PHABULOSA Mediates an Auxin Signaling Loop to Regulate Vascular Patterning in Arabidopsis

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Plant vascular tissues, xylem and phloem, differentiate in distinct patterns from procambial cells as an integral transport system for water, sugars, and signaling molecules. Procambium formation is promoted by high auxin levels activating class III homeodomain leucine zipper (HD-ZIP III) transcription factors (TFs). In the root of Arabidopsis (*Arabidopsis thaliana*), HD-ZIP III TFs dose-dependently govern the patterning of the xylem axis, with higher levels promoting metaxylem cell identity in the central axis and lower levels promoting protoxylem at its flanks. It is unclear, however, by what mechanisms the HD-ZIP III TFs control xylem axis patterning. Here, we present data suggesting that an important mechanism is their ability to moderate the auxin response. We found that changes in HD-ZIP III TF levels affect the expression of genes encoding core auxin response molecules. We show that one of the HD-ZIP III TFs, PHABULOSA, directly binds the promoter of both monopteros (MP)/auxin response factor 5, a key factor in vascular formation, and IAA20, encoding an auxin/indole acetic acid protein that is stable in the presence of auxin and able to interact with and repress MP activity. The double mutant of IAA20 and its closest homolog IAA30 forms ectopic protoxylem, while overexpression of IAA30 causes discontinuous protoxylem and occasional ectopic metaxylem, similar to a weak loss-of-function mutant. Our results provide evidence that HD-ZIP III TFs directly affect the auxin response and mediate a feed-forward loop formed by MP and IAA20 that may focus and stabilize the auxin response during vascular patterning and the differentiation of xylem cell types.

In multicellular organisms, tissue and organ development results from coordinated cell division and cell fate specifications, underlying the patterning of the developing tissue and organ. In plants, vascular tissues arise in intricate patterns, as they form in concert with other tissue types during the development of stems, leaves, and roots in order to ensure the transport of water, nutrients, and signaling molecules throughout the plant body (Scarpella and Helariutta, 2010; Lucas et al., 2013). In the Arabidopsis (*Arabidopsis thaliana*) primary root, the vascular pattern is relatively invariant and apparent already within the root meristem (Fig. 1A). Traversing the stele is an axis of xylem cells. In the xylem axis periphery, protoxylem forms and differentiates with annular or spiral secondary cell walls, while metaxylem forms centrally and is characterized by reticulate or pitted secondary cell walls. The xylem axis is flanked on each side by procambium and a strand of phloem tissue. The outermost layer of the stele is the pericycle, surrounded by consecutive layers of endodermis, cortex, and epidermis.

Root vascular patterning depends on class III homeodomain leucine zipper (HD-ZIP III) transcription factors (TFs) composed of five genes, *PHABULOSA* (PHB), *PHAVOLUTA* (PHV), *REVOLUTA* (REV), *CORONA* (CNA), and *Arabidopsis thaliana* homeobox gene8 (*ATHB8*). Their activity domains in the root are regulated through the activity of microRNA165 and microRNA166 (miR165/166). These microRNAs (miRNAs) move from their site of synthesis in the endodermis to restrict HD-ZIP III transcripts to the central stele (Carlsbecker et al., 2010; Miyashima et al., 2011). This results in higher levels of the HD-ZIP III proteins in
the central stele compared with its periphery (Lee et al., 2006; Carlsbecker et al., 2010; Miyashima et al., 2011). Analyses of various combinations of double, triple, and quadruple mutants as well as miR165-insensitive gain-of-function mutants suggest that HD-ZIP III TFs act redundantly and that their protein levels dose-dependently translate into xylem cell fate determination, such that high levels result in metaxylem formation and low levels result in protoxylem (Carlsbecker et al., 2010). In quadruple loss-of-function mutants, protoxylem develops throughout the xylem axis, while in gain-of-function phb-d mutants insensitive to miR165/166 regulation, metaxylem develops in peripheral xylem positions. Furthermore, HD-ZIP III TF levels appear to dose-dependently influence root stele cell number (Carlsbecker et al., 2010) and xylem strand continuity (Ilegems et al., 2010), suggesting that these factors not only redundantly determine xylem cell fate

Figure 1. Alterations in the levels of HD-ZIP III TFs lead to changes in auxin responses in the Arabidopsis root. A, Schematic representation of the Arabidopsis root meristem depicting the general pattern of stele cell type organization in longitudinal and radial views. B, Xylem pattern in pCRE1>>MIR165A roots after 0 and 24 h of β-estradiol induction. m, Metaxylem; p, protoxylem. C, Quantitative real-time PCR (qPCR) analysis of AHP6 and ACL5 expression in root tips after induction of pCRE1>>MIR165A. *, P < 0.05, Student’s t-test; error bars indicate SD. D, Radial (top) and longitudinal (bottom) confocal images of pAHP6::GFP in pCRE1>>MIR165A root meristems after 0, 15, and 40 h of β-estradiol induction. E, DR5::GUS staining in pCRE1>>MIR165A roots after 0 and 24 h of β-estradiol induction. F and G, DR5rev::GFP expression in Columbia (Col-0; F) and athb8 cna phb phv (G) as seen in longitudinal and radial confocal images. Dotted lines indicate positions of the radial cross sections at 20 and 60 μm above the QC. H and I, DR5::GUS staining in 3-d-old seedlings of Col-0 (H) and phb-7d (I) following 24 h of growth on mock or 1 μM IAA-containing plates. Cell walls are stained with propidium iodide and appear magenta in D, F, and G. Fractions indicate the frequency of the observed staining or fluorescence pattern. Yellow or black arrowheads mark the QC (longitudinal images), and white arrowheads mark the xylem axis (radial images). Bars = 25 μm, except in B, where bars = 10 μm.
but also act to suppress cell division in the stele and promote vascular continuity. The mechanisms by which the HD-ZIP III TFs act to control the root vascular pattern, however, are not clear, and direct transcriptional targets for these factors have just begun to be revealed (Brandt et al., 2012; Dello Ioio et al., 2012; Reinhart et al., 2013; Baima et al., 2014; Huang et al., 2014; Taylor-Teeples et al., 2015).

Vascular patterning depends on the hormone auxin (for review, see Scarpetta et al., 2010; Sawchuk and Scarpetta, 2013; De Rybel et al., 2014b). In the root meristem, auxin is laterally focused to the xylem axis through PIN-FORMED (PIN)-mediated transport (Bishopp et al., 2011). Here, it activates ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN6 (AHP6) in the protoxylem positions. AHP6 is required for protoxylem cell fate; its absence results in discontinuous protoxylem strands as a result of derepressed cytokinin signaling that promotes procambial fate (Mähönen et al., 2006). In addition, local auxin biosynthesis in the root is required for the formation of metaxylem (Ursache et al., 2014). With reduced auxin biosynthesis, HD-ZIP III genes have reduced expression insufficient to promote metaxylem formation; therefore, protoxylem forms in the central xylem axis. During leaf vascular patterning, an elevated auxin level in preprocambial cells is established through a self-organizing system where PIN1 mediates the canalization of auxin, activating the auxin response factor ARF5/MONOPTEROS (MP), which enhances PIN1 expression (Scarpella et al., 2006; Wenzel et al., 2007), and directly activates ATHB8 in a file of cells within its expression domain (Baima et al., 1995; Donner et al., 2009). Here, ATHB8 narrows the preprocambial domain, stabilizes these cells against auxin perturbations, and promotes xylem formation (Baima et al., 2001; Donner et al., 2009). Hence, vascular patterning requires auxin signaling to directly or indirectly induce HD-ZIP III expression.

