The *HALTED ROOT* gene encoding the 26S proteasome subunit RPT2a is essential for the maintenance of *Arabidopsis* meristems

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Summary

In higher plants, post-embryonic development is dependent on the activity of the root and shoot apical meristem (RAM and SAM). The quiescent center (QC) in the RAM and the organizing center (OC) in the SAM are known to be essential for the maintenance of meristematic activity. To understand the mechanism that maintains post-embryonic meristems, we isolated an *Arabidopsis* mutant, *halted root* (*hlr*). In this mutant, the cellular organization was disrupted in post-embryonic meristems both in the root and in the shoot, and their meristematic activity was reduced or became abnormal. We showed that the mutant RAM lost its QC identity after germination, which was specified during embryogenesis, whereas the identity of differentiated tissues was maintained. In the post-embryonic SAM, the expression pattern of a typical OC marker gene, *WUSCHEL*, was disturbed in the mutant. These observations indicate that the *HLR* gene is essential to maintain the cellular organization and normal nature of the RAM and SAM. The *HLR* gene encodes RPT2a, which is a subunit of the 26S proteasome that degrades key proteins in diverse cellular processes. We showed that the *HLR* gene was expressed both in the RAM and in the SAM, including in the QC and the OC, respectively, and that the activity of proteasomes were reduced in the mutant. We propose that proteasome-dependent programmed proteolysis is required to maintain the meristem integrity both in the shoot and in the root.

Key words: HALTED ROOT (HLR), *Arabidopsis*, Proteasome, Quiescent center, Organizing center, Meristem

Introduction

In higher plants, the shoot apical meristem (SAM) is the source of all aerial organs (Brand et al., 2001; Clark, 2001), whereas the root apical meristem (RAM) produces the below-ground structures (Dolan et al., 1993; Scheres et al., 1994). Both meristems are formed during embryogenesis, and start to produce cells and organs after germination (Jürgens, 1995; Scheres et al., 1994). In these post-embryonic meristems, cell division and cell differentiation are highly regulated, and the cellular organization is maintained in the meristem as it grows.

The *Arabidopsis* RAM has a typical cell arrangement that contains four central cells, known as the quiescent center (QC), surrounded by initial cells (Dolan et al., 1993). The QC is mitotically inactive, whereas the initial cells have stem cell-like activity, with regularly repeated cell divisions. During post-embryonic development, each initial cell divides in a plane parallel to that in which it touches the QC (Dolan et al., 1993). The daughter cell that is adjacent to the QC is kept as the initial, and the other daughter differentiates into a specific cell type according to its position (van den Berg et al., 1995). When the QC is laser ablated, the adjacent initial cells lose their stem cell-like activity and differentiate in the same way as their daughter cells (van den Berg et al., 1997). These observations revealed that the QC plays essential roles in the maintenance of the post-embryonic RAM. Several genes involved in the specification of QC identity have been isolated by analyses with *Arabidopsis* mutants. For example, in the mutant of a putative transcription factor SCARECROW, several QC-specific markers lost their expression, and the root growth was ceased prematurely (Di Laurenzio et al., 1996; Sabatini et al., 2003). In addition, the mutation of a putative auxin efflux carrier PIN4 disrupted both the expression pattern of QC-specific markers and the cellular organization of the RAM (Friml et al., 2002).

Similar to the role of QC in the RAM, the organizing center (OC) in the SAM is involved in the maintenance of meristematic activity (Laux et al., 1996; Mayer et al., 1998). The *Arabidopsis* SAM consists of a dome of cells, which is organized into a central zone (CZ) that harbors the OC and stem cells, and a peripheral zone wherein organ primordium are developed. The OC and the stem cells are located in the lower and the upper region of the CZ, respectively, and the OC is known to confer the stem cell state on its upper cells, which in turn restrict the size of the OC (Mayer et al., 1998; Laux,

2003). In this feedback regulation, a homeobox gene *WUSCHEL* (*WUS*) has an important role in maintaining the size and activity of the OC. The *WUS* gene is expressed in the OC, and it is known that enlargement of *WUS* expression domain causes expansion of the SAM (Schoof et al., 2000).

Despite of the similarity between the basic organization of the RAM and the SAM, mutations affecting both meristems have not been extensively analyzed. Here, we present evidence that the *HALTED ROOT* (*HLR*) gene is essential for maintenance of the post-embryonic RAM and SAM, and encodes a subunit of the 26S proteasome.

The proteasome is a huge complex that degrades various target proteins that are tagged with poly-ubiquitin chains (Hershko and Ciechanover, 1998). This ubiquitin-proteasome system is highly conserved in eukaryotes (Ferrell et al., 2000; Shibahara et al., 2002; Fu et al., 1999). It has been suggested that, in higher plants, the proteasome degrades various regulators in diverse cellular processes, including cell cycle progression (Genschik et al., 1998), auxin transport (Sieberer et al., 2000) and various hormone signaling pathways (Callis and Vierstra, 2000; Hellmann and Estelle, 2002). The 26S proteasome consists of a 20S catalytic 'core' and a 19S regulatory particle, which is divided further into two subcomplexes known as the 'base' and the 'lid' (Glickman et al., 1998). The base is made up of six ATPases, RPT1 to RPT6, and three non-ATPase subunits, RPN1, RPN2 and RPN10, whereas the lid consists of nine RPN subunits. The base plays multiple roles in proteasome functions, such as in the recognition of poly-ubiquitin chains, the unfolding of target proteins, the channel opening of the core, and the stuffing of the unfolded proteins into the core (Lam et al., 2002; Glickman et al., 1998; Kohler et al., 2001). Each RPT protein is known to play distinct roles (Ferrell et al., 2000). In particular, RPT2 is essential for the channel opening of the core, and thus it is necessary for proteasome activity in yeast (Rubin et al., 1998; Kohler et al., 2001). In *Arabidopsis*, the genes that encode the non-ATPase subunits, RPN10 and RPN12, of the lid were isolated, and these subunits were suggested to control specificity for the target proteins (Smalle et al., 2002; Smalle et al., 2003). However, the role of each subunit of the base in higher plants, particularly during morphogenesis, remains unknown.

Here, we demonstrate that the *HLR* gene that encodes the RPT2 protein is required for proteasome activity in *Arabidopsis*, and that this gene has important roles in meristem maintenance.

Materials and methods

Plant strains

The *hlr-1* mutant was identified in screens of T-DNA-transformed wild-type Wassilewskija (WS) plants, but no T-DNA was found in the mutant. The *hlr-2* mutant was isolated from the database of T-DNA-transformed wild-type Columbia (Col) plants made by the Salk Institute Genomic Analysis Laboratory. The WS ecotype was used as wild type.

