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

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Abstract

Complex organisms consist of a multitude of cell types arranged in 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 and 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 may also indirectly feed into established regulatory networks for the maintenance of the root meristem.

INTRODUCTION

Multicellular organisms must coordinate division and expansion of each tissue's constituent cell types to ensure organized development. Plants develop through 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 production of differentiated offspring are tightly controlled.

The root meristem 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 GRAS family transcription factors, SCARECROW (SCR) and SHORTROOT (SHR) (Sabatini et al., 2003) and the hormone auxin as mediated through the AP2 class transcription factors PLETHORA1 (PLT1) and PLT2 (Aida et al., 2004). 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

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Arabidopsis homologue of the Retinoblastoma gene, *RETINOBLASTOMA-RELATED (RBR)*, 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 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 other plants, such as wild-grown radish, may be diarch to heptarch (reviewed in (Turner and Sieburth, 2002)). Lateral roots are produced by postembryonic divisions in the pericycle. In 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*, critical 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-1* mutation have fewer cells in the stele and fail to produce phloem (Mahonen et al., 2000; Scheres et al., 1995). *WOL/CRE1* 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 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., 2006; Mahonen et al., 2006; Nishimura et al., 2004).

In this study we identify a new locus, *LONESOME HIGHWAY (LHW)* 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 scored for deviations in J0121::GFP pattern. All lines were backcrossed at least twice before further analysis. Through backcrosses and complementation crosses, five mutations that resulted in presence of a single J0121::GFP stripe were found to be recessive to WT 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 *lhw-5*, respectively. All *LHW* mutants were also crossed to Landberg *erecta* (Ler) to establish mapping populations.

Phenotypic characterization

Markers of cell fate used were: SCR::GFP (gift of J. Long, SALK), APLproAPL::GFP ((Bonke et al., 2003)), QC25::GUS (gift of B. Scheres, U. Utrecht), J0121::GFP (ABRC stock CS9090), Q1630::GFP (ABRC stock CS9227), VH1::GUS (Clay and Nelson, 2002) DR5::GUS (Ulmasov et al., 1997), CYCB1;1::GUS (Colon-Carmona et al., 1999; Donnelly et al., 1999). Unless otherwise indicated, the wild type control for experiments with *lhw-1* and *lhw-2* is the unmutagenized parental line CS9090 (C24 ecotype). Seedlings were grown vertically on plants containing 0.5X MS, 1% agar for indicated times. 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-dpg with the indicated concentration of hormone. Seedlings grown on plates containing 20 μ M 1-N-naphthylphthalamic acid (NPA) were scored at 7-dpg for rescue and at 21-dpg 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 ~800 F2 individuals from a mapping outcross of *lhw-1* to Ler and scored for additional SSLP markers, localizing *LHW* to an 80KB region on BAC F12K2. T-DNA insertion alleles for 24/34 of the genes in the region were screened for root phenotypes and SALK_079402 (At2g27230) exhibited a single xylem pole phenotype. Mutations leading to stop codons in the predicted open reading frame of At2g27230 were identified in 4 *LHW* alleles. Using numbering derived from AY035151, mutations were found in *lhw-1* g→a at 575; *lhw-2* g→a at 1944; *lhw-3* c→t at 1883; *lhw-4* g→a at 1066. *lhw-1* is predicted to truncate the protein at amino acid 23 and is likely a null. All phenotypic characterization was carried out with both *lhw-1* and *lhw-2*, and some tests with SALK_079402, to ensure that the *lhw* phenotype was not ecotype dependent. All alleles behaved similarly so only results from *lhw-1* are reported except as noted. Full length cDNA AY035151 was obtained from RIKEN, Japan. An error in the cDNA that introduced an additional G at position 918 was corrected by PCR. CaMV35S expression of this cDNA was capable of rescuing the xylem phenotype of *lhw-1* (Fig. 5B) in the T1 generation (6/14 independent lines).