Multiple observations suggest that certain and, in some cases, all HD-ZIP III TFs feed back on auxin signaling by interfering on several levels (for review, see Turchi et al., 2015). Polar auxin transport is affected by modified HD-ZIP III TF levels (Zhong and Ye, 2001; Izhaki and Bowman, 2007), while the direct control by REV of auxin biosynthesis (Brandt et al., 2012; Huang et al., 2014) and the auxin influx carriers LIKE AUXIN RESISTANT2 (LAX2) and LAX3 (Baima et al., 2014) were recently shown. In line with a modification in auxin accumulation patterns, Ilegems et al. (2010) showed that seedlings with elevated miR165 levels display enhanced auxin reporter activity. However, apparently in contrast, the expression of MP was found to be down-regulated after miR165 induction in seedlings (Baima et al., 2014). Hence, previous studies have shown a complex but tight integration of HD-ZIP III TF activity and the plant hormone auxin in many aspects of plant development, on the levels of auxin biosynthesis, transport, and, potentially, also response.

The auxin response is dependent on ARFs such as MP. In the absence of auxin, ARF activity is inhibited through interactions with auxin/indole acetic acid (AUX/IAA) transcriptional repressors (Guilfoyle, 2015). Upon elevated auxin levels, DII domain-containing AUX/IAA forms auxin receptor complexes with TRANSPORT INHIBITOR RESPONSE (TIR)/AUXIN SIGNALING F-BOX (AFB) proteins (Calderón Villalobos et al., 2012). As a consequence, the AUX/IAA protein is degraded, releasing the ARF from repression (for review, see Peer, 2013; Salehin et al., 2015). In contrast, the closely related IAA20 and IAA30 proteins (Remington et al., 2004) lack the canonical DII domain, rendering them stable even in the presence of auxin (Dreher et al., 2006). Because of this and the strong auxin-related defects upon overexpression of IAA20 and IAA30, it has been suggested that these proteins act to dampen the auxin response (Sato and Yamamoto, 2008a, 2008b).

This study reinforces the interconnection of auxin signaling and HD-ZIP III TFs and contributes additional points of integration. We show that HD-ZIP III TFs influence the auxin response, both positively and negatively, as they are needed for normal expression levels of MP as well as IAA20 and IAA30. We show that PHB in vivo localizes to promoters of IAA20 and MP. Furthermore, the iaa20 iaa30 double mutant displays additional strands of protoxylem, while ectopic IAA30 expression causes breaks in protoxylem and occasional ectopic metaxylem, demonstrating their importance for root vascular patterning. Our results suggest a mechanism in which PHB, possibly together with other HD-ZIP III TFs, focus and stabilize the auxin response within the xylem axis by activating MP, along with its repressors, IAA20 and IAA30, to secure the vascular patterning process.

RESULTS

HD-ZIP III TFs Affect Auxin Signaling in the Root Xylem Axis

Previously, we showed that the expression of AHP6 expands throughout the xylem axis in the athb8 cna phb phv mutant, correlating with ectopic protoxylem, while AHP6 is repressed in the gain-of-function phb-7d mutant, correlating with a loss of protoxylem and gain of metaxylem (Carlsbecker et al., 2010). Because AHP6 is a direct target of the auxin response (Bishopp et al., 2011), its altered expression suggests differences in auxin signaling within the xylem axis in the loss- and gain-of-function mutants. To test this hypothesis, we analyzed auxin reporters in loss- and gain-of-function HD-ZIP III mutants as well as upon induction of miR165. Because the class III HD-ZIP transcription factors govern multiple aspects of plant development, from early embryogenesis onward, and in many instances are known to function redundantly (McConnell et al., 2001; Emery et al., 2003; Prigge et al., 2005), multiple loss-of-function mutants may have defects established already during embryogenesis. To assess the effects of reduced HD-ZIP III TF activity specifically occurring during root vascular development, we established a line to induce elevated levels of miR165 in the root meristem and thereby...
repress HD-ZIP III transcript accumulation. With the pCRE1::MIR165A line, stele-specific transcription of MIR165A is induced upon β-estradiol application. Induction for 16 h resulted in the inhibition of metaxylem and the formation of protoxylem in the metaxylem position in more than 50% (n = 36 of 64) of roots analyzed, while induction for 24 h or longer resulted in complete inhibition in metaxylem formation, and protoxylem frequently formed in both protoxylem and metaxylem positions (n = 141; Fig. 1B; Supplemental Fig. S1, A and B). The induction of miR165 resulted in significant suppression of ATHB8, CNA, and PHB expression by 4 h and PHV expression by 24 h within the root meristem, while REV levels remained unchanged even after 24 h of induction (Supplemental Fig. S1, C and D). In contrast to loss-of-function mutants and stable pCRE1::MIR165A lines (Carlsbecker et al., 2010), miR165 induction in the postembryonic root was not sufficient to significantly increase procambial periclinal divisions in the postembryonic root (Supplemental Fig. S1E).

Correlating with the shift in xylem cell identity, we found that expression of the protoxylem-specific gene AHP6 rapidly increased in root tips while the metaxylem marker ACAULIS5 (ACL5; Muñiz et al., 2008) decreased upon miR165 induction (Fig. 1C), consistent with the elevated AHP6 expression and loss of ACL5 expression in the athb8 cna phb phv mutant (Carlsbecker et al., 2010). Tracking the activity of the pAHP6::GFP reporter (Mähönen et al., 2006) upon miR165 induction showed that it expanded into the neighboring xylem axis cells over time. With increased time of induction, expression was detected also in most central xylem cells (Fig. 1D). This is consistent with a graded distribution of HD-ZIP III transcripts peaking in the central stele (Carlsbecker et al., 2010; Miyashima et al., 2011), rendering the peripheral cells more susceptible to the increased miR165 levels over the stele upon pCRE1::MIR165A induction. To assess possible alterations in auxin signaling, we introduced the DR5::GUS reporter (Ulmasov et al., 1997) to the pCRE1::MIR165A line. After induction for 24 h, we clearly detected enhanced GUS staining in the stele in 38% of the roots compared with 7% in mock-treated plants (Fig. 1E), indicating increased auxin signaling in the stele upon reduced HD-ZIP III gene expression.

Similarly, in the athb8 cna phb phv mutant (Prigge et al., 2005), the expression of DR5rev::GFP (Friml et al., 2003) marked the xylem axis; however, in contrast to the wild type, where expression became reduced in the central axis approximately 40 to 60 μm above the quiescent center (QC; Fig. 1F; Bishopp et al., 2011), the mutant did not show a differential expression in peripheral versus central xylem positions (n = 18 of 19; Fig. 1G). Furthermore, in the wild type, DR5rev::GFP was restricted to a one-cell-file-wide axis, while the signal often expressed in a wider domain in the mutant. Similar to DR5rev::GFP, the pIAA2::GFP reporter (Bishopp et al., 2011) appeared relatively homogenous throughout the xylem axis in the athb8 cna phb phv mutant, when compared with the wild type, where its expression was reduced in the central xylem axis (Supplemental Fig. S2, A and B; Bishopp et al., 2011). Hence, auxin response reporters suggest that the central xylem axis has elevated and less focused auxin signaling in the athb8 cna phb phv mutant.

