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**UDP-D-galactose synthesis by UDP-glucose
4-epimerase 4 is required for organization of the
trans-Golgi network/early endosome in *Arabidopsis
thaliana* root epidermal cells**

Running head: UGE4 and organization of the *trans*-Golgi network

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Abstract

Endomembrane organization is essential for cell physiology. We previously identified an *Arabidopsis thaliana* mutant in which a plasma membrane (PM) marker GFP-NIP5;1 and *trans*-Golgi network/early endosome (TGN/EE) markers were accumulated in intracellular aggregates in epidermal cells of the root elongation zone. The mutant was identified as an allele of *UDP-glucose epimerase 4 (UGE4)/root hair defective 1/root epidermal bulgar 1*, which was previously described as a mutant with swollen root epidermal cells and has an altered sugar composition in cell wall polysaccharides. Importantly, these defects including aggregate formation

were restored by supplementation of D-galactose in the medium. These results suggested that UDP-D-galactose synthesis by UGE4 is important for endomembrane organization in addition to cell wall structure. Here, we further investigated the nature of the aggregates using various markers of endomembrane compartments and BOR1-GFP, which traffics from PM to vacuole in response to high-B supply. The markers of multi-vesicular bodies/late endosomes (MVB/LEs) and BOR1-GFP were strongly accumulated in the intracellular aggregates, while those of the endoplasmic reticulum (ER), the vacuolar membrane, and the Golgi were only slightly affected in the *uge4* mutant. The abnormal localizations of these markers in the *uge4* mutant differed from the effects of inhibitors of actin and microtubule polymerization, although they also affected endomembrane organization. Furthermore, electron microscopy analysis revealed accumulation of abnormal high-electron-density vesicles in elongating epidermal cells. The abnormal vesicles were often associated or interconnected with TGN/EEs and contained ADP-ribosylation factor 1, which is usually localized to the Golgi and the TGN/EEs. On the other hand, structures of the ER, Golgi apparatus, and MVB/LEs were apparently normal in *uge4* cells. Together, our data indicate the importance of UDP-D-galactose synthesis by UGE4 for the organization and function of endomembranes, especially TGN/EEs, which are a sorting station of the secretory and vacuolar pathways.

Keywords: *Arabidopsis thaliana* · UDP-D-galactose · UDP-D-glucose 4-epimerase · endomembrane organization · TGN/EEs

Abbreviations

UGE4 UDP-D-glucose-4 epimerase 4

rhd1 *Root hair defective (rhd) 1*

65	<i>reb1</i>	<i>Root epidermal bulgar</i>
66	PM	Plasma membrane
67	TGN	<i>Trans</i> -Golgi network
68	MVB	Multi-vesicular body
69	ER	Endoplasmic reticulum
70	GFP/YFP	Green/Yellow fluorescence protein
71	NIP	Nodulin 26-like intrinsic protein
72	EE	Early endosome
73	LE	Late endosome
74	HDVs	High-electron-density vesicles
75	BFA	Brefeldin A
76	ARF-GEF	ADP-ribosylation factor-Guanine nucleotide exchange factors
77	DIC	Differential interference contrast
78	FM4-64	N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium
79	Dibromide	

80 **Footnote:**

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88 **Introduction**

89 UDP-D-galactose is synthesized in the cytosol by UDP-D-glucose-4 epimerase isoforms (UGEs) and transported
90 into the Golgi apparatus to serve as a nucleotide sugar donor for the biosynthesis of cell wall polysaccharides
91 (Reiter and Vanzin 2001; Yin et al. 2011). The Arabidopsis genome encodes five *UGE* genes, and their
92 expression has been detected in every plant organ (Seifert et al. 2002). Among these genes, *UGE4* is most
93 highly expressed in epidermal cells of the root elongation zone, followed by *UGE1* (Barber et al. 2006; Rösti et
94 al. 2007). Mutants of *UGE4* are known as *root hair defective (rhd) 1*/*root epidermal bulgar (reb) 1*, which
95 exhibit defects in root hair development, root morphology, and susceptibility to nematodes (Baskin et al. 1992;
96 Baum et al. 2000; Schiefelbein and Somerville 1990). The trichoblasts (root hair-forming cells) of the mutants
97 become swollen and the base of the root hair shows a widened (bulbous) morphology (Schiefelbein and
98 Somerville 1990). In the swollen cells, microtubules were disordered or absent entirely (Andeme-Onzighi et al.
99 2002). *UGE4* is located close to the Golgi stacks and was shown to be specifically required for the
100 galactosylation of xyloglucan and type II arabinogalactan, but was not thought to be involved in galactolipid
101 biosynthesis (Barber et al. 2006; Rösti et al. 2007; Seifert et al. 2002).

