A Novel Endosomal Sorting Complex Required for Transport (ESCRT) Component in *Arabidopsis thaliana* Controls Cell Expansion and Development*

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Background: ESCRT mediates membrane remodeling in cellular processes such as endosomal sorting. The ATPase SKD1/VPS4 is essential for ESCRT function.

Results: PROS is a plant-specific ESCRT component that increases SKD1/VPS4 ATPase activity and is required for cell expansion.

Conclusion: Plants have evolved specific ESCRT components that are required for normal development.

Significance: Diversification of ESCRT components in eukaryotes may provide additional regulatory mechanisms for ESCRT-dependent processes.

ESCRT proteins mediate membrane remodeling and scission events and are essential for endosomal sorting of plasma membrane proteins for degradation. We have identified a novel, plant-specific ESCRT component called PROS (POSITIVE REGULATOR OF SKD1) in *Arabidopsis thaliana*. PROS has a strong positive effect on the *in vitro* ATPase activity of SKD1 (also known as Vacuolar Protein Sorting 4 or VPS4), a critical component required for ESCRT-III disassembly and endosomal vesiculation. PROS interacts with both SKD1 and the SKD1-positive regulator LIP5/VTA1. We have identified a putative MIM domain within PROS that mediate the interaction with the MIT domain of SKD1. Interestingly, whereas MIM domains are commonly found at the C terminus of ESCRT-III subunits, the PROS MIM domain is internal. The heterologous expression of PROS in yeast mutant cells lacking Vta1p partially rescues endosomal sorting defects. PROS is expressed in most tissues and cells types in *Arabidopsis thaliana*. Silencing of PROS leads to reduced cell expansion and abnormal organ growth.

Endosomal sorting of signaling receptors, transporters, and other plasma membrane proteins is a key process that controls plasma membrane protein composition and therefore, the ability of cells to respond to extracellular stimuli. Plasma membrane proteins are continuously internalized by endocytosis and delivered to endosomes. At the endosomal membranes, the endosomal sorting complex required for transport (ESCRT) bind and concentrate the ubiquitinated cargo and mediate their sorting into intraluminal vesicles, giving rise to multivesicular bodies (MVB). In fungi and metazoans, ESCRT-0, I, and II are assumed to recognize and concentrate the cargo proteins while initiating or stabilizing negative membrane curvature on the endosomal membrane. ESCRT-III polymerizes into long filaments and drives membrane deformation, constriction, and membrane scission *in vitro* (1–5). Finally, the AAA ATPase SKD1/Vps4p (Suppressor of K+ Transport Growth Defect 1/Vacuolar Protein Sorting 4) together with its cofactor LIP5/Vta1p disassemble and recycle the ESCRT coats back to the cytoplasm (6).

There are four core ESCRT-III subunits, Vps20p/CHMP6, Snf7p/CHMP4, Vps24p/CHMP3, and Vps2p/CHMP2 (with several isoforms in animal and plants) and three accessory subunits Did2p/CHMP1 (7, 8), Vps60p/CHMP5 (9–11), and IST1 (12, 13). The three first core subunits are sufficient for driving membrane scission *in vitro* (3) whereas Vps2p recruits the Vps4p/SKD1 complex to the endosomal membrane (14). The accessory ESCRT-III subunits play a modulatory effect on Vps4p/SKD1 function (7, 12).

ESCRT proteins are ancient components of the eukaryotic endomembrane machinery and are found in all five major groups of eukaryotes (15). Plants contain orthologs for most of the ESCRT-I to III proteins identified in metazoans and fungi (15, 16) but they also have undergone a significant diversification, exhibiting multiple isoforms of most ESCRT components (17–22).

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Here, we characterized a novel ESCRT component found in plants called PROS (POSITIVE REGULATOR OF SKD1). PROS interacts with Arabidopsis SKD1 and LIP5 and is able to induce a strong increase in the in vitro ATPase activity of both plant SKD1 and yeast Vps4p. Plants with reduced levels of PROS transcripts were dwarf and showed cell expansion and organ formation defects, indicating that PROS plays a key role on plant development.