The structure of *DR5::GFP* was cited by Ottenschläger et al. (Ottenschläger et al., 2003). The *cyclinB1;1p::destruction box-GUS* (*cycB1::GUS*) was provided by Dr P. Doerner (Colon-Carmona et al., 1999); *SHRp::GFP* and *SCRp::GFP* by Dr H. Fukaki and Dr P. Benfey (Wysocka-Diller et al., 2000; Helariutta et al., 2000); QC184 and QC46 by Dr B. Scheres (Sabatini et al., 1999; Sabatini et al., 2003); and *HS::AXR3NT-GUS* and *HS::axr3-1NT-GUS* by Dr S. Kepinski and Dr M. Estelle (Gray et al., 2001). J1092 that was made by Dr J. Haseloff was obtained from the Nottingham Arabidopsis Stock Centre (NASC) (http://www.plantsci.cam.ac.uk/haseloff/CATALOGUES/Jlines/index.html). To observe marker expression in the *hlr* mutant, we examined F3 seeds from a cross between *hlr-1* and plants harboring the marker for all lines except for *SCRp::GFP*, where the F2 seeds were used.

Growth conditions and measurement of root growth

Seeds were surface-sterilized and planted in square Petri dishes containing 1.5% agar medium as described previously (Okada and Shimura, 1992), except for in in situ immunolocalization experiments, where MS medium was used (0.1 g/l myo-inositol, MES, 4.33 g/l MS, 5 g/l sucrose, agar 16 g/l, pH 5.7). Seeds on plates were kept in a cold room at 4°C for 3-4 days and then were exposed to white light in plates placed vertically in an incubator kept at 22°C under continuous illumination.

For the measurement of primary roots, the date was counted after germination. Only seedlings that germinated at approximate time were used for root growth measurements.

Histological analysis and GUS assay

The whole-mount preparation for roots and embryos was performed as described by Yadegari et al. (Yadegari et al., 1994). For Lugol staining, roots were stained as described previously (Fukaki et al., 1998), with some modifications.

For SAM observations, resinous sections were prepared. Tissues fixed with FAA overnight were replaced with 50, 70, 90 and 100% ethanol, and these samples were embedded in Technovit 7100 resin (Kulzer, Heraeus) and sectioned (5-8 μm). Sections of embryos were stained with Astra Blue (Merk), and those of seedlings were stained with Toluidine Blue (Merk) (Scheres et al., 1994).

Tissues harboring *GUS* marker genes were stained at 37°C overnight, and then cleared as described previously (Malamy and Benfey, 1997).

For *HS::AXR3NT-GUS* and *HS::axr3-1NT-GUS*, the protocol described by Gray et al. (Gray et al., 2001) was modified. The 36-hour-old seedlings were heat shocked for 3 hours on plates or in water at 37°C. After incubation at room temperature for 0-120 minutes, samples were GUS-stained and cleared using the above procedures. The proteasome-inhibitor treatments were performed by adding 20 μM MG132 1 hour before the end of the heat-shock period.

For light microscopy, samples were observed using DIC optics on a Zeiss Axiophot 2microscope.

Imaging of GFP expression

To examine cell arrangement in the RAM, root tips were stained with 10 μg/ml propidium iodide (PI) solution and observed using a confocal laser scanning microscope (LSM410 or LSM510, Carl Zeiss) with an argon laser. The FITC channel (green: GFP) was overlaid onto the TRITC channel (red: PI) to permit identification of GFP-positive cells. Images were scanned into Adobe Photoshop.

Whole-mount in situ immunolocalization

Immunolocalization in roots was performed as described (Friml et al., 2002; Müller et al., 1998; Steinmann et al., 1999). Affinity-purified primary anti-PIN1, anti-PIN2 and anti-PIN4 antibodies were diluted 1:500, 1:400 and 1:400, respectively (Friml et al., 2002; Gälweiler et al., 1998). The secondary antibody, Alexa-488-conjugated anti-rabbit antibody, was diluted 1:300. Solutions, during the immunolocalization procedures, were changed using a pipetting robot (Insitu Pro, Intavis).

In situ RNA hybridization

Seedlings were fixed with 4% paraformaldehyde in PBS. Paraffin sections (8 μm thick) were hybridized with digoxigenin-labeled probes as described previously (Coen et al., 1990), with some

modifications. The *HLR* and *WUS* probes were prepared by subcloning part of the cDNA (corresponding to the region 1163-1544 of the *HLR* cDNA, and to 101-1202 of *WUS* cDNA, respectively) into pBluescript SK (Stratagene).

Map-based cloning

The *hlr-1* mutant was crossed to Col or Landsberg *erecta* and plants showing root expansion were identified in the F2 population. Commercially available PCR-based markers (SSLP and CAPS) were used to map the *HLR* gene to the bottom of chromosome 4. Additional SSLP and CAPS markers were developed using publicly available *Arabidopsis* genomic sequence at the Munich Information Centre for Protein Sequences (MIPS; http://www.mips.biochem.mpg.de/).

Complementation test

A 7.3 kb genomic fragment spanning the *HLR* gene, corresponding to region 18998-26309 of BAC clone F19B15, was subcloned into binary vector pPZP211 (Hajdukiewicz et al., 1994) and introduced into *Agrobacterium tumefaciens* C58C1. The binary vector containing the fragment was introduced into *hlr-1* plants and the phenotypes were examined for complementation.

Accession numbers

GenBank Accession Numbers for sequences described in this article are AB161192 (*HLR* cDNA) and AJ012310 (*WUS* cDNA). Accession Numbers for amino acid sequences compared with HLR are AY056335 (AtRPT2b), P46466 (PRS4-ORYSA), P48601 (PRS4-DROME), Q03527 (PRS4-HUMAN) and P40327 (PRS4-YEAST). The Accession Number of the BAC clone used for mapping is AL078470 (F19B15).

Results

The *halted root* (*hlr*) mutation reduces RAM activity after germination

The *hlr* mutant was isolated on the basis of its root phenotypes: retarded root growth and expansion of the root tip (Fig. 1A-C). Within two days of germination, the growth rate of the primary roots was reduced in the *hlr* mutant (Fig. 1J). In 10-day-old seedlings of the mutant, the average root length was 12.2±1.3 mm (n=100), which was only approximately one-fifth as long as in the wild type (60.9±6.3 mm [n=103]). In the mutant, the root cap was elongated and the root tip was expanded radially (Fig. 1B,C; brackets), suggesting disruption of the root structure. In one-month-old mutant seedlings, the lateral root primordia formed unusually close to the root tip (Fig. 1D,E; arrows). These features were observed not only in the major root, but also in the lateral root (Fig. 1A; data not shown).

We then examined whether the *hlr* mutant exhibited any structural or functional defects in the embryonic RAM. As stated above, the *Arabidopsis* RAM has a typical cell arrangement (Fig. 2A,C). Although a few embryos of the mutant reduced the cell layers of columella root cap, the QC and surrounding initial cells were observed in mature embryos of the mutant (Fig. 2B, white arrowheads). At 36 hours after germination, however, this pattern was disrupted in the mutant, and the QC could not be identified morphologically (Fig. 2D). This structural abnormality suggests that the *hlr* mutant lost normal QC identity and failed to regulate the cell division pattern in the post-embryonic RAM.