102 Based on fluorescence imaging-based screening using a plasma-membrane (PM) localized boric acid
103 channel fused with green fluorescence protein (GFP-NIP5;1), we identified a novel allele of *UDP-D-glucose*
104 *4-epimerase 4* (line 20-2, Uehara et al. 2014). The epidermal cells in both the elongation zone and root hair zone,
105 but not meristem zone, of the mutant line 20-2 showed intracellular aggregates containing GFP-NIP5;1. The
106 aggregates were stained by FM4-64, a lipophilic styryl dye that stains vesicles in the endocytic pathway, and
107 contained the *trans*-Golgi network (TGN) markers YFP-RabA1e and YFP-VTI12 (Uehara et al. 2014). TGN

functions as a major sorting hub for the secretory and endocytic pathways, and is thus referred to as TGN/early endosome (EE) in plant cells (Viotti et al. 2010; Uemura et al. 2012). Importantly, the addition of D-galactose to the medium decreased endomembrane aggregates and rescued growth defects of *uge4* mutants. Pharmacological analysis using oryzalin and latruncurin B on GFP-NIP5;1 localization showed that disruption of microtubules and the actin cytoskeleton caused endomembrane disorganization, but the effects were distinct from those observed in the *uge4* mutants. Furthermore, analysis of several mutants with reduced accumulation of cell wall polysaccharides such as xyloglucan, arabinogalactan proteins (AGPs), and pectin, combined with the use of β -D-glucosyl Yariv reagent which binds to AGPs did not show endomembrane disorganization or abnormal intracellular aggregates (Uehara et al. 2014). These results suggested that the intracellular aggregates containing TGN/EEs were not caused by disruption of the cell wall structure or cytoskeleton.

In this study, to verify the nature of the aggregates in *uge4* mutants, we performed fluorescence imaging of various endosome markers and electron microscopy analysis of the elongating epidermal cells of the *uge4* mutant. Our results show the accumulation of abnormal vesicles derived from TGN/EEs in *uge4* mutant cells, and suggest the importance of UDP-D-galactose for the organization and function of TGN/EEs.

Material and Methods

Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh. ecotype Col-0 was used as the wild-type strain in this study and was from our laboratory stock. Transgenic *Arabidopsis* plants expressing BOR1-GFP (Takano et al. 2010), ER-GFP, Vac-GFP (Nelson et al. 2007), YFP-Got1 (Geldner et al. 2009), and GFP-VAMP727 (Ebine et al. 2008) were described previously. SALK_080766, a T-DNA insertion allele of *uge4*, was provided by the *Arabidopsis*

Biological Resource Center (ABRC, Alonso et al. 2003) and the homozygous line was established (Uehara et al. 2014). SALK_080766 expressing GFP-VAMP727 was selected from the F2 progeny of a cross between a GFP-VAMP727 line and SALK_080766. Other markers were introduced into the SALK_080766 line using the *Agrobacterium*-mediated *in planta* transformation method (Clough and Bent, 1998). Plants were grown on solid media (Takano et al. 2005) containing 1 % (w/v) sucrose, 1.5 % (w/v) gellan gum (Wako Pure Chemical Industries), and 10 μ M boric acid for 4-5 days in a growth chamber at 22 °C under a light/dark cycle of 16 h/8 h.