Assessing auxin reporters in phb-7d (Carlsbecker et al., 2010), we found that, similar to the lack of pAHP6::GFP expression, DR5::GUS and pIAA2::GFP did not express or expressed at a much reduced level in the primary root meristem of the mutant (Fig. 1; Supplemental Fig. S2, C, D, and F). This may be caused by reduced auxin biosynthesis, altered transport, or an inhibited response. To distinguish these possibilities, we treated the phb-7d mutant with exogenous auxins. Neither application of IAA nor the transport-independent 1-naphthaleneacetic acid, both of which strongly induced the expression of the DR5::GUS and pAHP6::GFP reporters in the wild-type background, could induce the expression of these auxin reporters in the phb-7d mutant (Fig. 1, H and I; Supplemental Fig. S2, D–F), suggesting that the phb-7d mutant has an inhibited auxin response and not primarily defects in auxin biosynthesis or transport.

Taken together, our results suggest that elevated PHB activity reduces the auxin response in the root meristem, while reduced HD-ZIP III activity leads to an elevated and less focused auxin response along the xylem axis.

**Genome-Wide Survey of Responses to Reduced HD-ZIP III Activity in the Root Meristem**

To understand by what mechanisms alterations in the auxin response could be generated upon reduction in HD-ZIP III activity, we used Affymetrix microarrays to assess transcriptional gene expression changes in microdissected root meristems upon induction of miR165 for 6, 10, and 24 h (Supplemental Table S1). Considering ACL5 as a direct ATHB8 target (Baima et al., 2014), the early time point was determined by the time at which we detected significant ACL5 and AHP6 expression changes by qPCR (Fig. 1C). Ten hours was chosen as a time point just prior to major xylem phenotypic changes, and 24 h represents the time at which the roots clearly exhibited ectopic protoxylem (Supplemental Fig. S1B). For more comprehensive insight, we compared gene expression in root meristems of several loss-of-function HD-ZIP III mutants with the wild type, in addition. Gene expression differences in the root meristem of the athb8 cna phb phv mutant were compared with the wild type (Supplemental Table S2). In a separate experiment, we compared cna phb phv and cna phb phv rev with the wild type (Supplemental Table S3). To compare results over all three experiments, we conducted a Venn SuperSelector analysis (the Bio-Analytic Resource for Plant Biology; http://bar.utoronto.ca/) of genes determined to be significantly altered in each experiment (Supplemental Table S4).

Confirming the validity of the array analyses, we detected significantly reduced expression of CNA, PHB,
**PHV**, and **ATHB8** by miR165 induction along with both **ACL5** and **BUSHY AND DWARF2 (BUD2)**, ensuring that HD-ZIP III TF targets (Baima et al., 2014) could be identified (Table I). However, changes in **ACL5** and **BUD2** were not detected in microarrays of loss-of-function mutants, suggesting that these analyses were less sensitive than the inducible miR165 analyses and may reflect more long-term expression changes and potentially phenotypic differences that could obscure interpretations. We probed the list of genes for auxin signaling- and auxin response-related genes following Bargmann et al. (2013), particularly noting those genes expressed in xylem (Brady et al., 2007). A number of auxin-inducible genes with expression in developing or developing-maturing xylem were found up-regulated at 10 and 24 h of miR165 induction, including **AHF6**, **IAA2**, **IAA19**, **IAA29**, **TARGET OF MONOPTEROS-LIKE1** (TSL1), and **LAX3**. In addition, expression of the AUX/IAAs **IAA14**, **IAA17**, and **IAA28** was elevated, consistent with an enhanced auxin signaling upon reduced HD-ZIP III activity.

Contrary to our expectations, we found a number of genes known to be auxin induced that instead were suppressed. These included **IAA30**, which was down-regulated already at 6 h, paralleling **ACL5** and **BUD2**, and continued to be down-regulated at 10 and 24 h. Its sister gene, **IAA20**, was also down-regulated at 24 h. Furthermore, **MP**, **TARGET OF MONOPTEROS5** (TMO5), **LONELY GUY4** (LOG4), and **LAX2** were down-regulated, suggesting that these factors are normally promoted by HD-ZIP III TFs. In line with these results, REV was shown to directly bind to the LAX2 promoter (Baima et al., 2014). Specifically probing for factors that may contribute to altered levels of active auxin showed little support for alterations in auxin biosynthesis, with the exception of an increase in **YUCCA8** at 10 h of miR165 induction. We found no significant changes in PIN auxin efflux transporter activities.

![Table 1](https://plantphysiol.org)

_Genes were considered differently expressed if they had a log fold change equal to or larger than 0.5 (light face) or equal to or smaller than −0.5 (bold face), an adjusted P value of equal to or less than 0.05 for miR165 induction of 6, 10, and 24 h relative to the wild type and for cna phb phv (bvc) and cna phb phv rev (bvc) relative to the wild type, and an adjusted P value of equal to or less than 0.1 for athb8 cna phb phv (bvca). Expression in xylem is indicated with +._

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<td>PLT5</td>
<td>PLETHORAS/AINTEGUMENTA-LIKE5</td>
<td></td>
<td>−</td>
<td>−</td>
<td>0.52</td>
<td>0.66</td>
<td>−</td>
</tr>
<tr>
<td>At2g33310</td>
<td>IAA13</td>
<td>INDOLE-3-ACETIC ACID INDUCIBLE13</td>
<td></td>
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<td>−</td>
<td>0.49</td>
<td>0.55</td>
<td>−</td>
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<tr>
<td>At3g04730</td>
<td>IAA16</td>
<td>INDOLE-3-ACETIC ACID INDUCIBLE16</td>
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<td>−</td>
<td>−</td>
<td>0.87</td>
<td>−</td>
<td>+</td>
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<tr>
<td>At3g23050</td>
<td>IAA7</td>
<td>INDOLE-3-ACETIC ACID INDUCIBLE7</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>0.52</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

Examples of other genes changed by miR165 induction or HD-ZIP III mutation

| At5g19530 | ACL5          | ACAULIS5                               | −0.64 | −2.23 | −3.32 | −     | −     | +     |
| At5g18930 | BUD2          | BUSHY AND DWARF2/SAMDC4                 | −0.59 | −0.86 | −0.84 | −     | −     | +     |
| At5g64530 | XND1          | XYLEM NAC DOMAIN1                      | −     | −0.56 | −0.74 | −     | −     | +     |
| At5g66300 | VND3          | VASCULAR-RELATED NAC-DOMAIN3           | −     | −     | −0.68 | −     | −     | +     |
| At1g20850 | XCP2          | XYLEM CYSTEINE PEPTIDASE2              | 0.75  | 1.20  | −     | −     | −     | +     |
| At4g35330 | XCP1          | XYLEM CYSTEINE PEPTIDASE1              | 1.28  | 2.64  | −     | −     | −     | +     |

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expression levels. In summary, our array results suggested that inducing reduced HD-ZIP III TF levels within the root meristem had contrasting effects on the auxin response, with a number of auxin-responsive genes being up-regulated while another set of genes instead became down-regulated.

