BLOS1, a putative BLOC-1 subunit, interacts with SNX1 and modulates root growth in *Arabidopsis*

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Summary

Internalization and sorting of macromolecules are inherent properties of all eukaryotic cells that are achieved by vesicle trafficking. However, this process is relatively less understood in plants. An eight-subunit protein complex, BLOC-1, which is involved in endosomal transport from the endosomes to the lysosomes, has been identified in both human and mice. In this study, two homologous subunits of this complex, BLOS1 (or AtGCN5L1) and BLOS2, have been characterized in *Arabidopsis*. Both BLOS1 and BLOS2 interacted with SNX1 on the sorting endosomes. Inducible RNAi lines with reduced levels of BLOS1 had longer primary roots and more lateral roots. Consistently, PIN1 and PIN2 were increased in *BLOS1* RNAi lines, implicating an impaired transport from the endosomes to the vacuoles. These results suggest that a putative BLOC-1 complex in *Arabidopsis* might mediate the vacuolar degradative transport through direct interaction with SNX1 to regulate the homeostasis of PIN1 and PIN2, which is important for plant growth and development.

Key words: Biogenesis of lysosome-related organelles complex 1 (BLOC-1), Sorting nexin 1 (SNX1), Auxin efflux transporter, Root growth, Endosomal transport, Vacuole

Introduction

Vesicle trafficking performs conserved functions in eukaryotes. It is involved in communication among different cell compartments and the exchange of information between cells and their environment. Endosomal transport plays a central role in vesicle trafficking as it connects anterograde transport from the Golgi apparatus to the endosomes and the lysosomes, endocytic transport from the plasma membrane to the endosomes and retrograde transport from the endosomes to the Golgi (Bonifacino and Rojas, 2006; Li et al., 2007). Cargo is sorted from the early endosomes to the cell surface, recycling endosomes or late endosomes and lysosomes (Gruenberg, 2001; Bonifacino and Glick, 2004). Components destined for the lysosomes are sorted to the late endosomes, or multivesicular bodies, which contain whorls of membranes and vesicles. The late endosomes morph into the lysosomes with a highly regulated fusion machinery (Raposo et al., 2007). The lysosomes serve as the major degradative compartments in eukaryotic cells (Kornfeld and Mellman, 1989). In addition, the secretory lysosomes or some specialized lysosome-related organelles play important functions in secretion via exocytosis (Raposo et al., 2007).

In contrast to mammalian cells, plant vesicle trafficking is less well understood. The identification of genes encoding components of vesicle trafficking in plant genomes suggests the conserved role of vesicle trafficking in plants. Endosomal transport plays a key role in many processes in plants including embryo differentiation (Goh et al., 2007; Silady et al., 2008), cell fate specification (Shen et al., 2003; Tian et al., 2007), cell wall remodeling (Baluaska et al., 2002), gravitropism (Silady et al., 2004; Abas et al., 2006) and polar auxin transport (Gälweiler et al., 1998; Steinmann et al., 1999; Geldner et al., 2001; Jallais et al., 2006; Jallais et al., 2007; Jallais et al., 2008; Dhonukshe et al., 2008). Endosomal trafficking is essential for polar transport of the phytohormone auxin and its influx (AUX) and efflux (PIN) carriers (Paciorek et al., 2005; Müller et al., 2007). Targeting of PIN1 protein to the basal plasma membrane requires PIN1 recycling from the plasma membrane to the endosomes mediated by GNOM, an endosomal GDP and GTP exchange factor for ARF GTPases (Geldner et al., 2003). By contrast, PIN2 transport requires the SNX1-positive endosomes. SNX1 is a newly described plant-sorting nexin that is localized to the prevacuolar compartment (PVC) in plants (Jailais et al., 2006). Besides SNX1, another component of retromer, VPS29, is proposed to function in endosomal recycling of PINs. In the *vps29* mutant, intracellular accumulation of PIN1 and, to a lesser degree, of PIN2 and other membrane proteins was observed. VPS29 colocalizes with SNX1 and is required for maintaining the SNX1 endosomal morphology but not the GNOM endosomes. A current model is that PINs are internalized first through the GNOM endosomes and then routed to the SNX1 endosomes for recycling (Jailais et al., 2007). Nevertheless, the destination of the endocytosed PIN1 and PIN2 remains to be clarified.