EXPERIMENTAL PROCEDURES

Protein Purification and Interaction Assays—Recombinant proteins were expressed in Escherichia coli BL21. The cDNA fragments were cloned either in pGEX (GE Healthcare Life Sciences), pET or both using the restriction sites BamHI and EcoRI for all of them except for IST1 that was cloned between the Sall and NotI sites, to generate N terminus GST- or His6- fusion proteins. PROS and CHMP1A were amplified from the ABRC clones U11633 and U16228, respectively. VPS601 and IST1 were amplified from Col-0 WT cDNA; SKD1 was amplified using the clone described in (20) as a template. For the GST alone control, the pGEX vector was used. GST–fusion proteins were purified using glutathione-agarose beads (Sigma-Aldrich) and His6–fusion proteins were purified using Ni-NTA (nickel nitritriocetic acid)-agarose beads (Qiagen). Mutations in PROS (A1, A2, A3) and SKD1 (L63A) were introduced by PCR. Expression of His-SKD1, His-LIP5 (20); His–CHMP1A (19); His–Vta1p (23) and His–Vps4p (24) was performed as previously described. For the in vitro interaction pull-down experiments, equivalent amounts of the purified proteins were incubated 2 h at 4 °C in 20 mM Hepes, pH 7.4, 300 mM NaCl, 5 mM MgCl2, 10% (v/v) glycerol, and 0.02% (v/v) Nonidet P-40 (Input). The glutathione-agarose beads were then rinsed three times with the same buffer described above except that 0.2% (v/v) of Nonidet P-40 was used (Output). Both samples were denatured using Laemmli buffer, separated by SDS-PAGE and transferred onto nitrocellulose membrane. The proteins were detected using anti-His6 antibodies (Sigma) and anti-GST antibody (19).

Yeast Two-hybrid Assay—PROS and SKD1 cDNAs were cloned in-frame with the GAL4 DNA binding domain (BD) in the vector pBD-GAL4 and with the GAL4 DNA activation domain (AD) of the vector pAD-GAL4 (Stratagene), respectively. The yeast strain AH109 was sequentially transformed with the vector pBD-GAL4 and with the GAL4 DNA activation domain cloned in-frame with the GAL4 DNA binding domain (BD) in the pGBKT7 vector and cloned into the yeast expression vector pRS425-CPS (20). PROS was PCR-amplified from the ABRC clone U11633. For the expression of SKD1 fused to the C-terminal portion of YFP (SKD1–YC) the open reading frame of SKD1 was amplified from the clone U12244 (ABRC) using forward and reverse primers containing the restriction sites Sall and NotI, respectively, and cloned into the pSY738 vector. Both vectors were used to transfet protoplasts from 23-day-old seedlings as previously described (26).

ATPase Assay—For the in vitro ATPase assays, 2 μl His-SKD1, His–PROS, His–LIP5, His–CHMP1A, His–CHMP1MIM, His–Vps4p, and His–Vta1p were used for the assay. ATPase activity was measured using a modified malachite green-based colorimetric method (20).

Expression of PROS and Sorting Defect Analysis in Yeast—The His–PROS fragment was PCR-amplified from the pET–HisPROS vector and cloned into the yeast expression vector pGPD416 using the EcoRI and HindIII sites. Saccharomyces cerevisiae strain BY4742 (MATa, his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0), Δvta1ΔΔG418 and Δvps4 were kindly donated by David Katzmann, Mayo Clinic–Minnesota. The different yeast strains were transformed with the URA plasmids pGPD416-His–PROS or pGPD416-His–Vta1 and/or the LEU plasmid pRS425–CPS–GFP and were grown at 30 °C in liquid medium containing 0.67% (w/v) yeast nitrogen base, 2% (w/v) glucose and synthetic complete media (SCM)-LEU or SCM-URA-LEU. Transformation was performed using lithium acetate, followed by standard procedures. Yeast RNA was isolated using TRIzol (Invitrogen) following the manufacturer’s instructions and the retro transcripase (RT) reaction was performed using oligo-dT primers and AMV-Reverse Transcripase (Promega).
Novel ESCRT Component in Arabidopsis

