The Arabidopsis USL1 controls multiple aspects of development by affecting late endosome morphology

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Summary

- The polar transport of auxin controls many aspects of plant development. However, the molecular mechanisms underlying auxin transport regulation remain to be further elucidated.
- We identified a mutant named usl1 (unflattened and small leaves) in a genetic screen in Arabidopsis thaliana. The usl1 displayed multiple aspects of developmental defects in leaves, embryogenesis, cotyledons, siliques, phyllotaxy and lateral roots in addition to abnormal leaves. USL1 encodes a protein orthologous to the yeast vacuolar protein sorting (Vps) 38p and human UV RADIATION RESISTANCE-ASSOCIATED GENE (UVRAG). Cell biology, Co-IP/MS and yeast two-hybrid were used to identify the function of USL1.
- USL1 colocalizes at the subcellular level with VPS29, a key factor of the retromer complex that controls auxin transport. The morphology of the VPS29-associated late endosomes (LE) is altered from small dots in the wild-type to aberrant enlarged circles in the usl1 mutants. The usl1 mutant synergistically interacts with vps29. We also found that USL1 forms a complex with AtVPS30 and AtVPS34.
- We propose that USL1 controls multiple aspects of plant development by affecting late endosome morphology and by regulating the PIN1 polarity. Our findings provide a new layer of the understanding on the mechanisms of plant development regulation.

Introduction

The phytohormone auxin plays essential roles in control of many aspects of plant development from embryogenesis to postembryonic development as a morphogen by specific distribution through its biosynthesis, conjugation, metabolism and polar transport (Friml, 2003; Qin et al., 2005; Teale et al., 2006; Cheng et al., 2007; Baylis et al., 2013; Kazan, 2013; Bar & Ori, 2014; Kasprzewska et al., 2015; Tang et al., 2016). At the cellular level, the asymmetric subcellular distribution of the PIN-FORMED (PIN) auxin transport family proteins generate local auxin gradients key for plant development by driving auxin to be transported from cell to cell in a polar manner (Gälweiler et al., 1998; Müller et al., 1998; Tanaka et al., 2006). Therefore, the changes of the PIN family proteins in subcellular organelles indirectly regulate plant development by affecting auxin distribution.

PIN proteins undergo constitutive clathrin-mediated endocytosis to subsequently be recycled to different polar domains (Geldner et al., 2003; Dhonukshe et al., 2007; Tanaka et al., 2009; Feraru et al., 2012; Naramoto et al., 2014b) or to be delivered to vacuoles for degradation (Kleine-Vehn et al., 2008; Spitzer et al., 2009; Baster et al., 2013). Briefly, PIN proteins on the plasma membrane (PM) can be internalized by membrane invagination to form clathrin-coated vesicles (CCVs). CCVs first reach the trans-Golgi network/early endosomes (TGN/EE). From there, the PIN proteins are sorted back to the PM for reuse via recycling endosomes or delivered to vacuoles for degradation through late endosomes/multivesicular bodies/prevacuolar compartments (LE/MVB/PVC) (Grunewald & Friml, 2010). GNOM and ESCRT (endosomal sorting complex required for transport) complex has been identified to be essential for the recycling or degradation of the PIN proteins, respectively. GNOM encodes a guanine nucleotide exchange factor for ADP-ribosylation factors (ARF-GEF) (Shevell et al., 1994; Busch et al., 1996; Steinmann et al., 1999). The active GTP-bound ARFs are important for the formation, targeting and fusion of vesicles in endocytic trafficking because of their tight binding to the membrane in eukaryotic organisms (Donaldson & Jackson, 2000; Jürgens & Geldner, 2002). GNOM can activate ARFs by changing a GDP in the inactive ARFs to be a new GTP in control of the PIN1 endocytic cycling (Geldner et al., 2003). The disruption of GNOM causes abnormal PIN1 polarity and severe defects in plant embryogenesis. The ESCRT complex includes five subcomplexes including ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III and VPS4 that coordinate to sort ubiquitinated PM proteins into intraluminal vesicles (ILV) inside the lumen of the LE/MVB/PVC for their final degradation in vacuoles (Piper & Katzmann, 2007; Otegui & Spitzer, 2008; Reyes et al., 2011). Several reports have demonstrated that some subunits of the plant ESCRT complex play critical roles in mediating the
degradation of the PIN proteins in the vacuoles using the endosomal sorting pathway (Spitzer et al., 2009; Gao et al., 2014; Wang et al., 2017). In contrast to the function of the ESCRT complex, the retromer complex retrieves the PIN proteins from the LE/MVB/PVC to return them to the TGN/EE via the retrograde pathway to avoid PIN degradation in the lytic vacuoles (Kleine-Vehn et al., 2008).

The retromer is a conserved complex localized to the cytosolic face of the endosomes and is highly important for the intracellular sorting of PM transporters and receptors in eukaryotes (Seaman, 2005; Bonifacino & Rojas, 2006; Bonifacino & Hurley, 2008; Zelazny et al., 2013). The multimeric retromer is composed of two subcomplexes. One is the core subcomplex containing the trimer of VPS26, VPS29 and VPS35 that is proposed to bind directly to the cytosolic tail of cargo proteins. The other consists of the dimer of the sorting nexin proteins (SNXs). The Arabidopsis VPS29 is a single copy gene, and the vps29 loss-of-function mutant displays severe auxin-related phenotypes including defective embryogenesis, abnormal cotyledons, fewer lateral roots, dwarfism and agravitropism (Shimada et al., 2006; Jaillais et al., 2007). Genetic and molecular evidence indicates that VPS29 is required for the proper subcellular trafficking of the PIN proteins by maintaining the morphology of the LE/MVB/PVC (Jaillais et al., 2007). The Arabidopsis genome contains three VPS35 homologs designated as VPS35a, VPS35b and VPS35c. VPS35a plays the predominant role in PIN endocytic trafficking. Although the vps35a mutant exhibits no obvious phenotypes such as those observed in vps29, the altered morphology of the LE/MVB/PVC and the abnormal intracellular localization of the PIN proteins in vps35a are similar to those in vps29 (Nodzyński et al., 2013). In addition, the triple mutant vps35a vps35b vps35c is embryo-lethal, indicating that the core retromer complex is pivotal for plant development (Yamazaki et al., 2008). VPS26 has two copies denominated VPS26a and VPS26b in Arabidopsis. The double mutant vps26a vps26b displays defects in seedling development similar to those observed in vps29, further demonstrating that the subunits of the core retromer are indispensable for the function of the retromer core complex in PIN endocytic cycling and plant development (Zelazny et al., 2013). Indeed, tandem affinity purification (TAP) with VPS29 finds that the other components include VPS35a, VPS35b, VPS35c, VPS26a and VPS26b (Nodzyński et al., 2013). VPS29 has been confirmed to natively co-immunoprecipitate with VPS35a in the cytosol of plant cells, suggesting that the core retromer complex is first formed in the cytosol and is then recruited to the membrane of the endosomes (Jaillais et al., 2007). These biochemical data indicate that the composition of the subunits in the core retromer is conserved in eukaryotes. However, compared to mammals and yeasts that contain >30 and about 10 SNX proteins, respectively, only three SNXs including SNX1, SNX2a and SNX2b have been characterized in Arabidopsis (Pourcher et al., 2010). SNX1 colocalizes with VPS29 to the same endosomes (Jaillais et al., 2007). In the vps29 mutant, the SNX1-GFP-labeled endosomes display aberrant enlarged morphology (Jaillais et al., 2007). The snx1 or snx2a mutants display weak developmental phenotypes, whereas both of them interact synergistically with vps29, and snx1 vps29 or snx2a vps29 double mutants are lethal, indicating that the SNXs work together with VPS29 in the same plant developmental pathways (Pourcher et al., 2010). These findings demonstrate that the normal morphology of the LE/MVB/PVC and the retromer function are pivotal for PIN endocytic trafficking and plant development. However, the regulation of them during plant development is still unclear.

