



HOKKAIDO UNIVERSITY

Title	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
Author(s)	Uehara, M.; Wang, S.; Kamiya, T. et al.
Citation	Plant and Cell Physiology, 55(4), 704-714 https://doi.org/10.1093/pcp/pct191
Issue Date	2014-04
Doc URL	https://hdl.handle.net/2115/59480
Rights	This is a pre-copy-editing, author-produced PDF of an article accepted for publication in Plant & Cell Physiology following peer review. The definitive publisher-authenticated version Plant Cell Physiol (2014) 55 (4): 704-714 is available online at: http://pcp.oxfordjournals.org/content/55/4/704.full?sid=8fdda2c4-6f13-4ee6-b47f-b4cb758342b3
Type	journal article
File Information	Uehara2014PCP-HUSCAP.pdf



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

Running head:

Endomembrane organization is dependent on D-galactose

Corresponding author:

Dr. J. Takano

Graduate School of Agriculture, Hokkaido University, Kita-ku N9-W9, Sapporo 060-8589,

Japan

Tel: +81-11(706)3888, FAX: +81-11(706)4932, Email: jtakano@abs.agr.hokudai.ac.jp

Subject area:

Special Focus Issues: Plant Endomembranes.

Membrane and transport

Number of black and white figures: 1, Color figures: 6, Supplementary figures: 4,

Supplementary tables: 2

Title

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

Running Head

Endomembrane organization is dependent on D-galactose

Authors

Masataka Uehara¹, Sheliang Wang¹, Takehiro Kamiya², Shuji Shigenobu³, Katsushi Yamaguchi³, Toru Fujiwara², Satoshi Naito^{1,4}, Junpei Takano¹

¹ Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

² Graduate School of Agricultural and Life Science, The University of Tokyo, Tokyo 113-8657, Japan

³ National Institute for Basic Biology, Okazaki 444-8585, Japan

⁴ Graduate School of Life Science, Hokkaido University, Sapporo 060-0810, Japan

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AGP, arabinogalactan-protein;

B, boron; DIC, differential interference contrast; DMSO, dimethyl sulfoxide; 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, rhamnogalacturonan; RHD, root hair defective; SNP, single nucleotide polymorphism; SSLP, simple sequence length polymorphism; TGN, *trans*-Golgi network; UGE, UDP-D-glucose-4-epimerase; XXT, xylosyltransferase

Abstract

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 GFP-NIP5;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 (uge) 4/root hair defective (rhd) 1/root epidermal bulgar (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

59 polysaccharide xyloglucan and arabinogalactan proteins (AGPs). The endomembrane
60 aggregates in the mutants were absent in the presence of D-galactose, indicative of a
61 requirement for a D-galactose-containing component in endomembrane organization.
62 Genetic and pharmacological analyses suggested that the aggregates were not caused by the
63 disruption of cell wall polysaccharides or the cytoskeleton. Overall, our results suggest that
64 UGE4 activity in D-galactose synthesis is required for the structure of cell wall
65 polysaccharides and endomembrane organization.

66

67 **Key words:** *Arabidopsis thaliana*, cell wall, endomembrane, D-galactose,
68 UDP-D-glucose-4-epimerase

69

70

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, Löffke 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. 2010). 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, 2007). The interaction between KAM1, a Golgi membrane protein, with actin filaments was shown to be important for proper endomembrane organization. Another screen using a polar PM 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-nodulin 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 toward 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-D-glucose 4-epimerase (uge) 4/root hair defective (rhd) 1/root epidermal bulgar (reb) 1*, which was isolated as mutants of root hair development, root morphology, and susceptibility to nematodes (Schiefelbein and Somerville 1990, Baskin et al. 1992, Baum et al. 2000). UGE4 activity in D-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*