Yeast two hybrid assay and screen

LHW and other clones were PCR amplified from cDNA clones or by RT-PCR and cloned into 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 ~800,000 colonies was performed using the LHW bHLH 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 -gal expression was performed by transforming LHW variants into 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). 100ng of RNA was used in first strand synthesis with superscript III (Invitrogen), followed by PCR with gene specific primers *lhwr1f1-gatcgtgccaagagctgcg* and *lhwr1r1-ttcgaagcccatgttgctcc* and control primers *actinF-ggcgatgaagctcaatccaacg* and *actinR-ggtcagcaccagcaagatcaagacg* amplified for 32 and 25 cycles, respectively for 15 sec at 95° C, 30 sec at 52° C and 1 min 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 the LHW cDNA from 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 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 become visible in the elongation zone and extend to the root/hypocotyl junction (Fig 1B). Seedling roots were screened for alterations in the pattern of this marker 5–7 days post germination (dpg). Five completely recessive and allelic (see methods) mutations were found in that result in plants expressing J0121::GFP in only a single stripe (Fig. 1C and Fig. S1 in 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 only one side of the primary root (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. Two protoxylem strands normally run 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 metaxylem are made, and they bear the same spatial relationship to the protoxylem pole, but there appear 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 is seen in nuclei of two cell files corresponding to maturing protophloem (Fig. 1G) In *lhw*, only a single APLpro::APL-GFP marked file is visible (Fig. 1H). Despite the reduced cell number in the root vasculature, LHW plants are healthy and fertile. Plants with mutations in *LHW* do not exhibit dramatically altered phyllotaxis, nor do they have any gross morphological abnormalities in their leaf and floral organs (Fig. 1I–J). *lhw* mutations in the C24 background lead to plants that are slightly agravitropic as seen in waving of *lhw* roots as they grow down a slanted agar surface (Fig. 1K). In cotyledons, *lhw* vein development is delayed relative to WT (Fig. 1L–M) and xylem gaps are still visible in the mature organs (Fig 1N–O); however, leaf venation patterns appear normal (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) and 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. 2 B–C) through the mature zone (where root hairs are visible; Fig. 2H–I) and up into the hypocotyl (Fig. 2 J–K). In cross sections of a wild type root (30 μ m above tip), the epidermis consists of ~ 25 cells, the cortex and endodermal layers each consist of 8 cells and the stele (pericycle, xylem and phloem) is approximately 25 cells (Table 1). In *lhw* roots, the normal number of cortex and endodermal cells are present, the epidermal number is slightly reduced, but the number of cells in the *lhw* stele is reduced to half as many as wild type (Fig. 2; Table 1). This affects all cell types in the stele; in addition to the reduction in cells from which the xylem and phloem arise, the *lhw* pericycle is reduced from the normal 13 cells to 8 cells (Fig. 2; Table 1).

The total number of cells in the stele is a product of the initial pool in the embryo and divisions postembryonically. The number of stele cells visible in cross section of the *lhw* root at 30 μ m and 120 μ m is virtually unchanged (Table 1), suggesting that postembryonic divisions rarely occur. In the embryo, the stele is derived from the uppermost tier of the root meristem (Dolan et al., 1993). Early embryogenesis in *lhw* was indistinguishable from wild type in terms of orientation of cell divisions (Fig. S2A–B in supplementary material). The only defect seen at a significant frequency (3/15 *lhw* globular embryos and 11/15 *lhw* late heart stage embryos) was a delay relative to WT in divisions in the base of the embryo, in cells that will later become the RM (Fig. S1 C vs. D; E and G vs. F and H, in supplementary material). At the torpedo stage, *lhw* embryos appear to have a well formed vascular cylinder, but it is narrower in *lhw* than in wild type (Fig. S1 J vs. I in supplementary material).

Because *lhw* mutants still make some lateral roots, we could examine the organization of these post-embryonically 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 are indistinguishable between WT and *lhw* (Fig. S1K–P in supplementary material), *lhw* lateral roots generate 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* does respond to exogenous auxins (IAA and 2,4 D) by producing root hairs and lateral roots and inhibiting primary root elongation (Fig. S3 in supplementary material and not shown) yet these auxin treatments do not rescue the xylem or pericycle defects (Fig. 3C and not shown). We visualized local auxin response near the RM by scoring the expression pattern and intensity of markers *DR5::GUS* and *PIN4::GUS* (Friml et al., 2002; Ulmasov et al., 1997). In *lhw* plants, expression of both markers is similar to WT in intensity and in position of the maximum (Fig. 3G vs. F and I vs. H).