In yeast, Vps38p forms a complex with Vps30p, Vps15p and Vps34p to regulate retrograde transport by generating a specific pool of phosphatidylinositol 3-phosphate (PtdIns3P) for the function of the retromer complex (Burd et al., 2002). In animals, the PtdIns3p generated by the VPS34 complex is also central to the regulation of retromer function. For example, the murine VPS34 promotes the recycling of the IL-7Rα in the T lymphocyte by affecting the proper location of VPS36 and the retromer function (McLeod et al., 2011). More recently, the interaction of UVRAG and BECLIN 1/VPS30 has been proposed to be essential for the roles of BECLIN 1/VPS30 during the control of PtdIns3p distribution, late endosome formation and endocytosis in murine neurons (McKnight et al., 2014). These data indicate that VPS38/UVRAG plays a pivotal role in the regulation of retromer function and late endosome formation by associating with the VPS34 complex in both yeasts and animals.

We conducted a genetic screen for mutants with defects in leaf development using the sets of confirmed SALK lines. We obtained a leaf defective mutant denominated usl1 because we first observed that the mutant produced unflattened and small leaves. Further analysis showed that usl1-1 displayed pleiotropic developmental phenotypes. Genetic analysis confirmed that the disruption of USL1 function causes the developmental defects in usl1-1. USL1 encodes a protein with a domain structure similar to those in the yeast Vps38p and human UVRAG. We demonstrate that auxin homeostasis and the subcellular distribution of the PIN1 proteins are disrupted in usl1-1. USL1 colocalizes with VPS29 in the LE/MVB/PVC, and the VPS29-associated LE/MVB/PVC becomes enlarged and circular in usl1-1. We further demonstrate that USL1 interacts with AtVPS30 in vivo and in vitro. Our findings suggest that USL1 controls plant development by forming a complex with AtVPS30 and AtVPS34 to regulate late endosome morphology.

Materials and Methods

Plant materials and growth conditions

The model plant species Arabidopsis thaliana (L.) ecotype Columbia-0 (Col-0) was used. To carry out a screen for leaf defective mutants, we grew the sets of confirmed SALK lines (CS27941, CS27942 CS27943 and CS27944) one by one and observed the leaf defective phenotypes during plant growth. From the sets of CS27943, we found that SALK_094540 produced unflattened and small leaves, thus named it as usl1-1. The usl1-2 (SAIL_552_F02) was ordered from a public database. Arabidopsis seeds from the wild-type (WT), mutants, transgenic plants and crossed plants were placed on 0.5×
Murashige and Skoog (MS) medium with or without 50 μg ml⁻¹ or kanamycin 20 μg ml⁻¹ DL-phosphinothricin. The plates were kept at 4°C for 3 d to stratify seeds before being placed at 22°C under long-day conditions (16 h : 8 h, light : dark) for 7 d. Seedlings of *Arabidopsis* or *Nicotiana benthamiana* were transferred to soil and grown under the same conditions as described above.

**Genotyping analysis and gene expression assay**

All of the primers used in this study are listed in Supporting Information Table S1.

The genotyping of *SALK_094540* (*usl1-1*) was performed using *usl1-1*-LP, *usl1-1*-RP, LBB1.3 primers. *SAIL_552_F02* (*usl1-2*) was genotyped using *usl1-2*-LP, *usl1-2*-RP and LB3 primers. The *pin1-LP, pin1-RP* and LBB1.3 primers were used to genotype *SALK_047613* (*pin1*).

For semi-quantitative PCR, the total RNA from the WT and *usl1-1* seedlings were extracted using a plant total RNA purification kit (GeneMark, Taichung, Taiwan, cat. no. TR02-150). RNA was treated with a DNase as the protocol described in the RNA purification kit, and was then reverse transcribed using an M-MLV kit (Promega, cat. no. A5003) in a reaction volume of 20 μl. The cDNA was diluted and used as a template for semi-quantitative PCR. The cycling conditions of genotyping PCR were 94°C for 30 s, 55°C to 58°C for 30 s, and 72°C for 60 s to 120 s with 30 cycles, whereas semi-quantitative PCR was limited to 28 cycles with the above conditions.

**Generation of binary constructs and transformation**

The coding sequences of VPS29, ArVPS30, ArVPS34 and USL1 were amplified from *Arabidopsis* seedling cDNA. The products were cloned into pENTR/D-TOPO (Invitrogen) to generate pENTRY-VPS29, pENTRY-USL1pro, pENTRY-USL1pro-USL1-MYC, the full-length coding regions of AtVPS30 and USL1 were amplified from *Arabidopsis* genomic DNA using primers USL1proF/USL1proR and USL1Rnsc. The products were cloned into pENTR/D-TOPO to generate pENTRY-USL1-pro-USL1-CC1. The USL1pro-USL1CC1-GFP and USL1pro-USL1CC2-GFP were generated by LR reactions between pK7FWG0 and pENTRY-USL1pro-USL1-CC1 or pENTRY-USL1pro-USL1-CC2.