Laser scanning confocal microscopy

Laser scanning confocal microscopy was performed using a Leica TCS-SP8 equipped with HCX PL APO CS $\times$ 40 water immersion lens. Excitation and detection wavelengths were 488 nm and 500-530 nm for GFP, and 488 nm and 500–540 nm for YFP, 488 nm and 500-530 nm for Venus, and 488 nm and >640 nm for FM4-64, respectively. FM4-64 (Molecular Probes) was prepared as a 10 mM stock solution in water and used at 4 μ M. Plants grown for 4 days were treated with 10 μ M latrunculin B or 10 μ M oryzalin (Wako Pure Chemical Industries) in liquid medium for 2 days. The liquid medium was replaced every day. Control experiments were performed with 0.1 % DMSO. Image analysis was performed using the Image J software (Abràmoff et al. 2004). The fluorescence intensity profiles were quantified along a line drawn through the region of interest. For calculation of Pearson and Spearman correlation coefficients, a PSC colocalization plug-in (French et al. 2008) for ImageJ was used to quantify the linear Pearson's value (rp) and the nonlinear Spearman's value (rs) of GFP and FM4-64 fluorescent signals in selected regions. The values ranged from +1 (perfect positive correlation) to -1 (perfect negative correlation).

150

151 Electron microscopy

152 Root tips containing the elongation zone were obtained from 4-day-old plants of wild-type and *uge4* mutant
153 (SALK_080766). A high-pressure freezing system (Leica EM PACT2) was used to freeze samples, followed by
154 incubation with 2 % osmium tetroxide-acetone solution. Gradually thawed samples were washed with acetone at
155 room temperature and then suspended in Epon (Nisshin EM Corporation)-acetone solution series with 25, 50, 75
156 and 100 % Epon. Samples embedded in the Epon resin were polymerized at 60 °C for 48 h. Ultrathin sections
157 (120 nm) were cut using grass knives or a diamond knife (Sumi Knife 45°, Sumitomo Electric Industries) on an
158 ultramicrotome (Reichert-Nissei ULTRACUT N) and mounted on copper slot grids coated with Formvar film
159 (Nisshin EM Corporation). The cut sections were then stained with 2 % uranyl acetate and Reynold's lead
160 citrate and dried at 60 °C for 30 min. Electron micrographs were obtained with a transmission electron
161 microscope (JEM-2100, JEOL). To perform electron tomography, specimens prepared for transmission electron
162 microscopy were used and the side of the grid carrying the section was coated with Formvar. The grid was
163 coated with carbon on both sides using a Carbon Coater vacuum device (Nisshin EM Corporation). The
164 specimens were hydrophilized by an ion sputter (E101, Hitachi), after which 1 µl of 15-nm colloidal gold
165 solution was applied to each side and dried at 60 °C for 2 min. Electron tomography was performed using a
166 JEM-3200FS transmission electron microscope (JEOL), and the images were processed using the 3dmod
167 program of the IMOD package (Chanoca and Otegui 2014; Kremer et al. 1996).

168

169 Immunoelectron microscopy

High-pressure freeze substitution was performed for immunoelectron microscopy following the method of Chanoca and Otegui (2014) with slight modifications. The frozen samples were incubated in 0.2% glutaraldehyde with 0.2% uranyl acetate solution at -80°C for 3 days and warmed to -20°C for 20 h. Then, the samples were washed three times with acetone at -20°C followed by infiltration in a LR white resin-acetone solution series of 33, 66, and 100% with 24-h intervals. Polymerization was accelerated by adding LR white accelerator at -20°C under UV light for 72 h. Ultrathin sections (80 nm) were cut using a diamond knife (Sumi Knife 45°, Sumitomo Electric Industries) on an ultramicrotome (Reichert-Nissei ULTRACUT N). Sections were mounted on Formvar-coated nickel grids and blocked for 20 min with 5% (w/v) skim milk (Difco) in phosphate-buffered saline (PBS, pH 7.2) containing 0.1% Tween 20 and 0.1% NaN_3 . The samples were incubated in a blocking buffer containing 1:100 anti-ADP-ribosylation factor 1 (ARF1) antibody (Agrisera) overnight at 4°C . After rinsing three times for 1 min in PBS buffer containing 0.5% Tween 20, the sections were incubated in a blocking buffer containing 1:40 goat anti-rabbit IgG conjugated 10 nm gold (BBI Solutions) for 1.5 h. Then, the sections were washed in PBS buffer containing 0.5% Tween 20 twice and distilled water twice for 1 min each. The sections were stained with 2% uranyl acetate for 5 min and dried at 60°C for 30 min. Electron micrographs of the gold particles were obtained using a transmission electron microscope (JEM-2100, JEOL).