Germination and growth on media containing the auxin transport inhibitor NPA can lead to excessive root meristem proliferation and xylem production (Mattsson et al., 1999). *lhw* and WT plants grown on MS agar plates containing 20 μ M NPA were sampled at 7 and 21 dpv for xylem vessel formation and expression of the J0121::GFP marker in roots. At neither timepoint was expression of J0121::GFP seen in two stripes nor was the second xylem pole restored in *lhw* (Fig. 3B vs. 3A and not shown). Morphology of the root tip was strikingly different between wildtype and *lhw* at 21-dpv. In wild type, the roots became extensively fasciated and produced 8–10 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 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 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., 2006). The *wol-1* mutation in the *WOL/CRE1* 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 number of cells interior to the pericycle is reduced to less than 10, all of which become xylem (Mahonen et al., 2000; Table 1). Double mutants between *lhw* and *wol-1* exhibit the root length defect of *wol* 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 eliminate phloem production and result in a stele consisting solely of protoxylem. In terms of cell identity, the *wol-1* mutation is epistatic to *lhw* because the interior of the *lhw;wol-1* root resembles *wol-1*. The presence of multiple xylem poles in this double mutant indicates that there is no explicit requirement for *LHW* in 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, and transcriptional regulators (for example: Aida et al., 2004; Blilou et al., 2002; Blilou et al., 2005; Friml et al., 2002; Mahonen et al., 2006; Sabatini et al., 2003; Wildwater et al., 2005). To understand how *LHW* might control root development, we used map-based cloning approach and found that *LHW* corresponds to At2g27230, a locus that encodes a protein of 650 amino acids (see 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 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 Fig. S4 in 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 doing a comprehensive analysis 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 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 autoactivating portion of the protein (DB-bC) was co-transformed with variants of LHW fused to the GAL4 activation domain (AD). LHW (DB-bC) interacts strongly with full length LHW (AD-FL) and 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 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. 9 of the clones corresponded to 4 bHLH genes: At5g08130 (5 clones); At1g68810 (2 clones); At1g29950 (1 clone) and At3g25710 (1 clone). This suggests that LHW readily binds to typical bHLH proteins. The bHLH proteins that were identified in the 2-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 RT-PCR, LHW expression is highest in the meristematic regions of both the root and shoot, and is lowest in mature tissues (Fig. 5F). A transcriptional reporter containing 2.8 KB of sequence 5' of the 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 T1s (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 (not shown). Silencing of the LHW transgene was often observed in T2 lines as a reduction in GFP expression and the appearance of a single xylem pole in plants with a wild type genomic copy of *LHW* (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 that are important for regulating both radial and longitudinal growth. SCR is normally expressed in the QC, endodermal/cortex initials and in the maturing endodermis of the root (Di Lorenzo 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 it was present in the endodermis, but not in the QC (0/40 *lhw* plants compared to 38/40 wild type plants; Fig. 6B vs. 6A). In torpedo stage *lhw* embryos, however, SCRpro::GFP is present in QC cells (Fig. 6C), suggesting that SCR expression is lost over time.

Loss of SCR in the QC is reminiscent of the defects in *HOBBIT* (Blilou et al., 2002) and *SHR* (Helariutta et al., 2000) both required for meristem maintenance. The arrangement of SCRpro::GFP expressing cells in *lhw* was also similar to that in roots provided 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 expression of QC identity markers, the longitudinal extent of the zone of proliferation and whether *lhw* roots exhibit determinate growth.

In WT plants, QC25::GUS is expressed specifically in the QC (Sabatini et al., 2003). At 7-dpg, *lhw* mutants express QC25::GUS, but interestingly, the intensity of GUS expression in the QC cells is often asymmetric (14/20 *lhw* plants vs. 0/10 WT; (Fig. 6H–I). The intensity of staining does not appear to be correlated with the side on which protoxylem forms (not shown). Despite this asymmetry, at 7-dpg, the *lhw* RM appears to have normal proliferative capacity as assayed by root growth (Fig. 6Q) and presence of columella initials. Columella initials are identified as a layer of cells between the QC and cell expressing columella marker Q1630::GFP and starch granules (Fig. 6D–E and not shown). Several groups have used the expression of a mitotic cyclin (CYCB1;1 GUS) to measure the longitudinal extent of the proliferative zone (for example, Aida et al., 2004; Hutchison et al., 2006; Ioio et al., 2007). At 7-dpg, the region of CYCB1;1pro::GUS expressing cells is similar in WT and *lhw* (Fig. 6F–G; 119 $\mu\text{m} \pm 1.6$ SD in WT $n = 11$; 125 $\mu\text{m} \pm 1.73$ in *lhw*-1 $n = 10$). 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 fails over time. At 13 days, QC25::GUS is still expressed (Fig. 6J vs. K), but columella initials begin to differentiate and contain starch grains (star in Fig 6L). At 17 dpg, the meristem of *lhw* roots is visibly disorganized. In contrast to WT roots (Fig 6 M–N), *lhw* roots exhibit a variety of defects including a failure to express QC25::GUS (5/10; Fig. 6P), loss of columella initials (6/10; Fig 6O) and grossly abnormal QC morphology (4/10; Fig 6O). The RM abnormalities correlate with decreased growth; beginning approximately 10-dpg, *lhw* root growth slows relative to wild type and by 19 days, *lhw* primary roots cease 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 that, in the mature zone, is consistently half of the wild type number. All *lhw* primary and lateral roots produce single files of protoxylem, metaxylem, phloem, and lateral-root producing pericycle cells.