In order to generate 35S-AtVPS30-FLAG and 35S-USL1-MYC, the full-length coding regions of ArVPS30 and USL1 without a stop codon were amplified using primers VPS30F/VPS30R and USL1F/USL1R, respectively. The fragments were cloned into pENTR-D-TOPO to generate pENTRY-VPS30nsc and pENTRY-USL1nsc. LR reactions were conducted between pENTRY-VPS30nsc and pENTRY-USL1nsc. The USL1pro-USL1CC1-GFP and USL1pro-USL1CC2-GFP were generated by LR reactions between pK7FWG0 and pENTRY-USL1pro-USL1-CC1 or pENTRY-USL1pro-USL1-CC2.

In order to observe the truncated USL1 subcellular localization, the truncated genomic sequence USL1pro-USL1ΔCC1 for complementation was amplified from the *Arabidopsis* genomic DNA by overlap extension PCR using primers USL1proF/USL1ΔCC1genomR and USL1ΔCC1genomF/USL1Rnsc. The primers USL1proF/USL1ΔCC1genomR and USL1ΔCC1genomF/USL1Rnsc were used for the first round of PCR, and the primers USL1proF/USL1Rnsc were used for the second PCR using the first round PCR production as template. The same strategy was adopted to amplify USL1pro-USL1CC2 using primers USL1proF/USL1ΔCC2genomR, USL1ΔCC2genomF/USL1Rnsc. The products of the second PCR were cloned into pENTR/D-TOPO to generate pENTRY-USL1pro-USL1ΔCC1 and pENTRY-USL1pro-USL1ΔCC2. The USL1pro-USL1ΔCC1-GFP and USL1pro-USL1CC2-GFP were generated by LR reactions between pK7FWG0 and pENTRY-USL1pro-USL1ΔCC1 or pENTRY-USL1pro-USL1ΔCC2.

**GUS staining and venation observation**

The β-Glucuronidase (GUS) staining assay was conducted as described previously (Zhang et al., 2017). For venation observation, the leaves from 21-d-old plants were fixed with an ethanol : acetic acid (6 : 1) mixture for 24 h. The samples were then immersed in a chloral hydrate : glycerol : H₂O (8 g : 1 ml : 2 ml) mixture for at least 24 h. The venations were observed using a Leica M205FA stereoscope.

**Subcellular localization observation and chemical treatments**

The complementary transgenic T3 plants with USL1pro-USL1-GFP in *usl1-1* were crossed with the endomembrane marker lines (wave line markers) (Geldner et al., 2009). For the colocalization of VPS29 and USL1, 35S-VPS29-RFP was transformed into complementary transgenic T3 plants with USL1pro-USL1-GFP in *usl1-1*.

The fluorescence was observed with a Zeiss LSM 710 NLO. To quantify the colocalization, Pearson's Correlation and scatter-plot were generated using the VOLOCITY software. At least 20 individual cells for each experiment were chosen for the calculations.
For the observation of different endomembrane markers in the usl1, the Wave line was crossed with both usl1-1 and usl1-2. At least 10 independent lines were observed to confirm the results.

For the Wortmannin treatment, the 5-d-old cross seedlings of USL1-GFP with RABF2a-mCherry were used. The seedlings were treated with 33 μM Wortmannin (Invitrogen, 3.3 mM stock in DMSO, diluted with deionized and distilled water) for 1 h, and the control was treated with the same concentration of DMSO diluted with deionized and distilled water.

Scanning electron microscopy
The fifth leaves from 21-d-old WT and usl1-2 plants were isolated. Scanning electron microscopy (SEM) was conducted as described previously (Tao et al., 2013). Leaves were observed using a scanning electron microscope (Jeol JSM6610LV) following the manufacturer’s instructions. The areas of leaf epidermal cells were analyzed using ImageJ software, and the frequency of cells sizes was calculated.

Co-IP with mass spectrometry assay
Approximately 3 g leaf tissues from 20-d-old 35S-USL1-MYC and 35S-VPS30-FLAG transgenic plants were ground in liquid N2. The proteins were extracted and purified using the method described previously by Li et al. (2015). The purified proteins were separated using SDS-PAGE. The entire gel lane was excised and soaked in DMSO diluted with deionized and distilled water.

Firefly luciferase complementation imaging assay
In order to test the interaction of AtVPS30 and USL1 using the firefly luciferase complementation imaging assay, the pCAMBIA-USL1-nLUC was generated by an LR reaction between pCAMBIA-nLUC (Chen et al., 2008; Zhang et al., 2017) and pENTRY-USL1nsc. The pCAMBIA-cLUC-VPS30 was generated by an LR reaction between pCAMBIA-cLUC (Zhang et al., 2017) and pENTRY-VPS30nsc.

The constructs were transformed into Agrobacterium tumefaciens GV3101. The different combinations were co-infiltrated into N. benthamiana leaves. The leaves were observed under a low-light cooled CCD imaging apparatus (Lumazone 1300B; Roper Bioscience).

Split ubiquitin Y2H assay
For the yeast two-hybrid (Y2H) assay, the truncated USL1ΔC and USL1ΔN were amplified using the primers USL1F/USL1ΔC, USL1ΔN/USL1ΔCR and USL1ΔNF/USL1Rnsc. The USL1ΔCC1, USL1ΔCC2 were amplified by overlap extension PCR. The first PCR was conducted by primers USL1F/USL1ΔCC1R, USL1ΔCC1F/USL1Rnsc and USL1F/USL1ΔCC2R, USL1ΔCC2F/USL1Rnsc, respectively. The second PCR was then amplified using the primers USL1F/USL1Rnsc. The fragments were cloned into pENTR/D-TOPO to generate pENTRY-USL1ΔN, pENTRY-USL1ΔC, pENTRY-USL1ΔCC1 and pENTRY-USL1ΔCC2. All of the pENTRY vectors were cloned into MerYGate by the LR reaction to generate USL1-Cub, USL1ΔN-Cub, USL1ΔC-Cub, USL1ΔCC1-Cub and USL1ΔCC2-Cub. pENTRY-VPS30 and pENTRY-VPS34 were cloned into pPR3-N using the LR reaction to generate NubG-VPS30 and NubG-VPS34. pPR3-NubWT was used as the positive control, and pPR3-NubG was used as the negative control. The different combinations were cotransformed into the yeast strain NMY51 (Biotech), respectively.

Medium SD-Trp-Leu-His-Ade was used to verify the interaction between the different combinations.

RNA-sequencing (RNA-seq) analysis
Total RNAs were extracted from 7-d-old seedlings of usl1-2 or WT control using an RNA purification kit (GeneMark, Cat. no. TR02-150). The RNA samples were used to perform RNA-seq on Illumina Hi-seq 2500 sequencer in the Biodynamic Optical Imaging Center (BIOPIC) of Peking University. TopHat v.2.0.14 (Kim et al., 2013) was used to map reads with the Arabidopsis genome in TAIR10 (http://www.arabidopsis.org/download_files/Genes/TAIR10genomerelease/TAIR10chromosomesfiles/TAIR10_chr_all.fas). The CUFFDIFF (v.2.2.1; Trapnell et al., 2012) was used for stringent statistical analysis to normalize and find the differential expression levels of the RNAs using the FPKM values and the P-values in the output files. Gene ontology enrichment analysis was conducted on BMKcloud website (http://www.biocloud.net/). Part of the analysis was performed on the Computing Platform of the Center for Life Science of Peking University.

