Identification and Characterization of an Arabidopsis Mutant with Altered Localization of NIP5;1, a Plasma Membrane Boric Acid Channel, Reveals the Requirement for D-Galactose in Endomembrane Organization

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Endomembrane organization is important for various aspects of cell physiology, including membrane protein trafficking. To explore the molecular mechanisms regulating the trafficking of plasma membrane-localized proteins in plants, we screened for Arabidopsis mutants with defective localization of green fluorescent protein (GFP)–nodulin 26-like intrinsic protein (NIP)5;1. Fluorescence imaging-based screening led to the isolation of a mutant which accumulated abnormal intracellular aggregates labeled by GFP–NIP5;1. The aggregates appeared in epidermal cells in the root elongation zone and included the trans-Golgi network/early endosomes. Rough mapping and whole-genome sequencing identified the mutant as an allele of UDP-glucose 4-epimerase 4 (uge4)/root hair defective 1 (rhd1)/root epidermal bulgar 1 (reb 1), which was originally defined as a cell wall mutant. The responsible gene encodes UDP-glucose 4-epimerase 4 (UGE4), which functions in the biosynthesis of D-galactose, especially for the synthesis of the cell wall polysaccharide xyloglucan and arabinogalactan proteins (AGPs). The endomembrane aggregates in the mutants were absent in the presence of D-galactose, indicative of a requirement for a D-galactose-containing component in endomembrane organization. Genetic and pharmacological analyses suggested that the aggregates were not caused by the disruption of cell wall polysaccharides or the cytoskeleton. Overall, our results suggest that UGE4 activity in D-galactose synthesis is required for the structure of cell wall polysaccharides and endomembrane organization.

Keywords: Arabidopsis thaliana • Cell wall, endomembrane, D-galactose, UDP-D-glucose-4-epimerase.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AGP, arabinogalactan protein; B, boron; DIC, differential interference contrast; DMSO, dimethylsulfoxide; EMS, ethylmethane sulfonate; ER, endoplasmic reticulum; GFP, green fluorescent protein; KAM, katamari; MVB, multivesicular body; NIP, nodulin 26-like intrinsic protein; PM, plasma membrane; REB, root epidermal bulgar; RG, rhamnogalacturan; RHD, root hair defective; SNP, single nucleotide polymorphism; SSLP, simple sequence length polymorphism; TGN, trans-Golgi network; UGE, UDP-D-glucose-4-epimerase; UTR, untranslated region; WT, wild type; XXT, xylosyltransferase; YFP, yellow fluorescent protein.

Introduction

Membrane trafficking between endomembranes and the plasma membrane (PM) plays an important role in plant cells. Membrane proteins destined for the PM are inserted into the endoplasmic reticulum (ER) membrane, transported through Golgi stacks and the trans-Golgi network (TGN), and are then targeted to the PM. Increasing numbers of PM-localized proteins show polar localization in specific PM domains, possibly through different secretion and recycling pathways (Park and Jürgens 2012, Löfke et al. 2012). PM-localized proteins are selectively or non-selectively transported to early endosomes by endocytosis. It is now accepted that the TGN functions as an early endosome in plant cells, and thus serves as a major sorting hub for the secretory and endocytic pathways (Viotti et al. 2010, Uemura et al. 2012). From the TGN/early endosomes, membrane proteins can be recycled back to the PM or proceed to multivesicular bodies (MVBs)/late endosomes. At the limiting membrane of MVBs, the membrane proteins destined for degradation are transported to luminal vesicles (Spitzer et al. 2009, Viotti et al.
MVBS fuse with vacuoles and release their luminal vesicles into the lumen of a lytic vacuole, where the proteins are degraded by proteases (Scheuring et al. 2011).