It has been demonstrated that plants have evolved different vacuoles to adapt their metabolism. Vacuoles, including the protein storage vacuole (PSV) and the lytic vacuole (LV), are important structures in vesicle trafficking. The lytic vacuoles share lysosome-like properties but also function as the protein storage vacuoles for protein accumulation (Frigerio et al., 2008). LVs and PSVs have different pH in their lumen, as highlighted by staining with pH- or acidic protease-sensitive dyes. The general consensus stemming from extensive tonoplast intrinsic protein (TIP) isoform analysis is
that α-TIP is enriched in PSVs, γ-TIP in LVs and δ-TIP in storage vacuoles (Jauh et al., 1998; Jauh et al., 1999). However, the transport machinery of cargoes from the endosomes to LVs or PSVs is lacking. Meanwhile, the physiological role of LVs in the maintenance of the steady-state levels of the endocytosed proteins remains to be elucidated.

The BLOC-1 complex consists of at least eight subunits in both human and mice, including dysbindin, snapin, mutated, palladin, cappuccino, BLOS1, BLOS2 and BLOS3. It is involved in vesicle trafficking from the endosomes to the lysosomes in higher eukaryotes (Li et al., 2007; Raposo et al., 2007; Truschel et al., 2009). Human Hermansky-Pudlak syndrome (HPS) is caused by the mutations of genes encoding dysbindin or BLOS3 (Li et al., 2003; Morgan et al., 2006). HPS is characterized by the defects in the biogenesis of lysosome-related organelles due to impaired cargo transport from the endosomes to the lysosomes (Li et al., 2007; Raposo et al., 2007). Whether there exists a homologous BLOC-1 complex in plants is unknown. Sequence comparisons reveal putative homologs of BLOS1 (also known as AtGCN5L1) and BLOS2 in Arabidopsis. Whether these homologs play similar roles in endosomal transport in Arabidopsis requires investigation. We here report the first evidence of a putative BLOC-1 in Arabidopsis and its possible involvement in the transport of PIN1 and PIN2, mediated by SNX1, to control root development.

**Results**

**BLOS1 and BLOS2 are putative homologs of mammalian BLOC-1 subunits**

Multiple protein sequences from different species were aligned using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/) and GeneDoc (http://www.nrbsc.org/downloads/) (supplementary material Fig. S1). The percentage of identity between the Arabidopsis and human proteins was calculated using the BLASTP algorithm (http://blast.ncbi.nlm.nih.gov/). The calculated sequence identities to human homologs for BLOS1 (At2g30330) and BLOS2 (At5g49550) were 36% and 29%, respectively. Interaction between BLOS2 and BLOS1 was revealed by yeast two-hybrid assays (Fig. 1A) and split-YFP assays (Fig. 1B). Next, our work is mainly focused on the functional studies of BLOS1.

**BLOS1 is expressed in multiple tissues**

Tissue-specific expression of BLOS1 was tested by real-time PCR with RNA extracted from adult plants. BLOS1 expression was found in all major tested organs including the root, stem, leaf, flower and silique, with highest expression in the leaf (Fig. 1C). Western blotting by a polyclonal anti-BLOS1 antibody was adopted. Sixteen hours after cotransformation with different organellar markers, confocal images showed that BLOS1 partially colocalized with endosome markers ARA7 (Fig. 3A) and VAMP27 (Fig. 3B), PVC and vacuole markers PEP72 (Fig. 3C) and VAM3 (Fig. 3D). These data indicate that BLOS1 is probably localized at the endosomal compartments.

**BLOS1 is partially localized to the endosomal compartments**

To study the subcellular localization of BLOS1, a protoplast system was adopted. Sixteen hours after cotransformation with different vectors, pUC-SPYCEG–BLOS1 and pUC-SPYNEG–BLOS2, mediated by SNX1, to control root development.

![Image](https://via.placeholder.com/150)

Fig. 1. BLOS1 interacts with BLOS2 and is expressed in multiple tissues. (A) Interaction between BLOS1 and BLOS2 in yeast two-hybrid assays. BLOS2 exhibits directional interactions with BLOS1. No interaction is detected between BLOS2 and the empty pGBK7 vector. (B) BLOS1 and BLOS2 interaction in a split-YFP system. Subcloning of BLOS1 and BLOS2 into split-YFP gateway vectors pUC-SPYCEG–BLOS1 and pUC-SPYNEG–BLOS2 forms YFP C-terminal and N-terminal fusion proteins, respectively. (C) Tissue expression of BLOS1 in adult plants by real-time PCR. BLOS1 mRNA levels in wild-type plants were determined by real-time PCR using gene-specific primers and total RNA from the root, stem, leaf, flower and silique. Actin is used as an internal control. BLOS1 is expressed in all these tissues with different levels. Experiments were repeated three times independently. (D) Western blotting assay of BLOS1 in different tissues. Protein extract from the root, stem, leaf, flower and silique were loaded to 12% SDS-PAGE for separation and probed by purified BLOS1 antibody. Ponceau S staining was used as loading control.
BLOS1 interacted with SNX1

