *REV* has in
505 specification of terminal xylem differentiation is influenced by *KNOTTED-LIKE*
506 *HOMEODOMAIN OF ARABIDOPSIS THALIANA 7* (*KNAT7*) and *BEL1-LIKE*
507 *HOMEODOMAIN 6* (*BLH6*) (Figure 6). These homeodomain transcription factors form a
508 heterodimer that binds to the promoter of, and negatively regulates, *REV* expression.
509 Consequently, *knat7 blh6* double mutants demonstrate large increases in *REV* expression that
510 are accompanied by increases in secondary cell wall thickness (Liu et al., 2014). These
511 results support the role of *REV* as a positive regulator of xylem cell wall deposition,
512 specifically in fibres and are consistent with observations that *rev* mutants have reduced
513 secondary walls in fibres. Rather surprisingly, in contrast to fibre walls that are thicker in
514 *knat7 blh6* lines (Liu et al., 2014), vessel secondary walls, and in particular those of *knat7*
515 mutants are thinner than those of wild type counterparts, such that the vessels collapse due to
516 a failure to withstand the negative pressures of water transport (Li et al., 2012). The *KNAT7-*
517 *BLH6-REV* interaction consequently does not appear to act in xylem vessels, at least not in
518 the same way that it regulates wall deposition in fibres. One explanation of this phenotype is
519 that *KNAT7/BLH6* acts independently from *REV* in vessel element differentiation.

520

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

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

544

545 The roles of *HD-ZIP III* genes in xylem specification and differentiation have been confirmed
546 by experiments that have looked for HD-ZIP III regulators and targets. The phytohormone
547 brassinosteroid (BR) has been implicated as a regulator of wide-ranging aspects of vascular
548 development, both in terms of regulation of number and position of vascular bundles (Caño-
549 Delgado et al., 2010), and xylem differentiation (Cano-Delgado et al., 2004; Yamamoto et al.,
550 2001) (Figure 6). In xylogenic cultures, levels of BR have been shown to dramatically
551 increase at a time point corresponding to entry into the final stages of xylem differentiation.
552 Rapid induction of *REV* homologue transcripts, *ZeHB11* and *ZeHB12*, occurs upon BR
553 treatment. In contrast, expression of the same transcripts is repressed upon treatment with
554 uniconazole, a BR inhibitor (Ohashi-Ito and Fukuda, 2003; Yamamoto et al., 2001;
555 Yamamoto et al., 2007). Intriguingly, the behaviour of the *ATHB8* orthologue *ZeHB10* is
556 similarly regulated, and while *ZeHB13*, a *CNA* orthologue, is not repressed upon perturbation
557 of BR signalling, its expression is also increased upon BR induction (Ohashi-Ito and Fukuda,
558 2003). This begs the question of how apparently opposing functions of *ATHB8/CNA* and

559 *PHB/PHV/REV* might be reconciled? The answer likely lies in the position that each gene
560 controls within a complex network. *ACAULIS5 (ACL5)* is a gene encoding a thermospermine
561 synthase, which has been shown to negatively regulate xylem differentiation. *acl5* mutants
562 are characterised by early terminal differentiation of xylem that results in programmed cell
563 death prior to xylem expansion and deposition of a full secondary cell wall (Muñiz et al.,
564 2008). *ATHB8* acts together with auxin as a direct positive regulator of *ACL5* which, in turn,
565 slows xylem differentiation, in part by negative regulation of *REV* (Baima et al., 2014).
566 Intriguingly, it was recently found that *ACL5* also activates proteins capable of counteracting
567 the cell-proliferation promoting effect of *TMO5/LHW* (Vera-Sirera et al., 2015) (Figure 4C).
568 *ATHB8* together with auxin, therefore, also regulates vascular cell divisions.

569

570 Consistent with the idea that *ATHB8* is a negative regulator of xylem differentiation, other
571 signalling pathways that act in the procambium to maintain the vascular meristem are thought
572 to positively influence *ATHB8* expression. TRACHEARY ELEMENT DIFFERENTIATION
573 INHIBITORY FACTOR (TDIF) and PHLOEM INTERCALATED WITH XYLEM
574 (PXY)/TDIF RECEPTOR (TDR) are a ligand-receptor pair that act both to maintain cell
575 division in, and exclude xylem differentiation from, the procambium. Seedlings grown in
576 liquid media containing TDIF ligand, and plants overexpressing *CLAVATA3/ESR-RELATED*
577 *41 (CLE41)*; a gene from which TDIF is derived), demonstrate increases in *ATHB8* expression
578 (Etchells and Turner, 2010; Hirakawa et al., 2008; Ito et al., 2006) (Figure 6), which supports
579 the idea that *ATHB8* acts to slow xylem differentiation.

580

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

591 However, expression of *ATHB8/CNA* and consequently *ACL5* expression is lowered further

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

594

595 The HD-ZIP III transcription factors lie at the centre of a network that is required to fine-tune
596 dynamic changes in gene expression throughout vascular development. High throughput YIH
597 screens have recently been used to place *PHB*, *PHV* and *REV* in a network that regulates
598 secondary cell wall deposition. Interactions within this network include both *VND7* and *PHV*
599 binding to the promoter of *REV*, hinting at complex regulatory mechanisms. In particular,
600 *VND7* was reported to negatively regulate *REV* expression. *REV*, in turn, binds to the
601 promoter and negatively regulates the expression of *PHENYLALANINE AMMONIA LYASE4*
602 (*PAL4*) (Taylor-Teeples et al., 2015), a gene involved in lignin biosynthesis (Sewalt et al.,
603 1997). However, as *REV* has previously been reported to positively regulate expression of
604 *VND7* (Endo et al., 2015), these results suggest that an understanding at cell-type specific
605 resolution is required to understand how these interactions control commitment to xylem
606 differentiation, fibre formation, and deposition of the secondary cell wall.

607

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

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

626 seasonal growth. In hybrid aspen, miR166 has been shown to be seasonally regulated, with a
627 large peak in expression in the winter months. Elevated winter miR166 coincides with
628 reductions in expression of *PtaHBI*, a *REV* orthologue (Ko et al., 2006), suggesting that
629 seasonal control of *REV*-directed wood development is at least in part via miR166 regulation.
630 It may be interesting to observe the roles that HD-ZIP III's and miRNA's might have in
631 patterning of plants with unusual cambial organisations for example those with included
632 phloem such as *Avicennia* and *Bougainvillea* (Studholme and Philipson, 1966; Zamski,
633 1979), or plants that develop phloem wedges, such as members of the *Bignoniaceae* (Pace et al.,
634 2009; Spicer and Groover, 2010). Aside from miRNA mediated regulation of HD-ZIP III's,
635 other regulatory interactions are likely to be conserved across plants with differing growth
636 habits. One such regulatory interaction is that between *PttHB8* (an *ATHB8* orthologue) and
637 poplar *ACL5* (*POPACAULIS5*). *POPACAULIS5* represses *PttHB8* expression, while in
638 contrast *PttHB8* promotes expression of *POPACAULIS5* expression, suggesting that
639 thermospermine levels and *PttHB8* expression are balanced by feedback control (Milhinhos
640 et al., 2013). Conifers and other gymnosperms also display extensive secondary development,
641 and also here HD-ZIP III transcripts are associated with secondary xylem (Côté et al., 2010;
642 Duval et al., 2014). However, in conifers the xylem tissues contain only tracheids, while
643 vessels and fibres are missing. Potentially reflecting this, conifers have relatively few NAC-
644 domain-containing VND-homologues, while this gene family has expanded considerably in
645 angiosperms (Nystedt et al., 2013). A recent study employing *Agrobacterium* mediated
646 transformation of embryonic spruce cells to test for promoter-transcription factor interactions
647 in planta in a semi-high throughput manner found evidence for the regulation of multiple
648 genes regulating secondary cell wall formation by a NAC-domain transcription factor (Duval
649 et al., 2014), including interaction with a homologue to the angiosperm *HD-ZIP III* genes
650 from *Picea glauca*. However, the NAC-domain transcription factor most closely related to
651 the VNDs, which also displayed expression during secondary growth, did not show
652 interaction with the HD-ZIP promoters tested. Thus, despite the ca 300 million years of
653 separate evolution molecular circuits connecting HD-ZIP III's and NACs may be at least
654 partially conserved. It will be interesting to learn if the HD-ZIP III's are also important for
655 conifer tracheid formation.

656

657 **9. Perspectives and outlook**

658 While clearly a considerable amount is now known about the roles that HD-ZIP III's play in
659 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,

694 2006; Yip et al., 2016). In early vascular plants, lycophytes and ferns, *HD-ZIP III*'s are
695 associated with leaf development and procambium (Floyd and Bowman, 2006; Vasco et al.,
696 2016). It is conceivable that the HD-ZIP III-miR165/166 regulon evolved from an ancestral
697 function in leaf patterning and growth to also govern vascular differentiation with secondary
698 cell walls. Analyses of the molecular networks in which the moss and liverwort *HD-ZIP III*
699 homologues act will likely contribute not only to our understanding of vascular plant
700 evolution, but perhaps also to the function of the famous five in the complex processes of
701 patterning and differentiation of vascular tissues in *Arabidopsis*, and other vascular plants.