The QC identity is lost in the *hlr* mutant after germination

We next examined whether differences in the expression of QC-specific marker genes could be observed before and after germination. In the RAM of wild type, a QC-specific marker, QC184 (Sabatini et al., 2003), was detected in the QC of mature embryos but not in other cells (Fig. 3A). In addition, in wild type, expression of QC184 was lost in dry seeds, but was restored in 36-hour-old seedlings (Fig. 3C). In mature embryos of the mutant, although the cell shape of the QC was slightly aberrant, the QC184 marker was expressed in the QC (Fig. 3B). At 36 hours after germination, however, QC184 expression could not be detected in the mutant (Fig. 3D). We further examined the expression of another QC-specific marker, QC46 (Sabatini et al., 2003), which was expressed in the QC of 7-day-old wild-type seedlings (Fig. 3E). In *hlr* seedlings,

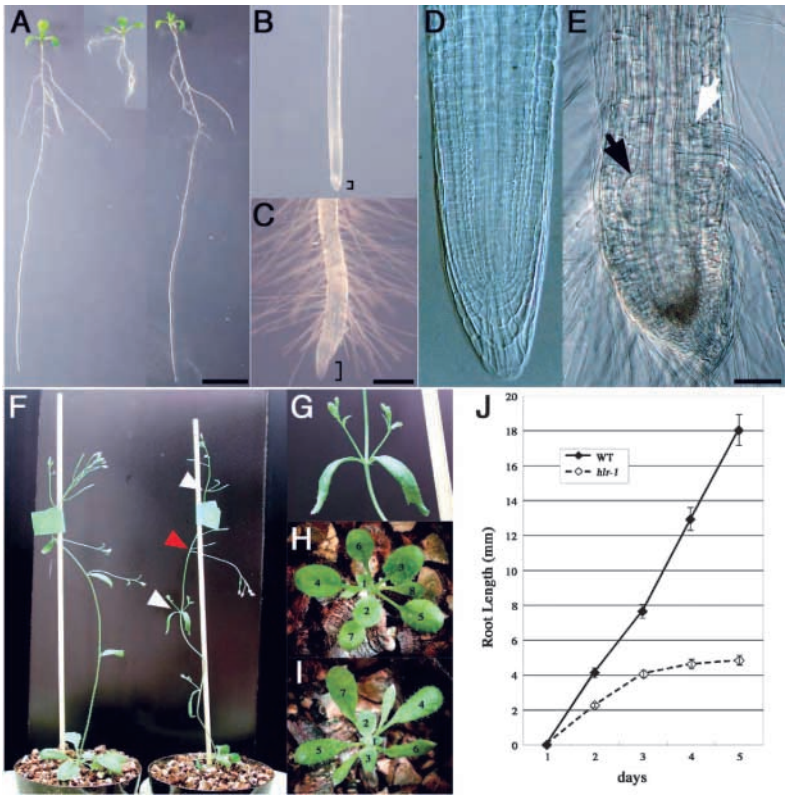

Fig. 1. The *halted root* (*hlr*) mutant is defective both in roots and shoots. Phenotypes of wild type (A,B,D,F,H) and *hlr-1* (A,C,E,F,G,I). (A) Ten-day-old seedlings of wild-type (WT; left), *hlr-1* mutant (center) and the *hlr-1* mutant possessing a genomic fragment of the wild-type *HLR* gene (right). (B,C) Primary roots of 12-day-old wild-type (B) and *hlr-1* (C) seedlings. Brackets indicate the area of the root cap. (D,E) Root tips of one-month-old seedlings. White and black arrows indicate the lateral root and the lateral root primordium, respectively. (F,G) Aerial parts of 42-day-old plants. Red arrowhead indicates the secondary inflorescence without cauline leaves, and white arrowheads indicate lateral organs with irregular internode length (higher magnification is shown in G). (H,I) Aerial parts of one-month-old plants. The number on each leaf indicates age order, from young to old leaves. (J) Elongation of primary roots. Each point represents the mean of 100 seedlings. Error bars indicate s.e. (n=103 for wild type; n=100 for *hlr-1*). Scale bars: 1 cm in A; 500 μm in B,C; 50 μm in D,E.

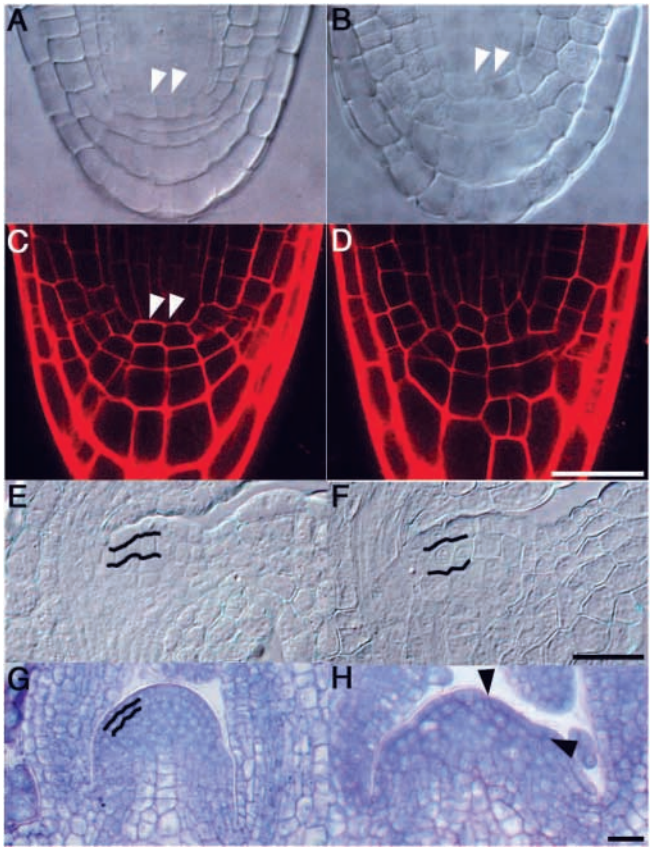

Fig. 2. The *HLR* gene is required for post-embryonic meristems. Structures of embryonic and post-embryonic meristems in wild type (A,C,E,G) and *hlr-1* mutants (B,D,F,H). (A-D) Whole-mount preparations of the root apical meristem (RAM) in mature embryos (A,B), and in PI-stained root tips of 36-hour-old seedlings (C,D). White arrowheads indicate the quiescent center (QC). (E-H) Longitudinal sections of the shoot apical meristem (SAM) in mature embryos (E,F) and in 8-day-old seedlings (G,H). Black lines and arrowheads indicate the boundaries between cell layers and the irregular cell division planes, respectively. Scale bars: 25 μm.