Size and symmetry can be mechanistically connected when pattern is generated through inhibitory signals from differentiating tissues or cells. The organization of vascular tissues in plants has been hypothesized to result from both feed-forward mechanisms that promote 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 *LHW* is required only for cell production, as the *lhw* mutants leave the lateral inhibition system intact.

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 WT. Several possible ways to account for this are: (1) the described alleles of *LHW* only partially reduce function; (2) *LHW* paralogues 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 *lhw* mutations are partial loss of function because at least six *LHW* alleles exhibit identical phenotypes and two of these mutations (*lhw-1* and SALK_079402) are expected to produce no functional protein. *In silico* expression patterns of *LHW* paralogues At1g06150, At1g64625 and At2g31280 are consistent with these genes playing a role in root development, however, no single mutant phenotypes have been observed for T-DNA insertion alleles of these genes (DCB, unpublished). It is still possible that multiple mutant combinations might reveal the role of these genes in relation to *LHW* and root development.

If stele cell number is controlled by *LHW* in parallel with other factors, then cytokinin is a likely candidate. Cytokinin signaling is required for repression of xylem differentiation and promotion of stele cell proliferation (Mahonen et al., 2006; Mahonen et al., 2006). Like cytokinin, *LHW* is required to promote cell proliferation in the stele, however, *LHW* is also required to promote protoxylem formation—a combination of phenotypes inconsistent with a simple loss or gain of cytokinin production or response. In addition, *lhw-1;wol-1* roots have significantly fewer stele cells in the mature zone than *lhw* mutants. The interpretation of this genetic result is complicated because *WOL* is one of three cytokinin receptors required for root vascular development (Higuchi et al., 2004; Mahonen et al., 2006). The *wol-1* mutation has been reported to mimic the loss of all three receptors in root vascular development (Mahonen et al., 2006; Mahonen et al., 2006); if *wol-1* eliminates cytokinin perception, then *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 *lhw* embryo and young lateral roots as meaning that the primary role of *LHW* is to produce the wild type number of stele initial cells in the radial direction. However, we also demonstrated that *LHW* is required to maintain growth in the longitudinal direction. *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 are not noticeably shorter than wild type until 10-dpg. Abnormalities in the QC cells, however, precede 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 fail to express *SCR* in the QC; by 7-dpg, the majority of *lhw* roots express QC25::GUS asymmetrically and a small fraction exhibit and morphological abnormalities in the QC cells. That *SCR* is missing from the QC earlier than other markers could indicate a specific requirement for *LHW* to promote expression of this gene. Alternatively, *LHW* might be indirectly required for the RM via its effects on *SHR* production. 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 radial direction of the stele and *SHR* RNA is produced exclusively in the stele, the loss of *SCR* and gradual slowing of root growth in *lhw* may be due to the reduction of the *SHR* source (Fig. 6R).