702

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707

References

- Baima, S., Forte, V., Possenti, M., Peñalosa, A., Leoni, G., Salvi, S., Felici, B., Ruberti, I., and Morelli, G.** 2014. Negative feedback regulation of auxin signaling by ATHB8/ACL5-BUD2 transcription module. *Molecular plant* 7, 1006-1025.
- Baima, S., Nobili, F., Sessa, G., Lucchetti, S., Ruberti, I., and Morelli, G.** 1995. The expression of the Athb-8 homeobox gene is restricted to provascular cells in *Arabidopsis thaliana*. *Development* 121, 4171-4182.
- Baima, S., Possenti, M., Matteucci, A., Wisman, E., Altamura, M.M., Ruberti, I., and Morelli, G.** 2001. The *Arabidopsis* ATHB-8 HD-Zip Protein Acts as a Differentiation-Promoting Transcription Factor of the Vascular Meristems. *Plant Physiology* 126, 643-655.
- Bishopp, A., Help, H., El-Showk, S., Weijers, D., Scheres, B., Friml, J., Benková, E., Mähönen, A.P., and Helariutta, Y.** 2011. A mutually inhibitory interaction between auxin and cytokinin specifies vascular pattern in roots. *Current biology* 21, 917-926.
- Bonke, M., Thitamadee, S., Mahonen, A.P., Hauser, M.T., and Helariutta, Y.** 2003. APL regulates vascular tissue identity in *Arabidopsis*. *Nature* 426, 181-186.
- Brady, S.M., Zhang, L., Megraw, M., et al.** 2011. A stele-enriched gene regulatory network in the *Arabidopsis* root. *Molecular Systems Biology* 7, 459
- Byrne, M.E., Barley, R., Curtis, M., Arroyo, J.M., Dunham, M., Hudson, A., and Martienssen, R.A.** 2000. *Asymmetric leaves1* mediates leaf patterning and stem cell function in *Arabidopsis*. *Nature* 408, 967-971.
- Caño-Delgado, A., Lee, J.-Y., and Demura, T.** 2010. Regulatory Mechanisms for Specification and Patterning of Plant Vascular Tissues. *Annual Review of Cell and Developmental Biology* 26, 605-637.

Cano-Delgado, A., Yin, Y.H., Yu, C., Vafeados, D., Mora-Garcia, S., Cheng, J.C., Nam, K.H., Li, J.M., and Chory, J. 2004. BRL1 and BRL3 are novel brassinosteroid receptors that function in vascular differentiation in Arabidopsis. *Development* *131*, 5341-5351.

Carlsbecker, A., Lee, J.-Y., Roberts, C.J., et al. 2010. Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. *Nature* *465*, 316-321.

Chandler, J.W., Cole, M., Flier, A., Grewe, B., and Werr, W. 2007. The AP2 transcription factors DORNROSCHEN and DORNROSCHEN-LIKE redundantly control Arabidopsis embryo patterning via interaction with PHAVOLUTA. *Development* *134*, 1653-1662.

Chitwood, D.H., Guo, M., Nogueira, F.T.S., and Timmermans, M.C.P. 2007. Establishing leaf polarity: the role of small RNAs and positional signals in the shoot apex. *Development* *134*, 813-823.

Comelli, R.N., and Gonzalez, D.H. 2007. Conserved homeodomain cysteines confer redox sensitivity and influence the DNA binding properties of plant class III HD-Zip proteins. *Archives of Biochemistry and Biophysics* *467*, 41-47.

Côté, C.L., Boileau, F., Roy, V., Ouellet, M., Levasseur, C., Morency, M.-J., Cooke, J.E., Séguin, A., and MacKay, J.J. 2010. Gene family structure, expression and functional analysis of HD-Zip III genes in angiosperm and gymnosperm forest trees. *BMC Plant Biology* *10*, 1-17.

De Rybel, B., Adibi, M., Breda, A.S., et al. 2014. Plant development. Integration of growth and patterning during vascular tissue formation in Arabidopsis. *Science* *345*, 1255215-1255215.

De Rybel, B., Möller, B., Yoshida, S., Grabowicz, I., Barbier de Reuille, P., Boeren, S., Smith, R.S., Borst, J.W., and Weijers, D. 2013. A bHLH Complex Controls Embryonic Vascular Tissue Establishment and Indeterminate Growth in Arabidopsis. *Developmental Cell* *24*, 426-437.

- Donner, T.J., Sherr, I., and Scarpella, E.** 2009. Regulation of preprocambial cell state acquisition by auxin signaling in Arabidopsis leaves. *Development* 136, 3235-3246.
- Du, J., Miura, E., Robischon, M., Martinez, C., and Groover, A.** 2011. The *Populus* Class III HD ZIP Transcription Factor *POPCORONA* Affects Cell Differentiation during Secondary Growth of Woody Stems. *PLoS ONE* 6, e17458.
- Du, Q., Avci, U., Li, S., Gallego-Giraldo, L., Pattathil, S., Qi, L., Hahn, M.G., and Wang, H.** 2015. Activation of miR165b represses AtHB15 expression and induces pith secondary wall development in Arabidopsis. *The Plant Journal* 83, 388-400.
- Duclercq, J., Assoumou Ndong, Y.P., Guerineau, F., Sangwan, R.S., and Catterou, M.** 2011. Arabidopsis shoot organogenesis is enhanced by an amino acid change in the ATHB15 transcription factor. *Plant Biology* 13, 317-324.
- Duval, I., Lachance, D., Giguère, I., Bomal, C., Morency, M.-J., Pelletier, G., Boyle, B., MacKay, J.J., and Séguin, A.** 2014. Large-scale screening of transcription factor–promoter interactions in spruce reveals a transcriptional network involved in vascular development. *Journal of Experimental Botany* 65, 2319-2333.
- El-Showk, S., Help-Rinta-Rahko, H., Blomster, T., Siligato, R., Marée, A.F.M., Mähönen, A.P., and Grieneisen, V.A.** 2015. Parsimonious Model of Vascular Patterning Links Transverse Hormone Fluxes to Lateral Root Initiation: Auxin Leads the Way, while Cytokinin Levels Out. *PLoS Computational Biology* 11, e1004450.
- Emery, J.F., Floyd, S.K., Alvarez, J., Eshed, Y., Hawker, N.P., Izhaki, A., Baum, S.F., and Bowman, J.L.** 2003. Radial Patterning of *Arabidopsis* Shoots by Class III HD-ZIP and KANADI Genes. *Current Biology* 13, 1768-1774.
- Endo, H., Yamaguchi, M., Tamura, et al.** 2015. Multiple Classes of Transcription Factors Regulate the Expression of VASCULAR-RELATED NAC-DOMAIN7, a Master Switch of Xylem Vessel Differentiation. *Plant and Cell Physiology* 56, 242-254.

Eshed, Y., Izhaki, A., Baum, S.F., Floyd, S.K., and Bowman, J.L. 2004. Asymmetric leaf development and blade expansion in *Arabidopsis* are mediated by KANADI and YABBY activities. *Development* *131*, 2997-3006.

Etchells, J.P., Mishra, Laxmi S., Kumar, M., Campbell, L., and Turner, Simon R. 2015. Wood Formation in Trees Is Increased by Manipulating PXY-Regulated Cell Division. *Current Biology* *25*, 1050-1055.

Etchells, J.P., and Turner, S.R. 2010. The PXY-CLE41 receptor ligand pair defines a multifunctional pathway that controls the rate and orientation of vascular cell division. *Development* *137*, 767-774.

Floyd, S.K., and Bowman, J.L. 2006. Distinct developmental mechanisms reflect the independent origins of leaves in vascular plants. *Current Biology* *16*, 1911-1917.

Floyd, S.K., Zalewski, C.S., and Bowman, J.L. 2006. Evolution of class III homeodomain-leucine zipper genes in streptophytes. *Genetics* *173*, 373-388.

Fu, Y., Xu, L., Xu, B., Yang, L., Ling, Q., Wang, H., and Huang, H. 2007. Genetic Interactions Between Leaf Polarity-Controlling Genes and ASYMMETRIC LEAVES1 and 2 in *Arabidopsis* Leaf Patterning. *Plant and Cell Physiology* *48*, 724-735.

Gardiner, J., Sherr, I., and Scarpella, E. 2010. Expression of DOF genes identifies early stages of vascular development in *Arabidopsis* leaves. *The International journal of developmental biology* *54*, 1389-1396.

Guo, Y., Qin, G., Gu, H., and Qu, L.-J. 2009. Dof5.6/HCA2, a Dof Transcription Factor Gene, Regulates Interfascicular Cambium Formation and Vascular Tissue Development in *Arabidopsis*. *The Plant Cell* *21*, 3518-3534.