The structural organization of the endomembrane is important for proper membrane trafficking and is fundamental for plant physiology. Previously, a fluorescence imaging-based screen using a transgenic Arabidopsis thaliana line expressing vacuole-targeted green fluorescent protein (GFP) identified the katamari (kam) mutants, which contain endomembrane aggregates in their cells (Tamura et al. 2005, Tamura et al. 2007). The interaction between KAM1, a Golgi membrane protein, and actin filaments was shown to be important for proper endomembrane organization. Another screen using a polar marker ProPIN1:PIN1–GFP identified the protein-affected trafficking2 and 4 (pat2 and pat4) mutants, which showed strong intracellular accumulation of PIN1–GFP (Feraru et al. 2010, Zwiewka et al. 2011). In these mutants, the morphology and function of lytic and protein storage vacuoles are defective and membrane proteins accumulate in the aberrant vacuolar structures. Map-based cloning identified PAT2 and PAT4 as putative β and δ subunits of the adaptor protein-3 complex, and suggested that the plant adaptor protein-3 complex plays a role in mediating lytic vacuole performance and in the transition of storage into lytic vacuoles. These studies indicate that the fluorescence imaging-based screening of Arabidopsis mutants can be used to identify important components of endomembrane organization.

In this study, to explore the mechanisms regulating membrane trafficking and endomembrane organization in plant cells, we screened Arabidopsis mutants with a defect in the localization of GFP–nodulein 26-like intrinsic protein (NIP)5;1 using fluorescence microscopy. NIP5;1 is a PM-localized boric acid channel belonging to the major intrinsic protein (aquaporin) family (Takano et al. 2006). NIP5;1 plays a role in boric acid uptake and is required for the normal growth of Arabidopsis plants under low boron (B) conditions (Takano et al. 2006). NIP5;1 is localized to the PM of root cap cells and root epidermal cells with polarity towards the outer (soil-facing) side of the cells (Takano et al. 2010). The polarity of NIP5;1 is thought to be associated with the limited lateral diffusion in the PM, but the mechanisms for polar trafficking and retention remain unclear. Here, we report the isolation of a mutant that accumulated endomembrane aggregates labeled by GFP–NIP5;1. We identified the mutant as a novel allele of UDP-α-glucose 4-epimerase 4 (uge4)/root hair defective 1 (rh1)/root epidermal bulgar 1 (reb1), which were isolated as mutants of root hair development, root morphology, root susceptibility to nematodes (Schiefelbein and Somerville 1990, Baskin et al. 1992, Baum et al. 2000). UGE4 activity in α-galactose synthesis is required for the structure of cell wall polysaccharides (Ding and Zhu 1997, Andème-Onzighi et al. 2002, Seifert et al. 2002, Nguema-Ona et al. 2006, Rösti et al. 2007). In this study, we demonstrate that UGE4 is also required for endomembrane organization, which is important for the proper localization of membrane proteins.

Results

Screening for mutants with altered localization of GFP–NIP5;1 identified mutants with intracellular aggregates

To identify novel factors involved in regulating the intracellular localization of NIP5;1, we performed fluorescence imaging-based screening to obtain mutants in which NIP5;1 was localized abnormally in cells. For this purpose, we used transgenic Arabidopsis harboring ProNIP5;1 (35′UTR):GFP–NIP5;1 as a transgene in the nip5;1-1 mutant. In the construct, we used the NIP5;1 promoter lacking the 3′-untranslated region (UTR) of NIP5;1, which is responsible for the B-dependent degradation of NIP5;1 mRNA, to obtain relatively strong expression of GFP–NIP5;1 irrespective of the B level (Tanaka et al. 2011). In the transgenic plants, GFP–NIP5;1 localized to the PM in root epidermal cells (Fig. 1A–C; Takano et al. 2010). For screening, approximately 25,000 GFP–NIP5;1 seeds were mutagenized with ethylmethane sulfonate (EMS), and 20 pools of M2 seeds were obtained. We screened approximately 40,000 M2 seeds from the 20 pools under fluorescence microscopes and isolated 17 mutant lines in which GFP–NIP5;1 showed aberrant localization. Among them, three mutants showed localization of GFP–NIP5;1 in intracellular aggregates. In this study, we focused on Line 20-2, in which GFP–NIP5;1 was often localized to abnormal intracellular aggregates in epidermal cells in the root elongation zone (Fig. 1A). These aggregates were also observed in epidermal cells in the root hair zone, but not in the root meristem zone (Supplementary Fig. S1A). In the mutant elongation zone, bulging of the epidermal cells started to appear, leading to swelling of the cells in the root hair zone (Fig. 1A). A cross-section of the elongation zone showed that the aggregates accumulated in trichoblasts (root hair-forming cells), which are in contact with two cortical cells, and in atrichoblasts, which are in contact with only one cortical cell (Fig. 1C). The polar localization of GFP–NIP5;1 in the plasma membrane was not affected in epidermal cells containing aggregates (Fig. 1D).

