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Auxin-associated initiation of vascular cell differentiation by LONESOME HIGHWAY

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SUMMARY

Plant vascular tissues are essential for the existence of land plants. Many studies of transcriptional regulation and cell-cell communication have revealed the process underlying the development of vascular tissues from vascular initial cells. However, the initiation of vascular cell differentiation is still a mystery. Here, we report that *LONESOME HIGHWAY* (*LHW*), which encodes a bHLH transcription factor, is expressed in pericycle-vascular mother cells at the globular embryo stage and is required for proper asymmetric cell division to generate vascular initial cells. In addition, ectopic expression of *LHW* elicits an ectopic auxin response. Moreover, *LHW* is required for the correct expression patterns of components related to auxin flow, such as *PIN-FORMED 1* (*PIN1*), *MONOPTEROS* (*MP*) and *ATHB-8*, and ATHB-8 partially rescues the vascular defects of *lhw.* These results suggest that LHW functions as a key regulator to initiate vascular cell differentiation in association with auxin regulation.

KEY WORDS: Auxin, LONESOME HIGHWAY, Root, Vascular development

INTRODUCTION

The vascular bundle is the long-distance transport pathway for water, nutrients and signalling molecules that connect all parts of the plant body. Because the vascular system is an important lifeline, the molecular mechanisms underlying vascular development have been keenly investigated for a long time. Recent genetic and physiological studies have identified many key factors, including transcription factors that govern vascular development, suggesting the importance of transcriptional regulation in the developmental process, especially during differentiation of specialized vascular cells (Caño-Delgado et al., 2010; Ohashi-Ito and Fukuda, 2010; Scarpella and Helariutta, 2010). By contrast, the mode of transcriptional regulation of the initiation of vascular initial cells is largely unknown.

We recently identified *LONESOME HIGHWAY* (*LHW*), which encodes a bHLH transcription factor. Mutations of this gene eliminate the bilateral symmetry of the vascular pattern and reduce the number of cells in root vasculatures, resulting in roots with the monoarch vasculature that contains only single xylem and phloem poles (Ohashi-Ito and Bergmann, 2007; Parizot et al., 2008). The *lhw* mutant also shows a weak auxin-related phenotype. Polar auxin flow mediated by auxin efflux carriers has been implicated as the earliest event in vascular differentiation (Scarpella et al., 2006; Donner et al., 2009; Wenzel et al., 2007). In this study, therefore, we investigated the role of *LHW* in the initiation of vascular formation, focusing on the association of *LHW* with the regulation of auxin flow.

MATERIALS AND METHODS

Growth conditions for *Arabidopsis*

A. thaliana ecotype Columbia was used for all experiments. Seedlings were germinated on half-strength MS agar plates and were cultured vertically in a Percival incubator under 24-hour light for 5 to 7 days at 22°C. For *LHW* induction, 7-day-old seedlings grown on MS plates were moved into liquid MS with or without 5 μM estrogen and incubated with rotation. For the NPA (1-N-Naphthylphthalamic acid)-treatment experiment, 10 μM NPA was added to liquid MS and incubated for 24 hours.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed according to a previously described method (Hejátko et al., 2006).

Histology

Sections of 1.5 μm were prepared as previously described (Hirakawa et al., 2010). Specimens were observed under a DIC microscope with a Charge-Coupled Device camera (BX51, DB70, Olympus, Tokyo, Japan).

Imaging

Fluorescent images were taken using previously described units (Kondo et al., 2011). Images were digitally analyzed using ImageJ. For staining of the plasma membrane, roots were incubated in 0.01 mg/ml propidium iodide. Three-dimensional images were constructed using the interactive 3D surface-plotting tool in ImageJ.

Quantitative RT-PCR

Quantitative RT-PCR was performed according to previously described methods (Ohashi-Ito et al., 2010).

Cloning

Vectors based on Gateway cloning technology (Invitrogen) were used for most manipulations. Promoter fragments were introduced into pBGYN (Kubo et al., 2005). To generate an estradiol-inducible *LHW*, the *LHW*-coding sequence was recombined with pMDC7 (Curtis and Grossniklaus, 2003). To produce *SHR::ATHB-8^{r}* and *SHR::PHB^{r}*, the 2.0 kb SHR promoter was ligated into the *Not*I sites within pENTR/D/TOPO immediately upstream of *ATHB-8^{r}* or *PHB^{r}*. Primers are listed in supplementary material Table S1.