Results and Discussion

Intracellular aggregates in the *uge4* mutant contained endomembrane markers in the secretory and vacuolar pathways

In this study, to investigate the effects of the *uge4* mutation on endomembrane organization, we employed

several markers of the secretory and vacuolar pathways. We previously showed that the abnormal aggregates that accumulated in root epidermal cells of *uge4* mutant contained TGN/EEs markers and were stained by FM4-64, a tracer for endocytosis, as well as the ER-staining dye ER-Tracker Red (Uehara et al. 2014). To verify whether the ER membrane is contained in the aggregates, we analyzed the localization of an ER marker that contains the signal peptide of AtWAK2 (Wall-Associated Kinase 2) at the N-terminus of the GFP and the ER retention signal His-Asp-Glu-Leu at its C-terminus (Nelson et al. 2007). In the wild-type plants, the ER-GFP marker showed network-like structure and brightly fluorescent fusiform bodies (ER bodies) in the elongating epidermal cells (Fig. 1a, Fig. S1a) (Nelson et al. 2007). In an *uge4* mutant line (SALK_080766), ER-GFP was mainly observed in the bright fusiform bodies (ER bodies), as was the case in the wild type, and also in the aggregates stained by FM4-64 (Fig. 1b-d, Fig. S1b-d). To quantify the level of colocalization of ER-GFP and FM4-64 in the aggregates, we calculated the linear Pearson (r_p) and the nonlinear Spearman's (r_s) correlation (PSC) coefficients for the pixels in the selected region (Fig. 1e). These values ranged from +1 (perfect positive correlation) to -1 (perfect negative correlation) (French et al. 2008). The PSC coefficients of $r_p = 0.77$ and $r_s = 0.85$ confirmed that the ER marker is present in the aggregates stained by FM4-64. Furthermore, a plot profile analysis supported the partial colocalization of the ER marker and FM4-64 (Fig. 1e, f). These data suggested that the lack of UGE4 function affected the structure of the ER or trafficking of the ER marker. Although C-terminal HDEL signal effectively retains secretory proteins in ER, protein that escapes from the ER can be secreted or transported into the vacuole (Gomord et al. 1997; Petruccioli et al. 2006). The presence of ER marker signals in the intracellular aggregates may indicate proteins that escaped from ER and were trapped in the abnormal endosomes. Then, the localization of a Golgi marker YFP-Got1p homolog (Geldner et al. 2009) was investigated. Similar to the wild type, the marker in the *uge4* mutant showed punctate structures often

associated with the aggregates labeled by FM4-64 (Fig. 1g-k, Fig. S1e-h). The PSC coefficients of $r_p = 0.29$ and $r_s = 0.45$ and the plot profile of GFP and FM4-64 signal intensity in the selected region containing the aggregates supported the association or partial colocalization (Fig. 1k, l). These results suggest that the structure of the Golgi apparatus was not largely affected, while the localization was slightly affected.