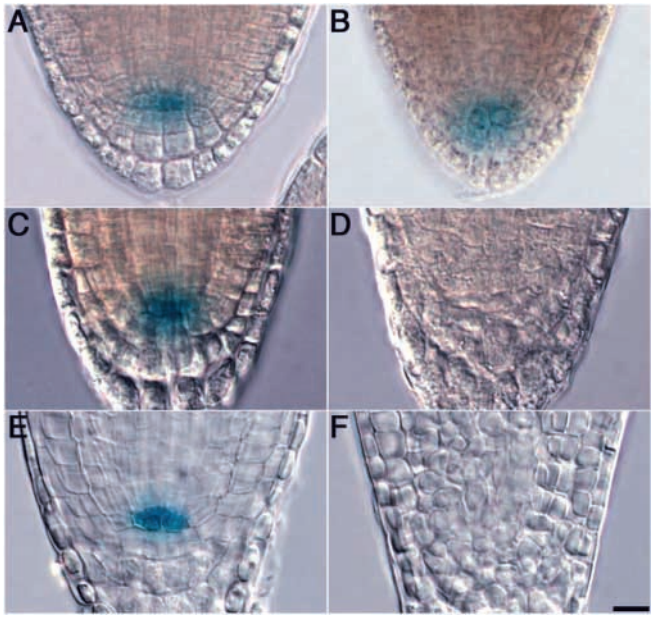

Fig. 3. QC identity is lost in the *hlr* mutant after germination. Expression of QC-specific markers in wild type (A,C,E) and *hlr-1* mutants (B,D,F). (A-D) Expression of QC184 in mature embryos (A,B) and in 36-hour-old seedlings (C,D). (E,F) Expression of QC46 in 7-day-old seedlings. Scale bar: 25 μm.

expression of the QC46 was not detected in the RAM (Fig. 3F). These results indicate that the post-embryonic RAM of the mutant fails to maintain its QC identity, which is specified during embryogenesis.

The identities of differentiated tissues are maintained in the *hlr* mutant

To examine whether the mutant roots also fail to maintain the specificity of differentiated tissues, we analyzed the expression of several cell-type-specific markers (Fig. 4). Cells in the root cap have starch granules, which can be visualized by Lugol-staining (Fukaki et al., 1998). In the root cap of the mutant, the cell arrangement was deformed (Fig. 4B), and, although these cells possessed starch granules as in wild type, the stained domain was enlarged covering the QC and the neighboring cells (Fig. 4C,D). The expression of a lateral root cap specific marker, J1092 (Long et al., 2002), was observed in the region corresponding to the lateral root cap, and was not expanded to the QC (Fig. 4E,F). Taken together, these data suggest that the QC and neighboring cells may take on the identity of columella root cap cells. As shown in Fig. 4G-J, the expression pattern of *SHRp::GFP*, a specific marker for the stele (Helariutta et al., 2000), and of *SCRp::GFP*, a marker for the endodermis, the cortex/endodermal initial cells and the QC (Wysocka-Diller et al., 2000), showed that these tissues maintained their identity in the mutant seedlings, except that the QC and the cortex/endodermal initial cells appeared to cease expression of the markers. These results indicate that the QC and surrounding initial cells lose their specificity, but that the other cells retain their identity. In addition, we noted that several cells in the mutant stele were stained with PI and Trypan Blue, showing that these cells were dead (Fig. 4B,F,H,J; data not shown). These dead cells may be related to the retardation of root growth and/or disruption of the cell arrangement in the RAM.

The *hlr* mutant is also defective in SAM structure and activity

Mutant plants form abnormally shaped leaves, such as dentate, narrow or asymmetrical leaves (Fig. 1H,I). In the vegetative phase, phyllotaxy in wild-type rosettes exhibited a regular spiral pattern, with each leaf separated by approximately 137.5° (Fig. 1H). By contrast, the *hlr* rosettes exhibited disordered phyllotaxy with irregular angles between the leaves (Fig. 1I). To quantify the abnormality in phyllotaxis, we counted the number of plants in which the angles between serial rosette leaves were greater than 170° or less than 100°. The frequency of abnormal *hlr* plants was 76.6% (n=193), much greater than that of wild type (1.8% [n=117]). Moreover, 23.8% (n=193) of the *hlr* plants ceased leaf production and died before bolting, whereas 100% (n=117) of wild-type plants succeeded in bolting. In the reproductive phase, the *hlr* plants

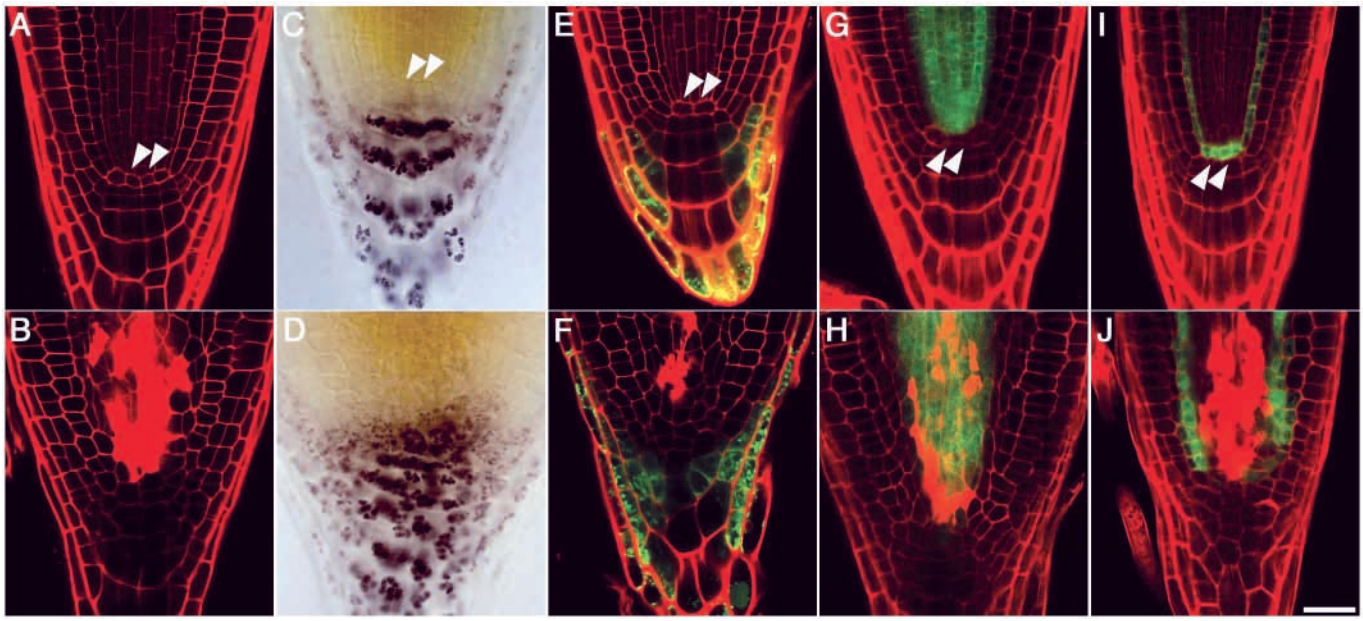

Fig. 4. The identities of differentiated tissues in the RAM remain in the *hlr* mutant. Cell arrangement and cell-type-specific markers in the post-embryonic RAM of wild type (A,C,E,G,I) and *hlr-1* mutants (B,D,F,H,J). Arrowheads indicate the QC. (A,B) PI-stained root tips of 6-day-old seedlings. (C,D) Lugol-stained root tips. Starch granules, a marker for the root cap, are visualized. (E,F) Expression of J1092, a marker for the lateral root cap, in 6-day-old seedlings. (G,H) Expression of *SHRp::GFP*, a marker for the stele, in 6-day-old seedlings. (I,J) Expression of *SCRp::GFP*, a marker for the QC, cortex/endodermal initial cells and endodermis, in 6-day-old seedlings. Scale bar: 25 μm.

frequently formed flowers or secondary inflorescences with irregular internode lengths, and secondary inflorescences that lacked cauline leaves (Fig. 1F,G). No wild-type plants exhibited both abnormalities (n=77), whereas 52.9% (n=68) of the mutant plants did. These results indicate that SAM activities are also abnormal in the *hlr* mutant.