To explore the function of BLOS1, full-length BLOS1 cDNA was used as a bait to screen an Arabidopsis cDNA library. SNX1 (At5g06140) was identified as a novel interactor (Fig. 5A) via the GCN5L domain of BLOS1 (Fig. 5B). Likewise, BLOS2 interacted with SNX1 in the yeast two-hybrid assay (Fig. 5C), further supporting that BLOS1 and BLOS2 might function in the same complex. In addition, BLOS1 and SNX1 colocalized in protoplast (Fig. 5D). To further confirm this interaction, we found that BLOS1 interacted with SNX1 in onion and protoplast by split-YFP assays (Fig. 5E,F).

Increase of PIN1 and PIN2 in BLOS1 RNAi lines

Considering a possible role for BLOS1 in endosomal trafficking, we hypothesized that BLOS1 recruits PINs sorted by SNX1 and transports a portion of PINs to the vacuoles for degradation. Immunocytochemical analysis of PIN1 and PIN2 in BLOS1 RNAi knockdown lines showed that the protein levels of PIN1 and PIN2 on the plasma membrane increased after induction (Fig. 6C,D) compared with wild type (Col-0) after induction (Fig. 6A,B). However, the polar distributions of both PIN1 and PIN2 were not apparently altered. To rule out the possibility of transcriptional upregulation of PIN1 and PIN2 by BLOS1, we performed real-time PCR and the results showed no significant increase of PIN1 or PIN2 transcripts in the BLOS1 RNAi-L4 after induction by estradiol (Fig. 6E).

To further study whether the accumulation of PIN1 and PIN2 on the plasma membrane is due to a defect in the endocytosis of these PINs, the recruitment to the endosomes after tracing for 15 minutes in L4 was examined. Recruitment was not apparently impaired as controlled by the effects of brefeldin A (BFA) treatment in FM4-64 endocytic assays (Fig. 7), suggesting that the endocytosis of PIN proteins from the plasma membrane to the endosomes might not be affected in these lines.

Discussion

Although the cellular function of the endosomal transport complex BLOC-1 in mammals requires further definition (Li et al., 2007), we here provide the first report of its possible involvement in endosomal trafficking in plants. BLOS1 and BLOS2 in Arabidopsis are probably two homologous subunits of BLOC-1, as revealed by sequence comparison, biochemical features and cellular function in endosomal transport. Downregulation of BLOS1 causes longer primary and lateral roots, suggesting that the homologous BLOC-1 complex might be involved in root development by negatively regulating the stability of PIN1 and PIN2 through the interaction with SNX1.

Thus far, at least eight subunits have been identified in the mammalian BLOC-1 complex, which is involved in cargo-specific sorting from the endosomes to the lysosomes (Li et al., 2007; Setty et al., 2007; Truschel et al., 2009). Sequence comparison reveals only two putative homologs (BLOS1 and BLOS2) of BLOC-1 subunits in Arabidopsis. Whether the composition of BLOC-1 is the same as its mammalian counterpart remains to be characterized biochemically. As shown in this study, BLOS1 and BLOS2 interact directly, suggesting a conservative role of BLOC-1 in protein trafficking from the endosome to the vacuole in plants. Cargo sorting from the trans-Golgi network (TGN) or from the plasma membrane (PM) to the lysosomes might share similar machinery. It is evident that BLOC-1 directs cargoes from the TGN or from the PM to the lysosome-related organelles or to the lysosomes for degradation (Setty et al., 2007; Iizuka et al., 2007; Truschel et al., 2009). In Arabidopsis, it is known that PIN proteins rapidly and constitutively cycle between the PM and the endosomes...
lines are counted. All plants grow vertically with 10 lateral root density of 8-day-old seedlings from the wild-type and transgenic between wild-type

Data shown are the averages + s.d. of 20 seedlings. * indicates statistically significant difference (Student’s t-test, P<0.05).

(Geldner et al., 2001). Internalization of PIN2 is clathrin-mediated (Dhonukshe et al., 2007) and it is then transferred to the sorting endosomes via SNX1 (Jaillais et al., 2006). However, the destination of the internalized PIN proteins remains uncertain. One possibility is the temporary storage in specific vesicles for fast recycling to the PM. Another possible fate is the proteasomal degradation of ubiquitinated PINs (Abas et al., 2006). Third, there is evidence that PINs could be targeted for vacuolar degradation in the absence of light (Laxmi et al., 2008), which might be mediated by the ESCRT complex (Spitzer et al., 2009). Nevertheless, the molecular aspects of this route are primitive. Currently, there are no direct interactions reported among the PINs, SNX1 and ESCRT. Here, we show for the first time physical interactions between the endosomes via SNX1 (Jaillais et al., 2006). However, the molecular aspects of this route are primitive. Currently, there are no direct interactions reported among the PINs, SNX1 and ESCRT. Here, we show for the first time physical interactions between SNX1 and BLOS1/2, which might provide insights into the complexity of PIN protein homeostasis.