Since intracellular trafficking pathways could have been disturbed, we stained the root cells with a lipophilic styryl dye (FM4-64) to examine the endocytic pathway. In wild-type (WT) epidermal cells, FM4-64 stained dotted endosomes within 30 min (Fig. 1D). However, in the epidermal cells of Line 20-2, FM4-64 stained aggregates containing GFP–NIP5;1 (Fig. 1D). These results suggest that the intracellular structure labeled with GFP–NIP5;1 contained membranes in the endocytic pathway. The use of FM4-64 also revealed that the aggregates were absent from leaf epidermal cells (Supplementary Fig. S1B).

Rough mapping and genome sequencing identified Line 20-2 as a novel allele of the uge4 mutant

To identify the gene responsible for Line 20-2, we performed genetic mapping and whole-genome sequencing. We
outcrossed the mutant (Col-0 background) with Ler plants and analyzed the phenotypes of F2 plants. Among 117 F2 plants, 28 contained aggregates with GFP–NIP5;1, indicating that the phenotype was caused by a single recessive mutation (P = 0.79 by a χ² test). Genotyping using the 28 F2 plants with 11 simple sequence length polymorphism (SSLP) markers roughly mapped the mutation to a region between 13.8 and 27 Mb on chromosome 1 (Fig. 2A; Supplementary Table S1).

We performed whole-genome sequencing of Line 20-2 on the SOLiD platform (Applied Biosystems). Sequence libraries were constructed from Line 20-2 and another three mutants obtained from the same screen. The read sequences were mapped to the A. thaliana Col-0 genome. Nine nucleotide changes were found in the exons of the genetically mapped region (between 13.8 and 27 Mb on chromosome 1) after subtracting shared single nucleotide polymorphisms (SNPs) among

Fig. 1 GFP–NIP5;1 in Line 20-2 accumulated in abnormal intracellular aggregates. (A, B) GFP–NIP5;1 in the roots of WT plants and Line 20-2. The root meristem and elongation zones (A), elongation zone (B) and cross-sections of the elongation zone (C) are shown. (D) Polar localization of GFP–NIP5;1 in WT plants and Line 20-2. GFP–NIP5;1 showed polar localization toward the soil side in the PM in comparison with FM4-64, which stained the PM in a non-polar manner. Plants were grown on solid medium containing 30 μM boric acid for 7–10 d. Roots were incubated with 4 μM FM4-64 for 30 min (D). The asterisk indicates a trichoblast. Scale bars = 50 μm (A), 10 μm (B, D) and 20 μm (C).
the four mutant lines, which could be originally present in the parental transgenic line. Of the nine mutations, seven were G/C to A/T transitions, which are known to arise from the EMS-mediated alkylation of guanine and adenine. Annotation of these seven EMS-induced mutations indicated that four were non-synonymous, one was nonsense and the rest were silent (Supplementary Table S2). The one nonsense mutation was in the gene AT1G64780 while the non-synonymous mutations were found in AT1G56570, AT1G64440, AT1G72950 and AT1G73160 (Fig. 2A).

In the candidate genes for the causative mutations, we identified UGE4/RHD1/REB1 (At1g64440), whose loss-of-function mutants showed similar root bulging phenotypes to Line 20-2 (Schiefelbein and Somerville 1990, Baskin et al. 1992). Line 20-2 had a single mutation (G1365A) in UGE4/RHD1/REB1, which resulted in the replacement of Gly223 (GGA) with arginine (AGA) (Fig. 2A; Supplementary Table S2). To determine whether Line 20-2 is an allele of the uge4 mutant, we first stained roots of rhd1-1 and SALK_080766, a T-DNA insertion allele of uge4 (Fig. 2A), with FM4-64 (Fig. 2B). In both alleles, FM4-64 stained aggregates in elongating epidermal cells, as in Line 20-2 (Fig. 1D). F1 plants between Line 20-2 and rhd1-1 or SALK_080766 were then produced and their roots were stained with FM4-64. The roots of the F1 plants contained aggregates stained by FM4-64 and contained GFP–NIP5;1 (Fig. 2C). These results indicated that Line 20-2 is allelic to the uge4 mutant.