Results
Identification of the usl1 mutants
In order to find regulators that control leaf development, we screened the sets of confirmed SALK lines for leaf defective mutants. In this study, we report a mutant denominated usl1-1 because it produces unflattened and small leaves (Fig. 1a,c). Compared to that of the WT control, usl1-1 produces leaves with wavy margins and uneven surfaces (Fig. 1a,c). The SALK number for usl1-1 is SALK_094540 that contains two T-DNA sequences inserted head to head in the fifth exon of At2g32760 (Fig. 1e). The T-DNA insertions disrupted the expression of At2g32760 in usl1-1 when we used the primer pair (F1 and R1) designed from the flanking sequences of T-DNA insertion site (Fig. 1e,f), because no specific bands were amplified. However, the truncated transcripts of At2g32760 were found in usl1-1 using the primer pair (F2 and R2) designed from the sequence upstream of T-
Fig. 1 The mutant unflattened and small leaves (usl1-1 and usl1-2) display severe leaf phenotypes in Arabidopsis. (a) The phenotypes of 21-d-old wild-type (WT) control and usl1-1. (b) The phenotypes of 21-d-old WT control and usl1-2. (c, d) Close-up views of the 7th and the 8th leaves from the 21-d-old WT control and the usl1-1 mutant. (e) Schematic representation of the T-DNA insertion sites in the usl1-1 and usl1-2 mutants. The F1 and R1 or F2 and R2 primer pairs used for the gene expression and genotyping analysis of usl1-1 or usl1-2, respectively, are indicated by the black lines with arrows. LB, T-DNA left border; RB, T-DNA right border. (f) Semi-quantitative polymerase chain reaction analysis of usl1-1 and usl1-2 showed that usl1-1 and usl1-2 were null mutants. ACTIN8 gene was used as a control. (g) Complementation of usl1-2 leaf phenotypes by USL1pro-USL1-GFP. (h, i) Scanning electron micrographs of the leaf epidermal cells of the 5th leaf from (h) 21-d-old WT, and (i) 21-d-old usl1-2. (j) Distribution of cell size of the leaf epidermal cells from usl1-2 and the WT control. Bars: (a, b, g) 1 cm; (c, d) 1 mm; (h, i) 20 μm.
DNA insertion site in usl1-1 (Fig. 1f). To test whether the disruption of At2g32760 function could lead to the small and curled leaves in usl1-1, we first identified the mutant allele SAIL_552_F02 in which the T-DNA was inserted in the second exon of At2g32760 (Fig. 1e). No specific bands were found when testing the expression of At2g32760 using F2 and R2 primers designed from the flanking sequences of T-DNA insertion site in SAIL_552_F02 (Fig. 1e,f). However, the truncated transcripts of At2g32760 were also observed when using F1 and R1 primers (Fig. 1e,f). The mutant displayed the small and curled leaf phenotypes resembling those observed in usl1-1 (Fig. 1b,d,f). We thus designated SAIL_552_F02 to be usl1-2. However, the phenotypes of usl1-2 were stronger than that of usl1-1, suggesting that the truncated transcripts of At2g32760 in usl1-1 might be partially functional (Fig. 1a–e). We then generated the construct USL1pro-USL1-GFP in which At2g32760 was fused to the gene encoding the green fluorescent protein (GFP) and was driven by its own promoter. We transformed the construct into the heterozygous usl1-1 or usl1-2 because the homozygous usl1 mutants also displayed dwarfism and low fertility as described below. The analysis of transgenic lines indicated that the expression of USL1-GFP completely complemented the developmental defects of the usl1 mutants (Figs 1g, S1). We thus designated At2g32760 to be USL1. In addition, we found that the leaf epidermal cells in usl1-2 were obviously smaller than those in the WT control (Fig. 1h–j). These data demonstrate that USL1 significantly affects leaf development by affecting cell expansion.

USL1 encodes a predicted protein containing 352 amino acid residues. Bioinformatical analysis suggests that USL1 shares 23.9% similarity with the yeast Vps38p and 20.3% similarity with the human UVRAG in its protein sequence. We further used the Protein Homology/analogY Recognition Engine v.2.0 (PHYRE) tool to predict the USL1 protein structure (Kelley et al., 2015). The results suggested that USL1 was homologous to the yeast Vps38p (Fig. S2a). The protein modeling of the USL1 structure based on the yeast Vps38p template suggested that USL1 contains two predicted CC (coiled-coil) domains in the N-terminus and a BARA (β-α repeated, autophagy-specific) domain in the C-terminus (Fig. S2b,c). These data suggest that USL1 could be structurally orthologous to Vps38p or UVRAG.

The usl1 mutants display pleiotropic developmental phenotypes

In addition to leaf developmental defects, the usl1 mutant also displayed other pleiotropic developmental phenotypes. First, the usl1-2 seedlings produced cotyledons abnormal in their number, position and shape (Fig. 2a–e,u). Second, in consistent with the abnormal cotyledons, the cell division and embryogenesis were compromised in the usl1-2 mutant (Fig. 2f–o,v). Third, the leaf venation of usl1-2 exhibited a simpler pattern with more open veins near the leaf margins displayed fewer secondary or higher order veins than those of the wild-type control (Fig. 2p–s,w). Fourth, the usl1-2 mutant produced fewer lateral roots than wild-type control (Fig. 2t,x). Finally, the usl1-2 mutant also displayed late flower, low fertility and abnormal silique phyllotaxy (Fig. S3). These data indicate that USL1 regulates multiple aspects during plant development.

USL1 is expressed in multiple organs

In order to identify the expression pattern of USL1, we cloned the 969 bp USL1 promoter from the TAA of the USL1 upstream gene to the start codon of USL1. We generated an USL1pro-GUS construct in which the β-glucuronidase (GUS) gene was driven by the USL1 promoter. Sixteen USL1pro-GUS transgenic plants showed similar GUS staining patterns. Histochemical analysis showed that USL1 was predominantly expressed in the shoot apical meristem (SAM) and in the leaf vascular tissues (Fig. 3a–d). Strong GUS activity was observed in the nascent leaves. Interestingly, as the leaves grew, the GUS staining was focused in the leaf vasculature and became stronger at the leaf tips or the hydathodes along the leaf margins (Fig. 3d–g). Strong GUS staining was also observed in the root tip, root vasculature, lateral root initiation sites, anther, pollen and pistils (Fig. 3a–c,h–j). These data demonstrate that USL1 is expressed in different organs. This is consistent with the observed pleiotropic developmental phenotypes in usl1, and the expression pattern suggests that USL1 plays an important function in plant development.