We then investigated the localization of markers in the vacuolar pathway in the *uge4* mutant. VAMP727 is a component of SNARE complex located on the multi-vesicular bodies/late endosomes (MVB/LEs) (Uemura and Ueda. 2014). Compared to wild-type cells (Fig. 2a, Fig. S1i), the number of punctate structures labeled by GFP-VAMP727 decreased in the epidermal cells as the intracellular aggregates appeared (Fig. 2b, Fig. S1j), and GFP-VAMP727 was accumulated in these aggregates together with FM4-64 (Fig. 2b-d, Fig. S1j-l). The PSC coefficients of $r_p = 0.97$ and $r_s = 0.97$ in the selected region containing aggregates (Fig. 2e) strongly supported the colocalization of GFP-VAMP727 and FM4-64. Similar plot profile patterns of GFP and FM4-64 also supported the strong colocalization (Fig. 2e,f). These results suggested that either MVB/LEs accumulated in the aggregates or GFP-VAMP727 miss-localized to the abnormal endosomes. We then observed a marker for vacuolar membrane, γ -TIP-GFP (vac-GFP) (Nelson et al. 2007) in the *uge4* mutant. The shape of the vacuole was apparently normal in the cells with the aggregates, but was not yet swollen (Fig. 2g, h, Fig. S1 m, n) (Uehara et al. 2014). The aggregates stained by FM4-64 contained weak GFP signals and were surrounded by stronger GFP signal (Fig. 2h-l, Fig. S1n-p). The PSC coefficients in the selected regions (Fig. 2k) were r_p value = -0.28 and r_s value = -0.27. The normal localization of γ -TIP-GFP on the vacuolar membrane and the abnormal accumulation in the aggregates were probably dependent on the timing of protein synthesis. The γ -TIP-GFP present on the vacuolar membrane was thought to represent those synthesized during the younger cell stages. As trafficking of γ -TIP/TIP1;1 to the vacuolar membrane is dependent on Golgi and TGN (Rivera-Serrano et al.

2012), the newly synthesized γ -TIP-GFP was likely trapped in the abnormal endosomes during trafficking to the vacuolar membrane.

As a PM marker that traffics through secretory and vacuolar pathways, a borate transporter BOR1 fused with GFP (BOR1-GFP) (Takano et al. 2010) was expressed in the *uge4* mutant. Compared with the exclusive PM localization of BOR1-GFP in wild-type plants under low boron conditions (Fig. 3a, Fig. S1q), BOR1-GFP was also accumulated in intracellular aggregates stained with FM4-64 in the *uge4* mutant (Fig. 3b-e, Fig. S1r-t), which is similar to the case of GFP-NIP5;1 (Uehara et al. 2014). The PSC coefficients of r_p value = 0.79 and r_s value = 0.83 and plot profiles of GFP and FM4-64 signal intensity (Fig. 3e, f) supported the colocalization of BOR1-GFP and FM4-64 in the aggregates. This may be due to accumulation of BOR1-GFP in the endomembranes of the secretory pathway or vacuolar pathway. We then performed a time-course analysis of BOR1-GFP trafficking from the PM to MVB/LE after high-boron supply (Takano et al. 2005, 2010). The BOR1-GFP signal decreased gradually from the PM of the wild-type and *uge4* cells without aggregate and appeared in punctate structures within 40 min (Fig. 3g-k), which was previously identified as MVB/LEs (Takano et al. 2010). In contrast, the BOR1-GFP signal less frequently appeared in punctate structures in the *uge4* mutant cells containing aggregates (Fig. 3l-p). These results suggest that either the endocytic pathway from the PM to the MVB/LE is defective or the MVB/LEs accumulated in the aggregates in the *uge4* mutant.

Disruption of the cytoskeleton is not a primary cause of the endomembrane aggregates in the *uge4* mutant

It has been shown that microtubules in the swollen cells were disordered or absent entirely in the *uge4* mutants (Andeme-Onzighi et al. 2002). Pharmacological analysis using latruncurin B and oryzalin showed that

disruption of actin and microtubule cytoskeleton, respectively, caused accumulation of GFP-NIP5;1 in spherical structures (Uehara et al. 2014). This indicated that the cytoskeletal disruption causes endomembrane disorganization, but the effect was distinct from that in the *uge4* mutants. To further compare the consequence of cytoskeletal disruption and lack of UDP-D-galactose synthesis in the *uge4* mutant, we investigated the effects of latrunculin B and oryzalin on localization of protein markers in the secretory and vacuolar pathways. Compared with the control (Figs. 4a and 5a), 48 hr treatment with 10 μ M latrunculin B or oryzalin significantly disrupted the organization of actin and microtubules, respectively, in the root epidermal cells (Figs. 4b and 5b). Under this condition, the large intracellular aggregates typical in the *uge4* mutant were not visualized by FM4-64 or any marker used in this study, although significant effects were observed in the localization of some markers (Figs. 4c-n and 5c-n). The Golgi marker YFP-Got1p homolog and the TGN marker YFP-VTI12 showed larger structures after latrunculin B treatment (Fig. 4f, h), and BOR1-GFP showed punctate structures in the cells after latrunculin B and oryzalin treatment (Figs. 4n and 5n). These effects can be attributed to the requirement of actin and microtubules for movement of organelle and organization of the endomembranes (Tamura et al. 2005; Brandizzi and Wasteneys 2013). These data suggest that cytoskeletal disruption is not the primary cause of intracellular aggregates in root epidermal cells of the *uge4* mutant.

