Regulation of the *Arabidopsis* root vascular initial population by *LONESOME HIGHWAY*

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Complex organisms consist of a multitude of cell types arranged in a precise spatial relation to each other. *Arabidopsis* roots generally exhibit radial tissue organization; however, within a tissue layer, cells are not identical. Specific vascular cell types are arranged in diametrically opposed longitudinal files that maximize the distance between them and create a bilaterally symmetric (diarch) root. Mutations in the *LONESOME HIGHWAY* (*LHW*) gene eliminate bilateral symmetry and reduce the number of cells in the center of the root, resulting in roots with only single xylem and phloem poles. *LHW* does not appear to be required for the creation of any specific cell type, but coordinately controls the number of all vascular cell types by regulating the size of the pool of cells from which they arise. We cloned *LHW* and found that it encodes a protein with weak sequence similarity to basic helix-loop-helix (bHLH)-domain proteins. *LHW* is a transcriptional activator in vitro. In plants, *LHW* is nuclear-localized and is expressed in the root meristems, where we hypothesize it acts independently of other known root-patterning genes to promote the production of stele cells, but might also indirectly feed into established regulatory networks for the maintenance of the root meristem.

**KEY WORDS:** *Arabidopsis*, Root pattern, Symmetry, Vasculature

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**INTRODUCTION**

Multicellular organisms must coordinate division and expansion of the constituent cell types of each tissue to ensure organized development. Plants develop via the activity of continuously dividing and self-renewing populations of cells called meristems. Two major meristematic populations, the shoot apical meristem (SAM) and root meristem (RM), are formed in the embryo, and generate and pattern the bulk of the above- and below-ground portions of the plant, respectively. Other populations, however, also generate new cells; these include shoot auxiliary meristems, lateral root meristems and dispersed groups of cells such as stomatal meristemoids. Each of these populations maintains a constant size during normal development, requiring that cell division and the production of differentiated offspring are tightly controlled.

The RM has a stem-cell population that divides asymmetrically to create each of the tissue layers. Cells in this population (initial cells) maintain competence to divide by their proximity to the quiescent center (QC), which is specified by the coordinate activity of the hormone auxin, as mediated via the AP2 class transcription factors PLETHORA1 (PLT1) and PLT2 (Aida et al., 2004), and the GRAS family transcription factors SCARECROW (SCR) and SHORTROOT (SHR) (Sabatini et al., 2003). Although the SHR protein is a transcription factor, it also serves as a positional cue by virtue of its regulated movement from the center of the root to the neighboring cell layers, where it activates SCR (Nakajima et al., 2001). Downstream of these regulators that position the root stem cells, the *Arabidopsis* homolog of the Retinoblastoma gene, *RETINOBLASTOMA-RELATED* (*RBR1*), appears to behave similarly to its animal counterparts in repressing cell divisions within the stem cell population (Wildwater et al., 2005). Although it appears that initial cells for each of the different tissue types are regulated by this common pathway, very little is known about the initial cells for central tissues in the root.

These central tissues are collectively referred to as the stele. Clonal analysis of *Arabidopsis* embryos has indicated that all of the tissues of the stele – the pericycle, vascular elements (xylem and phloem) and some ground tissue – share a common origin (Dolan et al., 1993; Kidner et al., 2000). When viewed in cross-section, the tissues in the stele exhibit a stereotyped, species-specific arrangement. The small *Arabidopsis* root invariably has two xylem poles diametrically opposed (diarch; Fig. 1A), whereas the roots of other plants, such as wild-grown radish, can vary from being diarch through to heptarch (reviewed in Turner and Sieburth, 2002). Lateral roots are produced by postembryonic divisions in the pericycle. In the roots of many species, including *Arabidopsis*, only the pericycle cells adjacent to the xylem poles are capable of initiating laterals. This leads to a predictable pattern of root growth somewhat analogous to the arrangement of organs in the shoot known as phyllotaxis.

Several genes and growth regulators have been implicated in root vascular development. *ALTERED PHLOEM DEVELOPMENT* (*APL*) encodes a MYB transcription factor required for the production of phloem. In the absence of *APL*, crucial proliferative divisions in the vascular cylinder do not take place and the phloem is not specified (Bonke et al., 2003). *WOODEN LEG* (*WOL*, also known as *CRE1* or *AHK4*) is also required for proliferation of the vascular cylinder. Plants homozygous for the *wol-I* mutation have fewer cells in the stele and fail to produce phloem (Mahonen et al., 2006a; Scheres et al., 1995). *WOL* encodes a histidine kinase that functions in cytokinin response (Inoue et al., 2001; Mahonen et al., 2000). Further work with this kinase family, as well as classic physiology experiments, has implicated cytokinins in the control of cell proliferation and cell fate in both shoot and root vascular development (de Leon et al., 2004; Higuchi et al., 2004; Mahonen et al., 2006a; Mahonen et al., 2006b; Nishimura et al., 2004).