129 harboring *ProNIP5;1 (Δ5'UTR)::GFP-NIP5;1* as a transgene in the *nip5;1-1* mutant. In the
130 construct, we used the NIP5;1 promoter lacking the 5'UTR of *NIP5;1*, which is responsible
131 for the B-dependent degradation of *NIP5;1* mRNA, to obtain relatively strong expression of
132 GFP-NIP5;1 irrespective of the B level (Tanaka et al. 2011). In the transgenic plants,
133 GFP-NIP5;1 localized to the PM in root epidermal cells (Fig. 1A-C and Takano et al. 2010).
134 For screening, approximately 25,000 GFP-NIP5;1 seeds were mutagenized with ethylmethane
135 sulfonate (EMS), and 20 pools of M2 seeds were obtained. We screened ~40,000 M2 seeds
136 from the 20 pools under fluorescence microscopes and isolated 17 mutant lines in which
137 GFP-NIP5;1 showed aberrant localization. Among them, three mutants showed localization
138 of GFP-NIP5;1 in intracellular aggregates. In this study, we focused on Line 20-2, in which
139 GFP-NIP5;1 was often localized to abnormal intracellular aggregates in epidermal cells in the
140 root elongation zone (Fig. 1A). These aggregates were also observed in epidermal cells in
141 the root hair zone, but not in the root meristem zone (Supplementary Fig. S1A). In the
142 mutant elongation zone, bulging of the epidermal cells started to appear, leading to swelling
143 of the cells in the root hair zone (Fig. 1A). A cross-section of the elongation zone showed
144 that the aggregates accumulated in trichoblasts (root hair-forming cells), which are in contact
145 with two cortical cells, and in atrichoblasts, which are in contact with only one cortical cell
146 (Fig. 1C). The polar localization of GFP-NIP5;1 in the plasma membrane was not affected
147 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 χ^2 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

167 three mutants obtained from the same screen. The read sequences were mapped to the *A.*
168 *thaliana* Col-0 genome. Nine nucleotide changes were found in the exons of the genetically
169 mapped region (between 13.8 and 27 Mb on chromosome 1) after subtracting shared single
170 nucleotide polymorphisms (SNPs) among the four mutant lines, which could be originally
171 present in the parental transgenic line. Of the 9 mutations, 7 were G/C-to-A/T transitions,
172 which are known to arise from the EMS-mediated alkylation of guanine and adenine.
173 Annotation of these 7 EMS-induced mutations indicated that 4 were nonsynonymous, 1 was
174 nonsense, and the rest were silent (Supplementary Table S2). The one nonsense mutation
175 was in the gene AT1G64780 while the nonsynonymous mutations were found in AT1G56570,
176 AT1G64440, AT1G72950, and AT1G73160 (Fig. 2A).

177 In the candidate genes for the causative mutations, we identified *UGE4/RHD1/REB1*
178 (*At1g64440*), whose loss-of-function mutants showed similar root bulging phenotypes to Line
179 20-2 (Schiefelbein and Somerville 1990, Baskin et al. 1992). Line 20-2 had a single
180 mutation (G1365A) in *UGE4/RHD1/REB1*, which resulted in the replacement of Gly²²³
181 (GGA) with Arg (AGA) (Fig. 2A, Supplementary Table S2). To determine whether Line
182 20-2 is an allele of the *uge4* mutant, we first stained roots of *rhdl-1* and SALK_080766, a
183 T-DNA insertion allele of *uge4* (Fig. 2A) with FM4-64 (Fig. 2B). In both alleles, FM4-64
184 stained aggregates in elongating epidermal cells, as in Line 20-2 (Fig. 1D). F₁ plants
185 between Line 20-2 and *rhdl-1* or SALK_080766 were then produced and their roots were

186 stained with FM4-64. The roots of the F₁ plants contained aggregates stained by FM4-64
187 and contained GFP-NIP5;1 (Fig. 2C). These results indicated that Line20-2 is allelic to the
188 *uge4* mutant.