Expression of IAA20 and IAA30 Is Controlled by HD-ZIP III TFs

We first approached the question of how certain auxin-activated genes might be up-regulated by the reduced activity of HD-ZIP III TFs. Among the genes responding to miR165 induction, we identified IAA20 and IAA30, which are putative repressors of the auxin response (Dreher et al., 2006; Sato and Yamamoto, 2008a). Thus, the down-regulation of IAA20 and IAA30 expression as seen upon miR165 induction may result in increased ARF activity leading to an increased auxin response, in line with the up-regulation of certain auxin-responsive genes and reporters that we observed after miR165 induction (Fig. 1; Table I).

By qPCR, we confirmed that IAA20 and IAA30 were rapidly down-regulated in root meristems after miR165 induction. Reduced expression was detected at 4 h of induction, and the expression of both genes continued to decrease over time, although after 2 d, expression may have normalized to some extent (Fig. 2A). In order to assess the possibility of cooperative regulation on these genes among the four HD-ZIP III TFs, we analyzed various double and multiple mutant combinations (Prigge et al., 2005; Carlsbecker et al., 2010). None of the double mutants had reduced IAA20 or IAA30 expression, and some even showed increased expression; however, in both the cua phb phw and athib8 cua phb phw mutants, IAA30 was down-regulated (Fig. 2B; Supplemental Table S5). The expression of the pIAA30::35S::GFP transcriptional reporter has previously been described and shown to be active in the columella, lateral root cap, and stele (Rademacher et al., 2011). Radial confocal imaging revealed that the IAA30 promoter was specific to the xylem axis (Fig. 2C). The expression of IAA20 is also confined to the xylem axis in a pattern very similar to IAA30 (C. Llavata-Peris and D. Weijers, personal communication). Hence, the expression of IAA20 and IAA30 is focused to domains of known high auxin and high HD-ZIP III TF activity levels. Together, these data suggest that multiple HD-ZIP III TF members are cooperatively needed for wild-type expression levels of these non-auxin-degradable AUX/IAA-encoded genes within the stele.

Because of the rapid reduction in IAA20 and IAA30 expression upon miR165 induction, paralleling or even preceding ACL5, a known HD-ZIP III TF target (Baima et al., 2014), we asked if IAA20 and/or IAA30 could be directly targeted by HD-ZIP III TFs. To investigate this, we performed chromatin immunoprecipitation (ChIP) with seedlings expressing a miRNA-insensitive version of PHB fused to GFP and driven by the PHB promoter (pPHB::PHB:GFP; Dello Ioio et al., 2012). Three different regions of the IAA20 promoter and gene, and two regions of the IAA30 promoter, were tested for PHB enrichment (Fig. 2D; Supplemental Figs. S3 and S4). We found that a fragment at position −200 bp upstream of the ATG start site of the IAA20 promoter was significantly enriched in four independent experiments, indicating a specific in vivo association of PHB to the IAA20 promoter (Fig. 2D; Supplemental Fig. S4A). On the contrary, we could not detect an enrichment of PHB on the IAA30 promoter (Supplemental Figs. S3 and S4B). To test if increased activity of PHB would be sufficient to activate the expression of IAA20 and IAA30, we created a line, pCRE1::>phb-1d, to induce the expression of a miRNA-resistant version of PHB within the root meristem stele. β-Estradiol induction strongly increased the expression of PHB (Supplemental Fig. S5, A and B). This was accompanied by breaks in protoxylem, additional protoxylem strands, and a conversion of protoxylem to metaxylem (Supplemental Fig. S5, C and D). Despite the strong increase in PHB expression, we could not detect a rapid increase in IAA20 or IAA30 expression; however, a 2-fold increase in IAA30 was detected after a 10-h induction (Fig. 2E), suggesting a potential requirement of additional factors for PHB-mediated activation of these genes. Significantly elevated expression of IAA20 and IAA30 was detected in the phb-7d gain-of-function mutant (Fig. 2F) and also in the CNA gain-of-function mutant cua4-1, which, similar to the phb-7d mutant, also forms ectopic metaxylem (Supplemental Fig. S6, A–C).

IAA20 and IAA30 Are Required for Proper Vascular Patterning

To assess the relevance of IAA20 and IAA30 downstream of PHB for their contribution to root vascular patterning, we analyzed the phenotypes of iaa20 iaa30 loss-of-function mutants, an IAA20 induction line, and IAA30 overexpression lines. Single-insertion mutants, iaa20-1, iaa20-2 (Supplemental Fig. S7A), and iaa30-1 (Zheng et al., 2009), displayed occasional phenotypic aberrations, including discontinuity of protoxylem and metaxylem strands and, infrequently, additional protoxylem strands (Fig. 3A; data not shown). As IAA20 and IAA30 are closely related, have overlapping expression patterns, and may act redundantly, we generated the double iaa20-2 iaa30-1 mutant. The double mutant had a substantially higher frequency of the formation of double protoxylem strands instead of a single protoxylem strand at the peripheral xylem axis (Fig. 3, A–C; Supplemental Fig. S7B). In addition, the iaa20-2 iaa30-1 mutant displayed significantly wider cotyledonary veins and longer roots compared with the Col-0 × Ler wild type generated by the cross of iaa20-2 and iaa30-1 (Supplemental Fig. S7, C–E).

It has been shown previously that ectopic IAA20 and IAA30 both yield phenotypes suggesting that they have reduced auxin signaling (Sato and Yamamoto, 2008a). We assessed the xylem phenotype of both an IAA20
Induction line from the TRANSPLANTA collection (Coego et al., 2014) as well as two independent 35S::IAA30 overexpression lines (Sato and Yamamoto, 2008a). Induction of IAA20 increased root expression about 3 times, but this did not result in any alteration to the xylem patterning (Supplemental Fig. S7, F and G). However, two 35S::IAA30 lines, in which IAA30 expression is strongly elevated, displayed major alterations in xylem patterning, predominantly breaks in or loss of protoxylem but also occasionally metaxylem forming in protoxylem positions or the formation of only metaxylem (Fig. 3, A and D; Supplemental Fig. S7, H and I). Hence, ectopic IAA30 activity primarily affected the formation of continuous protoxylem and appeared also to promote metaxylem formation. As increased IAA30 levels may cause suppressed MP activity, we compared these phenotypes with the weak mpS319 mutant (Cole et al., 2009). In this segregating weak mp allele, we detected primarily breaks in protoxylem but occasionally also breaks in metaxylem and the formation of double protoxylem (Fig. 3, A and E; Supplemental Fig. S7J). We conclude that its effect on xylem patterning is similar to that in the 35S::IAA30 lines, consistent with the possible suppression of MP activity in the 35S::IAA30 line. In addition, overexpression of IAA30 resulted in reduced expression of a number of auxin-induced genes, including MP itself, TMO5, and endogenous IAA30 (Fig. 3F; Supplemental Fig. S7H). Interestingly, in line with being auxin induced, the expression of all five HD-ZIP III genes was also down-regulated in the root tips of the 35S::IAA30 line (Fig. 3G), despite its phenotype of ectopic metaxylem resembling instead HD-ZIP III gain-of-function mutant phenotypes (Carlsbecker et al., 2010). Conversely,
in the iaa20-2 iaa30-1 mutant, the auxin targets AHP6 and IAA2 were significantly up-regulated (Fig. 3H), consistent with an enhanced auxin signaling.