In this study, we identify a new locus, *LONESOME HIGHWAY* (*LHW*), that is required to establish and maintain the normal vascular cell number and pattern in primary and lateral roots. Using a map-
based cloning approach, we identified the LHW gene and found that it defines the first member of a clade of plant-specific genes. Further characterization of protein localization and activity suggests that LHW encodes a transcriptional activator, suggesting that LHW plays a regulatory role in establishing a ‘set point’ for the radial extent of the root vascular population.

MATERIALS AND METHODS

Screen
An ethylmethane-sulfonate (EMS)-mutagenized population of approximately 4000 M1s was created from plants homozygous for the enhancer trap J0121::GFP (ABRC stock CS9090, C24 ecotype) using standard Arabidopsis mutagenesis procedures. Approximately 48,000 roots of 5-day-old M2 seedlings were screened for alterations in J0121::GFP pattern. All lines were backcrossed at least twice before further analysis. Via backcrosses and complementation crosses, five mutations that resulted in the presence of a single J0121::GFP stripe were found to be recessive to wild-type and allelic to each other. The locus defined by mutations w305, w279, w130, w123 and w116 was designated LONESOME HIGHWAY, and the mutant alleles renamed lhw-1 through to lhw-5, respectively. All LHW mutants were also crossed to Landsberg erecta (Ler) to establish mapping populations.

Phenotypic characterization
Markers of cell fate used were: SCR::GFP (gift of J. Long, SALK, San Diego, CA), AP3proAPL::GFP (Bonke et al., 2003), QC25::GUS (gift of B. Scheres, University of Utrecht, The Netherlands), J0121::GFP (ABRC stock CS9090), Q1630::GFP (ABRC stock CS9277), VH1::GUS (Clay and Nelson, 2002), DR5::GUS (Ulmasov et al., 1997) and CYCB1;1::GUS (Colon-Carmona et al., 1999; Donnelly et al., 1999). Unless otherwise indicated, the wild-type control for experiments with J01-1 and J01-2 is the unmutagenized parental line CS9090 (C24 ecotype). Seedlings were grown vertically on plates containing 0.5×MS, 1% agar. Expression of GFP markers was analyzed on a Bio-Rad 1024 confocal microscope, with propidium iodide counterstaining to observe cell morphology. Xylem was visualized by staining with 0.01% basic fuchsin. Root cross sections were prepared according to Scheres et al. (1995). Growth curves were performed by marking root lengths on the underside of plates every 24 hours during the growth of lhw and control parental plants grown side-by-side. Auxin analogue 2,4-dichlorophenoxyacetic acid (2,4D) and cytokinin (kinetin) effects on primary root growth were assayed at 5 days post germination (dpg). Seedlings grown on plates containing 20 µM 1-Naphthylphthalamic acid (NPA) were scored at 7 dpf for rescue and at 21 dpf for terminal phenotypes. Images were processed for figures using Adobe Photoshop consistent with guidelines for image manipulation specified in the instructions for authors.

Map-based cloning of LHW
All alleles were individually mapped using a standard set of PCR-based mapping primers (Lukowitz et al., 2000). Recombinants between CER459215 and CER460427 were identified from approximately 800 F2 individuals from a mapping outcross of lhw-1 and scored for alterations in the pattern of this marker at 5-7 days post germination. A screen of approximately 800,000 colonies was performed using the LHW HHL domain and C-terminus (DB-bC) as bait and a prey library in pACT constructed by Kim and Theologis (ABRC stock CD4-22). Positive colonies were tested to ensure a single plasmid was responsible for the interaction, sequenced, and then retransformed into a strain containing prey for confirmation of the interaction. Quantitative analysis of β-galactosidase (β-gal) expression was performed by transforming LHW variants into the yeast strain Y187 and following procedures in Clontech’s yeast protocol guide.