In the future, several lines of inquiry may illuminate whether *LHW* play an indirect or direct role in RM maintenance. For example, when *RETINOBLASTOMA-RELATED* function is inactivated specifically in the meristem (*rRBR*), a larger RM is created (Wildwater et al., 2005). If *rRBR* 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 *lhw* effects on longitudinal growth are largely indirect. If pattern and size are rescued, but the meristem terminates, then *LHW* may 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* orthologues retain a similar role in promoting vascular proliferation, and how this role manifests itself in a structurally diverse root system. As *LHW* represents a xylem promoting factor, its potential for promoting growth in a woody plant system might prove valuable in wood and biofuel production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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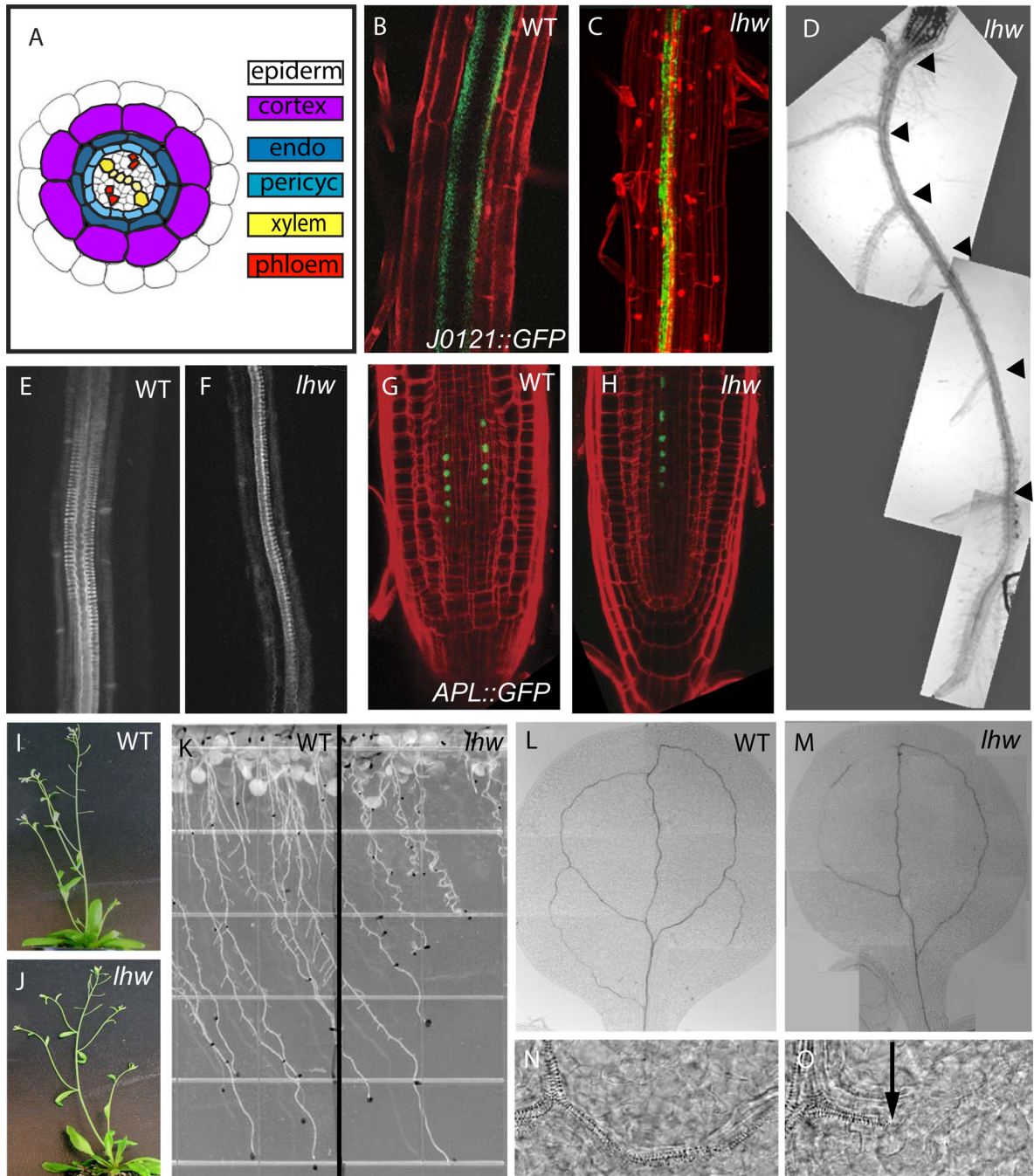


Figure 1. Phenotype of *LONESOME HIGHWAY* mutants

(A) Cross section diagram of a mature *Arabidopsis* root; tissues are arranged radially from outside in: epidermis (white), cortex (light purple), endodermis (dark blue) and stele. The stele consists of a ring of pericycle cells (light blue), surrounding xylem (yellow) and phloem (red) arranged in bilateral symmetry. (B–C) Confocal images of wild type (B) and *lhw-1* (C) expressing xylem-associated pericycle marker J0121::GFP in green. Roots are counterstained with propidium iodide (PI, red) to visualize outlines of cells. (D) Brightfield image of *lhw-1* root with all lateral roots (black arrowheads) emerging from single side of primary root. (E–F) Confocal image of basic fuchsin staining of xylem in wildtype (E) and *lhw-1* (F). (G–H) Confocal images of wild type (G) and *lhw-1* (H) expressing phloem

marker APLpro::APL-GFP in green. (I–J) Whole plant phenotypes of WT Col (I) and *lhw* SALK_079402 (J). (K) Root growth of wild type (left) and *lhw-1* (right) on agar plates at 20-dpg. Note root waving and short root phenotypes in *lhw-1*. (L–M) vascular pattern in mature (13 dpg) WT (L) and *lhw-1* (M) cotyledons (N–O) higher magnification images of xylem from images L and M, respectively. Black arrow points to end of mature xylem elements, to the right, elongated cells typical of procambium are still seen. For each marker, the WT and *lhw* image pair are at same magnification.