Helariutta, Y., Fukaki, H., Wysocka-Diller, J., Nakajima, K., Jung, J., Sena, G., Hauser, M.T., and Benfey, P.N. 2000. The SHORT-ROOT gene controls radial patterning of the *Arabidopsis* root through radial signaling. *Cell* *101*, 555-567.

Hirakawa, Y., Shinohara, H., Kondo, Y., Inoue, A., Nakanomyo, I., Ogawa, M., Sawa, S., Ohashi-Ito, K., Matsubayashi, Y., and Fukuda, H. 2008. Non-cell-autonomous control of vascular stem cell fate by a CLE peptide/receptor system. *Proceedings of the National Academy of Sciences, USA* *105*, 15208-15213.

Huang, T., Harrar, Y., Lin, C., Reinhart, B., Newell, N.R., Talavera-Rauh, F., Hokin, S.A., Barton, M.K., and Kerstetter, R.A. 2014. Arabidopsis KANADI1 acts as a transcriptional repressor by interacting with a specific cis-element and regulates auxin biosynthesis, transport, and signaling in opposition to HD-ZIPIII factors. *The Plant Cell* *26*, 246-262.

Husbands, A., Aggarwal, V., Ha, T., and Timmermans, M.C. 2016. In Planta Single-Molecule Pull-down SiMPull Reveals Tetrameric Stoichiometry of HD-ZIPIII:LITTLE ZIPPER Complexes. *The Plant Cell*, tpc.00289.02016.

Ilegems, M., Douet, V., Meylan-Bettex, M., Uyttewaal, M., Brand, L., Bowman, J.L., and Stieger, P.A. 2010. Interplay of auxin, KANADI and Class III HD-ZIP transcription factors in vascular tissue formation. *Development* *137*, 975-984.

Ito, Y., Nakanomyo, I., Motose, H., Iwamoto, K., Sawa, S., Dohmae, N., and Fukuda, H. 2006. Dodeca-CLE peptides as suppressors of plant stem cell differentiation. *Science* *313*, 842-845.

Izhaki, A., and Bowman, J.L. 2007. KANADI and Class III HD-Zip Gene Families Regulate Embryo Patterning and Modulate Auxin Flow during Embryogenesis in Arabidopsis. *The Plant Cell* *19*, 495-508.

Juarez, M.T., Kui, J.S., Thomas, J., Heller, B.A., and Timmermans, M.C.P. 2004a. microRNA-mediated repression of rolled leaf1 specifies maize leaf polarity. *Nature* *428*, 84-88.

Juarez, M.T., Twigg, R.W., and Timmermans, M.C.P. 2004b. Specification of adaxial cell fate during maize leaf development. *Development* *131*, 4533-4544.

- Kerstetter, R.A., Bollman, C., Bollman, K., Taylor, R.A., Bombles, K., and Poethig, S.R.** 2001. *KANADI* regulates organ polarity in *Arabidopsis*. *Nature* 411, 706-709.
- Kidner, C.A., and Martienssen, R.A.** 2005. The role of ARGONAUTE1 AGO1 in meristem formation and identity. *Developmental Biology* 280, 504-517.
- Kim, J., Jung, J.-H., Reyes, J.L., Kim, Y.-S., Kim, S.-Y., Chung, K.-S., Kim, J.A., Lee, M., Lee, Y., Narry Kim, V., et al.** 2005. microRNA-directed cleavage of ATHB15 mRNA regulates vascular development in *Arabidopsis* inflorescence stems. *The Plant Journal* 42, 84-94.
- Kim, Y.-S., Kim, S.-G., Lee, M., et al.** 2008. HD-ZIP III activity is modulated by competitive inhibitors via a feedback loop in *Arabidopsis* shoot apical meristem development. *The Plant Cell* 20, 920-933.
- Ko, J.H., Prassinis, C., and Han, K.H.** 2006. Developmental and seasonal expression of PtaHB1, a *Populus* gene encoding a class III HD-Zip protein, is closely associated with secondary growth and inversely correlated with the level of microRNA miR166. *New Phytologist* 169, 468-478.
- Kubo, M., Udagawa, M., Nishikubo, N., Horiguchi, G., Yamaguchi, M., Ito, J., Mimura, T., Fukuda, H., and Demura, T.** 2005. Transcription switches for protoxylem and metaxylem vessel formation. *Genes & Development* 19, 1855-1860.
- Le Hir, R., and Bellini, C.** 2013. The plant-specific Dof transcription factors family: new players involved in vascular system development and functioning in *Arabidopsis*. *Frontiers in Plant Science* 4, 10.3389/fpls.2013.00164.
- Lee, J.-Y., Colinas, J., Wang, J.Y., Mace, D., Ohler, U., and Benfey, P.N.** 2006. Transcriptional and posttranscriptional regulation of transcription factor expression in *Arabidopsis* roots. *Proceedings of the National Academy of Sciences, USA* 103, 6055-6060.

Lev-Yadun, S., Wyatt, S.E., and Flaishman, M.A. 2004. The Inflorescence Stem Fibers of *Arabidopsis thaliana* Revoluta (ifl1) Mutant. *Journal of Plant Growth Regulation* 23, 301-306.

Levesque, M.P., Vernoux, T., Busch, W., et al. 2006. Whole-genome analysis of the SHORT-ROOT developmental pathway in *Arabidopsis*. *PLoS Biology* 4, e143.

Li, E., Bhargava, A., Qiang, W., Friedmann, M.C., Forneris, N., Savidge, R.A., Johnson, L.A., Mansfield, S.D., Ellis, B.E., and Douglas, C.J. 2012. The Class II KNOX gene KNAT7 negatively regulates secondary wall formation in *Arabidopsis* and is functionally conserved in *Populus*. *New Phytologist* 194, 102-115.

Lin, W.C., Shuai, B., and Springer, P.S. 2003. The *Arabidopsis* LATERAL ORGAN BOUNDARIES-domain gene ASYMMETRIC LEAVES2 functions in the repression of KNOX gene expression and in adaxial-abaxial patterning. *The Plant Cell* 15, 2241-2252.

Liu, Q., Yao, X., Pi, L., Wang, H., Cui, X., and Huang, H. 2009. The ARGONAUTE10 gene modulates shoot apical meristem maintenance and establishment of leaf polarity by repressing miR165/166 in *Arabidopsis*. *The Plant Journal* 58, 27-40.

Liu, Y., You, S., Taylor-Teeples, M., Li, W.L., Schuetz, M., Brady, S.M., and Douglas, C.J. 2014. BEL1-LIKE HOMEODOMAIN6 and KNOTTED ARABIDOPSIS THALIANA7 Interact and Regulate Secondary Cell Wall Formation via Repression of REVOLUTA. *The Plant Cell* 26, 4843-4861.

Lobbes, D., Rallapalli, G., Schmidt, D.D., Martin, C., and Clarke, J. 2006. SERRATE: a new player on the plant microRNA scene. *EMBO Reports* 7, 1052-1058.

Lynn, K., Fernandez, A., Aida, M., Sedbrook, J., Tasaka, M., Masson, P., and Barton, M.K. 1999. The *PINHEAD/ZWILLE* gene acts pleiotropically in *Arabidopsis* development and has overlapping functions with the *ARGONAUTE1* gene. *Development* 126, 469-481.

- Magnani, E., and Barton, M.K.** 2011. A Per-ARNT-Sim-Like Sensor Domain Uniquely Regulates the Activity of the Homeodomain Leucine Zipper Transcription Factor REVOLUTA in Arabidopsis. *The Plant Cell* 23, 567-582.
- Mähönen, A.P., Bishopp, A., Higuchi, M., Nieminen, K.M., Kinoshita, K., Törmäkangas, K., Ikeda, Y., Oka, A., Kakimoto, T., and Helariutta, Y.** 2006. Cytokinin signaling and its inhibitor AHP6 regulate cell fate during vascular development. *Science* 311, 94-98.
- Mähönen, A.P., Bonke, M., Kauppinen, L., Riikonen, M., Benfey, P.N., and Helariutta, Y.** 2000. A novel two-component hybrid molecule regulates vascular morphogenesis of the Arabidopsis root. *Genes & Development* 14, 2938-2943.
- Mallory, A.C., Reinhart, B.J., Jones-Rhoades, M.W., Tang, G., Zamore, P.D., Barton, M.K., and Bartel, D.P.** 2004. MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5' region. *The EMBO Journal* 23, 3356-3364.
- Mattsson, J., Ckurshumova, W., and Berleth, T.** 2003. Auxin signaling in Arabidopsis leaf vascular development. *Plant Physiology* 131, 1327-1339.
- McConnell, J.R., and Barton, M.K.** 1998. Leaf polarity and meristem formation in Arabidopsis. *Development* 125, 2935-2942.
- McConnell, J.R., Emery, J., Eshed, Y., Bao, N., Bowman, J., and Barton, M.K.** 2001. Role of PHABULOSA and PHAVOLUTA in determining radial patterning in shoots. *Nature* 411, 709-713.
- Merelo, P., Xie, Y., Brand, L., Ott, F., Weigel, D., Bowman, J.L., Heisler, M.G., and Wenkel, S.** 2013. Genome-wide identification of KANADI1 target genes. *PLoS ONE* 8, e77341.
- Milhinhos, A., Prestele, J., Bollhöner, B., et al.** 2013. Thermospermine levels are controlled by an auxin-dependent feedback loop mechanism in Populus xylem. *The Plant Journal* 75, 685-698.