Living cells were imaged using a Zeiss 510 laser scanning confocal microscope and a 63× oil-immersion NA1.4 objective. GFP was excited with an Argon laser at 488 nm, 20% of intensity, and the emission was collected using a BP 500−530 IR filter and pinhole opening equivalent to 1 Airy unit. All cells with detectable signal in the vacuolar lumen were scored as wild type (WT). The experiment was designed as a blind study. Number-coded cultures containing the eight different yeast strains were placed on glass slides. Between 25 and 30 randomly selected fields of view were recorded at three different Z-positions. All cells contained in these images were scored either as mutant or WT for GFP-CPS sorting. The three Z-views were used to determine the localization of the GFP signal either at the vacuolar membrane or the vacuolar lumen. After the quantitative analysis was performed, the data from the number-coded cultures was assign to the eight yeast strains. The experiment was repeated five times with independently transformed yeast cultures.

Generation of Transgenic Plants—Artificial microRNAs (amiRNA) were designed using the Web microRNA designer (WMD2) tool and the amiRNAs were cloned as described in (29). Two amiRNAs were used, the first one against the coding region (A) TATCTAAGATCTCGGAAGGAG and the second one against the 3’-UTR (B) TATATATCGAGAAATGCGAC of PROS. The amiRNAs were sequenced and cloned in the binary vector pCAMBIA1300, where the Nos-terminator sequence was previously cloned between the sites KpnI and SmaI. For the analysis of PROS expression, a fragment consisting of the 1246 base pairs upstream the PROS coding region was amplified from genomic DNA and cloned into the pCAMBIA 1381 vector which already contains the amplified from genomic DNA and cloned into the pCAMBIA 0.5% Triton X-100, 0.1%Tween-20, 10 mM Na2-EDTA, and 2 mM 5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid sodium salt (X-Gluc) (Phytotechnology Laboratories). Incubations were performed at 37 °C for 15 h. Tissues were clarified with ethanol:acetic acid (3:1), washed in 70% ethanol, and visualized using a stereoscopic microscope (Nikon SMZ800), and imaged with a Nikon Coolpix 4500 CCD digital camera.

GUS Staining—For histochemical localization of β-glucuronidase (GUS) activity, tissues were vacuum-infiltrated with 50 mM Na-phosphate buffer pH 7.0 containing 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.5% Triton X-100, 0.1% Tween-20, 10 mM Na2-EDTA, and 2 mM 5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid sodium salt (X-Gluc) (Phytotechnology Laboratories). Incubations were performed at 37 °C for 15 h. Tissues were clarified with ethanol:acetic acid (3:1), washed in 70% ethanol, and visualized using a stereoscopic microscope (Nikon SMZ800), and imaged with a Nikon Coolpix 4500 CCD digital camera.

RNA Extraction, RT, and Quantitative PCR—RNA was isolated from 21-day-old plants using TRIzol reagent (Invitrogen) following the manufacturer instructions. 500 ng of total RNA was reversely transcribed to the first strand of the cDNA using AMV transcriptase (Promega) and oligo-dT primers in a 20 μl volume reaction. A 1/100 dilution was used as a template in reactions for PROS, ARCl2, and UBc9. A 1/10 dilution was used as a template in reactions for CYP71A13 and bHLH131. The SYBR GREEN PCR Master Mix (Applied Biosystems) was used for amplification and detection of transcripts in an ABI prism 7300 thermocycler. The data were analyzed using the LinRegPCR quantitative PCR data analysis program (version 12.4). Primers used for quantitative PCR analysis of transcripts are: GATCCGCTCCTGCTCTGTA and TCATCGCTATGCAATCTG for PROS; CATCTTGAAGGAGGACGTGGAAG and GGTTTTTGATCCAGTTAACAA for UBc9; ATACCAACGTAATGCCCTTCTC and TGAATCCGTTGAATCAAACCTTGG for ARCl2; GGTCTTCCGTTGCACTCTTCTC and CCGCCCAAGCTATTGATACCT for CYP71A13; and GAAACCAAGGCGGTGAG and CAAGGCACACTTGGTCTTCC for bHLH131.