Abnormal intracellular aggregates contain TGN/early endosomes

Since the GFP–NIP5;1-labeled aggregates were stained with FM4-64, an endocytic tracer, we assumed that the structure contained endomembranes along the endocytic pathway. As expected, significant amounts of two TGN/early endosome markers, yellow fluorescent protein (YFP)–VTI12 and YFP–RabA1e (Geldner et al. 2009), were localized to the aggregates using FM4-64 in the uge4 (SALK_080766) mutant (Fig. 3A).
We then stained the ER using ER-Tracker Red (Fig. 3B). In the cells with GFP–NIP5;1-labeled aggregates, ER-Tracker Red stained network structures, including the aggregates, suggesting that part of the ER membrane was included in the aggregates. Differential interference contrast (DIC) images showed apparently normal vacuolar structures in the cells containing aggregates (Fig. 1B). These observations suggested that some (but not all) of the endomembrane accumulated in the aggregates.

We then performed a time-course analysis of FM4-64 uptake to investigate the endocytic pathway from the PM to vacuolar membranes in uge4 mutants. In both the WT and the uge4 mutant, FM4-64 was observed as dotted structures after staining for 5 min, suggesting that endocytosis of FM4-64 from the PM was not impaired in the mutant (Fig. 4A). However, FM4-64 showed stained aggregates after 15 min, and the intensity of FM4-64 in the aggregates increased after 30 min in the uge4 mutant (Fig. 4A). After 160 min, FM4-64 clearly stained the vacuolar membrane in WT cells; however, the staining was less intense in the uge4 mutant (Fig. 4B). These results suggested that the uge4 mutant had a defect in the later endocytic pathway from TGN/early endosomes to vacuoles.

**Intracellular aggregates were absent after D-galactose and ethylene precursor treatment in uge4**

UGE4 encodes an isoform of UDP-D-glucose-4-epimerase, which interconverts UDP-D-glucose and UDP-D-galactose and is involved in D-galactose synthesis (Seifert et al. 2002). In uge4, the D-galactose content is decreased and the bulging of epidermal cells is rescued by the addition of D-galactose to the growth medium (Seifert et al. 2002). Thus, we grew our mutant in the

**Fig. 3** The intracellular aggregates include the TGN. (A) TGN markers, YFP–RabA1e (Wave34Y) and YFP–VTI12 (Wave13Y), in the epidermal cells of WT and SALK_080766 plants. Plant roots were stained with FM4-64 for 30–40 min. (B) ER-Tracker Red staining of epidermal cells from Line 20-2. Roots were incubated with 2 μM ER-Tracker Red for 1 min, washed with water and then observed. Scale bars = 10 μm.

**Fig. 4** The later endocytic pathway from the TGN/early endosome to the vacuole is affected in the uge4 mutant. (A, B) Time-course analysis of FM4-64 internalization in WT plants and Line 20-2 for 5, 15, 30 (A) and 160 min (B). Roots were stained with 4 μM FM4-64, washed with water and then observed after the indicated times. Scale bars = 10 μm.
presence of 10 mM D-galactose and found that D-galactose rescued the bulging and decreased endomembrane aggregation in epidermal cells (Fig. 5). This result indicated that D-galactose synthesized by UGE4 is required for endomembrane organization in epidermal cells of the root elongation zone. We next explored whether D-galactose decreased the number of existing aggregates. Based on our time-course analysis, 10 mM D-galactose did not affect existing aggregates within 110 min in the elongation zone (Supplementary Fig. S2A). After 19 h in the presence of D-galactose, aggregates were still observed in the epidermal cells in the root hair zone, but not in the elongation zone (Supplementary Fig. S2A). These results indicated that the external addition of D-galactose decreased the appearance of aggregates, but did not affect existing aggregates.

Previous reports have shown that root epidermal bulging in rhd1 is suppressed by ethylene or its precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (Seifert et al. 2004). The authors proposed that ethylene signaling channels UDP-D-galactose to specific UDP-D-galactose-requiring molecules. Therefore, we investigated whether ACC also suppressed aggregate formation. In epidermal cells after 5 μM ACC treatment for 2 d, the bulging and aggregates were not observed in Line 20-2 (Supplementary Fig. S3). This result suggested that ethylene increased the transfer of UDP-D-galactose synthesized by alternative UGE isoforms to a D-galactose-containing molecule required for endomembrane organization.