Miyashima, S., Koi, S., Hashimoto, T., and Nakajima, K. 2011. Non-cell-autonomous microRNA165 acts in a dose-dependent manner to regulate multiple differentiation status in the Arabidopsis root. *Development* *138*, 2303-2313.

Moussian, B., Schoof, H., Haecker, A., Jurgens, G., and Laux, T. 1998. Role of the *ZWILLE* gene in the regulation of central shoot meristem cell fate during *Arabidopsis* embryogenesis. *The EMBO Journal* *17*, 1799-1809.

Mukherjee, K., and Burglin, T.R. 2006. MEKHLA, a novel domain with similarity to PAS domains, is fused to plant homeodomain-leucine zipper III proteins. *Plant Physiology* *140*, 41142-41150.

Müller, C.J., Valdés, A.E., Wang, G., Ramachandran, P., Beste, L., Uddenberg, D., and Carlsbecker, A. 2016. PHABULOSA Mediates an Auxin Signaling Loop to Regulate Vascular Patterning in Arabidopsis. *Plant Physiology* *170*, 956-970.

Muñiz, L., Minguet, E.G., Singh, S.K., Pesquet, E., Vera-Sirera, F., Moreau-Courtois, C.L., Carbonell, J., Blázquez, M.A., and Tuominen, H. 2008. ACAULIS5 controls Arabidopsis xylem specification through the prevention of premature cell death. *Development* *135*, 2573-2582.

Muraro, D., Mellor, N., Pound, M.P., et al. 2014. Integration of hormonal signaling networks and mobile microRNAs is required for vascular patterning in Arabidopsis roots. *Proceedings of the National Academy of Sciences, USA* *111*, 857-862.

Nakajima, K., Sena, G., Nawy, T., and Benfey, P.N. 2001. Intercellular movement of the putative transcription factor SHR in root patterning. *Nature* *413*, 307-311.

Nogueira, F.T.S., Chitwood, D.H., Madi, S., Ohtsu, K., Schnable, P.S., Scanlon, M.J., and Timmermans, M.C.P. 2009. Regulation of Small RNA Accumulation in the Maize Shoot Apex. *PLoS Genetics* *5*, e1000320.

- Nogueira, F.T.S., Madi, S., Chitwood, D.H., Juarez, M.T., and Timmermans, M.C.P.** 2007. Two small regulatory RNAs establish opposing fates of a developmental axis. *Genes & Development* *21*, 750-755.
- Nystedt, B., Street, N.R., Wetterbom, A., et al.** 2013. The Norway spruce genome sequence and conifer genome evolution. *Nature* *497*, 579-584.
- Ochando, I., González-Reig, S., Ripoll, J.-J., Vera, A., and Martínez-Laborda, A.** 2008. Alteration of the shoot radial pattern in *Arabidopsis thaliana* by a gain-of-function allele of the class III HD-Zip gene *INCURVATA4*. *International Journal of Developmental Biology* *52*, 953-961.
- Ochando, I., Jover-Gil, S., Ripoll, J.J., Candela, H., Vera, A., Ponce, M.R., Martínez-Laborda, A., and Micol, J.L.** 2006. Mutations in the MicroRNA Complementarity Site of the *INCURVATA4* Gene Perturb Meristem Function and Adaxialize Lateral Organs in *Arabidopsis*. *Plant Physiology* *141*, 607-619.
- Ohashi-Ito, K., and Fukuda, H.** 2003. HD-Zip III Homeobox Genes that Include a Novel Member, *ZehB-13* *Zinnia*/*ATHB-15* *Arabidopsis*, are Involved in Procambium and Xylem Cell Differentiation. *Plant and Cell Physiology* *44*, 1350-1358.
- Ohashi-Ito, K., Matsukawa, M., and Fukuda, H.** 2013. An atypical bHLH transcription factor regulates early xylem development downstream of auxin. *Plant and Cell Physiology* *54*, 398-405.
- Ohashi-Ito, K., Saegusa, M., Iwamoto, K., Oda, Y., Katayama, H., Kojima, M., Sakakibara, H., and Fukuda, H.** 2014. A bHLH complex activates vascular cell division via cytokinin action in root apical meristem. *Current Biology* *24*, 2053-2058.
- Pace, M.R., Lohmann, L.G., and Angyalossy, V.** 2009. The rise and evolution of the cambial variant in *Bignoniaceae*. *Evolution & Development* *11*, 465-479.
- Porth, I., Klápště, J., McKown, A.D., et al.** 2014. Extensive Functional Pleiotropy of *REVOLUTA* Substantiated through Forward Genetics. *Plant Physiology* *164*, 548-554.

Prigge, M.J., and Clark, S.E. 2006. Evolution of the class III HD-Zip gene family in land plants. *Evolution & Development* 8, 350-361.

Prigge, M.J., Otsuga, D., Alonso, J.M., Ecker, J.R., Drews, G.N., and Clark, S.E. 2005. Class III homeodomain-leucine zipper gene family members have overlapping, antagonistic, and distinct roles in Arabidopsis development. *The Plant Cell* 17, 61-76.

Reinhart, B.J., Liu, T., Newell, N.R., Magnani, E., Huang, T., Kerstetter, R., Michaels, S., and Barton, M.K. 2013. Establishing a Framework for the Ad/Abaxial Regulatory Network of Arabidopsis: Ascertaining Targets of Class III HOMEODOMAIN LEUCINE ZIPPER and KANADI Regulation. *The Plant Cell* 25, 3228-3249.

Reinhart, B.J., Weinstein, E.G., Rhoades, M.W., Bartel, B., and Bartel, D.P. 2002. MicroRNAs in plants. *Genes & Development* 16, 1616-1626.

Rhoades, M.W., Reinhart, B.J., Lim, L.P., Burge, C.B., Bartel, B., and Bartel, D.P. 2002. Prediction of Plant MicroRNA Targets. *Cell* 110, 513-520.

Robischon, M., Du, J., Miura, E., and Groover, A. 2011. The Populus Class III HD ZIP, popREVOLUTA, Influences Cambium Initiation and Patterning of Woody Stems. *Plant Physiology* 155, 1214-1225.

Sato, A., and Yamamoto, K.T. 2008. Overexpression of the non-canonical Aux/IAA genes causes auxin-related aberrant phenotypes in Arabidopsis. *Physiologia Plantarum* 133, 397-405.

Scarpella, E., Marcos, D., Friml, J., and Berleth, T. 2006. Control of leaf vascular patterning by polar auxin transport. *Genes & Development* 20, 1015-1027.

Scheres, B., Di Lorenzo, L., Willemsen, V., Hauser, M.T., Janmaat, K., Weisbeek, P., and Benfey, P.N. 1995. Mutations affecting the radial organisation of the Arabidopsis root display specific defects throughout the embryonic axis. *Development* 121, 53-62.

- Sebastian, J., Ryu, K.H., Zhou, J., Tarkowská, D., Tarkowski, P., Cho, Y.-H., Yoo, S.-D., Kim, E.-S., and Lee, J.-Y.** 2015. PHABULOSA controls the quiescent center-independent root meristem activities in *Arabidopsis thaliana*. *PLoS Genetics* *11*, e1004973.
- Semiarti, E., Ueno, Y., Tsukaya, H., Iwakawa, H., Machida, C., and Machida, Y.** 2001. The *ASYMMETRIC LEAVES2* gene of *Arabidopsis thaliana* regulates formation of a symmetric lamina, establishment of venation and repression of meristem-related homeobox genes in leaves. *Development* *128*, 1771-1783.
- Sewalt, V., Ni, W., Blount, J.W., Jung, H.G., Masoud, S.A., Howles, P.A., Lamb, C., and Dixon, R.A.** 1997. Reduced Lignin Content and Altered Lignin Composition in Transgenic Tobacco Down-Regulated in Expression of L-Phenylalanine Ammonia-Lyase or Cinnamate 4-Hydroxylase. *Plant Physiology* *115*, 41-50.
- Smith, Z.R., and Long, J.A.** 2010. Control of *Arabidopsis* apical-basal embryo polarity by antagonistic transcription factors. *Nature* *464*, 423-426.
- Snow, M., and Snow, R.** 1959. THE DORSIVENTRALITY OF LEAF PRIMORDIA. *New Phytologist* *58*, 188-207.
- Spicer, R., and Groover, A.** 2010. Evolution of development of vascular cambia and secondary growth. *New Phytologist* *186*, 577-592.
- Studholme, W.P., and Philipson, W.R.** 1966. Woods with included phloem: Heimer *Llödendron Brunonianum* and *Avicennia Resinifera*. *New Zealand Journal of Botany* *4*, 355-365.
- Sussex, I.M.** 1954. Experiments on the cause of dorsiventrality in leaves. *Nature* *174*, 351-352.
- Sussex, I.M.** 1955. Experimental investigation of leaf dorsiventrality and orientation in the juvenile shoot. *Phytomorphology* *5*, 286-300.