Quantification of endogenous hormone levels

Roots of 5-day-old seedlings (~20 mg FW) were used for the quantification, which was performed according to Kojima et al. (Kojima et al., 2009).

RESULTS AND DISCUSSION

The defect of bilateral pattern formation in the vasculature of *lhw* roots occurred during early embryogenesis

The vasculatures of wild-type *Arabidopsis* roots contain two protoxylem cells and two protophloem cells that are arranged in

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bilateral symmetry. The *lhw* mutants fail to form the correct vascular pattern and lack bilateral symmetry in the vasculature of seedling roots. To uncover the origin of this defect, we first investigated *LHW* expression during embryogenesis using whole-mount in situ hybridization. The signal from *LHW* was first observed in globular-stage embryos, where it was restricted to the central cells (pericycle-vascular mother cells) that are destined to divide to produce vascular initial cells (Fig. 1A,B). After the heart stage, the *LHW* transcript accumulated in root vascular cells, especially near the root apical meristem (Fig. 1C-E).

Because *LHW* was expressed preferentially in pericycle-vascular mother cells during embryogenesis, we postulated that LHW controls the vascular patterning in embryos. To examine this possibility, we observed expression patterns of *TARGET OF MONOPTEROS5* (*TMO5*) and *TMO5-LIKE1* genes as pre-protoxylem markers in embryos. *TMO5* and *TMO5-LIKE1* signals formed two stripes in the provascular region of wild-type embryos after the early heart stage of development (Fig. 1F-I; supplementary material Fig. S1A-C), indicating that the pre-pattern of bilateral symmetry of vasculature is established as early as the heart stage during embryogenesis.

In *lhw* embryos, however, the signals from *TMO5* and *TMO5-LIKE1* were detected as a single stripe in the provascular region throughout embryogenesis (Fig. 1J-L; supplementary material Fig. S1D-F). These results indicate that the bilateral pattern of vasculature is already impaired in early embryos, and that the defect in the *lhw* mutant can be traced back to the early stage embryo.

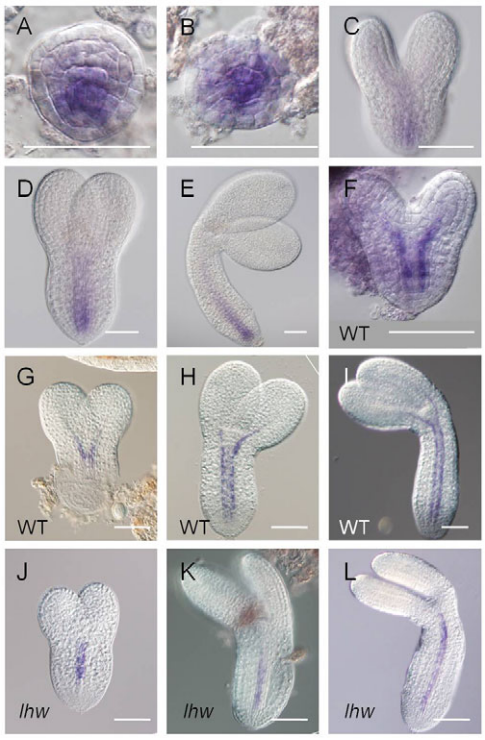

Fig. 1. LHW regulates bilateral symmetry in vasculature during embryogenesis. (**A-E**) Accumulation of *LHW* transcripts in globular stage embryos observed longitudinally (A) and transversely (B) in heart stage embryos (C), in a torpedo stage embryo (D) and in a bent cotyledon stage embryo (E). (**F-L**) Accumulation of *TMO5* transcripts. Wild-type embryos (F-I) and *lhw* mutant embryos (J-L). Scale bars: 50 μm.