USL1 is localized to the LE/MVB/PVC

In order to determine the subcellular localization of USL1, we crossed usl1-1 complemented by USL1pro-USL1-GFP to a series of mCherry-Wave (R) marker lines indicating different plant inner membrane compartments from the Golgi to the vacuole (Geldner et al., 2009). Our observations showed that the USL1-GFP-labeled compartments did not co-localize with the Golgi indicated by MEMB12-mCherry or the Golgi/endosome by RabD2a-mCherry (Fig. 4a,b). However, USL1 proteins could be seen close to the Golgi or Golgi/endosome. Further analysis showed that the USL1-associated compartments highly overlapped the RABF2a-mCherry-labeled LE/MVB/PVC (Fig. 4d; Movies S1), whereas it only partially overlapped the RabD1-mCherry-labeled post-Golgi/endosome (Fig. 4e) and RabG3f-mCherry-labeled late endosome/vacuole (Fig. 4e), respectively. USL1 was not localized to the vacuoles labeled by VAMP711-mCherry and the recycling endosomes labeled by RabA1e-mCherry (Fig. 4f,g). These data suggest that USL1 exerts its main function at the sites of the late endosomes/multivesicular bodies/pervacuolar compartments (LE/MVB/PVC) membranes.

USL1 is essential to maintain the morphology of the VPS29-associated endosomes

The retromer component VPS29 has been shown to strongly colocalize with the mRFP-RABF2b-labeled endosome (Jaillais et al., 2007), whereas our data showed that USL1 localized to the RABF2a-mCherry labeled endosome. RABF2a and RABF2b are canonical orthologs of animal RAB5 in Arabidopsis, and both are
Fig. 2 The *unflattened and small leaves* (*usl*1) mutants multiple developmental phenotypes in addition to leaf defects in Arabidopsis. (a–e) The cotyledons of (a) 10-d-old wild-type (WT) seedling and (b) *usl*1 seedlings with three cotyledons, (c, d) with abnormal position of cotyledons, or (e) with fused cup-shaped cotyledons. (f–o) Early embryogenesis from the four-cell stage to the globular stage in (f, h, j, l, n) the WT and (g, i, k, m, o) *usl*1-2 mutant. The developmental stages are indicated at the bottom of each picture. (p–s) The vasculature of the 1st and 5th leaf from 21-d-old (p, r) WT, or (q, s) *usl*1-2 plants. (t) The 15-d-old *usl*1-2 mutant produced fewer secondary roots than the WT. (u) The statistical analysis of abnormal cotyledons in (a–e) (*n* = 116). (v) The statistical analysis of abnormal embryos in (f–o) (WT, *n* = 147; *usl*1-2, *n* = 162). (w) The statistical analysis of open veins near the leaf margins in (p–s). Data are means ± SD (*n* = 8), (Student’s *t*-test): **, *P* < 0.005; ***, *P* < 0.001. (x) The statistical analysis of lateral roots in (t). Data are means ± SD (*n* = 12), (Student’s *t*-test): ***, *P* < 0.001. Bars: (a–e, p–s) 1 mm; (f–o) 20 μm; (t) 1 cm.
localized to the LE/MVB/PVC (Ueda et al., 2004). This implies that USL1 could be colocalized with VPS29 in the LE/MVB/PVC. To test this hypothesis, we expressed VPS29-RFP in usl1-1 complemented by USL1pro-USL1-GFP. The results clearly showed that the USL1-GFP-labeled endomembrane compartments largely overlapped with the VPS29-RFP-associated endosomes (Fig. 5a–d; Movies S2), suggesting that USL1 could regulate the function of the retromer. We then investigated the possible changes of the VPS29-RFP-labeled compartments in usl1-1. Interestingly, compared to the small dot morphology of VPS29-RFP-labeled endosomes in the WT control, the loss of USL1 function in usl1-1 caused the VPS29-RFP-labeled endosomes to become enlarged circles (Fig. 5e,f). Similarly, RABF2a-mCherry-labeled endosomes also displayed enlarged round morphology in usl1-1, whereas they were distributed in small dots in the WT control (Fig. 5g,h). However, the morphology of the Golgi labeled by MEMB12-mCherry or the recycling endosomes by RABA1g-mCherry in usl1-1 was not significantly different.
Fig. 4 The Arabidopsis Unflattened and Small Leaves (USL1) is localized to the late endosomes/multivesicular bodies/prevacuolar compartments (LE/PVC/MVB). The mCherry-Wave (R) marker lines were crossed with usl1-1 mutant complemented by USL1pro-USL1-GFP. (a) The fluorescence of the Golgi marker MEMB12-mCherry and USL1-GFP. (b) The fluorescence of the Golgi/endosome marker RABD2a-mCherry and USL1-GFP. (c) The fluorescence of the post-Golgi/endosome marker RABD1-mCherry and USL1-GFP. (d) The fluorescence of the late endosome/multivesicle body/prevacuolar compartments (LE/MVB/PVC) marker RABF2a-mCherry and USL1-GFP. (e) The fluorescence of the LE/vacuole marker RABG3f-mCherry and USL1-GFP. (f) The fluorescence of the recycling endosome marker RABA1e-mCherry and USL1-GFP. Scatterplots of the fluorescence values of the pixels of the two channels are provided. To quantify the colocalization, the Pearsons Correlation was calculated after analyzing the cytosolic areas of at least 20 cells. GFP, green fluorescent protein. Bars, 10 μm.
Fig. 5 The Arabidopsis UNFLATTENED AND SMALL LEAVES (USL1) controls the morphology of VPS29-related late endosomes (VPS, VACUOLAR PROTEIN SORTING). (a–d) USL1-GFP was colocalized with VPS29-RFP. (e, f) The morphology of VPS29-RFP-labeled endosomes in (e) wild-type (WT) plants and (f) usl1-1 mutants. (g, h) The morphology of RABF2a-mCherry-labeled endosomes in (g) WT plants and (h) usl1-1 mutants. (i, j) the MEMB12-mCherry-labeled endomembrane compartment in (i) WT plants and (j) usl1-1 mutants. (k, l) RABA1e-mCherry-labeled endomembrane compartment in (k) WT plants and (l) usl1-1 mutants. (m) Both usl1-2 and vps29-4 produced small and curled leaves. RFP, red fluorescent protein; GFP, green fluorescent protein. Bars: (a–l) 10 μm; (m) 1 cm; (m inset) 1 mm.
from those in the WT control (Fig. 5i–l). These results demonstrate that USL1 is specifically required to maintain the normal morphology of the VPS29-associated LE/MVB/PVC.