To test if we could restore the vascular defects of the phb-7d mutant with a reduction in IAA30 levels, we produced the iaa30-1 phb-7d double mutant. While more than 60% of phb-7d × Col-0 roots form only metaxylem, in the iaa30-1 phb-7d double mutant, this number was reduced to 50% ($n = 316$; Fig. 3, I and J), and the iaa30-1 phb-7d roots more frequently formed stretches of protoxylem and switches of metaxylem to protoxylem (38%) compared with phb-7d (C24 × Col-0) originating from the same cross (24%). In addition, the root length of the iaa30-1 phb-7d double mutant was slightly but significantly longer than that of phb-7d (C24 × Col-0; Supplemental Fig. S7K). Hence, these results suggest that an up-regulation of IAA30 by PHB in part explains the root developmental defects observed in phb-7d.

 Taken together, our results suggest that HD-ZIP III TFs exert control over the levels of IAA20 and IAA30 in the root meristem stele and that this regulation affects the patterning of vascular cells.

**MP Expression Is Controlled by HD-ZIP III TFs**

Intriguingly, upon miR165 induction, our array analyses revealed a down-regulation of MP, together with its direct target TMO5. LOG4 is directly activated by TMO5 (De Rybel et al., 2014a; Ohashi-Ito et al., 2014),
and in line with a loss of MP/TMO5 activity, LOG4 was also repressed after miR165 induction (Table I). Down-regulation of MP upon increased miR165 activity was also shown previously by Baima et al. (2014). This raised the question of how an auxin-regulated gene like MP could be down-regulated in parallel with an apparent reduction in IAA20 and IAA30 and enhanced auxin reporter activity and suggested that one or several of the HD-ZIP III TFs could be required for MP activation. We verified the results from the array by qPCR and found that miR165 induction reduced MP expression slightly, although this occurred rapidly and was maintained consistently over longer induction times (Fig. 4A). MP has a broadly similar expression domain in the stele to IAA20 and IAA30. The pMP::n3GFP reporter (Lau et al., 2011) expressed in the xylem axis and procambial cells neighboring the xylem axis (Fig. 4B). It was excluded from the QC and columella but expressed in the lateral root cap. Its expression domain in the stele and QC thus largely resembles the expression domain of most HD-ZIP III genes (Lee et al., 2006; Carlsbecker et al., 2010), consistent with a possible regulation by these factors. In agreement with a requirement for HD-ZIP III TFs to establish wild-type expression levels of MP, we found that, in the double mutants athb8 phb, athb8 phe, athb8 cna, and cna phe, MP expression was reduced to about half the wild-type levels; however, other loss-of-function combinations resulted in no change (cna phb) or even enhanced expression (phe phe), as was also seen in the athb8 cna phb phe mutant (Fig. 4C; Supplemental Table S5), suggesting that the combinatorial regulation of MP by HD-ZIP III TFs is complex. Alternatively, increased MP expression could be a consequence of the larger root meristem of these mutants (Dello Ioio et al., 2012; A.E. Valdés, C.J. Müller, and A. Carlsbecker, unpublished data), resulting in comparatively more MP-expressing cells. In phb-7d, the expression of MP was not significantly altered; however, TMO5 was down-regulated, while in icu4-1, MP expression was suppressed (Supplemental Fig. S6, D and E). This is consistent with the observation of severely reduced expression of known auxin reporters in phb-7d, possibly caused by elevated IAA20 and IAA30 expression. Induction of the pCRE1::>phb-1d line showed a significant increase in MP expression at 4 and 6 h versus 10 h of PHB induction (Fig. 4D). Accordingly, if PHB acts to induce MP expression, it appears not to be direct, or additional factors may be required for induction, since increased MP expression was delayed in relation to a rapid induction of PHB. To assess if PHB associates with the promoter of MP, we performed ChIP experiments with the pPHB::PHBd::GFP line, testing regions at −450 bp in the 5′ untranslated region and −700 bp within the promoter. In four independent experiments, we detected a significant enrichment over the control of the fragment at −450 bp upstream of the MP start site, while no significant enrichment was detected for the −700-bp fragment (Fig. 4E; Supplemental Fig. S4C), indicating a specific in vivo association of PHB to the MP 5′ untranslated region. Taken together, the rapid reduction in MP expression upon miR165 induction, its reduced expression in some HD-ZIP III double mutants, and the binding of PHB to the MP promoter suggest that PHB and potentially other members, such as ATHB8, are needed for the full activation of MP within the vasculature.

DISCUSSION

Auxin signaling has been implicated in essentially all aspects of plant development, and several lines of evidence strongly support that auxin acts upstream of HD-ZIP III genes. Here, we present data suggesting that HD-ZIP III TFs feed back on several auxin perception/response components. We show that normal expression levels of both MP and its putative inhibitors IAA20/IAA30 require HD-ZIP III TFs, that PHB directly binds the promoters of both MP and IAA20, and that altered activity of these factors results in distorted xylem axis formation. Based on these results, we propose that HD-ZIP III TFs act to stabilize the auxin response and that this is important to obtain and maintain a normal root vascular pattern (Fig. 4F).