Expression studies
Total RNA for semi-quantitative RT-PCR was isolated from plant tissues using a micro-midi RNA isolation kit (Invitrogen). RNA (100 ng) was used in first-strand synthesis with superscript III (Invitrogen), followed by PCR with the gene-specific primers (shown 5′-3′) lhwrtf1, GATCGT-GTCAAAGAGGCTGCG and lhwrt2, TTCGAAAGCCCATGTTGCTCC, and control primers actinF, GGCGATGAAGCTCAATCCAAACG and actinR, GGTCAGCAAGCCAGATCAAGACG. LHW and ACT were amplified for 32 and 25 cycles, respectively, for 15 seconds at 95°C, 30 seconds at 52°C and 1 minute at 68°C. A β-glucuronidase (GUS) reporter for LHW expression was created by PCR amplifying 2.8 kb of genomic sequence 5′ of the translational start site and cloning the piece into pCAMBIA 1303. Subcellular localization was determined by cloning the LHW cDNA from the translational start to one codon before the translational stop into pEZN (Cutler et al., 2000). Constructs were introduced into Arabidopsis plants via Agrobacterium-mediated transformation (Clough and Bent, 1998).

RESULTS

Identification of LONESOME HIGHWAY
To identify novel genes required for root cell fate specification, we screened for mutations that cause cell-identity defects within the seedling stele. The screen was facilitated by the use of the enhancer trap line J0121 to specifically mark xylem-adjacent pericycle cells (Laplaze et al., 2005). In wild-type roots, two J0121::GFP-positive stripes of cells became visible in the elongation zone and extended to the root-hypocotyl junction (Fig. 1B). Seedling roots were screened for alterations in the pattern of this marker at 5-7 days post germination (dpg). Five completely recessive and allelic (see Materials and methods) mutations were found that resulted in plants expressing J0121::GFP in only a single stripe (Fig. 1C and see Fig. S1 in the supplementary material). These five alleles define a new locus, LONESOME HIGHWAY (LHW). The absence of GFP expression correlates with a change in xylem-adjacent pericycle cell identity and/or function, as seen by the production of lateral roots from one side of the primary root only (Fig. 1D).

Phenotypic analysis of lhw defects
Closer examination of lhw roots revealed that the bilaterally symmetric (diarch) organization of the stele was reduced to a monarch arrangement. In wild-type plants, two protoxylem strands ran the length of the root (Fig. 1E). In lhw, only one protoxylem strand was observed and, in most cases (34/40), was displaced from the center of the root. J0121::GFP expression was always adjacent to the single remaining xylem strand. In mature parts of the root, 2-5 files of metaxylem elements are normally found between the two protoxylem poles (Mahonen et al., 2000). In lhw plants, cells with the morphological characteristics of

Yeast two-hybrid assay and screen
LHW and other clones were PCR amplified from cDNA clones or by reverse transcriptase (RT)-PCR and cloned into the Clontech Matchmaker vectors pGBK (bait) and pGAD (prey). Saccharomyces cerevisiae strains AH109 or Y187 were used as hosts. Bait clones were tested for transcriptional auto-activation by co-transformation with an empty prey vector. Direct interactions between plasmids were tested by retransformation of plasmids in pairwise comparisons. A screen of approximately 800,000 colonies was performed using the LHW HHL domain and C-terminus (DB-bC) as bait and a prey library in pACT constructed by Kim and Theologis (ABRC stock CD4-22). Positive clones were tested to ensure a single plasmid was responsible for the interaction, sequenced, and then retransformed into a strain containing prey for confirmation of the interaction. Quantitative analysis of β-galactosidase (β-gal) expression was performed by transforming LHW variants into the yeast strain Y187 and following procedures in Clontech’s yeast protocol guide.
metaxylem were made, and they had the same spatial relationship to the protoxylem pole, but there appeared to be only half as many metaxylem cells (Fig. 1F and Fig. 2). In addition to two xylem poles, Arabidopsis roots normally have two phloem poles. Phloem organization can be visualized by APLpro::APL-GFP expression (Bonke et al., 2003). In wild-type root tips, APLpro::APL-GFP was seen in nuclei of two cell files, corresponding to maturing protophloem (Fig. 1G). In lhw, only a single APLpro::APL-GFP-marked file was visible (Fig. 1H). Despite the reduced cell number in the root vasculature, lhw plants were healthy and fertile. Plants with mutations in LHW did not exhibit dramatically altered phyllotaxis, nor did they have any gross morphological abnormalities in their leaf and floral organs (Fig. 1I,J). lhw mutations in the C24 background led to plants that were slightly abnormal in their leaf and floral organs (Fig. 1I,J). In lhw, vein development was delayed relative to wild type (Fig. 1L,M); and xylem gaps were still visible in the mature organs (Fig. 1N,O); however, leaf venation patterns appeared normal (data not shown).