Next, we examined the cellular organization of the SAM before and after germination. In mature embryos of the mutant, both the size and cell arrangement of the SAM were normal, and cell layers were clearly observed (Fig. 2E,F; black lines). However, the SAM was enlarged and the cell layers were disrupted in 8-day-old mutant seedlings, possibly owing to their irregular cell division planes (Fig. 2H, arrowheads). These results indicate that *hlr* mutants form a SAM of normal structure during embryogenesis, but fail to maintain its cellular organization after germination.

To further examine whether the *hlr* mutant maintains the normal properties of cells in the SAM, we analyzed the expression pattern of the *WUSCHEL* (*WUS*) gene by in situ RNA hybridization (Fig. 5A). The *WUS* gene is a typical molecular marker for the OC, a small group of cells in the center of the SAM, underneath the three outer cell layers (Schoof et al., 2000). In 11-day-old mutant seedlings, the *WUS* expression domain was markedly enlarged, and was expanded to outer and/or inner cell layers (Fig. 5B). Furthermore, *WUS* mRNA accumulation was sometimes detected in some groups of cells at abnormal places (Fig. 5B, arrows). These observations suggest that the mutant SAM fails to maintain the proper size and position of the OC. These abnormalities of the OC were consistent with the enlargement and disruption of the cell arrangement of the SAM. From these results, it was concluded that the *HLR* gene is required to maintain the normal nature of the SAM, as well as that of the RAM.

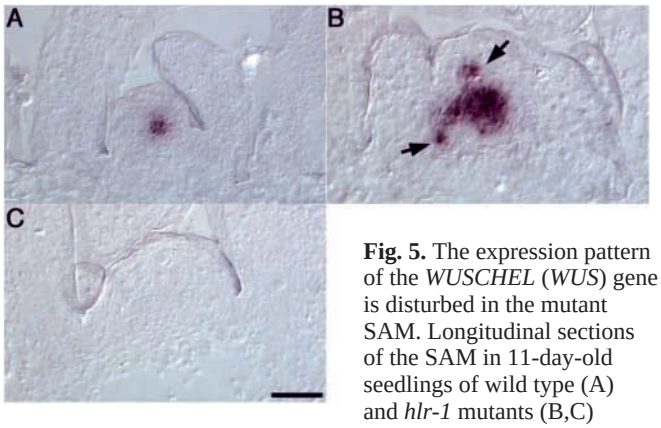

Fig. 5. The expression pattern of the *WUSCHEL* (*WUS*) gene is disturbed in the mutant SAM. Longitudinal sections of the SAM in 11-day-old seedlings of wild type (A) and *hlr-1* mutants (B,C) hybridized with an antisense (A,B) or sense (C) *WUS* probe. Arrows (B) indicate *WUS* mRNA accumulation in some groups of cells in abnormal places. Scale bar: 25 μm.

The *HLR* gene encodes a homolog of the 26S proteasome subunit RPT2 (RPT2a)

Using approximately 2000 recombinant chromosomes, we mapped the *HLR* gene on to the bottom of chromosome 4, in a 45 kb area between markers RPS2 and nga1139 (Fig. 6A). We sequenced this region in the mutant, and identified only one mutation site (a 13 bp deletion) in an open reading frame (ORF) At4g29040 with six exons (Fig. 6B). The deletion mutation was found in the first exon, which might cause a frameshift and generate a stop codon (Fig. 6C). In addition, we isolated a T-DNA insertional allele of *hlr* (*hlr-2*; Fig. 6B,C). All of the morphological features of *hlr-1* were also observed in *hlr-2*, although the phenotype was slightly milder than that of *hlr-1* (data not shown). Furthermore, a 7.3-kb genomic