Regulation of IAA20 and IAA30 Is Required for Normal Root Vascular Patterning

We found PHB and the other HD-ZIP III TFs to directly or indirectly affect the auxin response. Analyses of reporters and selected auxin-regulated genes suggested an increase in auxin response upon reduction in HD-ZIP III activity and suppression of the auxin response in phb-7d. Consistent with these findings, a previous study noted elevated and broadened DR5rev::GFP expression in embryos and seedlings with suppressed HD-ZIP III activity (Ilegems et al., 2010). However, among genes known to be directly and strongly induced by auxin, we noted that a subset of genes, including IAA20 and IAA30, were instead down-regulated upon miR165 induction and up-regulated in the gain-of-function mutants of PHB and CNA. This apparent contradiction suggested that IAA20 and IAA30 could be targets for regulation by certain HD-ZIP III TFs, and their specific feature of being potential suppressors of the auxin response indicated that these factors could provide a mechanistic explanation for the observed alteration in auxin response with altered HD-ZIP III TF levels. Because IAA20 and IAA30 lack the DII domain required for recognition by the TIR/AFB auxin receptor/ubiquitination complex, they are stable in the presence of auxin (Dreher et al., 2006), but similar to canonical auxin-degradable AUX/IAAs, they interact with transcriptionally activating ARFs such as MP (Vernoux et al., 2011). Thus, IAA20 and IAA30 can act as repressors of the auxin response, likely competing for interaction with MP and other ARFs with rapidly auxin-degradable AUX/IAAs (Vernoux et al., 2011), such as the well-known MP repressor BODENLOS (BDL)/IAA12 (Hamann et al., 2002; Weijers et al., 2005).
Figure 4. HD-ZIP III TFs mediate MP expression level, and PHB is localized to the MP promoter in vivo. A, qPCR analysis of MP expression in pCRE1::MIR165A root tips at the indicated induction times. B, Expression of pMP::n3GFP in Col-0 in longitudinal and radial confocal images at 20 and 80 μm above the QC. The yellow arrowhead marks the QC (longitudinal image), and white arrowheads mark the xylem axis (radial images). Dotted lines indicate positions of the radial cross sections. Bars = 25 μm. C, qPCR analysis of MP expression in three double loss-of-function HD-ZIP III mutants relative to the respective wild type (Col er-2) collected in parallel (wt−/wt−). D, qPCR analysis of MP expression in pCRE1::phb-1d root tips at the indicated induction times. In A, C, and D, error bars indicate sd; * P < 0.05, Student’s t test. E, At top is a schematic of the MP gene and upstream region indicating the ChIP-qPCR fragment A, positioned at −450 bp, and fragment B, positioned at −700 bp upstream of the ATG start site (not to scale). Untranslated regions are indicated in black, exons in white, and introns in grey. At bottom is a ChIP-qPCR analysis showing means of three or four independent experiments. Anti-GFP and anti-IgG antibodies were used to precipitate chromatin prepared from pPHB::PHBdGFP seedlings. Enrichment is shown relative to the signal of negative control sequences within the non-HD-ZIP III targets ABI2 and OTC. The anti-IgG was used as an antibody control to quantify nonspecific antibody binding. Error bars indicate the sd from four (for position A) or three (for position B) independent biological replicates; * P < 0.05 between the enrichment for the control IgG and the GFP enrichment (general linear model ANOVA test). F, A model summarizing our results in light of known interactions. Previously, it was shown that MP directly regulates its own expression (Lau et al. 2011) as well as the expression of IAA20 (Krogan et al. 2014), that IAA20 and IAA30, which are stable in the presence of auxin (Dreher et al. 2006), can interact with MP (Vernoux et al. 2011), and, therefore, that IAA20 and IAA30 likely repress MP function in their overlapping activity domains. Here, we show that HD-ZIP III TFs are needed for normal expression of IAA20/IAA30 and MP, and we hypothesize that PHB and MP cooperatively may activate IAA20 and IAA30. In conditions of changing auxin levels in the root.
Similar to MP and BDL (Lau et al., 2011; data not shown), IAA20 and IAA30 express in the root xylem axis, which is a site of likely very high auxin levels, reflected in a strong expression of auxin reporters in particular at the protoxylem position (Bishopp et al., 2011). Hence, the apparent increase in auxin response within the xylem axis upon reduced HD-ZIP III TFs and, conversely, the suppressed auxin response in PHB gain-of-function mutants may be attributed at least in part to their ability to interfere with the activity levels of IAA20 and IAA30 auxin response repressors.

Loss-of-function mutants of IAA20 and IAA30 as well as gain of IAA30 have substantial effects on xylem patterning within the Arabidopsis root vasculature. The iaa20 iaa30 mutant displays double protoxylem strands and several other vascular aberrations in the root. Double protoxylem is commonly found in phb athb8 or triple HD-ZIP III mutants (Carlsbecker et al., 2010). Extra protoxylem strands are also observed in conditions of suppressed cytokinin signaling (Mähönen et al., 2006; Yokoyama et al., 2007; Argyros et al., 2008), which presumably results in a shift in the auxin-cytokinin balance within the stele, favoring auxin signaling (Bishopp et al., 2011). In addition, the iaa20 iaa30 mutant has wider veins in its cotyledons and longer roots compared with the wild type. Wider cotyledonary and leaf veins are observed in cases of elevated auxin levels, such as under conditions of reduced auxin transport (Scarpella et al., 2010), and longer roots are seen under conditions of suppressed cytokinin signaling and enhanced auxin signaling within the root meristem (Pacifici et al., 2015). Thus, taken together, these phenotypes alone are suggestive of elevated auxin signaling in the iaa20 iaa30 double mutant. Elevated auxin signaling is further supported by enhanced expression of IAA2 and AHP6 in the iaa20 iaa30 mutant root tip.

Conversely, ectopic IAA30 expression results in a discontinuity or loss of protoxylem strands in roots. This is a phenotype observed in conditions of enhanced cytokinin signaling, such as in the aph6 mutant, that shifts the auxin-cytokinin balance toward reduced auxin signaling at the protoxylem position (Mähönen et al., 2006; Bishopp et al., 2011). The loss of protoxylem in 35S::IAA30 roots is accompanied by the reduced expression of a number of auxin-induced genes, and the xylem phenotype is similar to the weak loss-of-function mpS519 allele.

Also, the expression levels of the HD-ZIP III genes were down-regulated in the 35S::IAA30 line, consistent with auxin signaling being required for their proper expression. However, the root vascular phenotypic effect of 35S::IAA30 is more similar to phenotypes resulting from PHB or CNA gain of function than from HD-ZIP III loss of function. Furthermore, overexpression of IAA20 and IAA30 resulted in terminated root meristems (Sato and Yamamoto, 2008a), a phenotype also observed in 1-week-old phb-7d plants (Sebastian et al., 2015; A.E. Valdés, C.J. Müller, and A. Carlsbecker, unpublished data). We also note that the shoot phenotype of IAA20/IAA30-overexpressing plants shares many traits with phb-7d, including stunted growth and small rosettes (Sato and Yamamoto, 2008a). These similarities together suggest that the phb-7d mutant phenotype could at least in part be caused by elevated IAA20/IAA30 expression, a notion supported by the partial suppression of the phb-7d xylem phenotypes when combined with a loss of IAA30. It will be interesting to also assess if the HD-ZIP III TF-mediated regulation of shoot meristem and leaf development involves the regulation of IAA20/IAA30.

**PHB May Act to Stabilize the Auxin Response within the Xylem Axis through Both IAA20/IAA30 and MP**

As for most AUX/IAAs, auxin is known to rapidly induce the expression of IAA20 and IAA30 (Sato and Yamamoto, 2008a), and it was recently shown that MP is directly responsible for their activation (Krogan et al., 2014). The phenotypic effects generated by the overexpression of IAA20 and IAA30 are likely caused in part by sequestering of MP, as the introduction of a version of MP unable to interact with AUX/IAAs can partially suppress the strong phenotypes of a 35S::IAA20 line (Krogan et al., 2014). Hence, if IAA20 and IAA30 are dependent on MP for expression, while they also directly suppress the activity of MP, this generates a feedback system keeping these stable repressors to a certain level in the cell. We show here that this model is likely too simplistic and that HD-ZIP III TFs feed in and affect the levels of not only IAA20 and IAA30 but also MP. We found that the induction of miR165 suppressed the expression of MP, slightly but consistently, and that root meristems of most double loss-of-function HD-ZIP III mutants including various combinations with athb8 had a substantial reduction in MP expression, suggesting that MP expression is positively regulated by one or more of the HD-ZIP III TFs. The transcriptional reporter of MP in the root initially expresses in both xylem and neighboring procambium, while shoot ward, MP becomes more xylem specific. It is conceivable that the HD-ZIP III-mediated enhanced activation of MP contributes to its focusing to the xylem subdomain of the procambium. Such a positive feedback loop, if active in the leaf, may further focus the narrow activity domain of ATHB8 within the wider MP expression domain observed during procambium development in leaves (Donner et al., 2009).