The root vasculature phenotypes suggest that lhw does not have a defect in the production of any specific differentiated cell type, but that LHW is required to produce the normal arrangement and number of these cell types. In dicot roots, there is a strong correlation between the size of the stele and the number of xylem poles, and the experimental manipulation of cell number in some dicot roots leads to variation in vascular pole number (Torrey, 1955). In Arabidopsis primary roots, the stele is usually comprised of 12-13 pericycle cells (Dolan et al., 1993) and 25-28 internal cells at stages when mature xylem and phloem elements are found (Dolan et al., 1993) (Fig. 2F). To determine the number of cells in the lhw stele, we made cross-sections of roots from the level of the meristem (Fig. 2B,C) through the mature zone (where root hairs are visible; Fig. 2H,I) and up into the hypocotyl (Fig. 2L,K). In cross-sections of a wild-type root (30 μm above tip), the epidermis consisted of approximately 25 cells, the cortex and endodermal layers each consisted of 8 cells, and the stele (pericycle, xylem and phloem) consisted of approximately 33 cells (Table 1). In lhw roots, the normal number of cortex and endodermal cells was present and the epidermal number was slightly reduced, but the number of cells in the lhw stele was reduced to half as many as wild type (Fig. 2; Table 1). This affected all cell types in the stele; in addition to the reduction in cells from which the xylem and phloem arise, the lhw pericycle was reduced from the normal 13 cells to 8 cells (Fig. 2; Table 1).

The total number of cells in the seedling stele is a product of the initial pool in the embryo and cells created through postembryonic divisions. The number of stele cells visible in a cross-section of the lhw root at 30 μm and 120 μm is virtually unchanged (Table 1), suggesting that postembryonic divisions rarely occurred. In the embryo, the stele is derived from the uppermost tier of the RM (Dolan et al., 1993). Early embryogenesis in lhw was indistinguishable from wild type in terms of orientation of cell divisions (see Fig. S2A,B in the supplementary material). The only defect seen at a significant frequency (3/15 lhw) was a delay relative to wild type in divisions in the base of the embryo, in cells that would later become the RM (compare Fig. S1C with Fig. S1D, and Fig. S1E,G with Fig. S1F,H in the supplementary material). At the torpedo stage, lhw embryos appeared to have a well-formed vascular cylinder, but it was narrower in lhw than in wild type (compare Fig. S1J with Fig. S1I in the supplementary material).
Because lhw mutants still made some lateral roots, we could examine the organization of these postembryonically formed organs. Arabidopsis lateral roots originate from a stereotyped series of divisions in three pericycle cell files adjacent to a xylem pole. Lateral roots normally have the same tissue organization as primary roots, although control over the number of cells in the cortex and endodermis is somewhat relaxed (Dolan et al., 1993). Despite early division patterns that were indistinguishable between wild type and lhw (see Fig. S1K-P in the supplementary material), lhw lateral roots generated only a single protoxylem pole (100%, n=40), a single APLpro::APL-GFP-marked phloem pole (100%, n=20) and a single J0121::GFP-marked xylem-adjacent pericycle file (97%, n=40), suggesting that LHW is required to establish normal cell numbers in the stele of these organs.

The relationship between LHW and auxin
Defects in lateral root formation and xylem differentiation suggest that lhw might have defects in auxin synthesis, transport or perception. However, lhw mutants did respond to exogenous auxins (IAA and 2,4D) by producing root hairs and lateral roots and inhibiting primary root elongation (see Fig. S3 in the supplementary material), and data not shown), yet these auxin treatments did not rescue the xylem or pericycle defects (Fig. 3C and data not shown). We visualized local auxin response near the RM by scoring the expression pattern and intensity of the markers DR5::GUS and PIN4::GUS (Friml et al., 2002; Ulmasov et al., 1997). In lhw plants, expression of both markers was similar to wild type in intensity and in position of the maximum (compare Fig. 3G with Fig. 3F, and Fig. 3I with Fig. 3H).