LHW regulates the first step of vascular initial cell formation

To investigate the LHW function in early embryogenesis, we next analyzed the cell division pattern during early development of wild-type and *lhw* embryos in detail, with a special focus on vascular initial cell formation. Wild-type and *lhw* embryos at the globular stage showed very similar cell division patterns in all cells (supplementary material Fig. S2A-D). In wild-type embryos at the transition stage, four vascular initial cells were formed from four pericycle-vascular mother cells by asymmetric cell division (Fig. 2A; supplementary material Fig. S2E,F). Because all vascular cells, not including pericycle cells, are derived from these newly produced four cells, we defined these cells as vascular initial cells. In *lhw* embryos, however, the direction of division in pericycle-vascular mother cells was abnormal, although the division patterns of protodermal and ground cells were not different from those of the wild type (Fig. 2B; supplementary material Fig. S2G,H). As a result, *lhw* embryos possessed fewer vascular initial cells (3±0.37)

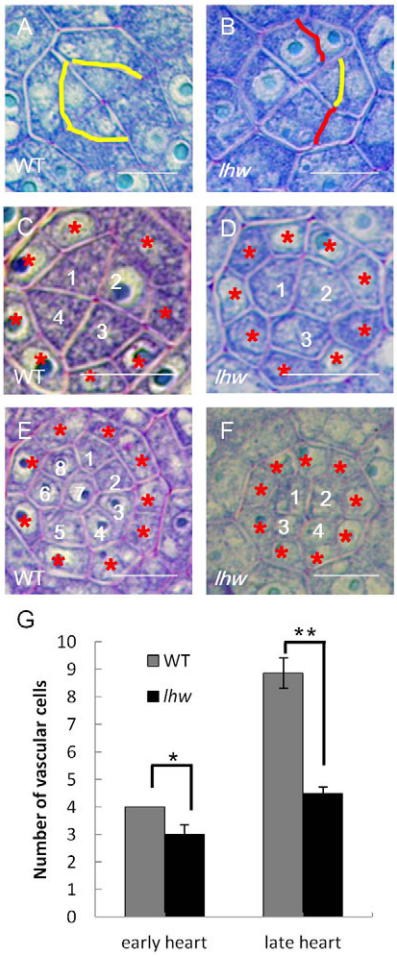

Fig. 2. LHW regulates vascular initial cell formation. Anatomical analysis of the central regions of embryos. (**A**,**B**) Transition stage embryos of wild type (A) and *lhw* (B). The yellow lines indicate a normal plane of cell division to create a vascular initial cell. The red lines indicate an abnormal plane of cell division. (**C**,**D**) Early heart stage embryos of wild type (C) and *lhw* (D). (**E**,**F**) Late heart stage embryos of wild type (E) and *lhw* (F). Stars indicate pericycle cells. Each cell is numbered. (**G**) The number of vascular cells in early heart and late heart stages embryos of wild type (gray) and *lhw* (black). Bars indicate s.d. $n>6$, $*P<0.05$, $**P<0.01$ (*t*-test). Scale bars: 10 μm.

and irregularly shaped cells in the center, whereas wild-type embryos had four uniform vascular initial cells (Fig. 2C,D,G). At the late heart stage, the number of vascular cells was 8.85±0.55 in the wild type but only 4.5±0.22 in *lhw*, indicating that the vascular initial cells in *lhw* embryos rarely divided (Fig. 2E-G). These results suggest that LHW plays a role in the establishment of vascular initial cells during early embryo development through the regulation of cell division, and the monoarch pattern in *lhw* may result from the reduced number of vascular cells, which are insufficient for creating the diarch pattern during the heart stage of embryogenesis.

The expressing region of *PIN1*

Because the early vascular development is associated with auxin flow, the expression pattern of an auxin efflux carrier, *PIN1::PIN1-GFP*, was examined in wild-type and *lhw* embryos (Gälweiler et al., 1998; Friml et al., 2003). It was difficult to find a difference in *PIN1* expression between these embryos before the transition stage (supplementary material Fig. S3). During the early heart stage, *PIN1* expression was restricted in two cotyledon primordia and provascular cells in wild-type embryos (Fig. 3A,C). At this stage, in *lhw* embryos, although the general pattern of *PIN1* expression was similar to that of wild type, the *PIN1*-expressing domain was enlarged (Fig. 3B,D). The enlarged *PIN1* expression was observed in cotyledon primordia where *LHW* was not expressed, suggesting non cell-autonomous effects of LHW. Altered *PIN1* expression was also observed during provascular tissue formation in lateral root primordia. The *PIN1* expression domain in lateral root primordia was gradually restricted to the provascular region and the root tip in wild type (Fig. 3E-G), whereas it was diffused more broadly and expanded to the cortex, endodermis and epidermal cells in *lhw* (Fig. 3I-K). To quantify the changes of the *PIN1*-expressing domain, we also observed the expression pattern of the *PIN1::YFP*-nuclear localization signal (nls) in wild-type and *lhw* lateral root primordia (Fig. 3H,L). The relative signal intensity of the central region (provascular region) to that of the peripheral region of wild-type primordia was considerably higher than was the case for *lhw* primordia (Fig. 3S). These results suggest that the restricted flow of auxin into the future provascular region may not be established in *lhw*.