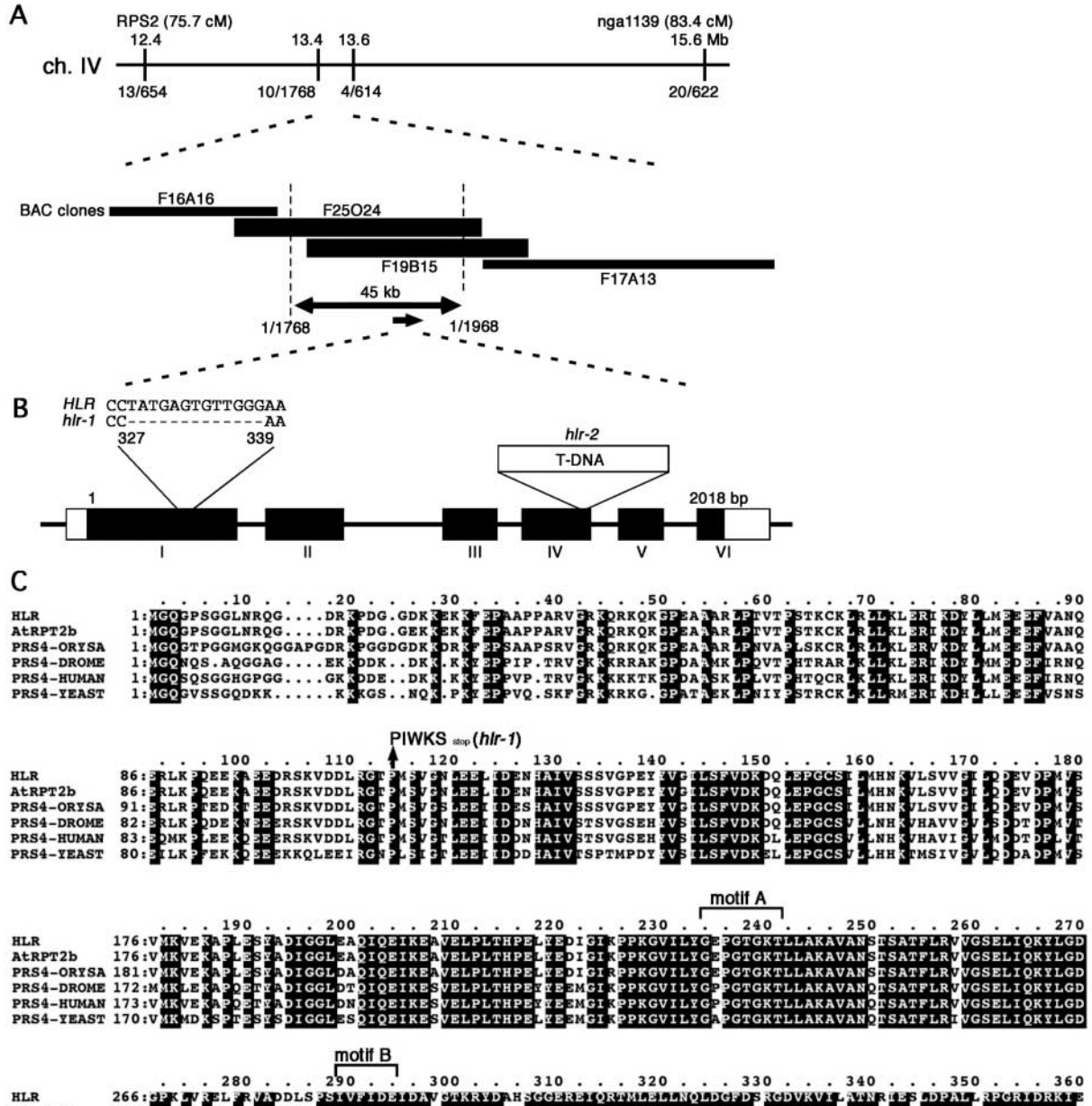

Fig. 6. The *HLR* gene encodes RPT2a, a homolog of the 26S proteasome subunit. Map-based cloning of the *HLR* gene. (A) Physical and genetic map of the *HLR* gene on chromosome 4. Molecular markers and their positions are shown above the line. Values under the lines indicate the frequency of recombinants. The bipolar arrow indicates the 45 kb region sequenced to find the mutation site in *hlr-1*. (B) Schematic structure of the *HLR* gene. Black and white rectangles indicate the protein coding regions and untranslated regions (UTRs), respectively. The 13 bp deletion found in *hlr-1* is shown. (C) Alignment of the deduced amino acid sequence of HLR with selected homologous proteins. Identical residues are shown in white on a black background. Major motifs and mutation sites found in the *hlr* mutant are shown. Orthologous proteins are from *Oryza sativa* (PRS4-ORYSA), *Drosophila melanogaster* (PRS4-DROME), *Homo sapiens* (PRS4-HUMAN) and *Saccharomyces cerevisiae* (PRS4-YEAST).

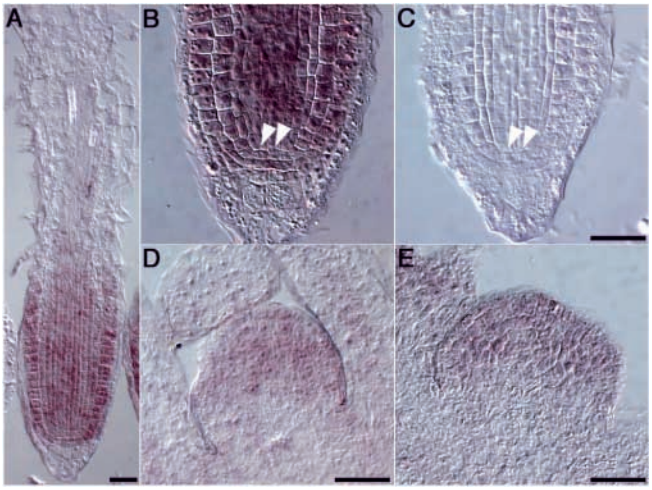

Fig. 7. The *HLR* gene is expressed both in the RAM and in the SAM. (A-C) Longitudinal sections of roots in 36-hour-old wild-type seedlings hybridized with an antisense (A,B) or sense (C) *HLR* probe. Arrowheads indicate the QC. (D,E) Longitudinal sections of the SAM in 11-day-old (D) and 32-day-old (E) seedlings hybridized with the antisense *HLR* probe. The SAM in D and E is in vegetative and reproductive phase, respectively. Scale bars: 25 μm.

fragment spanning this ORF, with 5 kb at the 5′ part of the ORF, complemented all of the phenotypes of the *hlr* mutant (Fig. 1A; data not shown). Based on these results, we concluded that this transcription unit represents the *HLR* gene.

We identified the *HLR* gene encoding a 443-amino acid protein, which is known as RPT2a (Fu et al., 1999). The RPT2 protein is a subunit of the 26S proteasome (subunit 4; Fig. 6C) and is conserved in eukaryotes. The protein carries the ATP/GTP-binding site P-loop (motif A) and AAA-protein family signature (motif B), which constitute the Walker-type ATPase domain conserved in the AAA ATPase family (Beyer, 1997). RPT2 has been shown to work in the 'base' subcomplex of the proteasome, and to be essential for proteasome function in yeast (Rubin et al., 1998; Kohler et al., 2001). These results suggest that the *hlr* mutant is defective in proteasome functions, and that proteasome activity is required to maintain the post-embryonic meristems.

The *HLR* gene is expressed both in the RAM and the SAM

RNA gel blot analysis indicated that *HLR* mRNA accumulates in all the organs that we tested (flower bud, stem, leaf and root; data not shown), which is consistent with the pleiotropic phenotype of the *hlr* mutant. In particular, mRNA accumulated greatly in organs containing meristems, suggesting that the *HLR* gene works throughout the entire plant, especially in the meristematic regions (data not shown).

We then examined expression of the *HLR* gene in meristems by in situ RNA hybridization experiments. In 36-hour-old wild-type seedlings, *HLR* mRNA accumulated in the root tip, including in the QC and all initial cells (Fig. 7A,B, arrowheads). *HLR* mRNA was detected in all kinds of tissues in the RAM, except for the columella root cap. A similar expression pattern was observed in older roots, although the signal was slightly weakened (data not shown). In the shoot, the *HLR* gene was expressed uniformly in the SAM in both the vegetative and the reproductive phase (Fig. 7D,E). The *HLR* mRNA accumulation in meristems was consistent with previous report that the subunits in the lid or core of the proteasome are strongly expressed both in the SAM and RAM of rice seedlings (Yanagawa et al., 2002b). These results suggest that the proteasome plays important roles in the meristems both in the shoot and in the root.

The activity of proteasome is reduced in the *hlr* mutant

To examine whether proteasomal activity is reduced in the mutant, we analyzed protein stability in the post-embryonic RAM by using AUX/IAA proteins. The AUX/IAA proteins are digested in response to auxin, and this turnover is suggested to depend on the proteasome (Dharmasiri and Estelle, 2002). To analyze the efficiency of their degradation in the mutant, we monitored the stability of a AXR3/IAA17-GUS reporter protein that was expressed under the control of a heat-shock promoter (*HS::AXR3NT-GUS*) (Gray et al., 2001).

In 36-hour-old seedlings of the wild type and the mutant, GUS stains were observed immediately after heat-shock induction, with the mutant staining rather more intensely than the wild type (Fig. 8A,B). At 60 minutes after the heat shock period, although GUS staining disappeared in wild-type seedlings, it was still observed in the *hlr* mutant (Fig.