It is probable that a portion of PIN proteins could be targeted for vacuolar degradation mediated by SNX1 and BLOS1/2, suggesting a conservative role of BLOC-1 in mammals and plants for vacuolar targeting. First, both BLOS1 and BLOS2 interact with SNX1, which probably occurs within the endosomal compartments. Second, SNX1 is reported to mediate PIN2 transport (Jaillais et al., 2006). Third, knockdown of BLOS1 leads to an increase of PIN1 and PIN2, which is probably caused by impaired vacuolar degradation as discussed below.

The augmentation of PIN1 and PIN2 could be a result of: (1) transcriptional upregulation of PIN1 and PIN2 by BLOS1; (2) impaired endocytosis of PIN1 and PIN2; and (3) delayed proteosomal or lysosomal protein degradation to facilitate membrane reinsertion of PIN1 and PIN2. As we showed in this study, BLOS1 is mostly expressed in the cytosol and the transcripts of PIN1 and PIN2 are not significantly increased in the BLOS1 RNAi lines (Fig. 6E); therefore, it is unlikely that BLOS1 acts as a transcription factor to regulate the expression of PIN1 and PIN2. In the FM4-64 traced assay, the endocytosis from the PM to the endosomes appears to be unaffected (Fig. 7). We have not ruled out the possibility that BLOS1 might affect the proteosomal degradation of ubiquinated PIN1 and PIN2. Based on the function of its mammalian counterpart, it is more likely that the impaired lysosomal targeting of PIN1 and PIN2 caused by the deficiency of BLOS1 might facilitate these PIN proteins to take an alternative route to the PM through membrane reinsertion. In agreement with this phenomenon, it has been reported that the melanosomal protein TYRP1 (Setty et al., 2007), the dopamine receptor D2 (Ji et al., 2009) or the NMDA receptor NR2A (Tang et al., 2009) are increased in the PM through a similar mechanism when BLOC-1 is deficient.
The timing and location of auxin accumulation determines spatiotemporal facets of developmental reprogramming. Localized increase of auxin in a single pericycle cell causes the initiation and formation of lateral roots (Dubrovsky et al., 2008). Moreover, the stabilization of PIN2 affects its abundance and distribution and leads to defects in auxin distribution and root development (Abas et al., 2006). Thus, the regulation of the homeostasis of PIN1 and PIN2 by vesicle trafficking is important to plant growth. The accumulation of PIN1 and PIN2 in the BLOS1 RNAi lines is probably caused by impaired degradation through the endosomal-vacuole pathway, which might, in turn, lead to an increase of auxin transport that initiates the development of longer primary roots and the formation of more lateral roots.

It must be re-emphasized that the root phenotypes of BLOS1 knockdown lines are opposite to the snx1 lines (Jaillais et al., 2006), although we have shown the physical interaction of BLOS1 with SNX1. One possible explanation is that SNX1 is responsible for the sorting of endocytosed PIN1 and PIN2 where they might be recycled to the PM via VPS29 or retromer (Jaillais et al., 2007). The recycled PIN1 and PIN2 are reduced in the snx1 lines (Kleine-Vehn et al., 2008) and thus show the delayed root development. The opposite root phenotypes in snx1 and in BLOS1 RNAi lines agree with the opposite changes in PIN1 and PIN2 stability. BLOS1/2 or BLOC-1 might act in a different pathway through the interaction with SNX1 for vacuolar degradation of PIN1 and PIN2. It is possible that this proportion of PIN1 and PIN2

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Fig. 6. Immunocytochemical analyses of PIN1 (green) and PIN2 (red) in BLOS1 RNAi knockdown lines. Immunocytochemical analysis of PIN1 and PIN2 in induced and uninduced wild-type (WT, Col-0; A,B) and BLOS1 RNAi L4 (C,D). Compared with WT and untreated lines, BLOS1 RNAi knockdown lines (RNAi-L4) treated with 10 μM beta-estradiol have a marked increase in cells without apparent changes in polar distribution. All seedlings are 5 days old and grow vertically in the indicated medium. Seedlings are representative of at least three independent experiments and each time more than 5 seedlings are calculated. Note that all the pictures are taken with the same settings and there is no brightness difference. (E) Real-time PCR shows no significant increase (P>0.05) in the transcripts of PIN1 and PIN2 with 10 μM beta-estradiol inducement after normalized by the untreated lines.