Ultrastructure analysis of the aggregates in the *uge4* mutant revealed the presence of high-electron-density vesicles derived from TGN/EEs

To further address the nature of the aggregates in the *uge4* mutants, we analyzed the ultrastructure using transmission electron microscopy. The structures including ER, Golgi stacks, TGN, MVB, vacuole, and mitochondria in elongating epidermal cells of wild-type plants are shown in Fig. 6a-c. In the *uge4* cells, the

structures of ER, Golgi, MVB, and vacuole were not apparently different from those of the wild-type cells (Fig. 6f, g). However, we noticed that the cytosol contained many large vesicles typically 150 to 250 nm in diameter and filled with high-electron-density materials (Fig. 6d, indicated by white triangles). The high-electron-density vesicles (HDVs) were observed in epidermal cells, but not in cortical cells of the mutant roots (Fig. 6d). We assume that the HDVs represent abnormally enlarged TGNs because (i) the mean size was greater than that of TGNs (diameter, 60–100 nm), (ii) they are closely associated with TGNs and some were interconnected (Fig. 6g), similar to the characteristics of TGN (Fig. 6b, c) (Otegui et al. 2006). We also found ER tubules in the region of HDVs accumulation (Fig. 6g, h) and Golgi around the region of HDVs (Fig. 6h). These results are consistent with the localization of the fluorescent ER marker in the aggregates (Fig. 1b-e) and the Golgi marker associated with the aggregates (Fig. 1h-k). We then investigated the spatial structure of HDVs in more detail using electron tomography. The three-dimensional analysis clearly illustrated that HDVs were interconnected with each other and occasionally also to TGNs (Fig. 7 and Supplemental movie). This observation strongly supports the assumption that the HDVs represent abnormally enlarged TGNs.

To further characterize the nature of the HDVs, we performed immunogold analysis of the ARF1 protein, which is localized to the Golgi and the TGN (Stierhof and Kasmi 2010; Robinson et al. 2011). The ARF1 antibody specifically labeled the Golgi apparatus and TGN/EEs in wild-type cells (Fig. 8a). The Golgi, the TGN/EEs, and the large vesicles were labeled in the *uge4* mutant cells (Fig. 8b). The large vesicles were observed only in *uge4* cells and thus most likely corresponded to the HDVs, although electron-dense materials were hardly observed without use of osmium acetate for the structural analysis (Fig. 6). These findings further suggest that the HDVs in the *uge4* mutant cells represent abnormally enlarged TGNs.

As the MVB/LE structures were not affected in *uge4* mutant cells, accumulation of FM4-64, the MVB/LEs marker GFP-VAMP727, the vacuolar membrane marker γ -TIP-GFP and BOR1-GFP in the aggregates (Figs. 2b-e, h-k, 3b-e, l-p, Fig. S1j-l, n-p, r-t) would probably have been trapped in the abnormal TGN/EEs before being transported to MVB/LEs and the vacuole.

Previously, accumulation of such HDVs was observed in an electron micrograph of brefeldin A (BFA)-treated elongating epidermal cells of Arabidopsis roots (Grebe et al. 2003). BFA is an inhibitor of a subset of guanine-nucleotide exchange factors for ADP-ribosylation factors (ARF-GEFs) and induces agglomeration of TGN/EEs in Arabidopsis roots (Geldner et al. 2003; Dettmer et al. 2006). These results suggest that the defect in membrane trafficking causes accumulation of HDVs derived from TGN/EEs in the elongating epidermal cells of Arabidopsis roots. The lack of UDP-D-galactose in *uge4* cells may cause insufficient glycosylation of some membrane glycoproteins in Golgi cisternae and TGN, which have essential functions during membrane trafficking from TGNs. Alternatively, insufficient glycosylation of some cargo proteins could cause aggregation of protein in the TGNs and affect proper TGN function.