Electron Microscopy—Fresh, unfixed, 41-day-old leaves of WT and PROS knock-down lines A and B were imaged in an Environmental Scanning Electron Microscope (ESEM) FEI Quanta 200 in environmental mode.

Morphometric Analysis of Pavement Cells—Fourth rosette leaves from 30-day-old plants were harvested from each genotype and fixed in 70% ethanol. Leaves were stained with a 10 μg/ml propidium iodide (MP Biochemicals) solution and imaged in a Zeiss LSM 510 META confocal laser scanning microscope using a 20x NA0.75 objective. Propidium iodide was excited with an HeNe laser at 543 nm, 19% of intensity, and the emission was collected using a LP 560 filter and a pinhole opening equivalent to 1 Airy unit. Whole leaves were imaged on an Epson Perfection 4870 PHOTO flatbed scanner for subsequent measurement of total leaf blade area. Adaxial pavement cells at the mid-third of the leaves were traced and measured with the aid of Fiji (28). The number of pavement cells at the adaxial epidermis for each leaf was extrapolated from the measurements of pavement cell area and leaf blade area for each leaf. Eight leaves and 100–200 pavement cells were measured for each genotype.

Accession Numbers—Sequence data from this article can be found in the GenBankTM/EMBL databases under the following accession numbers: PROS (At4g24370); SKD1 (At2g2760); VPS601 (At3g10640); IST1 (At1g34220); CHMP1A (At1g73030); UBc9 (At4g27960); LIP5 (At4g26750); ARCl2 (At1g69390), CYP71A13 (At2g30770), bHLH (At4G38070).

RESULTS

PROS (POSITIVE REGULATOR OF SKD1) Is a Plant-specific Protein That Interacts with SKD1 and LIP5—In a previous yeast two-hybrid (Y2H) screen using Arabidopsis SKD1 as bait (20), we identified AT4G24370 as a putative SKD1-interacting protein and named it PROS, for POSITIVE REGULATOR OF SKD1. The PROS coding region predicts a soluble protein of 164 amino acids, a molecular mass of 18.4 kDa, and two putative coiled coils domains (Fig. 1). Bioinformatics analysis with InterProScan did not predict the presence of any known functional domain. BLAST searches with the PROS protein sequence did not retrieve any previously identified ESCRT component in plants or in any other organisms. In addition, we were able to identify single copies of PROS-like sequences only in flowering plants (Fig. 1A).

PROS was confirmed to interact with Arabidopsis SKD1 in a directed Y2H (Fig. 1B) and in vitro pull-down assays using recombinant His-SKD1 and glutathione-S-transferase (GST)-PROS (Fig. 1C). To test whether PROS interacts with endogenous SKD1, we incubated total protein extracts from Arabidopsis leaves with GST and GST-PROS. GST-PROS but not GST
alone was able to pull-down endogenous *Arabidopsis* SKD1 (Fig. 1D). In addition, the co-expression in *Arabidopsis* protoplasts of PROS fused to the N-terminal portion of YFP and SKD1 fused to the C terminus of YFP was able to reconstitute YFP fluorescence above background levels in a BiFC assay (Fig. 1E), indicating that the two proteins are able to interact in *vivo*.