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Fig. 7. Intracellular trafficking from the plasma membrane to the endosomes in BLOS1 RNAi knockdown lines. Endocytic tracer FM4-64 binds to the membranes of endocytic compartments. FM4-64 is employed to trace intracellular trafficking from the plasma membrane to the endosomes within 15 minutes after FM4-64 uptake. No marked differences are observed in estradiol-treated BLOS1 RNAi line (L4) compared with wild-type (WT, Col-0) and untreated lines. As a positive control, both WT and RNAI-L4 show enlarged endosomes after BFA treatment.

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Fig. 8. A proposed model of SNX1-mediated transport of PIN proteins. In response to polar auxin transport, PIN1 and PIN2 are internalized first through the GNOM endosomes and then routed to the SNX1 endosomes for recycling. With the action of retromer, PIN1 and PIN2 are transported to the trans-Golgi network (TGN) then are reinserted into the plasma membrane (route 1). After being sorted by SNX1, PIN1 and PIN2 might be alternatively directed by BLOS1 and BLOS2 (or BLOC-1) for vacuolar degradation (route 2). SNX1 is sitting at the switch point of these two routes. When BLOC-1 is deficient or route 2 is blocked, PIN1 and PIN2 may take route 1 to be added to membrane.
PIN2 could be marked with mono-ubiquitin and recognized by 
ESCRT (Spitzer et al., 2009). To support this, our preliminary data 
shows that BLOC-1 interacted with ESCRT-1 in a yeast two-
hybrid assay (data not shown). In the mammalian cell, it is 
suggested that BLOC-1 acts downstream of ESCRT (Truschel et al.,
2009). Thus, we proposed a model for a dual role of SNX1, one 
for fast recycling of PIN1 and PIN2 via retromer (JailaaS et al.,
2007) and another for vacuolar targeting of PIN1 and PIN2 via 
BLOC-1 (Fig. 8). Similarly, in mammalian cells, the role of SNX1 
has been reported to be involved in both the retrograde endosomal 
transport via retromer (Gullapalli et al., 2004) and the lysosomal 
degradative pathway of cell surface receptors such as EGFR (Zhong 
et al., 2002) and PAR1 (Gullapalli et al., 2006). Our findings 
suggest that the vacuolar degradative pathway is important in 
the homeostasis of PIN1 and PIN2 to regulate the plant growth 
and development.

Materials and Methods
Plant growth conditions
Plants were grown under a 16:8-hour light:dark cycle at 22°C on solid 
Murashige and Skoog (MS) agar [half-strength MS salts, 3% (w/v) 
sucrose and 1% (w/v) agar]. For root analysis, seedlings were grown 
vertically on half MS medium in Petri dishes for the indicated period of 
time. For growing the inducible RNAi lines, experiments were 
carried out by using both wild-type (WT, Col-0) and transgenic 
plants treated with 10 μM beta-estradiol, whereas dimethyl sulfoxide 
(DMSO, a solvent for estradiol) was used as treatment for the control 
lines.

Yeast two-hybrid screen
The yeast two-hybrid screen was performed by following the user manual of the 
MATCHMAKER 3 Yeast Two-Hybrid System (Clontech, Mountain View, CA), with 
minor modifications. Full-length BLOS1 cDNA (At2g50330) was amplified by PCR 
using BLOS1-specific primers BLOS1-ACCGAGAGATCGAGAAAGGAAGCA-3' 
and 5'-GCGAATTCTGAGGATGATCTA-3'. The PCR product was cloned into the 
Ndel and PstI sites of pGBKT7 (binding domain, BD) and pGADT7 (activation 
domain, AD) in-frame. Yeast cells were first transformed with pGBK7-
BLOS1 and sequentially transformed with the Arabidopsis cDNA library constructed 
in the GAL4 AD vector pGAD-GH (Nie et al., 1999). The transformed cells were 
plated on dropout selection medium lacking Trp, Leu, His and adenine (Ade) and 
incubated at 30°C for 3–8 days.

RNA interference transgenic lines
To create inducible dsRNAi transgenic lines, primers 5'-GGCTCGAGACTA-
GTGATAAATCGGCGATG-3' and 5'-GGCTCGAGACTAATCGGCGATG-3' were used to amplify a 
400 bp BLOS1 cDNA PCR product. The remaining procedures were as described previously (Guo et al., 2003).