**Intracellular aggregates in uge4 mutants differ from the structures caused by cytoskeleton disruption**

Cortical microtubules are either disordered or absent in the enlarged trichoblasts of uge4 plants (Andème-Onzighi et al. 2002). To investigate the involvement of the cytoskeleton in endomembrane aggregation, we performed pharmacological inhibition of the assembly of actin and microtubules using latrunculin B and oryzalin, respectively, in transgenic WT plants expressing GFP–NIP5;1 (Fig. 6). Interestingly, both treatments promoted the accumulation of GFP–NIP5;1 in spherical structures that differed from the aggregates observed in the uge4 mutants (Fig. 6A–E, compared with Fig. 1B, D). For microtubule disruption, bulging was observed in epidermal cells of the elongation zone (Fig. 6C). These results suggest that cytoskeletal disruption causes endomembrane disorganization and affects the trafficking of GFP–NIP5;1, but that the effect differs in uge4 mutants.

**Genetic and pharmacological disturbances of cell wall polysaccharides did not induce endomembrane aggregates**

D-Galactose is a component of glycoproteins, glycolipids and cell wall polysaccharides in plant cells. Previous reports indicated that uge4 mutants contained reduced amounts of D-galactose, xyloglucan and arabinogalactan proteins (AGPs), while pectin was not affected (Ding and Zhu 1997, Andème-Onzighi et al. 2002, Seifert et al. 2002, Nguema-Ona et al. 2006). To explore whether the reduced amount of xyloglucan or AGPs causes endomembrane aggregation, we analyzed Arabidopsis mutants with a low AGP or xyloglucan content. In the roots of the mur1 mutants, the L-fucose content was reduced by 40% (Reiter et al. 1993), the content of fucosylated xyloglucan reactive to the monoclonal antibody CCRC-M1 was significantly reduced (Freshour et al. 2003) and fucosylated AGPs were decreased by 40% (van Hengel and Roberts 2002). The mur1 mutation decreased root cell elongation by approximately 50% and caused swelling at the base of the root hairs.

![Image](https://academic.oup.com/pcp/article-abstract/55/4/704/1813437)

Fig. 5 The intracellular aggregates in Line 20-2 were absent in the presence of D-galactose. GFP–NIP5;1 in root elongation zones of WT plants and Line 20-2 grown on solid medium containing 0 or 10 mM D-galactose (+D-gal) for 5 d. Scale bars = 20 μm.
In both mur1-1 and mur1-2 mutant plants, FM4-64 stained the PM and dotted endosomes, similar to the WT (Fig. 7A). This clearly differed from uge4 (SALK_080766), in which the aggregates were stained. These results suggest that the defect in root elongation in the mur1 mutants was not linked to endomembrane disorganization. We then used the mur2 mutant, which contained <2% of the WT level of fucosylated xyloglucan in its cell wall (although the growth and wall strength were normal) (Vanzin et al. 2002), and the xylosyltransferase 1 (xxt1) /xxt2 mutant, which contained no detectable xyloglucan and showed aberrant root hairs (Cavalier et al. 2008, Park and Cosgrove 2012). FM4-64 stained the PM and dotted endosomes in these mutants, suggesting that xyloglucan is not required for endomembrane organization (Fig. 7A). To increase our understanding of AGPs, we treated plant roots with b-D-glucosyl Yariv reagent, a synthetic phenylglycoside that specifically binds AGPs (Yariv et al. 1967, Nguema-Ona et al. 2012). As reported, treatment with 30 mM Yariv reagent for >20 h phenocopied the epidermal bulging seen in uge4 (Ding and Zhu 1997; Fig. 7B). However, Yariv reagent did not induce endomembrane aggregates with GFP–NIP5;1 or FM4-64 in WT plants (Fig. 7B), suggesting that the cell bulging caused by AGP dysfunction in the cell wall is not associated with endomembrane disorganization. To explore the possible additive effect of defects in cell wall polysaccharides and AGPs, we treated the mur1-2, mur2-1 and xxt1/xxt2 mutants with Yariv reagent (Fig. 7B). In the mutants treated with Yariv reagent, the FM4-64 staining patterns varied between independent plants, possibly because of severe cell wall damage. However, aggregates typically observed in the uge4 mutants were not apparent. In xxt1/xxt2 mutants treated with Yariv reagent, abnormal structures were occasionally stained by FM4-64 (Supplementary Fig. S4). However, the structures were smaller and apparently different in shape from the aggregates in the uge4 mutant. Although additional studies are required to clarify the effect of simultaneous defects in xyloglucan and AGPs, the reduced xyloglucan or dysfunction of AGPs in the cell wall was probably not the direct cause of endomembrane disorganization in uge4.