By *in vitro* pull-down of recombinant proteins, we also found that PROS interacted with His-LIP5 but not with the accessory

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**FIGURE 1.** PROS is a plant specific ESCRT component. A, amino acid sequence alignment of PROS-like proteins in flowering plants. Black indicates identical residues, and gray represents similar residues. The position of the two predicted coiled coils (CC1 and CC2) is also indicated. *A. thaliana* PROS is 49% identical (64% similar) to the castor bean (*Ricinus communis*) homolog (XP_002513129) and 37% identical (50% similar) to the rice (*Oryza sativa*) homolog (Os08g0151500). B, Y2H analysis of AD-SKD1 with BD-PROS. Transformed cells were spotted on SD-LW or SD-LWHA plates to test for auxotrophic growth. C, *in vitro* pull-down assays of recombinant *Arabidopsis* SKD1 and PROS proteins tagged either with GST or His6 and expressed in bacteria. D, pull-down assay of native SKD1 using recombinant GST-PROS and total protein extracts from *Arabidopsis* leaves. GST-PROS interacts with native *Arabidopsis* SKD1. E, BFC assay on *Arabidopsis* protoplasts. A vector containing a 35Sprom::CFP (pAVA574) reporter was co-transfected to assess transfection efficiency. Between 280 and 580 protoplasts were scored for each combination of vectors. F, *in vitro* pull-down assays of recombinant PROS and several ESCRT proteins tagged with either GST or His6, and expressed in bacteria. Recombinant PROS protein interacts with LIP5 but not with IST1, CHMP1, or VPS60. In all pull-down experiments, GST-tagged proteins bound to beads were incubated for 2 h with either His-tagged proteins or total leaf protein extracts (I, input). Beads were washed three times (O, out). Input and output fractions were loaded on an SDS-PAGE gel and transferred to a nitrocellulose membrane. Proteins were detected with specific antibodies anti-GST or anti-His6.
ESCRT-III components His-CHMP1A, His-IST1, or His-VPS60.1 (Fig. 1F).

**PROS Contains a Functional Internal MIM Domain**—By performing in vitro pull down assays of truncated PROS proteins, we determined that the region between amino acid residues 41–164 containing the second coiled coil interacted with both His-SKD1 and His-LIP5 whereas the PROS N-terminal fragment (residues 1 to 40) showed only barely detectable interaction with either His-SKD1 or His-LIP5 (Fig. 2A). We introduced simultaneously six alanine substitutions along the 18 amino acid residues predicted to be part of the coiled coil (Fig. 2B) and found that mutations in the first six residues disrupted the interactions with both His-SKD1 and His-LIP5 (Fig. 2B). A closer examination of the second coiled coil region revealed the presence of a putative MIM (MIT-interacting motif) domain between amino acid residues 38 and 54 (Fig. 2C). MIM domains in core ESCRT-III subunits are located at the C-terminal region, which is known to autoinhibit the assembly of the ESCRT-III complex and to maintain the soluble monomeric pool in closed state (30, 31). Upon association with the endosomal membrane, ESCRT-III subunits change their conformation to an “open state” and their MIM domains become available for interactions with MIT (microtubule interacting and transport) domains such as the ones present in SKD1 and LIP5 (32, 33). To test whether the SKD1 MIM domain is required for interaction with PROS, we introduced a change in a conserved leucine residue (Leu-63 in Arabidopsis SKD1) known to be required for the interaction between yeast Vps4p and the Vps2p MIM domain (33). The recombinant His-SKD1(L63A) protein failed to interact with GST-PROS (Fig. 2D), indicating that the SKD1 MIM domain is necessary for interaction with PROS.