The retromer mutant vps29 displays severe auxin-related phenotypes including abnormal cotyledons, defects in embryogenesis and fewer lateral roots as observed in usl1-2 (Jaillais et al., 2007; Fig. 2). The mutant allele GK-125H09 was previously reported to be a vps29-4 null mutant (Jaillais et al., 2007). To further show that USL1 is related to VPS29, we first observed the leaves of vps29-4. The vps29-4 mutant also produced small and uneven leaves similar to those observed in the usl1 mutants (Fig. 5m).

We then crossed usl1-2 to vps29-4. We genotyped 170 progenies from the F2 population and found no usl1-2 vps29-4 double mutant lines, suggesting that usl1-2 vps29-4 is gametophytic or embryo lethal. The similar auxin-related defects observed in both usl1-2 and vps29-4, plus the synergistic genetic interaction between usl1-2 and vps29-4 further suggest that USL1 and VPS29 act in parallel in auxin polar transport.

USL1 is required for PIN1 endocytic trafficking

In order to provide more evidence for the hypothesis that USL1 could function in controlling auxin polar transport, we first crossed the auxin-responsive DR5-GFP reporter line with usl1-2 to examine the auxin distribution in usl1-2. The results showed that the GFP signals were significantly reduced in the quiescent center (QC) but accumulated in the root cap in usl1-2 when compared to those in the WT control (Fig. 6a,b), indicating that auxin homeostasis was compromised in usl1-2. We then crossed the PIN1-GFP marker line to usl1-2. Our observation clearly showed that the PIN1-GFP signals were retained in the aberrant enlarged circular membrane compartments in usl1-2 (Fig. 6c–f), suggesting that USL1 was crucial for the proper PIN1 endocytic trafficking. In addition, we crossed usl1-2 to SALK_047613/ pin1. The leaves of usl1-2 pin1 were even smaller than those of the single usl1-1 or pin1 mutants (Fig. 6g–i), indicating that usl1 synergistically interacted with pin1. These data continue to demonstrate that USL1 regulates the endocytic trafficking of PIN1 auxin transporter.

USL1 interacts with the PI3K complex to regulate retromer function

In order to decipher the possible molecular mechanism by which USL1 regulates the morphology of the VPS29-associated LE/PVC/MVB, we first generated the construct 35S-USL1-MYC in which the USL1 fused with sequence encoding six MYC tags was driven by the CaMV 35S promoter. We used 35S-USL1-MYC transgenic plants as material to identify components of the USL1 complex by co-immunoprecipitation coupled to mass spectrometry (Co-IP/MS). The results showed that all of the main components of the PI3K complex including the AtVPS15, AtVPS30 and AtVPS34 isoforms were associated with USL1 (Table 1). To confirm that USL1 could interact with the PI3K complex, we generated 35S-VPS30-FLAG in which AtVPS30 fused with sequence encoding three FLAG tags was driven by the CaMV 35S

Fig. 6 The Arabidopsis UNFLATTENED AND SMALL LEAVES (USL1) is required for PIN1 endocytosis. (a, b) The fluorescence of DR5:GFP in (a) wild-type (WT) and (b) usl1 primary roots. (c–i) Subcellular localization of PIN1-GFP in (c, d) WT and (e, f) usl1-2. (g–i) Twenty-two-day-old (g) pin1, (h) usl1-2 and (i) usl1-2 pin1 double mutant. Bars: (a, b) 1 mm; (c–f) 10 μm; (g–i) 1 cm.
In order to further elucidate the mechanisms by which USL1 regulates plant development, we performed a genome-wide transcriptome analysis by RNA-seq using the RNA samples from usl1-2 and WT control. The results showed that the expression level of 1826 genes was altered in usl1-2 when compared to WT control (fold change ≥ 2; P-value < 0.05) (Notes S1, S2). Among them, 990 genes were upregulated and 836 ones were downregulated in usl1-2 mutant (Notes S1, S2). Gene ontology (GO) enrichment analysis showed that 114 biological processes were significantly upregulated and 129 ones were downregulated in usl1-2 (P-value < 0.05) (Notes S3, S4). These altered biological processes include ‘response to auxin’, ‘leaf senescence’, ‘lateral root development’, ‘vacuolar protein processing’, ‘cytoplasm-to-vacuole targeting (Cvt) pathway’, ‘protein targeting to membrane’ and so on (Notes S3, S4). This is consistent with the multiple developmental phenotypes displayed in usl1-2. We then searched our RNA-seq data for auxin-related genes. The results showed that the expression level of auxin-related genes including many SAUR genes was altered in usl1-2 (Tables S2, S3). We further searched our RNA-seq data for genes regulating leaf flattening or polarity. No leaf polarity-related genes were significantly regulated in usl1-2 (Notes S1, S2). However, we found some leaf flattening-related genes including WUSCHEL-RELATED HOMEOBOX1 (WOX1), PRESSED FLOWER (PRS)/WOX3 and TEOSINTE BRANCHED1/CYCLOIDEA/PCF17 (TCP) TCP17 were downregulated in usl1-2 (Table S4) (Tao et al., 2013; Guan et al., 2017). Interestingly, we found TCP Interactor containing EAR motif protein 1 (TIE1) and TIE4 were upregulated in usl1-2, consistent with the unflattened leaves in usl1-2 and the previous reports that the overexpression of TIE1 or TIE4 leads to leaves with wavy margins (Table S4) (Tao et al., 2013).