Germination and growth on media containing the auxin-transport inhibitor NPA can lead to excessive RM proliferation and xylem production (Mattsson et al., 1999). lhw and wild-type plants grown on MS agar plates containing 20 µM NPA were sampled at 7 and 21 dpg for xylem vessel formation and for expression of the J0121::GFP marker in roots. At neither time-point was expression of J0121::GFP seen in two stripes, nor was the second xylem pole restored in lhw plants (compare Fig. 3B with Fig. 3A, and data not shown). Morphology of the root tip was strikingly different between wild type and lhw at 21 dpg. In wild type, the roots became extensively fasciated and produced eight to ten xylem files (Fig. 3D). The lhw root tips were only slightly wider than untreated roots, failed to undergo excess cell proliferation and never produced more than a single differentiated xylem cell file (Fig. 3E). These data indicate that, although lhw plants appear to

Table 1. Cell numbers in the primary root

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total stele</th>
<th>Pericycle</th>
<th>Inside</th>
<th>Total stele</th>
<th>Pericycle</th>
<th>Inside</th>
<th>Total stele</th>
<th>Pericycle</th>
<th>Inside</th>
<th>Endodermis</th>
<th>Cortex</th>
<th>epidermis</th>
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</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>33</td>
<td>11.5</td>
<td>21.5</td>
<td>41±1.0</td>
<td>13.3±1.5</td>
<td>27.67±1.5</td>
<td>41.67±0.38</td>
<td>13±0.0</td>
<td>28±0.5</td>
<td>8±0.0</td>
<td>8±0.0</td>
<td>24.3±2.1</td>
</tr>
<tr>
<td>lhw-1</td>
<td>19±1.7</td>
<td>8.5±0.55</td>
<td>10.5±1.5</td>
<td>20.3±1.5</td>
<td>8.5±0.55</td>
<td>11.8±1.2</td>
<td>21.8±1.6</td>
<td>8.3±1.2</td>
<td>13.1±1.5</td>
<td>8±0.0</td>
<td>8±0.0</td>
<td>20.6±3.6</td>
</tr>
<tr>
<td>wol-1</td>
<td>16</td>
<td>8</td>
<td>8</td>
<td>19±0.9</td>
<td>9±0.9</td>
<td>10±0.9</td>
<td>15.3±2.9**</td>
<td>8±1.0</td>
<td>7±1.0</td>
<td>7.3±3.2</td>
<td></td>
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</tr>
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</table>

Comparison of cell numbers in the stele of wild-type, lhw and other mutant roots. Number of roots scored/genotype: wild type=3, lhw-1=8, wol-1=2, lhw-1; wol-1=3. Values are averages ±s.d. P values for difference between number of cells in lhw and lhw; wol stele: *P=0.08; **P=0.04. Lengths represent the distance from the root tip. ‘Inside’ refers to all cells interior to the ring of pericycle cells.
perceive auxin and respond in terms of primary root inhibition and production of root hairs, they are unable to respond to auxin in the formation of xylem and pericycle. The simplest explanation for these phenotypes is that LHW is not a core component of auxin signaling but that lhw mutants are defective in a downstream process.

The relationship between LHW and the cytokinin receptor WOL
Cytokinins, like auxin, are required for longitudinal proliferation in the root, but cytokinins also have significant roles in radial proliferation (Ferreira and Kieber, 2005; Mahonen et al., 2006a). The wol-1 mutation in the WOL gene, encoding a cytokinin receptor, severely reduces cell proliferation in the stele (Mahonen et al., 2000). We tested whether WOL and LHW acted in the same genetic pathway by constructing double mutants between the two. wol-1 roots are short and the number of cells interior to the pericycle is reduced to less than ten, all of which become xylem (Mahonen et al., 2000) (Table 1). Double mutants between lhw and wol-1 exhibited the root-length defect of wol-1; however, the presence of the wol-1 mutation further reduces the number of cell in the lhw stele (Fig. 2L-O; Table 1), suggesting that LHW promotes the production of stele cells in a somewhat WOL-independent manner. In addition to defects in cell proliferation, wol-1 mutations eliminated phloem production and resulted in a stele consisting solely of protoxylem. In terms of cell identity, the wol-1 mutation was epistatic to lhw, because the interior of the lhw;wol-1 root resembled wol-1. The presence of multiple xylem poles in this double mutant indicates that there is no explicit requirement for LHW in the production of this cell type.