**PROS Increases the in Vitro ATPase Activity of Arabidopsis SKD1 and Yeast Vps4p**—To determine the effects of PROS on the enzymatic activity of SKD1, we used recombinant His-SKD1 in the presence or absence of PROS and other SKD1-interacting proteins in a colorimetric in vitro ATPase assay (Fig. 3). At equimolar concentrations, His-LIP5 and His-PROS caused 1.2- and 1.5-fold increase, respectively, in SKD1 ATPase activity (Fig. 3A). In yeast, Vta1p/LIP5 acts as a positive regulator of Vps4p by interacting through the Vps4p C-terminal domain and stimulating its oligomerization (23, 34). However, the interaction between the MIT domain of human VPS4 and the MIM domain of ESCRT-III proteins can directly stimulate VPS4 ATPase activity (35). To test whether the increase in SKD1 ATPase activity by PROS is comparable to that caused by other Arabidopsis ESCRT-III proteins, we used His-CHMP1A, which interacts with SKD1 and contains a C-terminal MIM (19). The addition of His-CHMP1A to His-SKD1 caused a ~1.3 increase in Pi release (Fig. 3A). However, because ESCRT-III proteins in solution tend to adopt a closed conformation with their MIM domains hidden between the N-terminal α-helices, it is possible that only a small fraction of the His-CHMP1 molecules in our assay were in an open conformation and able to bind SKD1. In fact, isolated MIM domain fragments have been
showed to have a much stronger positive effect on human VPS4 ATPase activity than whole-length ESCRT-III proteins (35). Consistently, when we added a C-terminal CHMP1 fragment containing the CHMP1 MIM domain plus 72 amino acid residues of the upstream sequence (His-CHMP1 109–203) to His-SKD1, we observed a 1.5-fold increase in ATP hydrolysis (Fig. 3A), more similar to the activity increase caused by PROS. This is consistent with PROS having a constitutively exposed MIM domain. We also performed a similar in vitro ATPase assay using recombinant His-Vps4p from yeast and found that Arabidopsis PROS was also able to increase the activity of yeast Vps4p by ~4-fold (Fig. 3B).

Expression of PROS in Δvta1 Yeast Mutant Cells Partially Rescues MVB Sorting Defects—Although PROS is able to increase the in vitro ATPase activity of SKD1, it seems to act differently from the best characterized yeast Vps4p-positive regulator Vta1p. Whereas the C-terminal domain of yeast Vta1p binds the C-terminal domain of Vps4p promoting Vps4p oligomerization and stabilizing the Vps4p-Vta1p complex (23, 36), an internal MIM in PROS binds the MIT domain of SKD1. Nevertheless, we asked whether the expression of Arabidopsis PROS could alleviate endosomal sorting defects in a yeast Δvta1 mutant strain that mis-sorts GFP-CPS, an MVB cargo marker, to the vacuolar membrane (23). We scored the number of cells showing normal GFP-CPS distribution in the vacuolar lumen and those showing mis-sorting of GFP-CPS to the vacuolar membrane (Fig. 3C) in WT, Δvta1 cells, and also in Δvta1 cells transformed with vectors expressing His-Vta1p, His-PROS, or with an empty vector (Fig. 3C). The expression of His-Vta1p restored the normal sorting of GFP-CPS in almost 100% of the Δvta1 mutant cells, whereas the expression of PROS led to normal GFP-CPS localization in 60% of the cells. To determine whether PROS restores GFP-CPS localization in Δvta1 cells through a Vps4p-dependent mechanism, we expressed His-PROS in Δvps4 cells. PROS did not rescue the mis-sorting of GFP-CPS in this case, indicating that PROS requires Vps4p to partially restore the MVB sorting functions in the Δvta1 mutant (Fig. 3C). The expression of the His-PROS transgene in yeast cells was confirmed by RT-PCR (Fig. 3D).