- Talbert, P.B., Adler, H.T., Parks, D.W., and Comai, L.** 1995. The REVOLUTA gene is necessary for apical meristem development and for limiting cell divisions in the leaves and stems of *Arabidopsis thaliana*. *Development* *121*, 2723-2735.
- Tang, G., Reinhart, B.J., Bartel, D.P., and Zamore, P.D.** 2003. A biochemical framework for RNA silencing in plants. *Genes & Development* *17*, 49-63.
- Taylor-Teeple, M., Lin, L., de Lucas, M., et al.** 2015. An *Arabidopsis* gene regulatory network for secondary cell wall synthesis. *Nature* *517*, 571-575.
- Tucker, M.R., Hinze, A., Tucker, E.J., Takada, S., Jürgens, G., and Laux, T.** 2008. Vascular signalling mediated by ZWILLE potentiates WUSCHEL function during shoot meristem stem cell development in the *Arabidopsis* embryo. *Development* *135*, 2839-2843.
- Turchi, L., Baima, S., Morelli, G., and Ruberti, I.** 2015. Interplay of HD-Zip II and III transcription factors in auxin-regulated plant development. *Journal of Experimental Botany*, *66*, 5043-5053.
- Ursache, R., Miyashima, S., Chen, Q., Vatén, A., Nakajima, K., Carlsbecker, A., Zhao, Y., Helariutta, Y., and Dettmer, J.** 2014. Tryptophan-dependent auxin biosynthesis is required for HD-ZIP III-mediated xylem patterning. *Development* *141*, 1250-1259.
- Vasco, A., Smalls, T.L., Graham, S.W., Cooper, E.D., Wong, G.K.-S., Stevenson, D.W., Moran, R.C., and Ambrose, B.A.** 2016. Challenging the paradigms of leaf evolution: Class III HD-Zips in ferns and lycophytes. *New Phytologist*, DOI: 10.1111/nph.14075.
- Vatén, A., Dettmer, J., Wu, S., et al.** 2011. Callose biosynthesis regulates symplastic trafficking during root development. *Developmental Cell* *21*, 1144-1155.
- Vera-Sirera, F., De Rybel, B., Úrbez, C., et al.** 2015. A bHLH-Based Feedback Loop Restricts Vascular Cell Proliferation in Plants. *Developmental Cell* *35*, 432-443.
- Waites, R., and Hudson, A.** 1995. *phantastica*: a gene required for dorsoventrality of leaves in *Antirrhinum majus*. *Development* *121*, 2143-2154.

- Waites, R., Selvadurai, H.R.N., Oliver, I.R., and Hudson, A.** 1998. The *PHANTASTICA* gene encodes a MYB transcription factor involved in growth and dorsoventrality of lateral organs in *Antirrhinum*. *Cell* *93*, 779-789.
- Wenkel, S., Emery, J., Hou, B.-H., Evans, M.M.S., and Barton, M.K.** 2007. A Feedback Regulatory Module Formed by LITTLE ZIPPER and HD-ZIPIII Genes. *The Plant Cell* *19*, 3379-3390.
- Williams, L., Grigg, S.P., Xie, M., Christensen, S., and Fletcher, J.C.** 2005. Regulation of Arabidopsis shoot apical meristem and lateral organ formation by microRNA miR166g and its AtHD-ZIP target genes. *Development* *132*, 3657-3668.
- Wu, G., Lin, W.-c., Huang, T., Poethig, R.S., Springer, P.S., and Kerstetter, R.A.** 2008. KANADI1 regulates adaxial–abaxial polarity in Arabidopsis by directly repressing the transcription of ASYMMETRIC LEAVES2. *Proceedings of the National Academy of Sciences, USA* *105*, 16392-16397.
- Xie, Y., Huhn, K., Brandt, R., Potschin, M., Bieker, S., Straub, D., Doll, J., Drechsler, T., Zentgraf, U., and Wenkel, S.** 2014. REVOLUTA and WRKY53 connect early and late leaf development in Arabidopsis. *Development* *141*, 4772-4783.
- Xu, L., Xu, Y., Dong, A.W., Sun, Y., Pi, L.M., Xu, Y.Q., and Huang, H.** 2003. Novel *as1* and *as2* defects in leaf adaxial-abaxial polarity reveal the requirement for *ASYMMETRIC LEAVES1* and 2 and *ERECTA* functions in specifying leaf adaxial identity. *Development* *130*, 4097-4107.
- Yamamoto, R., Fujioka, S., Demura, T., Takatsuto, S., Yoshida, S., and Fukuda, H.** 2001. Brassinosteroid Levels Increase Drastically Prior to Morphogenesis of Tracheary Elements. *Plant Physiology* *125*, 556-563.
- Yamamoto, R., Fujioka, S., Iwamoto, K., Demura, T., Takatsuto, S., Yoshida, S., and Fukuda, H.** 2007. Co-regulation of brassinosteroid biosynthesis-related genes during xylem cell differentiation. *Plant & Cell Physiology* *48*, 74-83.

Yip, H.K., Floyd, S.K., Sakakibara, K., and Bowman, J.L. 2016. Class III HD-Zip activity coordinates leaf development in *Physcomitrella patens*. *Developmental Biology*.

<http://dx.doi.org/10.1016/j.ydbio.2016.01.012>

Zamski, E. 1979. The Mode of Secondary Growth and the Three-Dimensional Structure of the Phloem in *Avicennia*. *Botanical Gazette* *140*, 67-76.

Zhang, Z., and Zhang, X. 2012. Argonautes compete for miR165/166 to regulate shoot apical meristem development. *Current Opinion in Plant Biology* *15*, 652-658.

Zhong, R., Taylor, J.J., and Ye, Z.H. 1997. Disruption of Interfascicular Fiber Differentiation in an *Arabidopsis* Mutant. *The Plant Cell* *9*, 2159-2170.

Zhong, R., and Ye, Z.-H. 1999. IFL1, a Gene Regulating Interfascicular Fiber Differentiation in *Arabidopsis*, Encodes a Homeodomain-Leucine Zipper Protein. *The Plant Cell* *11*, 2139-2152.

Zhou, G.-K., Kubo, M., Zhong, R., Demura, T., and Ye, Z.-H. 2007. Overexpression of miR165 Affects Apical Meristem Formation, Organ Polarity Establishment and Vascular Development in *Arabidopsis*. *Plant and Cell Physiology* *48*, 391-404.

Zhou, Y., Honda, M., Zhu, H., et al. 2015. Spatiotemporal Sequestration of miR165/166 by *Arabidopsis* Argonaute10 Promotes Shoot Apical Meristem Maintenance. *Cell Reports* *10*, 1819-1827.

Zhu, H., Hu, F., Wang, R., Zhou, X., Sze, S.-H., Liou, Lisa W., Barefoot, A., Dickman, M., and Zhang, X. 2011. *Arabidopsis* Argonaute10 Specifically Sequesters miR166/165 to Regulate Shoot Apical Meristem Development. *Cell* *145*, 242-256.

Zhu, Y., Song, D., Sun, J., Wang, X., and Li, L. 2013. PtrHB7, a class III HD-Zip Gene, Plays a Critical Role in Regulation of Vascular Cambium Differentiation in *Populus*. *Molecular Plant* *6*, 1331-1343.

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 overlaid. 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- δ miRNA*) 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) Cartoon 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. Overlaid 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.