LHW encodes a member of a novel, plant-specific, family of proteins
LHW appears to play a central role in defining the number of stele cells. In the root, a variety of biochemical functions have been defined by mutational analysis to be required for patterning the RM and balancing cell proliferation and differentiation. These include: core cell cycle regulators, components of cell signaling and hormone perception pathways, and transcriptional regulators (e.g. Aida et al., 2004; Blilou et al., 2002; Bilou et al., 2005; Friml et al., 2002; Mahonen et al., 2006a; Sabatini et al., 2003; Wildwater et al., 2005). To understand how LHW might control root development, we used a map-based cloning approach and found that LHW corresponds to At2g27230, a locus that encodes a protein of 650 amino acids (see Materials and methods). Initial searches of databases with LHW revealed that it was a plant-specific protein of unknown function. LHW is closely related to three other uncharacterized proteins in Arabidopsis (encoded by At1g06150, At1g64625 and At2g31280) and to two proteins in rice (encoded by Os12g06330 and Os11g06010). The highest similarities among these proteins are in an N-terminal and a C-terminal region (Fig. 4B and see Fig. S4 in the supplementary material). Although the N-terminal region does not resemble any domains of known biochemical function, part of the C-terminal domain is weakly similar to basic helix-loop-helix (bHLH) transcription factors (Fig. 4B). Alignments of LHW with typical bHLHs (At1g66470 and At5g37800) revealed that this similarity is most convincing in the predicted dimerization domain (boxed in Fig. 4B); however, the canonical DNA-contacting residues are not conserved in LHW, and LHW was not considered a bHLH by two independent groups conducting comprehensive analyses of the family (Heim et al., 2003; Toledo-Ortiz et al., 2003).

Transcriptional activation and HLH dimerization activity of LHW
Proteins in the bHLH class generally interact with DNA and regulate transcription as dimers. They can partner with a variety of protein classes, including a class of proteins [the inhibitor of differentiation (Id) proteins] that have HLH dimerization domains but that lack a
DNA-binding domain and antagonize bHLH function (Chen et al., 1996). We examined whether LHW had any properties consistent with it acting as a transcriptional regulator and/or interacting with canonical bHLH proteins.

LHW can activate transcription when fused to a DNA-binding domain in a yeast two-hybrid assay (DB-FL; Fig. 4C,D). A series of deletion constructs established that the N-terminus (DB-N) was responsible for this activity (Fig. 4D). Neither the bHLH and C-terminal domain (DB-bC), nor the bHLH domain alone (DB-b1 and DB-b2), could activate transcription. To test whether LHW could homodimerize, a non-auto-activating portion of the protein (DB-bC) was co-transformed with variants of LHW fused to the GAL4 activation domain (AD). LHW (DB-bC) interacted strongly with full-length LHW (AD-FL) and with LHW missing the N-terminus (AD-bC), and weakly with versions of LHW containing only the bHLH domain (AD-b1 and AD-b2) (Fig. 4C).

We then performed a two-hybrid screen using a library made from seedling cDNA (see Materials and methods) to identify other potential partners of LHW. In a screen of 800,000 colonies, 12 prey constructs interacted with LHW (DB-bC) under stringent conditions. Nine of the clones corresponded to four bHLH genes: At5g08130 (five clones); At1g68810 (two clones); At1g29950 (one clone) and At3g25710 (one clone). This suggests that LHW readily binds to typical bHLH proteins. The bHLH proteins that were identified in the two-hybrid screen have not been extensively characterized. However, it is interesting that, in silico, transcripts...
for each of these bHLHs are enriched in root tips or found in xylem cell populations (Birnbaum et al., 2005) (http://bbc.botany.utoronto.ca/efp)

**Expression pattern of LHW**

The defects seen in lhw mutants suggested that LHW would be required in the RM, particularly in the vascular initials. By semi-quantitative reverse transcriptase (RT)-PCR, LHW expression was seen to be highest in the meristematic regions of both the root and shoot, and was lowest in mature tissues (Fig. 5F). A transcriptional reporter, containing 2.8 kb of the sequence 5′ of the LHW start codon fused to GUS, was expressed in the root tip, including, but not exclusively within, the meristem (Fig. 5C-E). This expression pattern is consistent with root transcriptional profiling data that finds At2g27230 to be enriched in stage-1 (closest to the meristem) roots (Birnbaum et al., 2003) and enriched in the QC cell population relative to other cell types (Nawy et al., 2005).

To determine the subcellular localization of LHW, roots of Arabidopsis stably transformed with 35Spro::LHW-GFP were examined. GFP expression was visible in nuclei (Fig. 5A,B). Expression of these constructs in lhw−1 mutant plants was sufficient to rescue the xylem pole defect in T1 plants (Fig. 5B), but expression in wild type did not result in any obvious phenotypes in root length, vasculature or overall plant morphology in T1 plants (data not shown). Silencing of the LHW transgene was often observed in T2 lines as a reduction in GFP expression and by the appearance of a single xylem pole in plants with a wild-type genomic copy of LHW (data not shown).