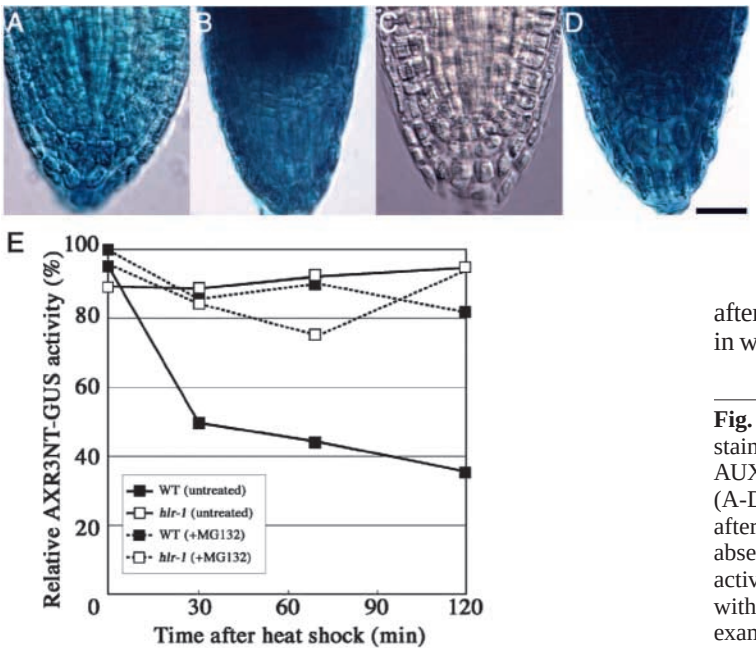

Fig. 8. Proteasome activity is reduced in the *hlr* mutant. GUS staining of *HS::AXR3 NT-GUS*, a marker for the degradation of AUX/IAA proteins, in wild type (A,C) and in the *hlr-1* mutant (B,D). (A-D) 36-hour-old seedlings stained for 0 (A,B) or 60 minutes (C,D) after the end of heat-shock induction. (E) GUS activity in the absence or presence of the proteasome inhibitor MG132. Relative activity was calculated as a percentage of the number of seedlings with GUS staining in the roots per total number of seedlings examined. Scale bar: 25 μm.

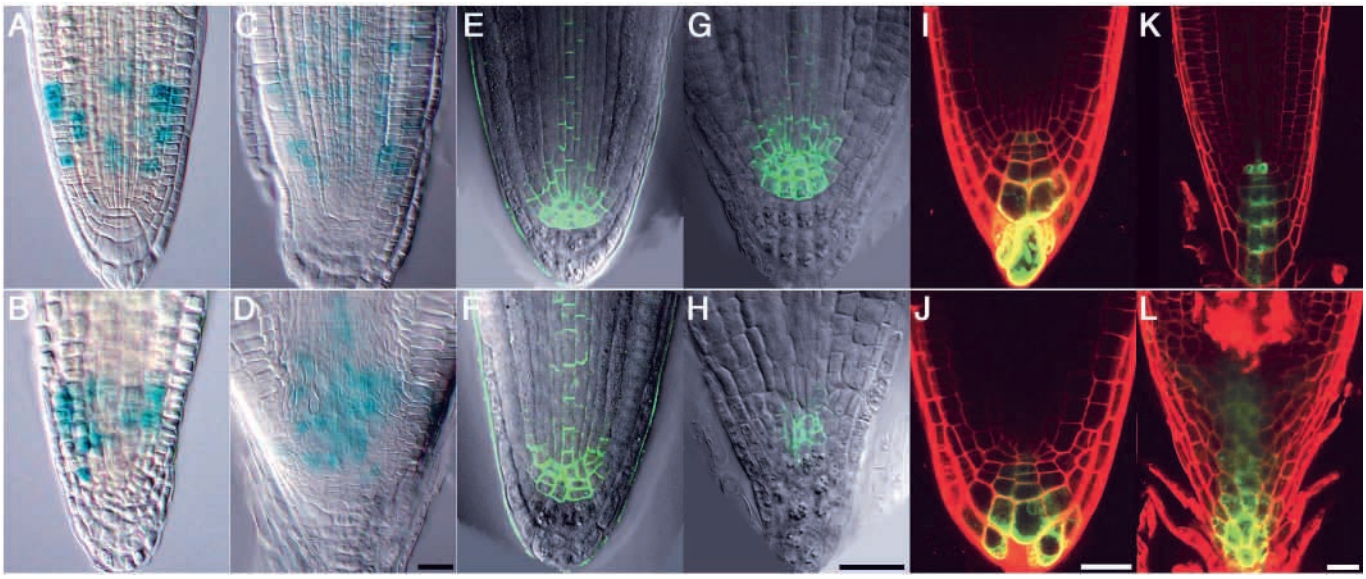

Fig. 9. Disruption of RAM organization in the *hlr* mutant is not caused by the defective degradation of cyclin B1 or PIN4. Marker gene expression and localization of putative target proteins for proteasome-mediated degradation in 36-hour-old (A,B,E,F,I,J) and 10-day-old (C,D,G,H,K,L) seedlings of wild type (A,C,E,G,I,K) and *hlr-1* mutants (B,D,F,H,J,L). (A-D) GUS staining of *cycB1::GUS*, a marker for the degradation of cyclin B1. (E-H) Whole-mount in situ immunolocalization of the PIN4 protein, a putative auxin efflux carrier. (I-L) Expression of *DR5::GFP*, a marker for auxin accumulation. Scale bars: 25 μm.

8C,D). Increased stability was observed in the wild type when a proteasome inhibitor, MG132, was added to the seedlings (Fig. 8E). Furthermore, stability was increased in both the wild type and the mutant when we used another marker protein, axr3-1NT-GUS (Gray et al., 2001), which has a mutation in the domain responsible for its degradation (data not shown). These results suggest that the mutation in the *HLR* gene stabilizes the AUX/IAA proteins, and that the *HLR* gene is required for proteasome activity in the post-embryonic meristem.

Disruption of cellular organization in the mutant RAM is not related to the failure to degrade cyclin B1 or PIN proteins

To study the role of proteasomes in the maintenance of meristems, we analyzed several proteins known or suggested to be regulated by proteasome-mediated degradation and known to be required for the meristematic activity. First, we analyzed the turnover of cyclin. Cyclin is digested by the proteasome at a particular phase in cell cycle, and its degradation is essential for cell division. We examined an M-phase cyclin, cyclin B1, because cell cycle-dependent synthesis and degradation of cyclin B1 are required for cell propagation in roots (Doerner et al., 1996). Wild-type plants carrying *cyclin B1;1p::destruction box-GUS* (*cyc B1::GUS*) showed a spotted pattern in their roots (Fig. 9A,C), possibly showing cells in M phase (Colon-Carmona et al., 1999). In 36-hour-old mutant seedlings, in which the QC was not identified and cell arrangement was disrupted, GUS-stained cells showed a similar spotted pattern to that of wild type (Fig. 9B). The same spotted pattern was observed in 10-day-old seedlings (Fig. 9D), suggesting that cell proliferation did not stop in the mutant. These observations suggest that phase-specific expression and degradation of cyclin B1 are normal in the mutant, and that loss of QC identity and disruption of cellular organization are therefore not related to the failure of cyclin B1 digestion. This result is consistent with previous reports that the cell arrangement in the RAM is not disrupted when cyclin is overproduced (Doerner et al., 1996), or when cell cycle progression is inhibited (Umeda et al., 2000).