**Discussion**

In this study, we performed a forward genetic analysis using transgenic Arabidopsis plants expressing GFP–NIP5;1. GFP–NIP5;1 is localized to the PM of epidermal and lateral root cap cells with polarity toward the outer (soil-facing) side of the roots (Takano et al. 2010). Our approach can be used to identify the mechanisms underlying the trafficking of PM-localized proteins, including ER exit, secretion, polar targeting, endocytosis and vacuolar trafficking. We identified mutants in which GFP–NIP5;1 was localized to the ER, the vacuolar membrane (our unpublished results) and intracellular aggregates (Fig. 1). Our data indicate that mutants with intracellular aggregates are defective in general endomembrane organization rather than in NIP5;1-specific trafficking mechanisms. Identification of the responsible gene led to re-discovery of the cell wall mutant rhd1/reb1/uge4 as a mutant of endomembrane organization.

Previously, the Arabidopsis mutant kam1 was identified in a screen based on vacuole-targeted GFP–2sc (Tamura et al. 2005). This mutant contains endomembrane aggregates with various organelles in the perinuclear region of its cells. However, the structure of the aggregates differed between uge4 and kam1. In kam1, endosomes stained with FM4-64 were a minor component of the aggregates (Tamura et al. 2005),

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**Fig. 6** Intact actin filaments and microtubules are required for the organization of endomembranes. GFP–NIP5;1 in root epidermal cells of WT plants incubated in liquid medium containing 1% sucrose and 0.2% DMSO (control, A), 10 μM latrunculin B (B, D) or 10 μM oryzalin (C, E) for 2 d. Scale bars = 10 μm.
while in uge4, the entire aggregate stained with FM4-64 (Fig. 1D). KAM1 is a Golgi membrane protein that interacts with actin filaments. This interaction, rather than its activity as a xyloglucan galactosyltransferase, is responsible for proper endomembrane organization. When leaf epidermal cells were treated with latrunculin B, an actin-depolymerizing reagent, GFP–2sc accumulated in aggregates, similar to the kam1 mutant (Tamura et al. 2005). However, in root epidermal cells, latrunculin B induced the accumulation of GFP–NIP5;1 in spherical structures (Fig. 6D), apparently different from the aggregates in uge4. Therefore, the endomembrane aggregation probably differs in kam1 and uge4. Interestingly, the disruption of microtubules by oryzalin also resulted in GFP–NIP5;1 accumulation in spherical structures (Fig. 6E). A previous report on reb1-1 (uge4) mutants showed that the microtubules in the swollen trichoblasts were either disordered or entirely absent, while they were apparently normal in atrichoblasts (Andéme-Onzighi et al. 2002). In our analysis, aggregation occurred in both trichoblasts and atrichoblasts in uge4 mutants (Fig. 1C). These observations suggest that disorganization of actin and microtubules is not likely to be the cause of endomembrane aggregation in uge4.

UGE4 encodes an isoform of UGE, which functions in the biosynthesis of UDP-α-galactose (Seifert et al. 2002). There are five isoforms of UGE with distinct enzymatic properties, and only UGE4 influenced plant growth when a single isoform was mutated (Barber et al. 2006, Rösti et al. 2007). Among the five isoforms, UGE1 and UGE4

![Fig. 7](https://academic.oup.com/pcp/article-abstract/55/4/704/1813437)
are expressed in the root elongation zone (Barber et al. 2006, Rösti et al. 2007). Both UGE1 and UGE4 are present in the cytoplasm, whereas UGE4 is additionally enriched close to Golgi stacks (Barber et al. 2006). The distinct enzymatic properties and intracellular localization would explain the occurrence of endomembrane aggregates in the root elongation zone of single uge4 mutants (Fig. 1).