Through ChIP, we found evidence that PHB is associated with the promoters of both MP and IAA20. However, induction of PHB was not sufficient to
activate the expression of \( IAA20 \), and expression of \( MP \) and \( IAA30 \) was elevated only after several hours of induction. These results suggest that PHB, likely together with other HD-ZIP III TFs, is required at the \( MP \) and \( IAA20 \) promoters for their full activation, but PHB is not sufficient to activate their transcription without additional factor(s). In an attempt to assess the combinatorial effects of the HD-ZIP III TFs, we analyzed the expression of \( MP \), \( IAA20 \), and \( IAA30 \) in various multiple loss-of-function mutants. Assessments of all possible double mutant combinations of \( ATHB8 \), \( CNA \), \( PHB \), and \( PHV \) revealed a potentially complex interaction between these factors in the regulation of \( MP \), \( IAA20 \), and \( IAA30 \), where in certain combinations they even appeared to act oppositely to the effect of HD-ZIP III down-regulation by miR165 induction (Supplemental Table S5). It is conceivable that this is, in part, a consequence of the evident cross-regulation observed between the HD-ZIP III gene family as indicated by our array results, where \( ATHB8 \) expression was up-regulated in the \( cna phb phiw \) mutant and \( REV \) was up-regulated in the \( athb8 cna phb phiw \) mutant. These results indicate that the molecular nature of the putative combinatorial action of the HD-ZIP III TFs is particularly complex and deserves further attention.

Potentially, the role for PHB and additional HD-ZIP III TFs is to facilitate auxin activation through \( MP \) (Lau et al., 2011; Krogan et al., 2014). We note that binding of PHB to the \( IAA20 \) promoter appears close to the auxin response element at \(-200\) bp, which previously was shown to attract MP (Krogan et al., 2014), and close to several auxin response elements within the \(-500\)-bp region of the MP promoter (Lau et al., 2011). Recently, a role for HD-ZIP III TFs in promoting auxin activation at the promoter of \( ACL5 \) was shown (Baima et al., 2014). It is plausible that HD-ZIP III TFs play a similar role in the context of the \( MP \) and \( IAA20 \) promoters.

We hypothesize that, through their promotion of \( MP \), but also its inhibitors \( IAA20 \) and \( IAA30 \), the HD-ZIP III TFs may add an additional layer of auxin response regulation. Phenotypic analyses upon the perturbation of \( MP \), \( IAA20 \), or \( IAA30 \) suggest that a delicate balance of these factors is required to maintain the normal xylem pattern of the Arabidopsis root. Thus, the role of HD-ZIP III TFs in this context may be to keep a moderate level of auxin response in the vasculature, allowing proper patterning and differentiation of the various cell types. Given that various environmental conditions may strongly affect auxin levels and signaling in the plant (Park et al., 2011; Kazan, 2013), it will be interesting to investigate if the observed interplay of HD-ZIP III TFs and auxin response factors affects the plant’s ability to respond to and adapt to changing environmental stress conditions.

**CONCLUSION**

We have shown previously that HD-ZIP III TFs determine xylem patterning in the Arabidopsis root. However, the mechanisms for this have been elusive. Here, we provide data to show that HD-ZIP III TFs affect xylem patterning through their direct influence on the auxin signaling components \( IAA20 \), \( IAA30 \), and \( MP \). Based on the numerous levels of interaction, we propose a model in which the HD-ZIP III factors would activate \( IAA20/IAA30 \) and \( MP \), while \( IAA20/IAA30 \) interacts with \( MP \) to repress its function in a loop that we show is ultimately vital for correct vascular patterning in the root. These results present novel direct targets of PHB regulation and sandwich the HD-ZIP III factors both upstream and downstream of auxin signaling.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

All Arabidopsis (Arabidopsis thaliana) seeds used in this study were surface sterilized (10% [v/v] commercial bleach with 0.15% [v/v] SDS for 15 min, then 70% [v/v] ethanol for 1 min), plated on square plates containing 0.5% Murashige and Skoog (MS) medium, with 1% (w/v) Suc and 1% (w/v) plant agar or 1% (w/v) Phytagel, and grown vertically in a Sanyo MLR growth cabinet at 22°C with 18 h of light and 6 h of dark (145–150 μmol m−2 s−1). The following alleles have been described previously: \( athb11 \), \( cna-2 \), \( phiw-13 \), and \( phiw-11 \) in the Col-0 ecotype (Prigge et al., 2005), \( phb-7d \) in the C24 ecotype (Carlebecker et al., 2010), \( icv1-1 \) in the En-2 ecotype (Ochando et al., 2006), \( mp39-2 \) (Cole et al., 2009), and \( iaa30-1 \) in the Col-0 ecotype (Zheng et al., 2009). G1T3391 (here renamed as \( iaa20-1 \)) and ET8075 (here renamed as \( iaa20-2 \)) are gene trap and enhancer trap lines, respectively, and were obtained from Cold Spring Harbor Laboratory. The \( iaa20-2 \) \( iaa30-1 \) and \( iaa30-1 \) \( phiw-7d \) double mutants were generated by crossing. The resulting \( Col-0 \times \) ler and \( Col-2 \times \) Col-0 controls as well as \( phb-7d \) (C24 \( \times \) Col-0) were used when comparing phenotypes. The following transgenic lines have been described elsewhere: \( DR5:GFP \) (Frimal et al., 2003), \( DRE:GUS \) (Ulmason et al., 1997), \( pIAA2:GUS:GFP \) (Bishopp et al., 2011), \( pAHpH:GFP \) (Mähönen et al., 2006), \( 35S:IAA30 \) (Sato and Yamamoto, 2008a), \( pAA30:3GFP \) (Rademacher et al., 2012), \( pMP:3C0:GFP \) (Lau et al., 2011), and \( pPBH:PBH::GFP \) (Dello Ioso et al., 2012). The \( pERGWIAA30 \) line is from the TRANSPLANTA collection (Coego et al., 2014) and has identifier number TPT_2,46990.1D. Transgenes were introduced into mutants by crossing, unless noted otherwise.

Seedsling were grown on 25-μm pore Sefar Nites 03-25/19 mesh (Sefar) placed on the agar for inductions and RNA collection, facilitating seedling transfer and providing resistance for root-tip dissection. 17-β-Estradiol stock was prepared in ethanol and added to the medium at a final concentration of 5 μM, which resulted in the equivalent volume of ethanol only. For hormone treatments and inductions, plants were germinated on MS plates containing 100 nM 1-naphthaleneacetic acid, 1 μM IAA, or 5 μM 17-β-estradiol for the time specified. All seedlings were analyzed at 4 d post germination (dpg) unless noted otherwise.

**Plant Transformation and Genotyping**

The inducible system used is based on pER8, an estradiol receptor-based chemical-inducible system (Zuo et al., 2000) modified to become Gateway compatible by Vatén et al. (2011). The \( CRE1 \) promoter sequence (Bonke et al., 2003) and the \( MIR165A \) stem-loop sequence (Carlsbecker et al., 2010) were amplified from genomic wild-type DNA and cloned into pDONR221 (Life Technologies). The \( phb-7d \) and \( iaa30-1 \) constructs were cloned using MultiSite Gateway LR recombination (Life Technologies) into pGBK7 (GFP) (Mähönen et al., 2008b), p35S:IAA30 (Sato and Yamamoto, 2008a), pAA30:3GFP (Rademacher et al., 2012), and pPBH:PBH::GFP (Dello Ioso et al., 2012). The \( pPBH:GFP:ER8 \) construct (Bishopp et al., 2011) was transformed into the \( athb11 \) \( cna-2 \) \( phiw-13 \) \( phiw-11 \) mutant using the same method.