To investigate whether the broad *PIN1* expression domain in *lhw* is associated with changes in auxin distribution, we next examined *DR5::GFP* expression as a marker of the auxin maximum (Benková et al., 2003). Although DR5 expression was concentrated to the central region of the meristem in the emerging wild-type lateral root primordia, the peak of DR5 signal in *lhw* primordia was not restricted to the central region (Fig. 3M-R). These results indicate that the *lhw* plant fails to form a proper *PIN1* expression domain and the proper auxin maximum in the central region of the primordium.

LHW induces the auxin response

To further elucidate how the function of *LHW* relates to auxin flow, we analyzed the auxin response in roots of *LHW* gain-of-function seedlings using *DR5::GFP*, in which *LHW* expression was temporally induced by the addition of estrogen. The addition of estrogen induced the overproduction of the *LHW* transcript (supplementary material Fig. S4A). Without the addition of estrogen, *DR5::GFP* was detected only in the stele and root tip (Fig. 4A; supplementary material Fig. S5A). The addition of estrogen caused ectopic *DR5::GFP* expression in the entire root, which contained the cortex, the endodermis and the epidermis, as

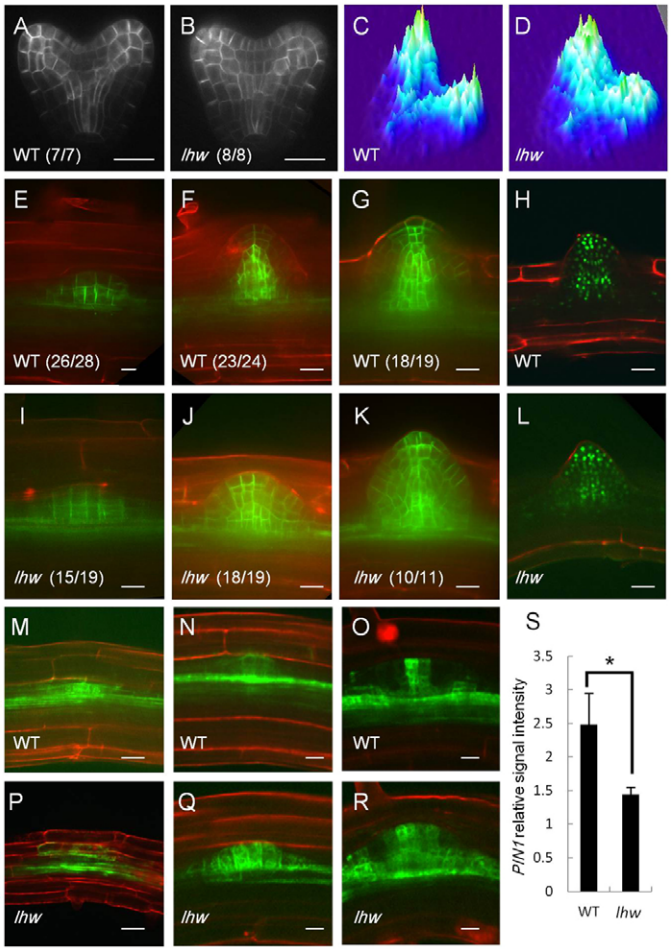

Fig. 3. LHW regulates the establishment of the correct *PIN1* expression pattern and the auxin maximum. (**A**,**B**) *PIN1::PIN1-GFP* expression in heart stage embryos of wild type (A) and *lhw* (B). (**C**,**D**) 3D images displaying signal intensities of A and B, respectively. (**E-G**,**I-K**) *PIN1::PIN1-GFP* expression patterns in lateral root primordia of wild type (E-G) and *lhw* (I-K). Fraction of samples showing a similar pattern are shown in the image. (**H**,**L**) *PIN1::YFP-nls* expression images in lateral root primordia of wild type (H) and *lhw* (L). (**M-R**) *DR5::GFP* expression patterns in lateral root primordia of wild type (M-O) and *lhw* (P-R). (**S**) Relative *PIN1::YFP-nls* signal intensity in stele region versus ground tissue region. Bars indicate s.d. n=5, *P<0.01 (t-test). Scale bars: 20 μm.