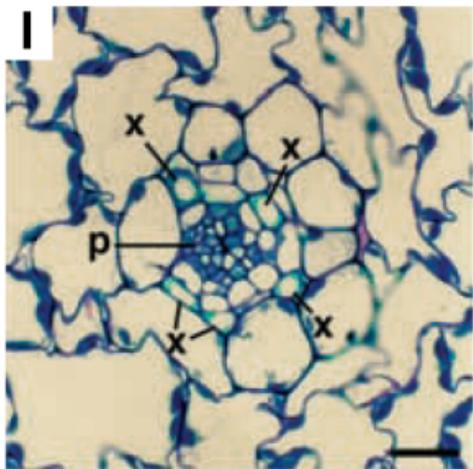
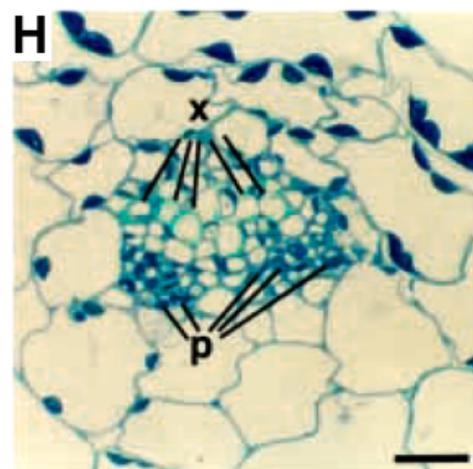
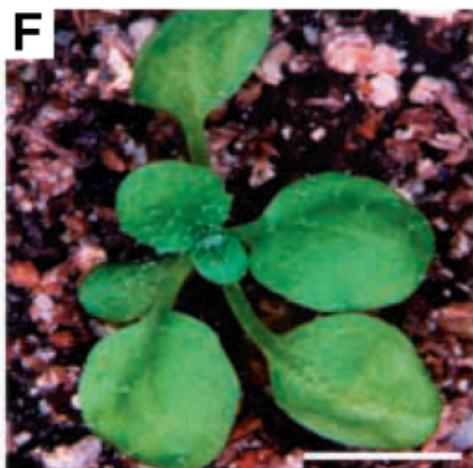
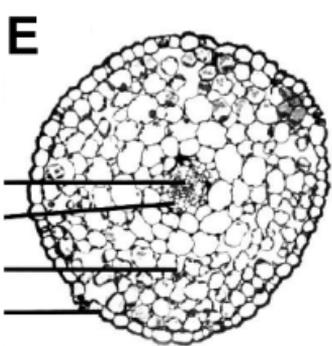
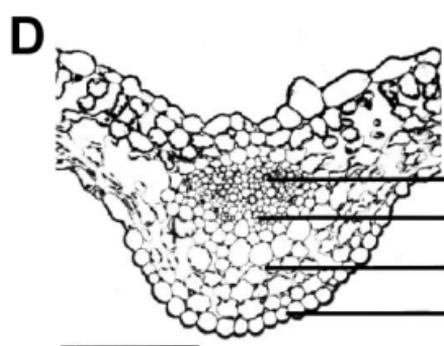
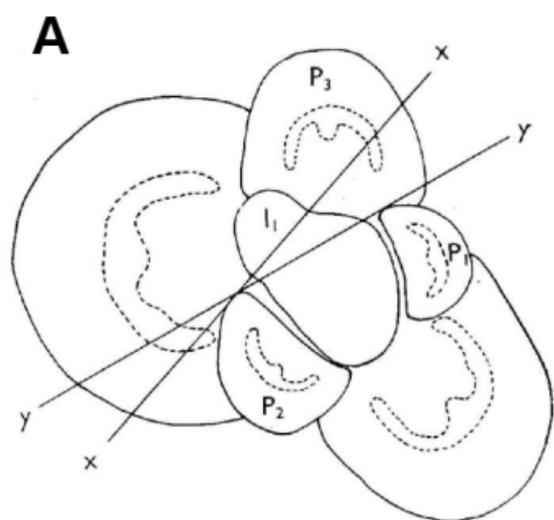
Figure 5. Xylem phenotypes of *rev-6* mutants in inflorescence stem.

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.