190 **Abnormal intracellular aggregates contain TGN/early endosomes**

191 Since the GFP-NIP5;1-labeled aggregates were stained with FM4-64, an endocytic
192 tracer, we assumed that the structure contained endomembranes along the endocytic pathway.
193 As expected, significant amounts of two TGN/early endosome markers, YFP-VTI12 and
194 YFP-RabA1e (Geldner et al. 2009), were localized to the aggregates using FM4-64 in the
195 *uge4* (SALK_080766) mutant (Fig. 3A). We then stained the ER using ER tracker Red (Fig.
196 3B). In the cells with GFP-NIP5;1-labeled aggregates, ER tracker Red stained network
197 structures, including the aggregates, suggesting that part of the ER membrane was included in
198 the aggregates. Differential interference contrast (DIC) images showed apparently normal
199 vacuolar structures in the cells containing aggregates (Fig. 1B). These observations
200 suggested that some (but not all) of the endomembrane accumulated in the aggregates.

201 We then performed time-course analysis of FM4-64 uptake to investigate the
202 endocytic pathway from PM to vacuolar membranes in *uge4* mutants. In both wild-type and
203 the *uge4* mutant, FM4-64 was observed as dotty structures after staining for 5 min, suggesting
204 that endocytosis of FM4-64 from the plasma membrane was not impaired in the mutant (Fig.

205 4A). However, FM4-64 showed stained aggregates after 15 min, and the intensity of
206 FM4-64 in the aggregates increased after 30 min in the *uge4* mutant (Fig. 4A). After 160
207 min, FM4-64 clearly stained the vacuolar membrane in wild-type cells; however, the staining
208 was less intense in the *uge4* mutant (Fig. 4B). These results suggested that the *uge4* mutant
209 had a defect in the later endocytic pathway from TGN/early endosomes to vacuoles.

210

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

213 *UGE4* encodes an isoform of UDP-D-glucose-4-epimerase, which interconverts
214 UDP-D-glucose and UDP-D-galactose and is involved in D-galactose synthesis (Seifert et al.
215 2002). In *uge4*, the D-galactose content is decreased and the bulging of epidermal cells is
216 rescued by the addition of D-galactose to the growth medium (Seifert et al. 2002). Thus, we
217 grew our mutant in the presence of 10 mM D-galactose and found that D-galactose rescued the
218 bulging and decreased endomembrane aggregation in epidermal cells (Fig. 5). This result
219 indicated that D-galactose synthesized by UGE4 is required for endomembrane organization
220 in epidermal cells of the root elongation zone. We next explored whether D-galactose
221 decreased the number of existing aggregates. Based on our time-course analysis, 10 mM
222 D-galactose did not affect existing aggregates within 110 min in the elongation zone
223 (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 μ M ACC treatment for 2 days, 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 to 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

262 elongation by ~50% and caused swelling at the base of the root hairs (van Hengel and Roberts
263 2002). In both *mur1-1* and *mur1-2* mutant plants, FM4-64 stained the PM and dotted
264 endosomes, similar to wild type (Fig. 7A). This clearly differed from *uge4* (SALK_080766),
265 in which the aggregates were stained. These results suggest that the defect in root elongation
266 in the *mur1* mutants was not linked to endomembrane disorganization. We then used the
267 *mur2* mutant, which contained less than 2% of the WT level of fucosylated xyloglucan in its
268 cell wall (although the growth and wall strength were normal) (Vanzin *et al.* 2002), and the
269 *xylosyltransferase (xxt) 1/xxt2* mutant, which contained no detectable xyloglucan and showed
270 aberrant root hairs (Cavalier *et al.* 2008). FM4-64 stained the PM and dotted endosomes in
271 these mutants, suggesting that xyloglucan is not required for endomembrane organization (Fig.
272 7A). To increase our understanding of AGPs, we treated plant roots with β -D-glucosyl Yariv
273 reagent, a synthetic phenylglycoside that specifically binds AGPs (Yariv *et al.* 1967,
274 Nguema-Ona *et al.* 2012). As reported, treatment with 10 μ M Yariv reagent for 7 days
275 phenocopied the epidermal bulging seen in *uge4* (Ding and Zhu 1997 and Fig. 7B).
276 However, Yariv reagent did not induce endomembrane aggregates with GFP-NIP5;1 or
277 FM4-64 in WT plants (Fig. 7B), suggesting that the cell bulging caused by AGP dysfunction
278 in the cell wall is not associated with endomembrane disorganization. To explore the
279 possible additive effect of defects in cell wall polysaccharides and AGPs, we treated the
280 *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 not likely 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 *rhdl/rebl/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), 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 likely 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 *rebl-1* (*uge4*) mutants showed that the microtubules in the swollen trichoblasts were either disordered or absent entirely, while they were apparently normal in atrichoblasts (Andème-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 the cause of endomembrane aggregation in *uge4*.