In contrast, the absence of aggregates in cotyledons of the uge4 mutants (Supplementary Fig. S1B) may be due to the redundant expression of all five UGE isoforms in leaves (Rösti et al. 2007).

Since d-galactose in the media reduced endomembrane aggregation (Fig. 5), the reduced amount or incorrect delivery of UDP-d-galactose in the cell caused the aggregation. UDP-d-galactose is a donor nucleotide sugar for the biosynthesis of cell wall polysaccharides, including xylolglucan, rhamnogalacturonan (RG)-I and RG-II pectins, AGPs and chloroplast galactolipids (Reiter et al. 2001). However, our results do not support the involvement of any known d-galactose-containing components in UGE4-dependent endomembrane organization. It was suggested that UGE4 is not required for galactolipid synthesis based on a thin-layer chromatographic analysis of lipids from roots or cotyledons (Seifert et al. 2002). A structural analysis revealed no changes in the galactosylation of RG-I and RG-II in uge4 roots (Ngueuma-Ona et al. 2006). Therefore, we focused on the involvement of AGPs and xylolglucan in endomembrane organization. When we treated WT plants with β-d-glucosyl Yariv reagent, which reacts with AGPs, epidermal bulging occurred, in agreement with previous results (Ding and Zhu 1997, Andéme-Onzighi et al. 2002, Seifert et al. 2002, Ngueuma-Ona et al. 2006). A structural analysis revealed no changes in the galactosylation of RG-I and RG-II in uge4 mutants compared with the WT (Ding and Zhu 1997, Andéme-Onzighi et al. 2002, Seifert et al. 2002, Ngueuma-Ona et al. 2006). A structural analysis revealed no changes in the galactosylation of RG-I and RG-II in uge4 roots (Ngueuma-Ona et al. 2006). Therefore, we focused on the involvement of AGPs and xylolglucan in endomembrane organization. When we treated WT plants with β-d-glucosyl Yariv reagent, which reacts with AGPs, epidermal bulging occurred, in agreement with previous results (Ding and Zhu 1997). However, endomembrane aggregation was not observed (Fig. 7B). Therefore, the function of AGPs in the cell wall is not linked to endomembrane organization. The involvement of xylolglucan was examined using the mur2 mutant, which contains <2% of the WT amount of fucosylated xylolglucan (Vanzin et al. 2002), and the xxt1/xxt2 mutant, which lacks detectable xylolglucan (Cavalier et al. 2008, Park and Cosgrove 2012). In these mutants, endomembrane aggregation was not observed (Fig. 7A). The treatment of mur2-1 and xxt1/xxt2 with β-d-glucosyl Yariv reagent also did not induce the typical aggregates observed in uge4 plants, suggesting that neither AGPs nor xylolglucan in the cell wall is responsible for endomembrane organization. However, the binding of Yariv reagent to AGPs and the lack of fucosyltransferase (MUR2) or xylosyltransferase (XXT1/2) should affect AGPs and xylolglucan differently in the uge4 mutant, in which the supply of d-galactose to AGPs and xylolglucan is reduced. Therefore, it is possible that the reduced synthesis of AGPs or altered structures of AGPs and/or xylolglucan cause endomembrane disorganization in uge4. It is also possible that there is an unknown d-galactose-containing component specifically required for endomembrane organization. The suppression of aggregates in the presence of the ethylene precursor ACC (Supplementary Fig. S3), which is thought to modulate the transfer of UDP-d-galactose to specific d-galactose-requiring components (Seifert et al. 2004), may increase our understanding of the mechanism of endomembrane aggregation in uge4 mutants.

In conclusion, we isolated a mutant with abnormal endomembrane aggregates and identified the responsible gene as uge4, encoding an enzyme for d-galactose synthesis. UGE4 activity is required for proper cell wall structure and endomembrane organization.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Ler was obtained from our laboratory stocks. ProNIP5;1 (−5′UTR):GFP-NIP5;1 (Tanaka et al. 2011), Wave 13Y, 34Y (Geldner et al. 2009) and the mur1 (Reiter et al. 1993), mur2 (Reiter et al. 1997) and xxt1/xxt2 (Cavalier et al. 2008) mutants of A. thaliana were described previously. All plants were grown on vertically placed solid medium (Takano et al. 2005) containing 1% (w/v) sucrose, 1.5% (w/v) gellan gum and 30 μM boric acid for 4–10 d in growth chambers at 22°C under fluorescent lamps with a 16 h/8 h light/dark cycle. For experiments with d-galactose, 10 mM d-galactose was added to the solid medium.