well as the stele, within 6 hours (Fig. 4B). Twenty four hours after the addition of estrogen, an ectopic, strong DR5 signal was maintained in the entire root (Fig. 4D; supplementary material Fig. S4B). These results suggest that ectopic *LHW* induces ectopic auxin response. Although NPA treatment altered the *DR5::GFP* expression pattern in root tips (supplementary material Fig. S5A-D), the treatment did not interfere with the ectopic expression of *DR5::GFP* in *LHW*-overexpressing roots (supplementary material Fig. S5E-G). Therefore, LHW may not promote *DR5* expression by the inhibition of auxin transport through efflux transporters.

Next, to determine whether LHW promotes auxin biosynthesis, we quantified endogenous phytohormone levels, including auxin levels, in the roots of wild type and *lhw* and in roots in which *LHW* was induced for 6 hours and 24 hours (supplementary material Table S2). The free IAA level did not change in these roots (supplementary material Fig. S6). These results suggest that ectopic

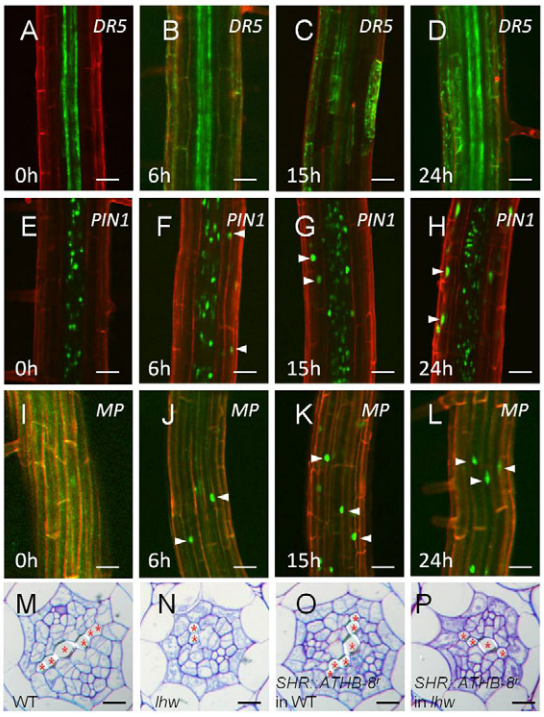

Fig. 4. LHW induces the auxin response. (**A-L**) Changes in *DR5::GFP* (A-D), *PIN1::YFP-nls* (E-H) and *MP::YFP-nls* (I-L) signals in estrogen-inducible *LHW* plants. Images were taken 0 hours (A,E,I), 6 hours (B,F,J), 15 hours (C,G,K) and 24 hours (D,H,L) after the addition of estrogen. Arrowheads indicate ectopic signals that emerged after *LHW* induction. Images of *MP::YFP-nls* were taken with a focus on the root surface to emphasize ectopic signals. Scale bars: 50 μm. (**M-P**) Root sections of wild type (M), *lhw* (N), *SHR::ATHB-8*^{r} in wild type (O) and *SHR::ATHB-8*^{r} in *lhw* (P). Stars indicate xylem vessel cells. Scale bars: 10 μm.

DR5 expression caused by *LHW* overexpression may not result from ectopic auxin biosynthesis. Similarly, there were no significant differences in the levels of other hormones in the roots of wild-type, *lhw* or *LHW*-induced plants (supplementary material Table S2).