Figures

**FIGURE 3.** **PROS increases in vitro SKD1 and Vps4p ATPase activity.** A, in vitro ATPase activity of SKD1 alone or in combination with equimolar concentrations of LIP5, PROS, CHMP1, and a peptide containing the MIM of CHMP1 (CHMP1 109–203). B, in vitro ATPase assays of Vps4p with Vta1p and PROS. In both A and B, recombinant proteins expressed in bacteria were purified and used in a malachite green-based assay to measure Pi released from ATP. Bars represent standardized average values (n = 8) from two independent experiments. Letters above the bars represent statistical significance (one-way ANOVA followed by Tukey, p < 0.05); bars sharing a letter are not significantly different from one another. Error bars represent S.D. C, expression of His-PROS in Δvta1 but not in Δvps4 mutant strains is able to partially rescue the mis-sorting of the ESCRT cargo GFP-CPS. Δvta1 and Δvps4 mutant expressing GFP-CPS were transformed with empty plasmids, with His-Vta1p, or His-PROS. Cells showing GFP signal in the vacuolar lumen were scored as “wild type phenotype” and those showing GFP signal on the vacuolar membrane, as “mutant phenotype”. At least 100 cells for each genotype were analyzed. Scale bar, 5 μm. D, RT-PCR of PROS transcripts in the yeast strains used in C.
CYP71A13 was strongly up-regulated not only in the amiRNA B lines but also in the amiRNA A plants (Fig. 5B). Since CYP71A13 is involved in conferring resistance to fungal pathogens (37) and it is up-regulated under both biotic and abiotic stress conditions, the increased levels of CYP71A13 transcripts in both amiRNA A and B plants is likely a indirect effect due to the silencing of PROS. Consistent with our gene expression analysis (Fig. 4), plants expressing PROS amiRNAs showed general morphological defects in all organs. PROS knock-down plants were dwarf, with wrinkled rosette leaves, and twisted inflorescence stems (Fig. 5, C–J). Their flowers had all floral parts in normal position and number but did not fully complete expansion (Fig. 5, G and J). On PROS knock-down rosette leaves, we observed regions of abnormally small pavement cells with reduced interdigitation compared with WT leaves (Fig. 5, K–L). In addition, trichomes with normal numbers of branches (3–4) but half the size of WT trichomes (Fig. 5, M and N) were common in these regions. To determine whether the reduction in leaf size was solely due to smaller leaf cells or to an additional reduction in cell number, we estimated the number of adaxial pavement cells per leaf blade of 30-day-old PROS knock-down plants and found it not to be different from WT controls (Fig. 4, O–Q), indicating that defective cell expansion is the main underlying cause for reduced leaf blade size.

DISCUSSION

ESCRT components were originally identified for their participation in MVB sorting but they are now known to control many other cellular functions such as cytokinesis, autophagy, cell polarity, and migration, miRNA activity, and mRNA transport (38). Consistently, when their ESCRT machinery is not functional, multicellular organisms show very strong and pleiotropic developmental defects that often lead to lethality. Many of these prominent developmental defects in ESCRT mutants can be correlated to alterations in receptor-mediated signaling due to MVB sorting defects. The most general defect is signaling up-regulation due to improper receptor sorting and degradation. However, mutations in different ESCRT components do not lead to identical developmental and signaling defects, and a current major challenge is to understand how the ESCRT machinery interacts with different signaling pathways, in specific cellular and developmental contexts, and in different organisms.

Like other ESCRT-III proteins, PROS contains a MIM domain that mediates its interaction with SKD1. ESCRT-III subunits are assumed to cycle between a closed, inactive, monomeric state in the cytoplasm and open, oligomeric state when associated with endosomal membranes (30, 31, 39). The MIM domains located at the C terminus of ESCRT-III subunits such as CHMP1 and VPS2 only become available for interactions with SKD1/VPS4 when the proteins are in open conformation. The fact that whole length PROS and a fragment containing the MIM domain of CHMP1 increase the in vitro SKD1 ATPase activity at comparable levels, suggests that the internal MIM domain of PROS may be constitutionally exposed. Although we do not know whether PROS is part of the SKD1-LIP5 complex in plants, the fact that PROS can partially suppress MVB sorting defects in the yeast \Delta vtal
mutant suggests that it can work as a positive regulator of SKD1/VPS4 in vivo.

The cell expansion and growth defects seen in PROS knock-down lines are consistent with defects in endosomal trafficking (40). However, we cannot exclude the possibility that PROS plays other ESCRT-independent functions in plants that affect cell and organ expansion.

In conclusion, we have identified a novel ESCRT component that seems to have evolved within the plant lineage. Its presence in plants but not in other organisms is a clear indication of the almost unexplored variations of ESCRT functions across eukaryotes and the multiple regulatory mechanisms that control MVB sorting and the vacuolar degradation of plasma membrane proteins.

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