UGE4 encodes an isoform of UGE, which functions in the biosynthesis of UDP-D-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 *-4* are expressed in the root elongation zone (Rösti et al. 2007, Barber et al. 2006). Both *UGE1* and *-4* 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 cotyledon 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 improper 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 xyloglucan, rhamnogalacturonan (RG)-I and -II pectins, AGPs, and chloroplast galactolipids (Reiter and Vanzin, 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). It is also unlikely that *uge4* mutations affect the N-glycosylation of glycoproteins, because in the N-glycans of most glycoproteins D-galactose exists as the trisaccharide Gal β (1 $\rightarrow$ 3)[Fuc α (1 $\rightarrow$ 4)]GlcNAc, a structure absent from the N-glycans of *A. thaliana* (Fitchette et al. 1999). Among D-galactose-containing polysaccharides, AGPs and xyloglucan (but not RG-I and -II pectins) are reduced in *uge4* mutants compared to wild type (Ding and Zhu 1997, Andème-Onzighi et al. 2002, Seifert et al. 2002, Nguema-Ona et al. 2006). A structural analysis revealed no changes in the galactosylation of RG-I and -II in *uge4* roots (Nguema-Ona et al. 2006). Therefore, we focused on the involvement of AGPs and xyloglucan 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 xyloglucan was examined using the *mur2* mutant, which contains less than 2% of the WT amount of fucosylated xyloglucan (Vanzin et al. 2002), and the *xxt1/xxt2* mutant, which lacks detectable xyloglucan (Cavalier et al. 2008). 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 xyloglucan 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 xyloglucan differently in the *uge4* mutant, in which the supply of D-galactose to AGPs and xyloglucan is reduced. Therefore, it is possible that the reduced synthesis of AGPs or altered structures of AGPs and/or xyloglucan 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 *xtt1/xtt2* (Park and Cosgrove 2012) 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 μ M boric acid for 4-10 days 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 μ M boric acid for 7-10 days were observed under the epifluorescence microscope with a 20 $\times$ 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 wild type (10,000 plants/30,000 seeds). Half-way through the selection procedure, we examined the localization of GFP-NIP5;1 in every plant (10,000 seeds). We screened approximately 40,000 M₂ seeds and observed ~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: F14J9, F28J9, NGA280, and NGA111 for chromosome 1; T12J2 and NGA168 for chromosome 2; NGA162 and CIW4 for chromosome 3; NGA8 for chromosome 4; and MBK20 and K20J1 for chromosome 5 (Supplementary Table S1). We referred to the TAIR

marker database
(http://www.arabidopsis.org/servlets/Search?action=new_search&type=marker) and Cereon
database (<http://www.arabidopsis.org/cereon/>) in selecting our SSLPs.

Whole-genome re-sequencing and data analysis

Genomic DNA of an M₃ 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× IMM 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 long-pass for FM4-64; and 552 and >600 nm long-pass 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 4 μ M for 5-160 min. ER tracker Red (Molecular Probes) was prepared as a 1 mM stock in dimethyl sulfoxide (DMSO) and used at 2 μ M for 1 min and washed with water. To disrupt actin filaments or microtubules, latrunculin B or oryzalin was used, respectively, at 10 μ M for 2 days. Latrunculin B and 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 -D-glucosyl Yariv reagent was dissolved in 0.15 M NaCl at 2 mg/ml as a stock solution. Plants were incubated in liquid medium containing 1.5 mM NaCl as a control experiment or 30 μ M Yariv reagent for more than 20 h in a growth chamber at 22°C. The ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC, Sigma) was prepared as a 10 mM stock solution in water and used at 5 μ M in solid medium.