Next, we analyzed the turnover of PIN proteins, which mediate auxin transport. Auxin is transported towards the root tips, and the proper auxin gradient is known to be important for QC identity (Sabatini et al., 1999; Friml et al., 2002). It has also been reported that a PIN protein, PIN2, is digested by proteasome (Sieberer et al., 2000). Thus, we determined the location and amount of PIN proteins by using in situ immunolocalization (Fig. 9E-H). In wild-type roots, PIN4 proteins localize on the cell membranes of QC-surrounding cells (Fig. 9E,G). In 36-hour-old mutant seedlings, where QC identity was already lost, the localization and signal intensity of PIN4 was almost identical to that of wild-type plants (Fig. 9F). At 10 days after germination, however, both the localization area and the signal intensity were significantly reduced in the mutant (Fig. 9H).

The localization and intensity of other PIN proteins, PIN1 and PIN2, were also normal in 36-hour-old mutant seedlings, but the amount of PIN1 at the QC and neighboring cells was reduced at 10 days (data not shown). However, expression of PIN2 in the differentiated cells of the epidermis and cortex was normally observed in 10-day-old seedlings (data not shown). These observations showed that the location and intensity of PIN proteins were not affected in the 36-hour-old seedlings, when the cell arrangement was already disrupted. They also indicate that PIN proteins were reduced in the QC and the surrounding cells only after meristematic activity was decreased. These results suggest that the loss of QC identity and the abnormal cell arrangement of the RAM in the mutant do not correspond to the failure to degrade PIN proteins.

To confirm that the turnover of PIN proteins was normal in 36-hour-old seedlings, we further examined auxin distribution by using *DR5::GFP*, a marker for auxin accumulation (Ottenschläger et al., 2003). In the wild type, GFP expression was observed in the QC and the columella root cap, where auxin is accumulated at a maximum level (Fig. 9I,K). A similar pattern was observed in 36-hour-old mutant seedlings (Fig. 9J), although an enlargement of the GFP expression domain was observed at 10 days (Fig. 9L). These observations were highly consistent with the pattern of PIN proteins, supporting the hypothesis that the loss of QC identity and the disruption of cellular organization in the mutant RAM is not caused by the failure to degrade PIN proteins, or to form a proper auxin gradient.

Discussion

The *HLR* gene is essential for the post-embryonic meristems

The structure of the SAM and RAM is formed during embryogenesis, and after germination, both meristems start to produce cells, and maintain their structure and activity (Jürgens, 1995; Scheres et al., 1994). In the *hlr* mutant, cell arrangement was disrupted in the post-embryonic SAM and RAM, and their meristematic activity was reduced or became abnormal. In the post-embryonic SAM, the expression pattern of the *WUSCHEL* (*WUS*) gene was disturbed in the mutant. In the RAM, expression of a QC-specific marker, QC184, disappeared after germination, although its expression was initiated normally in the embryos. These observations indicate that the *HLR* gene is indispensable for maintaining the cellular organization and the normal nature of the post-embryonic meristems. This also suggests that maintenance of the SAM and RAM is supported by a similar genetic system.

The *HLR* gene has a role in controlling the division pattern of stem cells

In the RAM of the *hlr* mutant, the cell arrangement around the QC was disrupted after germination, although roots continued to grow for several days. This observation indicates that the *HLR* gene is responsible for the timing and/or direction of cell division of the initial cells and their daughters. Similarly in the SAM, irregular cell division planes were observed, indicating that the *HLR* gene is also required to regulate the cell division pattern in the SAM. The orientation of division is known to be dependent on the proper localization of preprophase bands (PPBs) and phragmoplasts. It is reported that the proteasome is involved in the formation of both structures. The proteasome localizes at PPBs and phragmoplasts during cell cycle progression, and the application of proteasome inhibitor causes the arrest of PPB formation and the collapse of phragmoplasts in tobacco BY-2 cells (Yanagawa et al., 2002a). Therefore, HLR may be required for dividing cells in the meristems to control the position of PPBs and phragmoplasts. Another fascinating possibility is that the *HLR* gene acts on the QC and OC to regulate the normal division of neighboring stem cells, because disordered division in the RAM occurs at the same time as loss of QC identity in the mutant.

Roles of the *HLR* gene in the post-embryonic SAM

We noticed that the expression pattern of the *WUS* gene was disturbed in the post-embryonic SAM. Because the group of *WUS*-expressing cells acts as an OC to direct the fate of the overlying stem cells (Mayer et al., 1998), the enlargement of the *WUS* expression domain may increase the stem cell population, causing the expansion of the mutant SAM. In addition, the *WUS* expression domain expanded into another cell layers, and *WUS* mRNA was detected in some groups of cells at abnormal places in the SAM. These observations suggest that the mutant SAM may also fail to maintain the identity of cells in the peripheral region, causing the disruption of the position and/or timing of organ development. These notions are consistent with the pleiotropic defects in mutant shoots, such as the formation of abnormally shaped leaves and the disruption of phyllotaxy, and with the reports that these defects were observed in *clavata 1* or *fasciata* (*fas1* and *fas2*) mutants, which show enlargement of the *WUS* expression domain and expansion of the SAM (Leyser and Furner, 1992; Reinholz, 1966; Kaya et al., 2000; Schoof et al., 2000).

Regulatory network of the RAM-specific genes

It was recently demonstrated that the QC requires the expression of *SCARECROW* (*SCR*), a gene responsible for generation of cortex and endodermis, to maintain the QC identity (Di Laurenzio et al., 1996; Sabatini et al., 2003). In the *scr* mutant, the QC loses the expression of a QC-specific marker, QC46, but retains another marker, QC184 (Sabatini et al., 2003). In contrast to the *scr* mutant, the *hlr* mutant lost the expression of both QC46 and QC184 markers in the QC after germination. *SCRp::GFP* expression also disappeared in the QC and cortex/endodermal initial cells of the *hlr* mutant. Taken together, these data suggest that the *HLR* gene is required to promote the expression of *SCR* in the QC and the initial cells. In the *hlr* roots, however, *SCRp::GFP* was expressed in tissues that had already differentiated when the QC activity was reduced. Thus it is likely that the activity of HLR is required for the expression of *SCR* only in the QC and the neighboring initial cells, although the *HLR* gene is expressed not only in the QC and initial cells, but also in differentiated tissues. The requirement of the *HLR* gene in the QC and its neighboring cells is supported by the observation that the putative auxin efflux carriers, PIN proteins, were reduced only in cells surrounding the QC in the *hlr* mutant. This reduction of SCR and/or PIN proteins may be responsible for the retardation of root growth in the *hlr* mutant, because *scr* and *pin4* mutants also show retarded root growth (Sabatini et al., 2003; Friml et al., 2002). Furthermore, the decrease of PIN proteins around the QC may enlarge the auxin-accumulated domain, as shown in the DR5-GFP expression (Fig. 9L), and the regional increase of auxin concentration may change the fate of the QC and neighboring initial cells to that of the columella root cap cells that form starch granules (Fig. 4D).

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