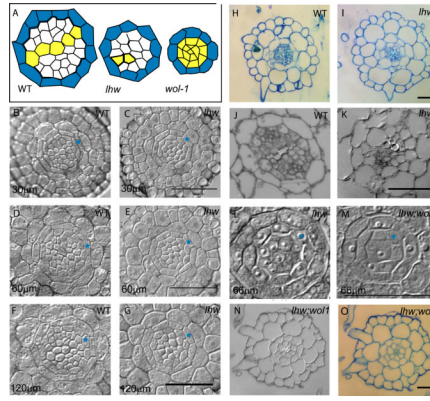


Figure 2. Cross sections of *lhw* roots and hypocotyls

(A) Schematic of stele in cross sections from WT, *lhw* and *wol-1*; pericycle in blue and xylem in yellow; WT and *lhw* were traced from 2F and 2G, respectively. (B–K) Brightfield images of WT and *lhw* root sections taken at increasing distance from tip of root; one pericycle cell marked with blue star for orientation. (B) WT, 30 μm (C) *lhw-2*, 30 μm (D) WT, 60 μm (E) *lhw-2*, 60 μm (F) WT, 120 μm (G) *lhw-2*, 120 μm . (H–I) Toluidine blue staining of WT (H) and *lhw-2* (I) in mature zone. (J–K) section through lower third of hypocotyl in WT (J) and *lhw-2* (K). (L) Center of *wol-1* root, 66 μm from tip (M) Center of *wol-1;lhw-1* root, 66 μm from tip. (N–O) DIC and toluidine blue images of *wol-1;lhw-1* mature roots showing the very reduced stele filled with xylem elements. Each image pair is at the same magnification. Scale bars = 20 μm .