Western blot analysis
Two peptides LEKKESEEESTLE and DNDRRSSLQLEKRTSREKAE selected from 
BLOS1 were synthesized to generate rabbit polyclonal antibodies. The 
specificity of purified BLOS1 antibody from peptide DNDRRSSLQLEKRTSREKAE 
was examined in protoplast protein extracts. Expression levels of 
BLOS1 in different tissues were analyzed by purified BLOS1 antibody from peptide DDNRRSSLQL-
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400 bp BLOS1 cDNA PCR product. The remaining procedures were as described previously (Guo et al., 2003).

Northern blotting
Total RNA from 2-week-old seedlings were isolated by the Plant RNAeasy Prep Kit 
(Qiagen, Valencia, CA) according to the manufacturer’s instructions. RNA northern 
blot analysis was performed (20 μg of total RNA per lane) with a 32P-radiolabeled full-
lengt BLOS1 probe. The procedure was carried out as described (Sun et al., 2003).

Bimolecular fluorescence complementation (BiFC)
The open reading frame sequences of BLOSI, BLOSI and SNXI were amplified by PCR with gateway-specific primer pairs PwBHOSI 
5'-AAAAAACGGACCGTGATGATATACCCTGACACTC-3', 5'-AGAGGAGC-
GTTGATTATTGTGATTTCTCAGATGATGTC-3', PwBHOS2 5'-AAAAAACGGAC-
GTTGATTATTGTGATTTCTCAGATGATGTC-3', 5'-AGAGGAGCAGGGAT-
TGAACACAGAATCTATGTTC-3', PwSNXI 5'-AAAAAACGGACCGTGATGATATACCCTGACACTC-3', 5'- 
AGAGGAGCAGGGAT-TGAACACAGAATCTATGTTC-3'. The resulting PCR products were cloned into plasmids pUC-
SPYCE-wg and pUC-DYN-dn-wg to give rise to pUC-SPYCE-wg-BLOSI1, pUC-
DYN-dn-wg-BLOSI2 and pUC-DYN-dn-wg-SNXI. pUC-DYN-dn-wg contains N-terminal 
YFP modified from pUC-SPYNE-wg. To generate the gateway constructs, we 
followed the gateway cloning protocol (Invitrogen, Carlsbad, CA).

Histochemical localization of GUS activity
For the promoter-GUS assay, a 2187 bp genomic fragment downstream of the 
BLOS1 TAA stop codon, together with a 1148 bp genomic fragment downstream of the 
BLOS1 TAA stop codon, were PCR-amplified with the primers Sin17 
5'-GGCTCGAGACTAATCGGCGATG-3' and P1148 5'-GGCTCGAGACTAATCGGCGATG-3' and 
used as template for the PCR reactions. The 2187 bp (Pst/BamHI) and 1148 bp (Sac/EcoRI) fragments were cloned into the pBI101 vector in frame to produce the 
BLOS1-GUS fusion construct. The construct was 
introduced into Arabidopsis wild-type plants by Agrobacterium-mediated vacuum 
infiltration (Bectold and Pelletier, 1998). After selection on MS agar plates 
containing 50 mg/ml kanamycin, the resulting transgenic plants were stained in GUS 
staining buffer (0.1% Triton X-100, 5 mM Fe2+CN, 5 mM Fe3+CN and 1 mg/ml X-
Gluc; Sigma-Aldrich, St Louis, MO) in 50 mM sodium phosphate buffer, pH 7.0 
and mounted in 50% glycerol.

Subcellular localization
A 783 bp DsRed fragment was amplified from pDsRed2N1 (Clontech) by prime PCR with 
primers 5'-AGTAGAACGCTGATCCCTGCTA-3' and 5'-GCTTCAAGATGCCGTATGC-
TAGT-3'. After digestion with KpnI and XbaI, the resulting PCR product was cloned into 
pWM101. The BLOSI and BLOSI cDNAs were amplified from wild-type 
Columbia (Col-0) with the primers 5'-CGAGGTCCTGATCCCTGCTA-3' and 
5'-GCTTCAAGATGCCGTATGC-TAGT-3'. For the promoter-GUS assay, the primers 5'-GGCTCGAGACTAATCGGCGATG-3' 
and 5'-AGGGTACCTGATCCCTGCTA-3' were used to amplify the 
BLOS1-GUS fusion construct. The 2187 bp (Pst/BamHI) and 1148 bp (Sac/EcoRI) fragments were cloned into the pBI101 vector in frame to produce the 
BLOS1-GUS fusion construct. The construct was 
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staining buffer (0.1% Triton X-100, 5 mM Fe2+CN, 5 mM Fe3+CN and 1 mg/ml X-
Gluc; Sigma-Aldrich, St Louis, MO) in 50 mM sodium phosphate buffer, pH 7.0 
and mounted in 50% glycerol.