Funding

This work is supported by the NEXT program (to J. T.) and Grant-in-Aid for Scientific Research (Kiban S, to T.F.) from the Japanese Society for the Promotion of Science (JSPS).

Acknowledgements

We acknowledge Masahira Hattori and Kenshiro Oshima (University of Tokyo) for genome sequencing, Tomoko Shimizu and Kayo Konishi for technical assistance, and Shinji Wakuta and Akira Yoshinari for critical reading. We thank Mayuki Tanaka (University of Tokyo), Niko Geldner (University of Lausanne), Malcolm O'Neill (University of Georgia), and

Nottingham Arabidopsis Stock Centre and Arabidopsis Biological Resource Center for providing materials.

References

Andème-Onzighi, C., Sivaguru, M., Judy-March, J., Baskin, T.I. and Driouich, A. (2002) The *reb1-1* mutation of Arabidopsis alters the morphology of trichoblasts, the expression of arabinogalactan-proteins and the organization of cortical microtubules. *Planta* 215: 949–958.

Barber, C., Rösti, J., Rawat, A., Findlay, K., Roberts, K. and Seifert, G.J. (2006) Distinct properties of the five UDP-D-glucose/UDP-D-galactose 4-epimerase isoforms of Arabidopsis thaliana. *J. Biol. Chem.* 281: 17276–17285.

Baskin, T., Betzner, A., Hoggard, R., Cork, A. and Williamson, R.E. (1992) Root morphology mutants in *Arabidopsis thaliana*. *Aust. J. Plant Physiol.* 19: 427–437.

Baum, T.J., Wubben, M.J.E., Hardy, Y.K.A., Su, H. and Rodermel, S.R. (2000) A screen for *Arabidopsis thaliana* mutants with altered susceptibility to *Heterodera schachtii*. *J. Nematol* 32: 166–173.

Cavalier, D.M., Lerouxel, O., Neumetzler, L., Yamauchi, K., Reinecke, A., Freshour, G. et al. (2008) Disrupting two *Arabidopsis thaliana* xylosyltransferase genes results in plants deficient in xyloglucan, a major primary cell wall component. *Plant Cell* 20: 1519–1537.

Ding, L. and Zhu, J.K. (1997) A role for arabinogalactan-proteins in root epidermal cell expansion. *Planta* 203: 289–294.

479 Feraru, E., Paciorek, T., Feraru, M.I., Zwiewka, M., De Groodt, R., De Rycke, R. et al. (2010)
 480 The AP-3 β adaptin mediates the biogenesis and function of lytic vacuoles in Arabidopsis.
 481 *Plant Cell* 28: 12-24.
 482
 483 Fitchette, A.C., Cabanes-Macheteau, M., Marvin, L., Martin, B., Satiat-Jeunemaitre, B.,
 484 Gomord, V. et al. (1999) Biosynthesis and immunolocalization of Lewis a-containing
 485 N-glycans in the plant cell. *Plant Physiol.* 121: 333–344.
 486
 487 Freshour, G., Bonin, C.P., Reiter, W.D., Albersheim, P., Darvill, A.G. and Hahn, M.G. (2003)
 488 Distribution of fucose-containing xyloglucans in cell walls of the *mur1* mutant of Arabidopsis.
 489 *Plant Physiol.* 131: 1602–1612.
 490
 491 Geldner, N., Dénervaud-Tendon, V., Hyman, D.L., Mayer, U., Stierhof, Y.D. and Chory, J.
 492 (2009) Rapid, combinatorial analysis of membrane compartments in intact plants with a
 493 multicolor marker set. *Plant J.* 59(1): 169–178.
 494
 495 Löffke, C., Luschig, C. and Kleine-Vehn, J. (2012) Posttranslational modification and
 496 trafficking of PIN auxin efflux carriers. *Mech. Dev.* 130(1): 1–13.
 497
 498 Nguema-Ona, E., Andème-Onzighi, C., Aboughe-Angone, S., Bardor, M., Ishii, T., Lerouge,
 499 P. et al. (2006) The *reb1-1* mutation of Arabidopsis. Effect on the structure and localization of
 500 galactose-containing cell wall polysaccharides. *Plant Physiol.* 140: 1406–1417.
 501
 502 Nguema-Ona, E., Coimbra, S., Vitré-Gibouin, M., Mollet, J.C. and Driouich, A. (2012)
 503 Arabinogalactan proteins in root and pollen-tube cells: distribution and functional aspects.
 504 *Ann. Bot.* 110(2): 383–404.