LHW modulates the auxin signalling

To further understand the role of *LHW* in vascular differentiation in relation to auxin, we examined the involvement of *LHW* in the expression of transcription factors regulating auxin signalling in vasculature. *MP* and *ATHB-8* are transcription factors that are involved in auxin signalling, and they are expressed in the provascular region (Hardtke and Berleth, 1998, Donner et al., 2009; Baima et al., 1995; Ohashi-Ito and Fukuda, 2010). First, we analyzed expression patterns of *MP* and *ATHB-8*. The *MP* signal was observed in the provascular region in wild-type heart stage embryos, as expected (supplementary material Fig. S7A). In *lhw* embryos, the *MP* signal was much weaker than that in the wild-type, especially in the provascular region (supplementary material Fig. S7B). The *ATHB-8* signal was also weaker in *lhw* embryos (supplementary material Fig. S8A,B). To confirm the reduction of *MP* expression in the provascular region in *lhw*, the expression of *MP::YFP-nls* was examined in the lateral root primordia and main roots of seedlings. Indeed, the *MP* signals in the vasculature were diminished in both root primordia and the main root of *lhw* (supplementary material Fig. S7C-G). Quantitative PCR indicated that the transcript levels of *MP* and *ATHB-8* were lower in *lhw* roots than in wild-type roots (supplementary material Fig. S8C). These results clearly indicate that *LHW* is required for promoting the expression of *MP* and *ATHB-8* in the provascular region.

Next, we analyzed the effects of LHW induction on *MP* and *PIN1* expression. Because the ectopic *DR5::GFP* signal was clearly seen 6 hours after *LHW* induction, followed by an increase until 24 hours after induction (Fig. 4B-D), we observed the expression patterns of *MP::YFP-nls* and *PIN1::YFP-nls* at the same time points after *LHW* induction. Ectopic signals of *MP::YFP-nls* and *PIN1::YFP-nls* were indeed seen 6 hours after *LHW* induction, after which time the signals gradually increased (Fig. 4E-L).

To clarify whether LHW regulates auxin signalling via ATHB-8, we examined the genetic interaction between *lhw* and a gain-of-function mutant of *ATHB-8*. Because *ATHB-8* mRNA levels are repressed by miR165/166, we introduced substitutions into the *ATHB-8* sequence to produce a mutant that was resistant to miR165/166 without introducing amino acid changes (*ATHB-8*^{r}) (Mallory et al., 2004; Emery et al., 2003). We also employed the *SHR* promoter to continuously overexpress *ATHB-8*^{r} in provascular tissues because *SHR* is specifically expressed in the provascular region from the late globular stage of embryos (Helariutta et al., 2000). *SHR::PHABULOSA*^{r} (*PHB*^{r}), which is another HD-ZIP III transcription factor but is not induced by auxin, was used as a control. The introduction of *SHR::PHB*^{r} into *lhw* plants did not alter the monoarch vascular pattern in *lhw* roots (supplementary material Fig. S8E). By contrast, *SHR::ATHB-8*^{r} rescued the *lhw* phenotype and produced the bilateral symmetry pattern in the vasculature of roots, although the rescue of this phenotype was only partial (9/34 plants, Fig. 4M-P; supplementary material Fig. S8D). These data strongly suggest that LHW functions in early vascular pattern determination through the enhanced expression of *ATHB-8*.

Our results suggest that LHW functions as a key regulator to generate vascular initial cells. In addition, they also suggest that the LHW function is closely related to the modulation of auxin response. Although how LHW is connected to the auxin response remains obscure, several possibilities are considered. Our favorite hypothesis is that LHW may be the first factor to initiate the auxin response by inducing auxin canalization. According to this hypothesis, *LHW* is expressed and induces the auxin response in the future vascular region, and then this restricted auxin response enhances the auxin positive-feedback loops consisting of PIN1, MP and ATHB-8, followed by auxin canalization, which in turn initiates vascular development (Scarpella et al., 2006; Wenzel et al., 2007; Donner et al., 2009; Ohashi-Ito and Fukuda, 2010). Another possible hypothesis is that LHW directly regulates auxin signalling components such as MP and ATHB-8 or regulators that modulate the PIN1 localization. It is well known that cytokinin, along with auxin, regulates vascular pattern formation (Bishopp et al., 2011a; Bishopp et al., 2011b; Cui et al., 2011). Therefore, we cannot deny the possibility that LHW induces the auxin response by modulating cytokinin signalling. Further investigation of the target genes of LHW may lead to an answer to the relationship between LHW and auxin.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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