Conclusions

Our analysis showed that the intracellular aggregates in elongating epidermal cells of the *uge4* mutant were mainly composed of abnormal TGN/EEs, and at a lower frequency ER tubules, and were associated with the Golgi apparatus. The abnormal TGN/EEs exhibited larger sizes and spatial interconnections, and contain high-electron-density materials, likely to be proteins, membrane components, and cell wall polysaccharides. The disorganization of TGN/EEs is not likely a consequence of the defect of cytoskeleton in the *uge4* cells. Although the mechanism requires further elucidation, our analysis established the requirement for

UDP-D-galactose synthesis by UGE4 for the organization and function of TGN/EEs.

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Figure legends

Fig. 1 Localization of an endoplasmic reticulum marker and Golgi marker in root epidermal cells of the *uge4* mutant. ER-GFP (**a-f**) and YFP-Got1p homolog (**g-l**) in the wild-type (**a, g**) and *uge4* mutant (**b-e, h-k**) cells were imaged. Plant roots were stained with FM4-64 for 30-40 min (**c, i**). Merged GFP and FM4-64 images are shown (**d, e, j, k**). White rectangles in **d** and **j** are five-fold enlargements (**e** and **k**) and were used for the correlation coefficient analysis (PSC coefficients shown in text). Lines in **e** and **k** were used for the plot profile analysis (**f** and **l**). Scale bars = 20 μ m. Enlarged images in a-d and g-j are shown in Fig. S1 a-d and e-h, respectively.

Fig. 2 Localization of the MVB/LEs marker and the vacuolar membrane marker in *uge4* mutant root epidermal cells. GFP-VAMP727 (**a-f**) and γ -TIP-GFP (**g-l**) in wild-type (**a, g**) and *uge4* mutant cells (**b-e, h-k**) were imaged. Plant roots were stained with FM4-64 for 30-40 min (**c, i**). Merged images of GFP and FM4-64 are shown (**d, e, j, k**). White rectangles in **d** and **j** are five-fold enlargements (**e** and **k**) and used for correlation coefficient analysis (PSC coefficients shown in text). Lines in **e** and **k** are used for plot profile analysis (**f** and **l**). Scale bars = 20 μ m. Enlarged images in **a-d** and **g-j** are shown in Fig. S1 **i-l** and **m-p**, respectively.

Fig. 3 Localization and vacuolar trafficking of BOR1-GFP in root epidermal cells of the *uge4* mutant. BOR1-GFP localization under low-boron (3 μ M B) conditions in the wild-type (**a**) and *uge4* mutant (**b-e**). Intracellular aggregates containing BOR1-GFP were observed in the *uge4* mutant (**b-d**). Plant roots were stained with FM4-64 for 30-40 min (**c-e**). White rectangles in **d** are fivefold enlargements (**e**) and used for correlation coefficient analysis (PSC coefficients shown in text). A line in **e** is used for plot profile analysis (**f**). Trafficking of BOR1-GFP after shifting from a low B condition (0.6 μ M) to a high B condition (500 μ M) in the wild-type (**g-k**) and *uge4* mutant (**l-p**). Asterisks indicate cells with aggregates. Scale bars = 20 μ m. Enlarged images in **a-d** are shown in Fig. S1 **q-t**.

Fig. 4 Effects of disruption of actin polymerization on the localization of endomembrane markers. Endomembrane markers in the wild-type background were imaged after treatment with 10 μ M of latrunculin B. DMSO (0.1 %) was used as a control. FM 4-64 was employed to label intracellular aggregates. Lifeact-Venus (**a, b**), ER-GFP (**c, d**), YFP-Got1p homolog (**e, f**), YFP-VTI12 (**g, h**), GFP-VAMP727 (**i, j**), γ -TIP-GFP (**k, l**), and BOR1-GFP (**m, n**) were used. Scale bar = 20 μ m.

448

449 **Fig. 5** Effects of disrupting microtubule polymerization on localization of endomembrane markers.