**Analysis of the requirement for LHW in root meristem maintenance**

Given its identity, activities and expression pattern, we hypothesized that LHW was required in the meristem to promote cell divisions that establish the normal size of the stele. Similar roles are played by transcriptional regulators such as SCR and SHR, which are important for regulating both radial and longitudinal growth. SCR is normally expressed in the QC, in endodermal/cortex initials and in the maturing endodermis of the root (Di Laurenzio et al., 1996). Mosaic analysis revealed that SCR has a cell-autonomous role in maintaining the QC (Heidstra et al., 2004; Sabatini et al., 2003). When we examined the expression of SCRpro::GFP in 7-dpg lhw, we found, unexpectedly, that the reporter was present in QC cells (Fig. 6C), although SCR expression is lost over time.

Loss of SCR expression in the QC is reminiscent of the phenotypes of hobbit (Blilou et al., 2002) and shr (Helariutta et al., 2000) mutants; both HOBBIT and SHR are required for meristem maintenance. The arrangement of SCRpro::GFP-expressing cells in lhw mutants was also similar to that in roots with only endodermal expression of SCR (Sabatini et al., 2003). Because roots lacking SCR in the QC often have a compromised RM (Sabatini et al., 2003), we examined several other markers of ‘meristem health’ in lhw, including the expression of QC identity markers, the longitudinal extent of the zone of proliferation and whether lhw roots exhibited determinate growth.

In wild-type plants, QC25::GUS is expressed specifically in the QC (Sabatini et al., 2003). At 7 dpg, lhw mutants expressed QC25::GUS but, interestingly, the intensity of GUS expression in the QC cells was often asymmetric (14/20 lhw plants versus 0/10 wild type; Fig. 6H,I). The intensity of staining did not appear to be correlated with the side on which the protoxylem formed (data not shown). Despite this asymmetry, at 7 dpg, the lhw RM appeared to have normal proliferative capacity, as assayed by root growth (Fig. 6Q) and by the presence of columella initials. Columella initials are identified as a layer of cells between the QC and the cells expressing the columella marker Q1630::GFP and starch granules (Fig. 6D,E and data not shown). Several groups have used the expression of a mitotic cyclin (CYCB1;1) to measure the longitudinal extent of the proliferative zone (e.g. Aida et al., 2004; Hutchison et al., 2006; Ioio et al., 2007). At 7 dpg, the region of CYCB1;1pro::GUS-expressing cells was similar in wild-type and lhw (Fig. 6F,G; 119 μm±1.6 s.d. in wild type, n=11; 125 μm±1.73 in lhw, n=10). Together, these data suggest that LHW is not required for the establishment of a functioning RM.

Despite the normal early development, maintenance of the lhw RM failed over time. At 13 days, QC25::GUS was still expressed (Fig. 6L versus 6K), but columella initials began to differentiate and contain starch grains (Fig. 6L, star). At 17 dpg, the meristem of lhw roots was visibly disorganized. In contrast to wild-type roots (Fig. 6M,N), lhw roots exhibited a variety of defects, including a failure to express QC25::GUS (5/10; Fig. 6P), the loss of columella initials (6/10; Fig. 6O) and grossly abnormal QC morphology (4/10; Fig. 6O). The RM abnormalities correlated with decreased growth; beginning at approximately 10 dpg, lhw root growth slowed relative to wild type and, by 19 days, lhw primary roots ceased growing (Fig. 6Q).

**DISCUSSION**

We have identified, characterized and cloned a new regulator of development in Arabidopsis. LHW positively regulates the size of the stele cell population and is required to establish the normal diarch pattern of root vascular tissues. One of the most striking aspects of the lhw mutant phenotype is that the vascular cylinder is not just reduced, but that lhw roots seem to have a new ‘set point’ for the number of cells in the stele, which, in the mature zone, is consistently half of the wild-type number. All lhw primary and
lateral roots produced single files of protoxylem, metaxylem, phloem and lateral-root-producing pericycle cells. Size and symmetry can be mechanistically connected when pattern is generated via inhibitory signals from differentiating tissues or cells. The organization of vascular tissues in plants has been hypothesized to result from feed-forward mechanisms that promote both the formation of continuous vascular strands (canalization) and lateral inhibition that creates spaces between the strands (reviewed in Turner and Sieburth, 2002). If this hypothesis is correct, then it suggests that \textit{LHW} is required only for cell production, because the \textit{lhw} mutants leave the lateral-inhibition system intact.