Genotyping the HD-ZIP III loss-of-function mutants was done as described previously (Prigge et al., 2005). The \( mp39-2 \) \( iaa20-1 \), \( iaa30-1 \), and \( iaa20-2 \) alleles were genotyped using gene-specific and transfer DNA-specific primers. \( phb-7d \) was genotyped by PCR with gene-specific primers and a subsequent digestion with \( HpaII \), which cleaves the wild-type \( PHB \) allele but not \( phb-7d \), due to the
C-to-T substitution at base pair 1,462. All primers used in this study can be found in Supplemental Table S6.

**Histochemical and Histological Analyses**

To visualize xylem, roots were either stained with basic fuchsin as described (Mähönen et al., 2000) or mounted directly in 8:2:1 (w/v/v) chloral hydrate:glycerol:water. Histochemical Gus staining was performed as described (Jefferson et al., 1987; Miyawaki et al., 2004). For stele cell quantification, roots were embedded as described (Mähönen et al., 2000), sectioned at 5 μm with a motorized microtome (Microm; HM350), and stained with 0.5% Toluidine Blue. For venation width analysis, cotyledons of 5-dpg seedlings were cleared with 8:2:1 (w/v/v) chloral hydrate:glycerol:water, and measurements were taken right before the first vein bifurcation in the cotyledon blade (Supplemental Materials and Methods S1).

Light microscopy was performed with a Zeiss Axioscope microscope at 20× or 40× magnification and imaged using differential interference contrast optics with a DFC-295 (Leica) camera. Confocal imaging was performed using a Leica SPS 5 with solid-state argon laser (488 nm) or a Zeiss 780 inverted Axio Observer with supersensitive GaAsP detector, and cell walls were counterstained with 100 μg mL⁻¹ propidium iodide. Images were processed in Photoshop (Adobe), and any changes in brightness and contrast were the over the whole image and applied equally to all compared images.

**RNA Analyses**

Root tips (0.5 mm) were hand dissected from 4-dpg seedlings and collected in RLT buffer from the Plant ReNaSy Kit (Qiagen) containing β-mercaptoethanol according to kit instructions. About 200 root tips were collected per replicate. Tissue was immediately homogenized with glass beads using a Fastprep homogenizer (Savant), and RNA extraction including on-column RNase-free DNase digestion (Qiagen) was performed following the manufacturer’s protocol. RNA quantity was calculated using the BR RNA Assay Kit with Qubit 2.0 (Life Technologies). cDNA was synthesized with the iScript cDNA Synthesis Kit (Bio-Rad) using 1 μg of total RNA following the manufacturer’s instructions. qPCR was carried out using iQ SYBR Green Supermix (Bio-Rad) and gene-specific primer pairs in each run. Gene normalization was done according to Pfaffl (2001). Two previously described normalization genes, ADENINE PHOSPHORIBOSYL TRANSFERASE1 (2001). Two previously described normalization genes, ADENINE PHOSPHORIBOSYL TRANSFERASE1 and PROTEIN PHOSPHATASE2A SUBUNIT A3 (Czechowski et al., 2005; Gutierrez et al., 2008), were assessed and used as qPCR normalization controls, as they displayed unaltered patterns of expression according to kit instructions. About 200 root tips were collected per replicate. The raw data were normalized in the free software Expression Console (Affymetrix) using BioarraySuite (www.bioarraysuite.com) using the robust multi-array average method (Li and Wong, 2001; Irizarry et al., 2003). Subsequent analysis of the gene expression data was carried out in R (http://www.r-project.org) using Bioconductor packages (www.bioconductor.org). To search for differentially expressed genes between the different biological replicate groups within each experiment, an empirical Bayes-moderated Student’s t test was then applied (Smyth, 2004) using the limma package (Smyth et al., 2005). To address the problem with multiple testing, the P values were adjusted using the method of Benjamini and Hochberg (1995).

The microarray data are deposited into the Gene Expression Omnibus repository under accession number GSE75612.

**ChiP and qPCR**

ChiP was performed following Bowler et al. (2004) as detailed previously by Valdes et al. (2012). Briefly, 4-d-old seedlings from pPHB::PHB::GFP transgenic plants were freeze-dried with formaldehyde at 1% (v/v), and nuclei were extracted. After lysis of the nuclei, DNA was sheared by sonication to approximately 0.2- to 1-kb fragments, which were later precleared by incubation with protein A-agarose beads. A sample fraction corresponding approximately to 15% of total chromatin was kept as input (positive control for the presence of chromatin). The rest was divided into two equal fractions: the mock sample that was immunoprecipitated with a normal rabbit IgG antibody, acting as a negative control to determine the background signal level, and the ChiP sample, which was immunoprecipitated with a GFP-specific antibody. DNA-protein complexes were eluted, RNaseA treated, and reverse cross-linked. After protein degradation by proteinase K treatment, DNA was phenol purified and resuspended in 10 μl Tris-HCl buffer. Immunoprecipitations were done in at least three independent biological replicates.

The purified DNA was analyzed by qPCR using the SYBR Green detection system (Bio-Rad). One microliter of ChiP and mock DNA per reaction was used, running in parallel with a serial input DNA dilution (1, 1.25, and 1.125). qPCR conditions were 95°C for 3 min; 40 cycles of 95°C for 10 s, 57°C for 15 s, and 72°C for 10 s; followed by a melting curve analysis. Cycle threshold values were obtained in each case, and a standard curve for each primer pair was obtained based on the values of the input serial dilution. The amount of ChiP DNA (arbitrary units) was inferred from the curve for each analyzed region: two negative binding control regions for the non-HD-ZIP III targets ABA INSENSITIVE2 (ABIZ) and ORNITHINE CARBOXYLYTRANSFERASE (OTC), and at least two upstream promoter regions within each putative target, MP, IAA20, and IAA30. Data normalization was done relative to negative control sequences (Haring et al., 2007) using the mean value of their absolute signal as a threshold, and the fold enrichment for the regions of interest were calculated by dividing their absolute signals by the mean signal of negative regions. All reactions on each PCR plate were performed in duplicate at minimum. At least three independent biological replicates were used.

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Characterization of the effect of pCRE1::MIR165A induction.

**Supplemental Figure S2.** HD-ZIP III mutants have altered expression of auxin response reporters.

**Supplemental Figure S3.** ChiP indicates that PHB is not localized to the promoter of IAA30.

**Supplemental Figure S4.** Individual biological replicates for the ChiP experiments.

**Supplemental Figure S5.** Characterization of the pCRE1::MIR165A::gfp-1d transgenic line.

**Supplemental Figure S6.** The CNA gain-of-function icd-1 mutant affects vascular patterning and the expression of auxin signaling genes similarly to phb-7d.

**Supplemental Figure S7.** IAA20 and IAA30 affect xylem patterning.

**Supplemental Table S1.** Comparison of gene expression in 0.5-mm root tips of Arabidopsis upon induction of pCRE1::MIR165A.

**Supplemental Table S2.** Comparison of gene expression between athh8 cna phb phe and the wild type in 0.5-mm root tips of Arabidopsis.

**Supplemental Table S3.** Comparison of gene expression between cna phb phe, cna phb phe rev, and the wild type in 0.5-mm root tips of Arabidopsis.
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