## Discussion

In this study, we identified an important factor UNFLATTENED AND SMALL LEAVES (USL1) that plays essential roles in plant development by regulating retromer function. The expression pattern of USL1 in different organs indicates that USL1 is important for plant organ development. The loss of USL1 function in usl1 mutants leads to small and uneven leaves by repressing leaf cell expansion. Our results also proved that usl1

### Table 1 Mass spectrometry analysis of proteins co-immunoprecipitated with UNFLATTENED AND SMALL LEAVES (USL1)-MYC in Arabidopsis

| Protein accession | Gene ID | Gene product | Score | Mass  | Spectra | Unique peptides | Seq. Cov (%) |
|-------------------|---------|---------------|-------|-------|---------|-----------------|--------------|
| IPI00516293       | At2g32760 | USL1          | 1462  | 40062 | 49      | 19              | 62.8         |
| IPI00532518       | At4g29380 | VPS15         | 752   | 17070 | 27      | 19              | 18.7         |
| IPI00521940       | At3g61710 | VPS30         | 546   | 59151 | 22      | 11              | 26.5         |
| IPI00517736       | At1g60490 | VPS34         | 64    | 94294 | 2       | 2               | 2.5          |

### Table 2 Mass spectrometry analysis of proteins co-immunoprecipitated with VACUOLAR PROTEIN SORTING (VPS)30-FLAG in Arabidopsis

| Protein accession | Gene ID | Gene product | Score | Mass  | Spectra | Unique peptides | Seq. Cov (%) |
|-------------------|---------|---------------|-------|-------|---------|-----------------|--------------|
| IPI00532518       | At4g29380 | VPS15         | 1416  | 17070 | 53      | 45              | 41.5         |
| IPI00517736       | At1g60490 | VPS34         | 820   | 94294 | 24      | 20              | 34.4         |
| IPI00521940       | At3g61710 | VPS30         | 802   | 59151 | 25      | 21              | 41.4         |
| IPI00516293       | At2g32760 | USL1          | 274   | 40062 | 11      | 10              | 38.9         |
results in defects in leaf vein development. The subcellular localization of USL1 demonstrates that this factor acts at the RABF2a-labeled endosome. USL1 interacts with the PI3K complex. The CC1 and CC2 domains at the N-terminus of USL1 are essential for its subcellular localization and interaction with AtVPS30 in the PI3K complex. The disruption of USL1 in the usl1 mutants causes enlarged aberrant VPS29-associated endosomes. Based on these data, we propose a model for USL1 in regulating leaf development (Fig. 7).

Fig. 7. The Arabidopsis UNFLATTENED AND SMALL LEAVES (USL1) regulates the function of PI3K by forming a complex with AtVPS30 and AtVPS34 (VPS, VACUOLAR PROTEIN SORTING). (a) Yeast two-hybrid (Y2H) assays of USL1 with AtVPS30 and AtVPS34. NubWT represents the wild-type (WT) N-terminal half of ubiquitin. NubG represents the mutated N-terminal half of ubiquitin. Transformed yeasts were spotted on control medium (-2: SD-Leu-Trp) or selective medium (-4: SD-Leu-Trp-His-Ade) at dilutions of 10-, 100-, and 1000-fold. (b) The firefly luciferase (LUC) complementation imaging assays show that USL1 interacted with AtVPS30. LUC signals were detected in the combination of USL1-nLUC and cLUC-AtVPS30, but not in the control combinations including USL1-nLUC and cLUC, nLUC and cLUC-AtVPS30 and nLUC and cLUC. (c) The schematic representation of the USL1 deletions. (d) Y2H assays between differently truncated USL1 and AtVPS30. (e–g) The subcellular location of (e) the USL1-GFP protein, (f) USL1ΔCC1-GFP protein, and (g) USL1ΔCC2-GFP protein. (h–m) The morphological changes of late endosome/multiple vesicle body/prevacuolar compartments (LE/MVB/PVC) following treatment with the PI3K inhibitor Wortmannin. The fluorescence of (h) USL1-GFP, (i) RABF2a-mCherry and (j) the merged picture after the mock treatment. (k) The fluorescence of USL1-GFP, (l) RABF2a-mCherry and (m) the merged picture after treatment with Wortmannin. GFP, green fluorescent protein. Bars, 10 µm.
In yeast, Vps38p was identified to be necessary to sort the vacuolar hydrolase carboxypeptidase Y (CPY) (Raymond et al., 1992). Vps38p forms a complex with PI3K in CPY targeting (Kihara et al., 2001). Recently, the crystal structure of the PI3K complex revealed that Vps38p interacts with Vps30p to form a parallel heterodimer that fits like a bracket around the Vps15p and Vps34p heterodimer (Rostislavleva et al., 2015). Vps38p is highly conserved in euukaryotes (Itakura et al., 2008; Sun et al., 2008; Matsunaga et al., 2009). In mammals, the Vps38p ortholog UVRAG was initially identified by partially complementing the UV sensitivity of xeroderma pigmentosum cells (Perelman et al., 1997), and UVRAG participates in the development of a variety of human malignancies including breast and colon cancer (Bekri et al., 1997; Goi et al., 2003; Liang et al., 2007; He & Liang, 2015). UVARG also forms a PI3K complex containing VPS34, Bclin1/VPS30 and VPS15 in mammals (Liang et al., 2006; Takahashi et al., 2007; Itakura et al., 2008; Matsunaga et al., 2009; Zhong et al., 2009). In Arabidopsis, we previously found that AtVPS15 and AtVPS30 are essential for the development of male gametophytes (Qin et al., 2007; Wang et al., 2012). No homozygous mutants of atvps15 or atvps30 could be obtained in the progeny of the heterozygous +/atvps15 or +/atvps30 (Fujiki et al., 2007; Qin et al., 2007; Xu et al., 2011; Wang et al., 2012). The disruption of AtVPS34 also causes male gametophytes to be lethal (Lee et al., 2008), indicating that the PI3K complex is pivotal for plant reproduction. In this study, we identified that USL1 was the possible ortholog of Vps38/ UVRAG in Arabidopsis. USL1 contains a CC1 and a CC2 domain in the N-terminus and a BARA domain in the C-terminus similar to those in Vps38/UVRAG. We also demonstrate that USL1 forms a complex with AtVPS15, AtVPS30 and AtVPS34. USL1 directly interacts with AtVPS30 using the CC1 and CC2 domains. These data suggest that the function of Vps38p/UVRAG/USL1 is conserved not only in yeast and animals, but also in plants. However, unlike AtVPS15, AtVPS30 or ArVPS34, the disruption of USL1 does not cause the gametophytes to be lethal. We obtained homozygous usl1 mutants, and they displayed severe vegetative phenotypes including the production of small and curled leaves. These results imply that some additional proteins could play redundant or independent functions in the regulation of PI3K during plant pollen development, because PI3K can also form an independent complex with Atg14 in addition to that with Vps38/UVRAG in yeast and animals (Kihara et al., 2001; Itakura et al., 2008). Alternatively, the roles of USL1 in leaf and other organ development implied that AtVPS15, AtVPS30 or ArVPS34 could also not only be essential for gametophyte development, but also for vegetative organ development in plants.

Membrane trafficking plays an important role in plant development and growth. During endocytosis, the membrane proteins are internalized to the cytoplasm and then transported to the early endosomes (EE). The EE then mature to become the LE/MVB/PVC where the cargo proteins are either transported for degradation or back to the EE for reuse. USL1 forms a complex with AtVPS15, AtVPS30 and AtVPS34 at the LE/MVB/PVC and regulates the morphology of LE/MVB/PVC. (b) In usl1 mutant cells, the LE/MVB/PVC are abnormally enlarged and the retromer cannot function normally, so that PIN1 cannot recycle back to the EE.