Immunocytochemical analysis
Immunocytochemical analysis of PIN1 and PIN2 was performed on whole-mount 
roots of 5-day-old seedlings. Mouse anti-PIN1 (Gälweiler et al., 1998) and guinea 
pig anti-PIN2 (Müller et al., 1998) were diluted to 1:50 and 1:100, respectively. For 
the secondary antibodies, Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 555 goat 
anti-guinea pig IgG (Invitrogen) were diluted to 1:500. The detailed procedures 
were previously described (Friml et al., 2002; Friml et al., 2002b). Imaging was performed using a Zeiss LSM 510 Meta inverting confocal microscope 
and accompanying software.

For FM4-64 treatments, seedlings were incubated for 15 minutes at room 
temperature in half-lipid medium containing FM4-64 (5 μM) and rinsed three times 
in half-lipid medium. BFA (25 μM in DMSO) treatments were performed in half-
lipid medium for 1 hour before observation.

Statistics
Quantitative data in paired groups was analyzed using the Student’s t-test. Qualitative 
data was compared using the χ2 test.

Accession numbers
Sequence data from this article can be found in the EMBL, GenBank databases under the following accession numbers: BLOSI (At2g50330, NM_128586), BLOSI (At5g49550, NM_124332) and SNXI (At5g06140, NM_126096). The accession numbers of the homologs of BLOSI and BLOSI2 are listed in Figure S1 in the supplementary material.
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References

Abas, L., Benjamins, R., Malenica, N., Paciorek, T., Wisniewska, J., Moulinier-Anzola, J. C., Sieberer, T., Friml, J., and Luschnig, C. (2006). Intracellular trafficking and proteinolyis of the Arabidopsis auxin-efflux facilitator PIN2 is involved in root gravitropism. Nat. Cell Biol. 8, 249-256.

Baluska, F., Hlavacka, A., Samaj, J., Palme, K., Robinson, D. G., Matoh, T., McCurdy, D. W., Menzel, D. and Volkmann, D. (2002). F-actin-dependent endocytosis of cell wall pectins in meristematic root cells. Insights from brefeldin A induced compartments. Plant Physiol. 130, 422-431.

Bechtold, N. and Pelletier, G. (1998). In planta Agrobacterium mediated transformation of adult Arabidopsis thaliana plants by vacuum infiltration. Methods Mol. Biol. 82, 259-266.

Bonifacino, J. S. and Rojas, R. (2008). Multiple vacuoles in plant cells: rule or exception? Nat. Rev. Mol. Cell Biol. 9, 153-166.

Bonifacino, J. S. and Traub, M. A. (2003). A chemical-regulated inducible RNAi system in plants. Plant Cell 15, 1891-1899.

Brakhage, O., Herren, A., Rohwer, T., Zuo, J. and Weber, K. (2007). Evidence for distinct functions of sorting nexin 1 and 2 in protein trafficking. Mol. Biol. Cell 18, 678-780.

Braun, S. and Glick, B. S. (2004). The mechanisms of vesicle budding and fusion. Cell 116, 153-166.

Bonifacino, J. S. and Rojas, R. (2006). Retrograde traffic from endosomes to the trans-Golgi network. Nat. Rev. Mol. Cell Biol. 7, 568-579.

Dubrovsky, J. G., Sauer, M., Napsucialy-Mendivil, S., Ivanchenko, M., Friml, J., Stierhof, Y., Jurgens, G. and Palme, K. (2007). VPS9a, the common activator for two distinct ESCRT-related organelles complex 1 (BLOC-1). Nat. Genet. 39, 84-89.

Li, W., Meng, Y. F., Hao, C. J., Guo, X. L., Cui, Y. Y., He, M. and He, X. (2007). The BLOC-1 interactome forms a network in endosomal transport. J. Genetomics 34, 68-82.

Morgan, N. V., Pasha, S., Johnson, C. A., Ainsworth, J. R., Eady, R. A., Dawood, B., McKeown, C., Trembath, R. C., Wilde, J., Watson, S. P. et al. (2006). A germline mutation in BLOC1S3/ reduced pigmentation causes a novel variant of Hermansky-Pudlak syndrome (HPS8). Am. J. Hum. Genet. 78, 160-166.

Müller, A., Guan, C., Huijts, P., Marchant, A., Parry, G., Bennett, M., Wisman, E. and Palme, K. (1998). AIP2 defines a locus of Arabidopsis for root gravitropism control. EMBO J. 17, 6903-6911.