Mutant screening

For mutant screening, 25,000 T4 seeds of GFP–NIP5;1 were mutagenized with 0.3% EMS for 17 h and 20 pools of M2 seeds were obtained. M2 seedlings grown on solid medium containing 30 μM boric acid for 7–10 d were observed under an epifluorescence microscope with a ×20 dry lens (Leica HC PL APO CS), and candidates showing abnormal GFP–NIP5;1 localization were selected. At the beginning of the mutant selection process, we observed plants showing growth levels lower than the WT (10,000 plants/30,000 seeds). Half way through the selection procedure, we examined plants showing growth levels lower than the WT (10,000 plants/30,000 seeds). We screened approximately 40,000 M2 seeds and observed approximately 20,000 seedlings.

Genetic mapping

For genetic mapping, Line 20-2 (Col-0 background) was crossed with the Ler ecotype and F2 seeds were obtained. Genomic DNA was extracted from F2 plants exhibiting the mutant phenotype of GFP–NIP5;1. For rough mapping, SSLP markers were used as follows: F149, F289, NGA280 and NGA111 for chromosome 1; T12J2 and NGA168 for chromosome 2; NGA162 and CIW4 for chromosome 3; NGA8 for chromosome
Whole-genome re-sequencing and data analysis

Genomic DNA of an M_3 homozygous mutant was isolated using a DNeasy Plant Mini Kit (Qiagen). Library construction and emulsion PCR were performed as described in the SOLiD fragment library construction kit (Applied Biosystems). The libraries were sequenced to 75 bp using the Applied Biosystems 5500xl SOLiD System with an Exact Call Chemistry module.

Color space reads produced by the SOLiD sequencer were mapped to the A. thaliana genome reference TAIR10 using LifeScope 2.1 software (Life Technologies) with default parameters. SNPs were called using a diBayes SNP caller, a component of LifeScope. diBayes was executed with a parameter setting defined as ‘medium call stringency’. Integrative Genomics Viewer (Robinson et al. 2011) was used to visualize the mapped reads and called SNPs, along with gene models. To predict the functional impact of the SNPs, we categorized them into coding (synonymous or non-synonymous), intronic, intergenic and splicing sites using custom scripts (Tabata et al. 2012). Gene models and the annotations were based on TAIR10.

Imaging analysis

Laser scanning confocal microscopy was performed using Leica TCS SP5 and SP8 (equipped with an HC PL APO CS2 ×20 water immersion lens and HCX PL APO CS ×40 water immersion lens) with the following excitation and detection wavelengths: 488 and 500–530 or 500–550 nm for GFP; 488 and 500–540 nm for YFP; 488 and >640 nm for FM4-64; and 552 and >600 nm for ER-Tracker Red. DIC was used to image cell structures. FM4-64 (Molecular Probes) was prepared as a 10 mM stock solution in water and used at 5 μM in solid medium. FM4-64 (Molecular Probes) was prepared as a 1 mM stock in dimethylsulfoxide (DMSO) and used at 2 μM for 1 min and washed with water. To disrupt actin filaments or microtubules, latrunculin B or oryzalin was used, respectively, at 10 μM for 2 d. L-tritumclacin B or oryzalin (Wako Pure Chemicals) were prepared at 10 mM in DMSO. Plants were transferred from solid to liquid medium containing the dye or inhibitors and incubated at room temperature or in the growth chamber at 22°C, respectively. The liquid medium was replaced each day. Control treatments for the inhibitor experiments were performed with 0.2% DMSO. β-1,2-Glucosyl Yariv reagent was dissolved in 0.15 M NaCl at 2 mg ml⁻¹ as a stock solution. Plants were incubated in liquid medium containing 1.5 mM NaCl as a control experiment or 30 μM Yariv reagent for >20 h in a growth chamber at 22°C. The ethylene precursor ACC (Sigma) was prepared as a 10 mM stock solution in water and used at 5 μM in solid medium.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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