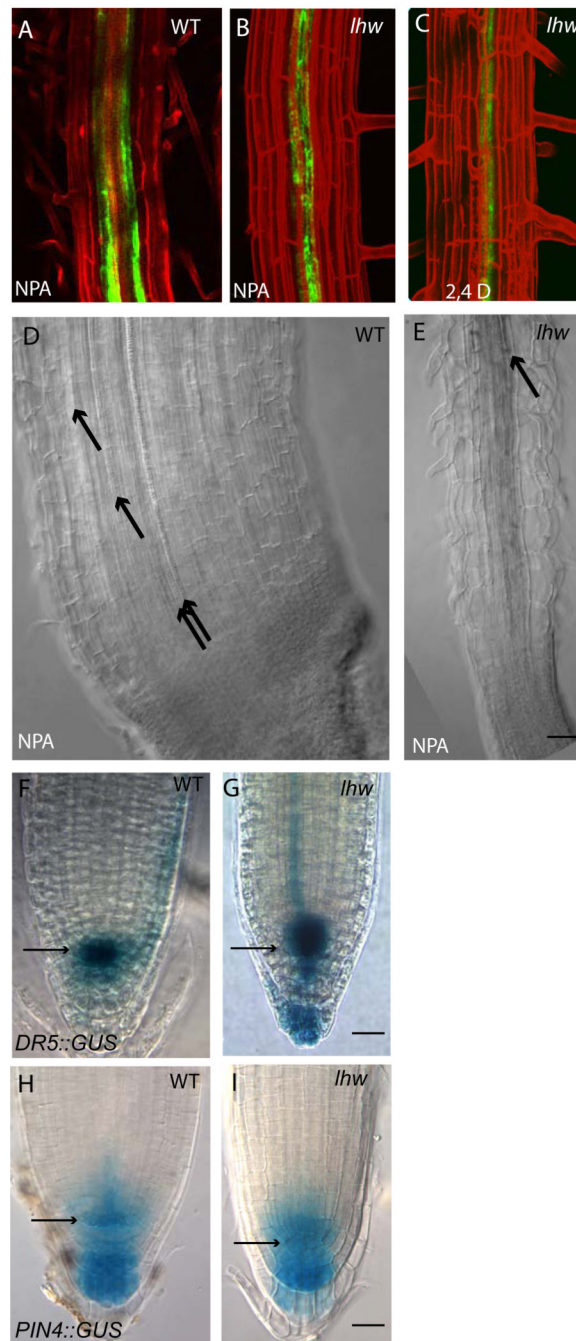


Figure 3. Measures of auxin response in *lhw*

(A–C) confocal images of WT (A) and *lhw-1* (B–C) expressing J0121::GFP. (A–B) treatment with 20 μ M NPA for 7 days (C) treatment with 30nM 2,4D for seven days. (D–E) DIC images of roots treated with 20 μ M NPA for 21 days. Black arrows point to xylem elements. (F–I) Brightfield images 7-dpg seedling root tips; (F–G) DR5::GUS expression; (H–I) PIN4::GUS expression. Each image pair (and A–C) is at the same magnification. Scale bars = 20 μ m.

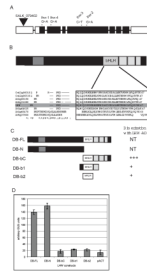


Figure 4. LHW gene and protein structure and behavior in two hybrid screen

(A) *LHW* gene structure with exons represented as boxes and introns as lines. *LHW* coding region is in black. The location and nature of *lhw* mutant alleles is indicated above the exons. (B) *LHW* protein structure. Two domains conserved with other plant proteins are indicated as grey boxes. Part of the C-terminal conserved region resembles the bHLH domain of transcriptional regulators. A sequence alignment of the putative bHLH domain (boxed) is diagrammed for *LHW* and related proteins from Arabidopsis and rice. (C) Graphical representation of *LHW* protein fragments used in yeast two hybrid assay and their ability to dimerize with full length *LHW*. (D) GAL4 transcriptional activation activity of *LHW* variants; pACT is included as a negative control. GUS measurements based on 4 replicates/sample. Error bars \pm SEM.

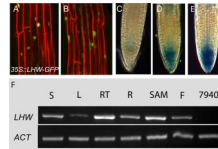


Figure 5. *LHW* expression

(A–B) Confocal images of *lhw-2* roots expressing 35S::LHW-GFP in nuclei (green). Expression of this transgene is sufficient to rescue the *lhw-2* xylem phenotype (B). (C–E) LHWpro::GUS reporter expression in root tips, staining for 1hr, 4hr and 8hr, respectively (F). RT-PCR of *LHW* and *actin* (*ACT*) expression. S, 14-dpg seedling; L, expanded rosette leaf; RT, 5mm of 14-dpg root tip; R, whole 14-dpg root; SAM, transition SAM and youngest leaves; F, flower stage 8-15; 79402, 14-dpg seedling of SALK_79402.