Müller, J., Mettbach, U., Menzel, D. and Samaj, J. (2007). Molecular dissection of endosomal compartments in plants. Plant Physiol. 145, 293-304.

Paciorek, T., Zazimalova, E., Rutherford, N., Petrasek, J., Stierhof, Y.-D., Klein-Vehne, J., Morris, D. A., Cutler, D. F. (2007). Lysosome-related organelles: driving organelle compartments into specialisation. Curr. Opin. Cell Biol. 19, 394-401.

Seyt, S. R., Tenza, D., Truschel, S. T., Chou, E., Salter, S., Theos, A. C., Sviderskaya, E. V., Theos, A. C., Mundt, D., Kato, T., Tasaka, M. and Somerville, C. R. (2009). The ESCRT-related CHMP1A and B proteins mediate multivesicular body pathway and is required for vacuolar membrane flow. Nat. Cell Biol. 12, 229-230.

Shen, B., Li, C., Min, Z., Meeley, R. B., Traczynski, M. C. and Olsen, O.-A. (2003). Salt1 determines the number of aleurone cell layers in maize endosperm and encodes a class E vacuolar sorting protein. Proc. Natl. Acad. Sci. USA 100, 6552-6557.

Silady, R. A., Kato, T., Lukowitz, W., Sieber, P., Tasaka, M. and Somerville, C. R. (2007). The gravitropism defective 2 mutants of Arabidopsis are deficient in a protein that mediates endosomal sorting from early endosomes to the limiting membrane. Plant Physiol. 144, 1318-1336.

Setty, S. R., Tenza, D., Truschel, S. T., Chou, E., Sviderskaya, E. V., Theos, A. C., Lamoreux, M. L., Di Pietro, S. M., Starcevic, M., Bennett, D. C. et al. (2007). BLOC-1 is required for cargo-specific sorting from vacuolar early endosomes toward lysosome-related organelles. Mol. Biol. Cell 18, 768-780.

Steinhann, T., Gehrke, M., Grebe, M., Mandol, S., Jackson, C. L., Paris, S., Gälweiler, L., Palme, K. and Jürgens, G. (1999). Coordinated polar localization of auxin efflux carrier PIN2 by GNOM ARF GEF. Mol. Biol. Cell 10, 106-109.

Sun, J., Niu, Q. W., Tarkowski, P., Zheng, B., Tarkowska, D., Schun, G., Chua, N. H. and Zuo, J. (2003). The Arabidopsis AIP1/PRG1 gene encodes an isopentenyl transferase that is involved in de novo cytokinin biosynthesis. Plant Physiol. 131, 167-176.

Tang, T. T., Yang, F., Chen, B. S., Lu, Y., Ji, Y., Roche, K. W. and Lu, B. (2009). Dybsinind regulates hippocampal LTP by controlling NMDA receptor surface expression. Proc. Natl. Acad. Sci. USA 106, 21395-21400.

Qian, T., Olsen, L., Sun, B., Lid, S. E., Brown, R. C., Lemmon, B. E., Fonse, K., Graus, D., Oppahl-Sorezteb, H.-G., Otegui, M. S. et al. (2007). Subcellular localization and functional domain studies of DEFECTIVE KERNE1 in maize and Arabidopsis suggest a model for aleurone cell fate specification involving CRINKLY4 and SUPERNUMERARY ALEURONE LAYER1. Plant Cell 19, 3127-3145.

Tscherusch, T., Sinosy, S., Setty, S. R., Harper, D. C., Tenza, D., Thomas, P. C., Ainsworth, J. R., Jurgens, G., Geldner, N. and Palme, K. (2008). The GRV2/RME-8 protein of Arabidopsis functions in the late endocytic pathways and mutant dysbindin, a member of the biogenesis of lysosome-related organelles complex 1 (BLOC-1). Nat. Genet. 35, 105-107.

Watanabe, M., Nakamura, A., Ueda, T., Nakano, A. and Jurgens, G. (2001). Auxin transport in Arabidopsis thaliana. Science 294, 425-428.

Wu, H., Feng, Y. Q., Hao, C. J., Guo, X. L., Cui, Y. Y., He, M. and He, X. (2007). Dybsinind regulates hippocampal LTP by controlling NMDA receptor surface expression. Proc. Natl. Acad. Sci. USA 106, 21395-21400.

Zhong, Q., Lazar, C. S., Tronchère, H., Sato, T., Mei, X., Yeon, M., Songyang, Z., Emr, S. D. and Gill, G. N. (2002). Endosomal localization and function of sorting nexin 1. Proc. Natl. Acad. Sci. USA 99, 6676-6677.