\textit{LHW} exhibits several characteristics consistent with it being a transcription factor; it is likely, therefore, to play a regulatory role upstream in a division/differentiation pathway. It is somewhat mysterious how mutations in such a factor consistently reduce the size of the stele cell population to half that of wild type. Several possible ways to account for this are: (1) the described alleles of \textit{LHW} only partially reduce function; (2) \textit{LHW} paralogs partially compensate for function and/or; (3) additional inputs from unrelated transcription factors or signaling systems contribute to stele size. We think it unlikely that the \textit{lhw} mutations are partial loss-of-function, because at least six \textit{LHW} alleles exhibit identical phenotypes and two of these mutations (\textit{lhw}-1 and SALK\_079402) are expected to produce no functional protein. In silico expression patterns of the \textit{LHW} paralogs \textit{At1g06150}, \textit{At1g64625} and \textit{At2g31280} are consistent with these genes playing a role in root development; however, no single-mutant phenotypes have been observed for \textit{T}-DNA insertion alleles of these genes (D.C.B., unpublished). It is still possible that multiple-mutant combinations might reveal the role of these genes in relation to \textit{LHW} and root development.

If stele cell number is controlled by \textit{LHW} in parallel with other factors, then cytokinin is a likely candidate. Cytokinin signaling is required for the repression of xylem differentiation and for the promotion of stele cell proliferation (Mahonen et al., 2006a; Mahonen et al., 2006b). Like cytokinin, \textit{LHW} is required to promote cell proliferation in the stele; however, \textit{LHW} is also required to promote protoxylem formation – a combination of phenotypes inconsistent with a simple increase or reduction in cytokinin synthesis or response. In addition, \textit{lhw}-1;\textit{wol}-1 roots have significantly fewer stele cells in the mature zone than do \textit{lhw} mutants. The interpretation of this genetic result is complicated because \textit{WOL} is one of three cytokinin receptors required for root vascular development (Higuchi et al., 2004; Mahonen et al., 2006b). The \textit{wol}-1 mutation has been reported to mimic the loss of all three receptors in root vascular development (Mahonen et al., 2006a; Mahonen et al., 2006b); if \textit{wol}-1 eliminates cytokinin perception, then \textit{LHW} and cytokinin are likely to be two of several inputs that promote proliferation of the stele independently.

We interpret the appearance of a smaller provascular region in the \textit{lhw} embryo and young lateral roots as meaning that the primary role of \textit{LHW} is to produce the wild-type number of stele initial cells in the radial direction. However, we also demonstrated that \textit{LHW} is required to maintain growth in the longitudinal direction. \textit{LHW} could have a direct or indirect role in maintaining the RM. In
contrast to other root-patterning mutants that exhibit a clear ‘short root’ phenotype, lhw mutant roots were not noticeably shorter than wild type until 10 dpg. Abnormalities in the QC cells, however, preceded this growth defect, and previous studies have shown that the self-renewing properties of the RM initials are maintained through interactions with the QC cells (Aida et al., 2004; Sabatini et al., 2003; van den Berg et al., 1997; Wildwater et al., 2005). By 5 dpg, lhw roots failed to express SCR in the QC; by 7 dpg, the majority of lhw roots expressed QCF25::GUS asymmetrically and a small fraction exhibited morphological abnormalities in the QC cells. The finding that SCR is missing from the QC earlier than other markers could indicate a specific requirement for LHW to promote the expression of this gene. Alternatively, LHW might be indirectly required for the RM via its effects on SHRs production. The disappearance of SCR from the QC is seen in reduction-of-function mutations of SHR (Sabatini et al., 2003). Because LHW acts early to establish the number of cells in the radial direction of the stele and SHR RNA is produced exclusively in the stele, the loss of SCR and the gradual slowing of root growth in lhw mutants might be due to the reduction of the SCR source (Fig. 6R).

In the future, several lines of inquiry might illuminate whether LHW plays an indirect or direct role in RM maintenance. For example, when RETINOBLASTOMA-RELATED function is inactivated specifically in the meristem (rBRRs), a larger RM is created (Wildwater et al., 2005). If rBRr can rescue lhw stele size, pattern and premature termination, then it is likely that LHW acts through this cell cycle controller to reach the balance of cells in the stele, and that the effects of lhw on longitudinal growth are largely indirect. If pattern and size are rescued, but the meristem terminates, then LHW might have a direct and independent role in creating and maintaining a functional stem cell pool in the root.

LHW is the first characterized member of a clade of proteins that represent potential transcriptional regulators in Arabidopsis and in other plants, including rice, a monocot, and poplar, a woody species. Root architecture is significantly different between monocots and dicots; therefore, it would be particularly interesting to see whether LHW orthologs retain a similar role in promoting vascular proliferation, and how this role manifests itself in a structurally diverse root system. Because LHW represents a xylem-promoting factor, its potential for promoting growth in trees might prove valuable for wood and biofuel production.

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