Adaxial side

Xylem

AS1

AS2

PNH

REV1

PHV

PHB

ATHB8

CNA

Phloem

miR165/
miR166

APL

CLE41

PIN1

Auxin

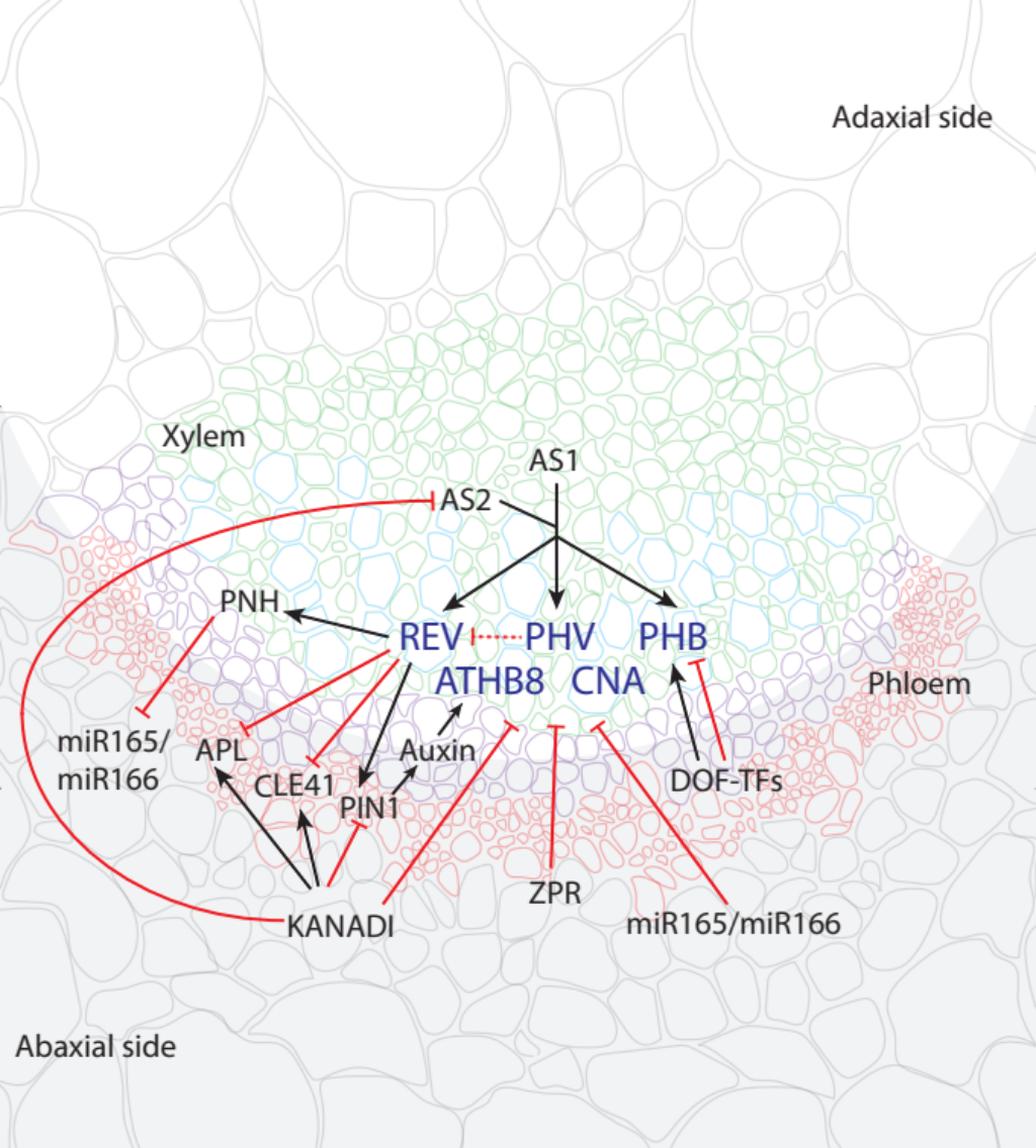
DOF-TFs

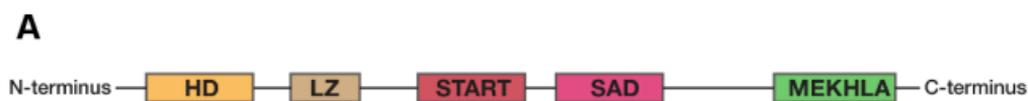
KANADI

ZPR

miR165/miR166

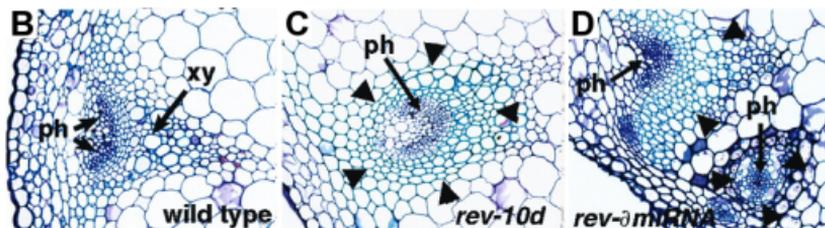
Abaxial side



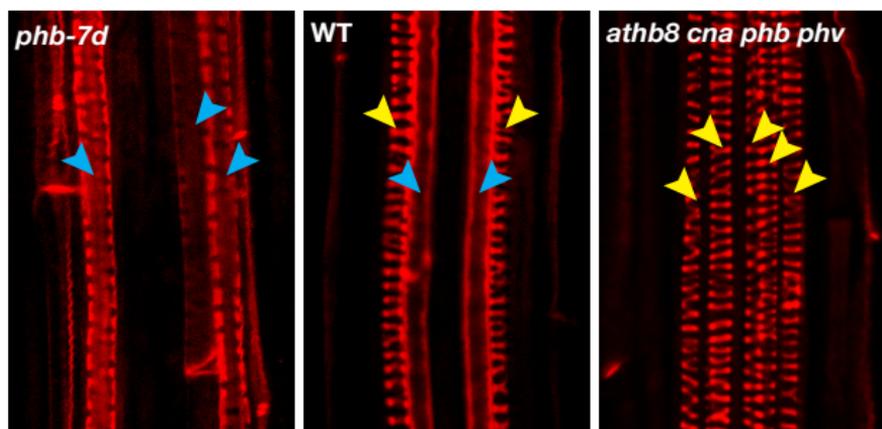


miR165 complementary site

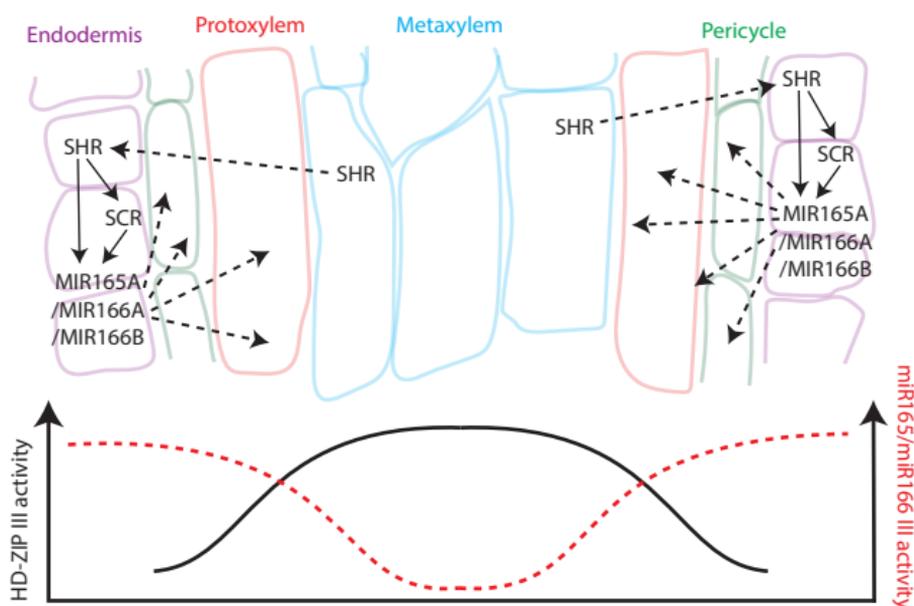
	G	M	K	P	G	P	D	
PHB, PHV, REV	GGG	AUG	AAG	CCU	GGU	CCG	GAU	
ICU/CNA	GGG	AUG	AAG	CCU	GGU	CCG	GAU	
<i>phb-1d, 2d</i>	G	M	K	--11aa insertion--	P	G	P	D
	GGG	AUG	AAG	--33nt insertion--	CCU	GGU	CCG	GAU
<i>phb-3d, 4d, 5d</i>	G	M	K	P	D	P	D	
	GGG	AUG	AAG	CCU	GAU	CCG	GAU	
<i>phb-7d</i>	G	M	K	P	G	L	D	
	GGG	AUG	AAG	CCU	GGU	CUG	GAU	
<i>phv-1d, 2d, 3d, 4d</i>	G	M	K	P	D	P	D	
	GGG	AUG	AAG	CCU	GAU	CCG	GAU	
<i>icu4-1, icu4-2</i>	G	M	K	P	D	P	D	
	GGG	AUG	AAG	CCU	GAU	CCG	GAU	
<i>rev-10d</i>	G	M	K	P	G	L	D	
	GGG	AUG	AAG	CCU	GGU	CUG	GAU	
<i>rev-δmiRNA</i>	G	M	K	P	G	P	D	
	GGG	AUG	AAG	CCU	GG	CC	GAU	



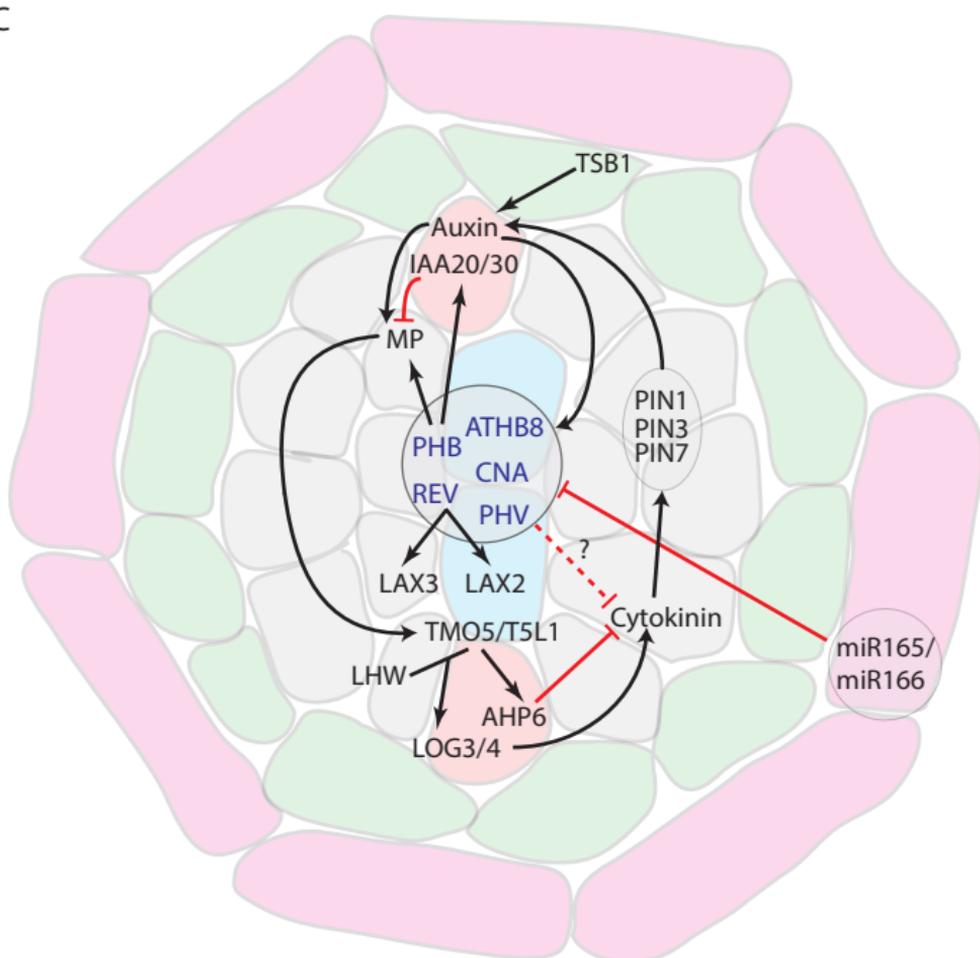
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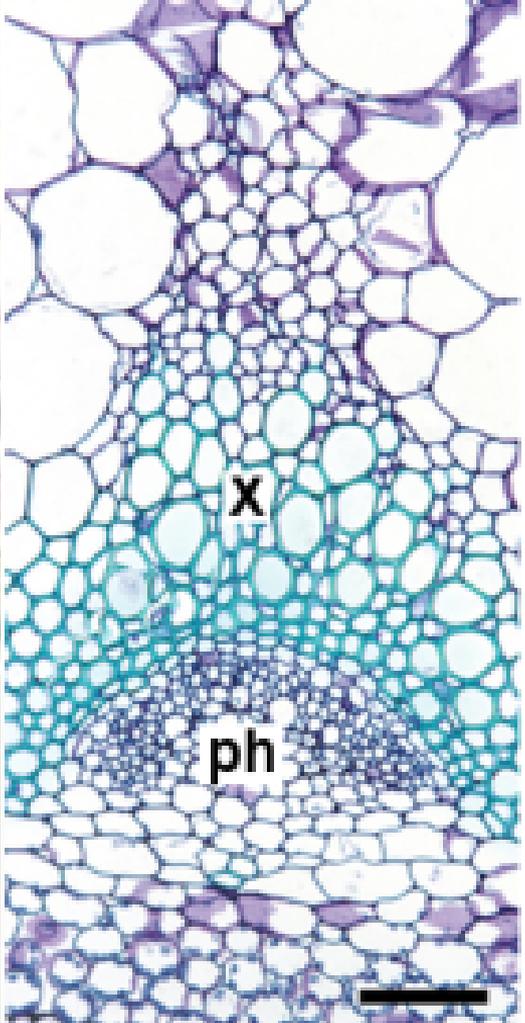
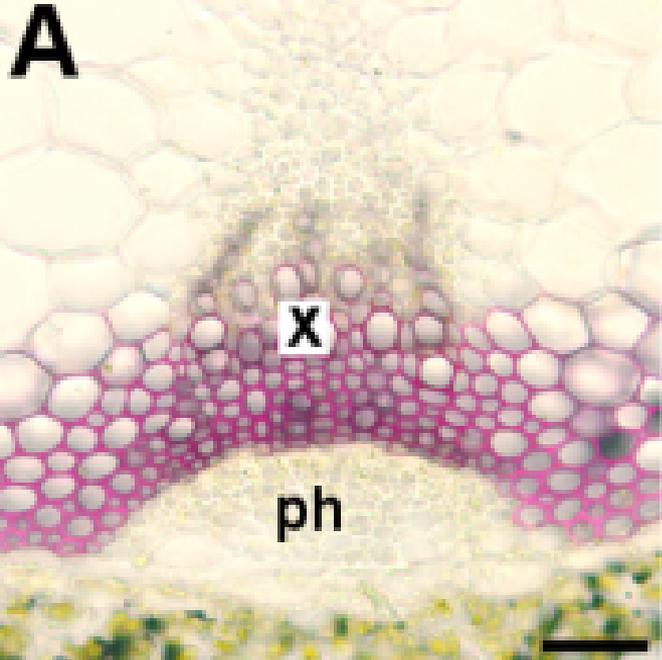
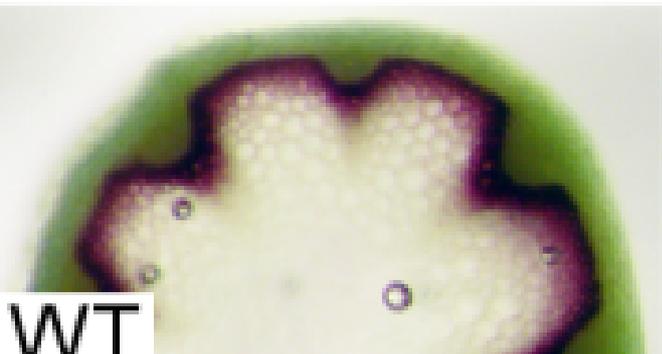
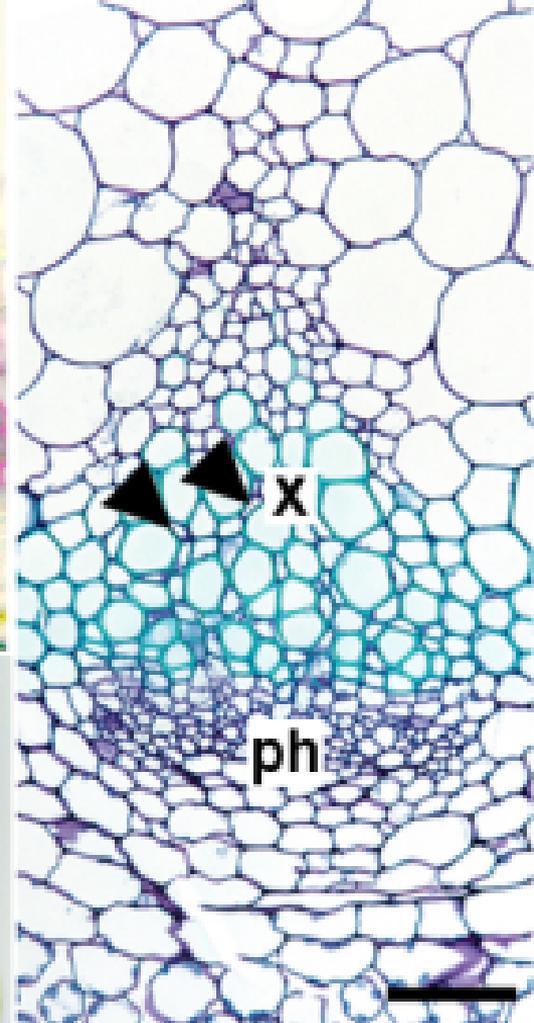
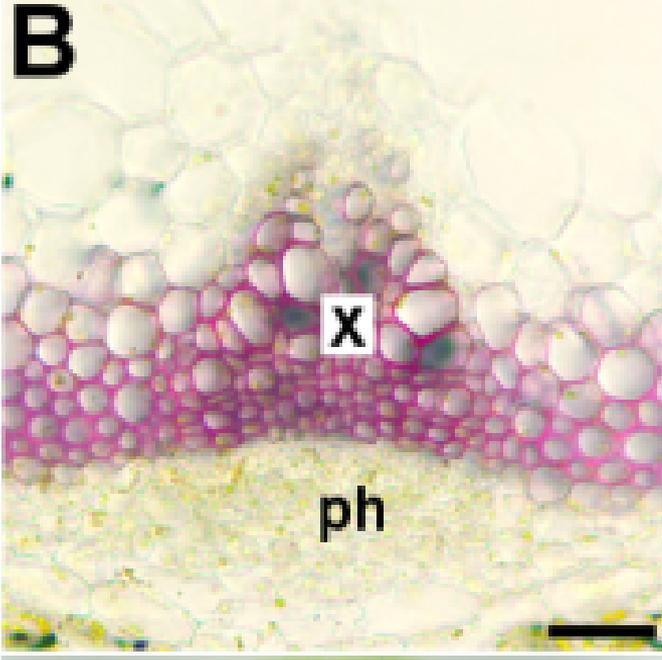
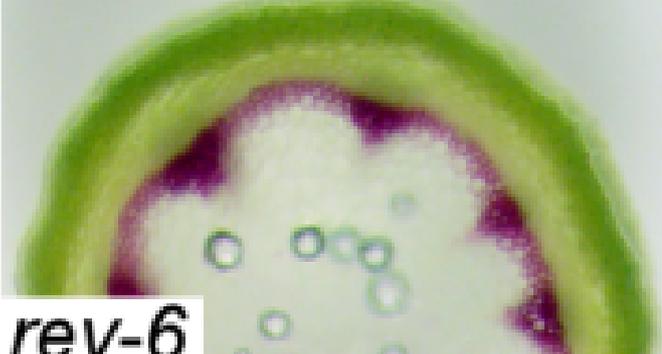