505

506 Park, M. and Jürgens, G. (2012) Membrane traffic and fusion at post-Golgi compartments.
507 *Front. Plant Sci.* 2: 1–15.

508

509 Park, Y.B. and Cosgrove, D.J. (2012) Changes in cell wall biomechanical properties in the
510 xyloglucan-deficient *xxt1/xt2* mutant of *Arabidopsis*. *Plant Physiol.* 158: 465–475.

511

512 Reiter, W.D., Chapple, C.C. and Somerville, C.R. (1993) Altered growth and cell walls in a
513 fucose-deficient mutant of *Arabidopsis*. *Science* 261: 1032–1035.

514

515 Reiter, W.D., Chapple, C. and Somerville, C.R. (1997) Mutants of *Arabidopsis thaliana* with
516 altered cell wall polysaccharide composition. *Plant J.* 12: 335–345.

517

518 Reiter, W.D. and Vanzin, G.F. (2001) Molecular genetics of nucleotide sugar interconversion
519 pathways in plants. *Plant Mol. Biol.* 47: 95–113.

520

521 Robinson, J., Thorvaldsdóttir, H., Wendy, W., Mitchell, G., Eric, S.L., Gad, G. et al. (2011)
522 Integrative genomics viewer. *Nat. Biotechnol.* 29(1): 24–26.

523

524 Rösti, J., Barton, C.J., Albrecht, S., Dupree, P., Pauly, M., Findlay, K. et al. (2007)
525 UDP-glucose 4-epimerase isoforms UGE2 and UGE4 cooperate in providing UDP-galactose
526 for cell wall biosynthesis and growth of *Arabidopsis thaliana*. *Plant Cell* 19: 1565–1579.

527

528 Scheuring, D., Viotti, C., Krüger, F., Künzl, F., Sturm, S., Bubeck, J. et al. (2011)
529 Multivesicular bodies mature from the trans-Golgi network/early endosome in *Arabidopsis*.
530 *Plant Cell* 23: 3463–3481.

531

532 Schiefelbein, J. and Somerville, C. (1990) Genetic control of root hair development in
533 *Arabidopsis thaliana*. *Plant Cell* 2: 235–243.

534

535 Seifert, G.J., Barber, C., Wells, B., Dolan, L., and Roberts, K. (2002) Galactose biosynthesis
536 in *Arabidopsis*: genetic evidence for substrate channeling from UDP-D-galactose into cell
537 wall polymers. *Current Biol.* 12: 1840–1845.

538

539 Seifert, G.J., Barber, C., Wells, B. and Roberts, K. (2004) Growth Regulators and the Control
540 of Nucleotide Sugar Flux. *Plant Cell* 16: 723–730.

541

542 Spitzer, C., Reyes, F.C., Buono, R., Sliwinski, M.K., Haas, T.J. and Otegui, M.S. (2009) The
543 ESCRT-related CHMP1A and B proteins mediate multivesicular body sorting of auxin
544 carriers in *Arabidopsis* and are required for plant development. *Plant Cell* 21: 749–766.