Fig. 8 A work model of the Arabidopsis UNFLATTENED AND SMALL LEAVES (USL1). (a) In wild-type cells, PIN1 proteins on plasma membrane (PM) can be internalized by membrane invagination to form clathrin-coated vesicles (CCV). CCVs first reach early endosome (EE). Then from there, PIN1 proteins are sorted back to PM for reuse via recycling endosome (RE), or are delivered to vacuoles for degradation through late endosome/multiple vesicle body/prevacuolar compartments (LE/MVB/PVC). The retromer complex including VPS29, VPS26 and VPS35 acts at the LE/MVB/PVC to help PIN1 back to the EE for reuse. USL1 forms a complex with AtVPS15, AtVPS30 and AtVPS34 at the LE/MVB/PVC and regulates the morphology of LE/MVB/PVC. (b) In usl1 mutant cells, the LE/MVB/PVC are abnormally enlarged and the retromer cannot function normally, so that PIN1 cannot recycle back to the EE.
Inactivation of the RAB GTPase requires the GTPase activating protein (GAP). Interestingly, TB2C that is a RAB GAP has recently been found to be recruited by VPS34 to inactivate RAB5 Rab GTPase during endosome maturation in Caenorhabditis elegans (Law et al., 2017). Although the exact mechanisms by which USL1 regulates the morphology of the LE/PVC/MVB remain unclear, we speculate that the loss of function of USL1 might cause the abnormal function of AtVPS34 to affect the recruitment of Rab GAPs to inactivate RABF2a/2b, leading to the ring-like LE/PVC/MVB resulting from the constitutive activation of RABF2a/2b.

Auxin acts as a master regulator of plant development. The polar auxin transport mediated by the PIN efflux carriers is critical to form the auxin gradient essential for plant organ development (Grunewald & Friml, 2010). PIN1 is one of the most important PIN proteins that is asymmetrically localized to the cell PM, causing auxin flow direction in plant tissues. PIN1 proteins are recycled between the PM and the cytosolic membrane, and the polar localization of the PIN1 is regulated by membrane trafficking. During the past decades, several important components of endocytic trafficking have been identified to be important for PIN1 trafficking and localization during plant leaf and other organ development. In addition to the GNOM protein and ESCRT complex that is important for PIN1 trafficking (Geldner et al., 2003; Reyes et al., 2011; Gao et al., 2014), VAN4 encoding a GEF for the RAB GTPase controls plant vascular development by mediating PIN1 trafficking (Narimoto et al., 2014a). Additional factors including BEX1/ARF1A/1C, BEX5/RabA1b, BEN1 and BEN2/VPS45 also participate in the membrane trafficking of PIN proteins (Tanaka et al., 2009, 2014; Feraru et al., 2012). VPS51 regulates leaf shape and vein patterning by targeting PIN1 to the lytic vacuole for degradation (Pahari et al., 2014), whereas VPS29 and SNX1 are the retromer components that are required for the retrieval of PIN1 from the LE/PVC/MVB back to the EE for recycling (Jaillais et al., 2006, 2007). Both of these are localized to the LE/PVC/MVB. The disruption of VPS29 causes severe auxin-related plant organ development phenotypes by affecting PIN1 recycling, indicating that the retromer plays a central role in the regulation of PIN1 trafficking (Jaillais et al., 2006, 2007). In this study, we found that USL1 co-localized with VPS29. The morphology of the VPS29-labeled endosome was aberrant in the usl1 mutant. The usl1 mutants display developmental defects similar to those observed in the vps29 mutant. The homozygous vps29 usl1 double mutants are lethal, whereas the homozygous vps29 or usl1 mutants are essential, indicating that USL1 acts in parallel with VPS29. We also found that usl1 interacts synergistically with pin1. These data suggest that USL1 acts in the same pathway with VPS29 and PIN1 and plays a pivotal role in regulating the morphology of LE, the retromer function and PIN1 recycling. Interestingly, in yeast, the USL1 ortholog Vps38p was also found to regulate the retromer function (Burda et al., 2002). The Vps30p–Vps38p complex binds to Vps34p for the proper localization of the retromer Vps5p–Vps17p complex (Burda et al., 2002). In animals, the PI3K complex also controls the retromer function during plgR-plgA transcytosis in epithelial cells (Vergès et al., 2007). These data provide further evidence that the function of USL1/Vps38p/UVRAG is highly conserved in yeasts, animals and plants.

In summary, we identified the important factor USL1 that plays pivotal roles in plant development by regulating the PI3K complex, retromer function and the maintenance of the LE/PVC/MVB morphology and auxin distribution. As sessile organisms, plants evolve morphological adaptations in response to different growth conditions. USL1 may control the polarity and abundance of the PIN1 proteins and other proteins that depend on retromer function and the normal morphology of LE, therefore affecting plant development in response to internal and external signals. It will be very interesting to identify how USL1 is regulated by different developmental or environmental cues in the future.

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**Author contributions**

G.Q. conceived the project; G.Q. and R.Y. designed the experiments; R.Y., J.L., Y.F., H.Y., J.Z. and G.Q. performed the experiments; G.Q., R.Y. and J.H. analyzed the data; and G.Q. and R.Y. wrote the paper.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article:

Fig. S1 The 50-d-old wild-type and rescued usl1-1 with USL1pro-USL1-GFP in Arabidopsis.

Fig. S2 Domain analysis of USL1 in Arabidopsis.

Fig. S3 The other phenotypes of the Arabidopsis mutant usl1-2.

Table S1 The list of primers used in this study
Table S2 The auxin-related genes upregulated in the Arabidopsis mutant *usl1-2*.

Table S3 The auxin-related genes downregulated in the Arabidopsis mutant *usl1-2*.

Table S4 The leaf flattening-related genes regulated in the Arabidopsis mutant *usl1-2*.

Notes S1 The genes upregulated in the Arabidopsis mutant *usl1-2*.

Notes S2 The genes downregulated in the Arabidopsis mutant *usl1-2*.

Notes S3 The gene ontology (GO) enrichment analysis of upregulated genes in the Arabidopsis mutant *usl1-2*.

Notes S4 The gene ontology (GO) enrichment analysis of downregulated genes in the Arabidopsis mutant *usl1-2*.

Movies S1 Colocalization of USL1-GFP and RABF2a-mCherry in Arabidopsis.

Movies S2 Colocalization of USL1-GFP and VPS29-RFP in Arabidopsis.

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