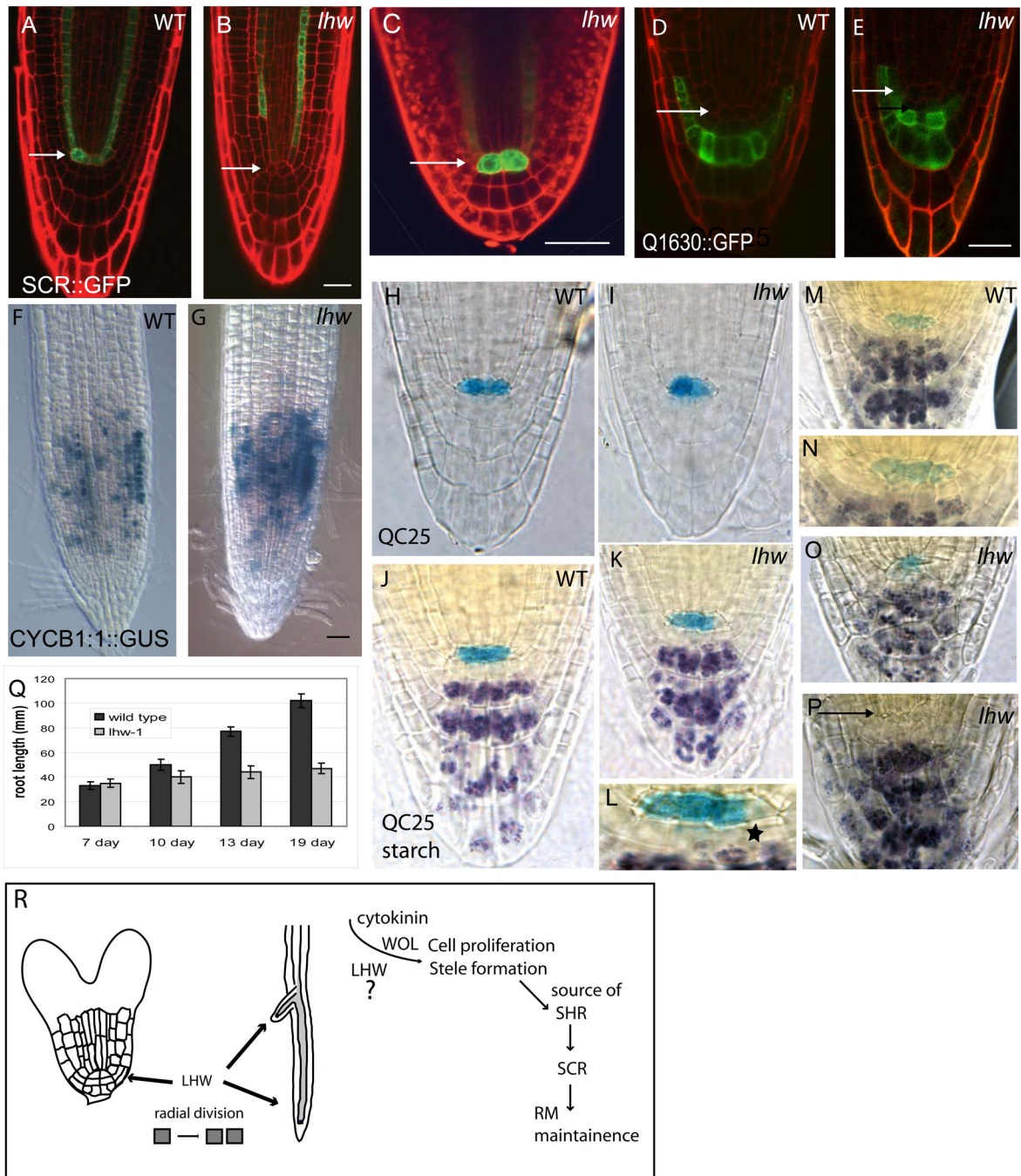


Figure 6. LHW effects on RM meristem establishment and maintenance

(A–I) Meristem markers in WT and *lhw-1* 7-dpf seedlings (A–B) *SCR::GFP* expression (C) *SCR::GFP* in torpedo stage *lhw-1* embryo (D–E) Columella differentiation marker Q1630::GFP. (F–G) *CYCB1;2::GUS* expression (H–P) *QC25::GUS* (blue) and starch granules (purple) mark degeneration of *lhw* meristem over time (H–I; 7dpf, J–L; 13 dpf; M–P; 18 dpf). L is higher magnification image of K; start indicates starch granule containing cells adjacent to *QC25* marked cells. N is higher magnification of M. (Q) Graph of WT (dark grey) and *lhw-1* (light grey) root growth over time. For each marker, the WT and *lhw* image pair are the same magnification. Arrows point to QC cells. (R) Model for LHW action in generating vascular pattern. *LHW* is required to establish the radial extent of

the root vascular tissues in the embryo and promotes postembryonic divisions in these tissues. *LHW* therefore acts as a meristem size-control protein for the center of the root. *LHW* and *WOL* are both required for these cell divisions, but appear to act at least somewhat independently. We propose that the eventual slowing down of longitudinal growth in *lhw* mutant roots is not due to direct requirement for *LHW* in meristem maintenance, but because *LHW* is required to create the tissue that normally produce SHR. Without adequate levels of SHR, SCR is not maintained in the QC and meristems eventually terminate. Each image pair (A-K and M, O, P) at the same magnification. Scale bars = 30 μ m. Error bars in Q \pm SEM

Table 1

Cell numbers in the primary root

	30µm			60 µm			120µm					
	total stele	pericycle	inside	total stele	pericycle	inside	total stele	pericycle	inside	endodermis	cortex	epidermis
WT	33	11.5	21.5	41±1	13.33±1.5	27.67±1.5	41.67±.58	13±0	28.67±.58	8±0	8±0	24.3±2.1
<i>lhw-1</i>	19±1.7	8.5±.55	10.5±1.5	20.33±1.5	8.5±.55	11.83±1.2	21.83±1.6	8.33±1.2	13.17±1.5	8±0	8±0	20.6±3.6
<i>wol-1</i>	16	8	8	19	9	10	19	9	10			
<i>lhw-1;wol-1</i>	14±3	7.67±1.5	6.33±1.5	14.67±3.2*	8±1	6.67±2.3	15.33±2.9**	8±1	7.33±2.1			

Number of roots scored/genotype: WT = 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