545

546 Tabata, R., Kamiya, T., Shigenobu, S., Yamaguchi, K., Yamada, M., Hasebe, M. et al. (2012)
547 Identification of an EMS-induced causal mutation in a gene required for boron-mediated root
548 development by low-coverage genome re-sequencing in *Arabidopsis*. *Plant Signal Behav.*
549 8(1): e22534.

550

551 Takano, J., Miwa, K., Yuan, L., von Wirén, N. and Fujiwara, T. (2005) Endocytosis and
552 degradation of BOR1, a boron transporter of *Arabidopsis thaliana*, regulated by boron
553 availability. *Proc. Natl. Acad. Sci. USA* 102: 12276–12281.

554

555 Takano, J., Wada, M., Ludewig, U., Schaaf, G., von Wirén, N. and Fujiwara, T. (2006) The
556 *Arabidopsis* major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant

557 development under boron limitation. *Plant Cell* 18: 1498–1509.

558

559 Takano, J., Tanaka, M., Toyoda, A., Miwa, K., Kasai, K., Fuji, K. et al. (2010) Polar
560 localization and degradation of Arabidopsis boron transporters through distinct trafficking
561 pathways. *Proc. Natl. Acad. Sci. USA* 107: 5220–5225.

562

563 Tamura, K., Shimada, T., Kondo, M., Nishimura, M. and Hara-Nishimura, I. (2005)
564 KATAMARI1/MURUS3 is a novel golgi membrane protein that is required for
565 endomembrane organization in Arabidopsis. *Plant Cell* 17: 1764–1776.

566

567 Tamura, K., Takahashi, H., Kunieda, T., Fuji, K., Shimada, T., and Hara-Nishimura, I. (2007).
568 Arabidopsis KAM2/GRV2 is required for proper endosome formation and functions in
569 vacuolar sorting and determination of the embryo growth axis. *Plant Cell* 19: 320–332.

570

571 Tanaka, M., Takano, J., Chiba, Y., Lombardo, F., Ogasawara, Y., Onouchi, H. et al. (2011)
572 Boron-dependent degradation of NIP5;1 mRNA for acclimation to excess boron conditions in
573 Arabidopsis. *Plant Cell* 23: 1–14.

574

575 Uemura, T., Kim, H., Saito, C., Ebine, K., Ueda, T., Schulze-lefert, P. et al. (2012).
576 Qa-SNAREs localized to the trans -Golgi network regulate multiple transport pathways and
577 extracellular disease resistance in plants. *Proc. Natl. Acad. Sci. USA* 109(5): 1784–1789.

578

579 van Hengel, A.J. and Roberts, K. (2002) Fucosylated arabinogalactan-proteins are required
580 for full root cell elongation in arabidopsis. *Plant J.* 32: 105–113.

581

582 Vanzin, G.F., Madson, M., Carpita, N.C., Raikhel, N. V., Keegstra, K. and Reiter, W.D.

(2002) The *mur2* mutant of *Arabidopsis thaliana* lacks fucosylated xyloglucan because of a lesion in fucosyltransferase AtFUT1. *Proc. Natl. Acad. Sci. USA* 99(5): 3340–3335.

Viotti, C., Bubeck, J., Stierhof, Y.D., Krebs, M., Langhans, M., van den Berg, W. et al. (2010) Endocytic and secretory traffic in Arabidopsis merge in the trans-golgi network/early endosome, an independent and highly dynamic organelle. *Plant Cell* 22: 1344–1357.

Yariv, J., Lis, H. and Katchalski, E. (1967) Precipitation of arabic acid and some seed polysaccharides by glycosylphenylazo dyes. *Biochem. J.* 105: 1c-2c

Zwiewka, M., Feraru, E., Möller, B., Hwang, I., Feraru, M.I., Kleine-Vehn, J. et al. (2011) The AP-3 adaptor complex is required for vacuolar function in Arabidopsis. *Cell Res.* 21: 1–12.