450 Endomembrane markers in the wild-type background were imaged after treatment with 10 μ M oryzalin. DMSO
451 (0.1%) was used as a control. FM 4-64 was employed to label intracellular aggregates. GFP-TUB (**a, b**),
452 ER-GFP (**c, d**), YFP-Got1p homolog (**e, f**), YFP-VTI12 (**g, h**), GFP-VAMP727 (**i, j**), γ -TIP-GFP (**k, l**) and
453 BOR1-GFP (**m, n**) were used. Scale bar = 20 μ m.

454

455 **Fig. 6** Ultrastructure analysis of elongating epidermal cells of the *uge4* mutant. Electron micrographs of
456 elongating epidermal cells of the wild-type (**a-c**) and the *uge4* mutant roots (**d-h**). The cells of the *uge4* mutant
457 develop abnormal high-electron-density vesicles (HDVs) in the cytoplasm (d and e, white arrowhead in **d**).
458 HDVs are closely located to TGN/EEs (**g, h**). An enlarged image of the region indicated by a rectangle (e) is
459 shown in (h). G: Golgi; TGN: *trans*-Golgi network; ER: endoplasmic reticulum; MT: mitochondrion; MVB:
460 multivesicle body; Vac: vacuole; HDVs: high-electron-density vesicles; co: cortex cells; ep: epidermal cells.

461

462 **Fig. 7** Electron tomography analysis of high-electron-density vesicles in root epidermal cells of the *uge4* mutant.

463 An ultrathin section (~300 nm thickness) was imaged at 10,000 $\times$ from +60.0 $^\circ$ to -60.0 $^\circ$ at 1.0 $^\circ$ intervals. A

464 3D-model processed by IMOD software (**a**) and a tomographic slice (**b**) were selected to represent the spatial

465 interconnection of the high-density vesicles (HDVs). Sequential images are shown in a Supplemental movie. G:

466 Golgi; TGN: *trans*-Golgi network; MT: mitochondrion; HDVs: high-electron-density vesicles.

467

468 Figure. 8 Immuno-electron microscopy of the ARF1 protein in *uge4* mutant root epidermal cells. An anti-ARF1
469 antibody and 10-nm gold-conjugated secondary antibody were used for the immunogold analysis. The Golgi
470 stacks and the TGNs in wild-type cells were labeled with 10-nm gold particles (**a**). Besides the Golgi and the
471 TGN, large vesicles were also labeled in the *uge4* mutant (**b**). T: *trans*-Golgi network; G: Golgi; L: large
472 vesicles.

473

474 Figure S1 Localization of various markers in root epidermal cells of the *uge4* mutant. ER-GFP in the wild-type
475 (**a**) and *uge4* mutant (**b-d**), YFP-Got1p homolog in the wild-type (**e**) and *uge4* mutant (**f-h**), GFP-VAMP727 in
476 the wild-type (**i**) and *uge4* mutant (**j-l**), γ -TIP-GFP in the wild-type (**m**) and *uge4* mutant (**n-p**), and BOR1-GFP
477 in the wild-type (**q**) and *uge4* mutant (**r-t**). Plant roots were stained with FM4-64 for 30-40 min prior to image
478 with confocal microscopy.

479

480 Supplemental movie Electron tomography analysis of high-electron-density vesicles in root epidermal cells of
481 the *uge4* mutant. An ultrathin section (~300 nm thickness) was imaged at 10,000 $\times$ from +60.0 $^{\circ}$ to -60.0 $^{\circ}$ at 1.0 $^{\circ}$
482 intervals.

Latrunculin B

a

G

T

T

500 nm

b

L

G

T

500 nm

Figure S1 Localization of various markers in root epidermal cells of the *uge4* mutant. ER-GFP in the wild-type (a) and *uge4* mutant (b-d), YFP-Got1p homolog in the wild-type (e) and *uge4* mutant (f-h), GFP-VAMP727 in the wild-type (i) and *uge4* mutant (j-l), γ -TIP-GFP in the wild-type (m) and *uge4* mutant (n-p), and BOR1-GFP in the wild-type (q) and *uge4* mutant (r-t). Plant roots were stained with FM4-64 for 30-40 min prior to image with confocal microscopy.