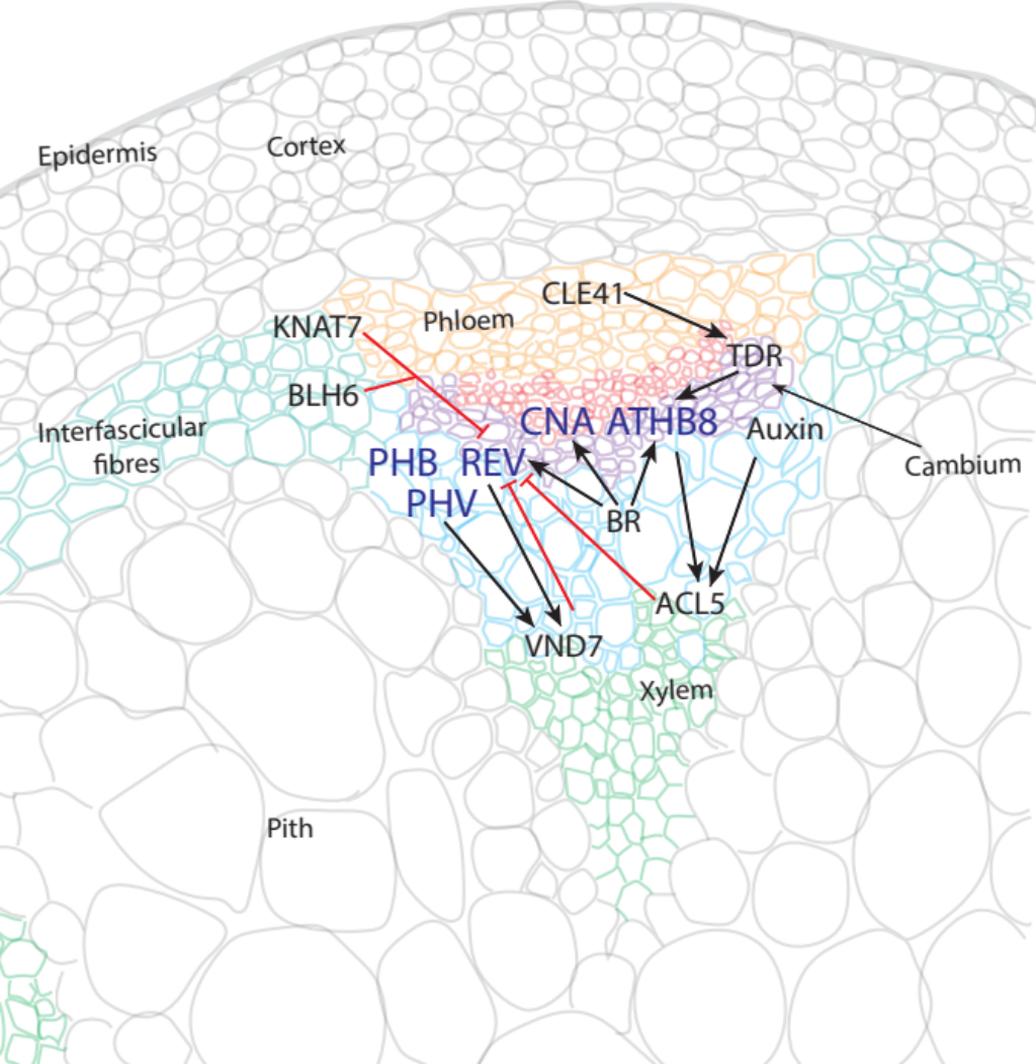
B



C



A**B****WT*****rev-6***



Epidermis

Cortex

KNAT7

Phloem

CLE41

BLH6

Interfascicular
fibres

PHB

CNA

ATHB8

Auxin

Cambium

REV

BR

ACL5

PHV

VND7

Xylem

Pith