Figure legends

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 soil-side in PM in comparison to FM4-64, which stained the PM in a non-polar manner. Plants were grown on solid medium containing 30 μ M boric acid for 7-10 days. Roots were incubated with 4 μ M FM4-64 for 30 min (D). *: trichoblast. Bars = 50 μ m (A), 10 μ m (B, D), and 20 μ m (C).

Fig. 2. Rough mapping and genome sequencing revealed that Line 20-2 is allelic to

uge4/rhd1/reb1. (A) Procedure for the identification of the responsible gene in Line 20-2 and the structure of *uge4/rhd1/reb1* (At1g64440), which has 9 exons. (B, C) FM4-64-stained epidermal cells from the root elongation zone of *rhd1-1* and SALK_080766 plants (B) and F₁ plants from a cross between Line 20-2 and SALK080766 or *rhd1-1* (C). Plants were grown on solid medium containing 30 μ M boric acid for 7-10 days. Roots were incubated with 4 μ M FM4-64 for 30 min. Bars = 10 μ m.

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 μ M ER tracker Red for 1 min, washed with water, and then observed. Bars = 10 μ m.

Fig. 4. The later endocytic pathway from 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 μ M FM4-64, washed with water, and then observed after the indicated times. Bars = 10 μ m.

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 days. Bars = 20 μ m.

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 μ M latrunculin B (B, D), or

10 μ M oryzalin (C, E) for 2 days. Bars = 10 μ m.

Fig. 7. A lack of xyloglucan and AGP dysfunction did not induce endomembrane aggregation. (A) FM4-64-stained roots of WT, *mur1-1*, *mur1-2*, *mur2-1*, and *xxt1/xxt2* plants. Note that in a cell in SALK_080766, vacuolar membranes were stained. This is probably because of direct penetrance by FM4-64 due to disruption of the PM. (B) GFP-NIP5;1 localization and FM4-64 staining pattern in roots of WT, *mur1-2*, *mu2-1*, and *xxt1/xxt2* plants treated with Yariv reagent. Plants were incubated in liquid medium containing 30 μ M β -D-glucosyl Yariv reagent for more than 20 h and then stained with 4 μ M FM4-64 for 30 min. Control treatment was performed with 1.5 mM NaCl. Bars = 10 μ m.

Figure legends for supplementary figures

Supplementary Fig. S1

Aggregates were observed in epidermal cells in the root hair zone, but not in the leaves, of Line 20-2. A) In epidermal cells of the root hair zone, GFP-NIP5;1 was localized in the aggregates. Note that the signal of GFP-NIP5;1 on the PM was low in this area. White arrows indicate the aggregates. B) No aggregate stained with FM4-64 in the epidermal cells of cotyledon in both the WT and Line 20-2. Plant leaves were stained with 4 μ M FM4-64 for 30 min. Bars = 100 μ m.

Supplementary Fig. S2

The presence of 10 mM D-galactose did not affect existing aggregates. (A) The aggregates (indicated by arrows) remained evident after 110 min.

(B) The aggregates remained evident in epidermal cells in root hair zone, while they were not observed in the root elongation zone after 19 h in the presence of D-galactose. Bars = (A)

661 20 μm and (B) 50 μm .

662

663 Supplementary Fig. S3

664 Ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) treatment rescued the
665 aggregates. Five-day-old seedlings of Line 20-2 were transferred to solid medium containing
666 30 μM boric acid (Control) or 30 μM boric acid and 5 μM ACC for 2 days. Bars = 10 μm .

667

668 Supplementary Fig. S4

669 The abnormal structures observed in some of the roots of the *xxt1/xxt2* mutant treated with
670 Yariv reagent. The plants were treated with 30 μM β -D-glucosyl for 24 h and then stained
671 with 4 μM FM4-64 for 30 min (A-C). Enlarged images with FM4-64 and DIC are shown in
672 the right panels. In some of the abnormal structures, single spots with high intensity were
673 observed (A and B, indicated by white arrows). Bars = 10 μm .

Fig. 1

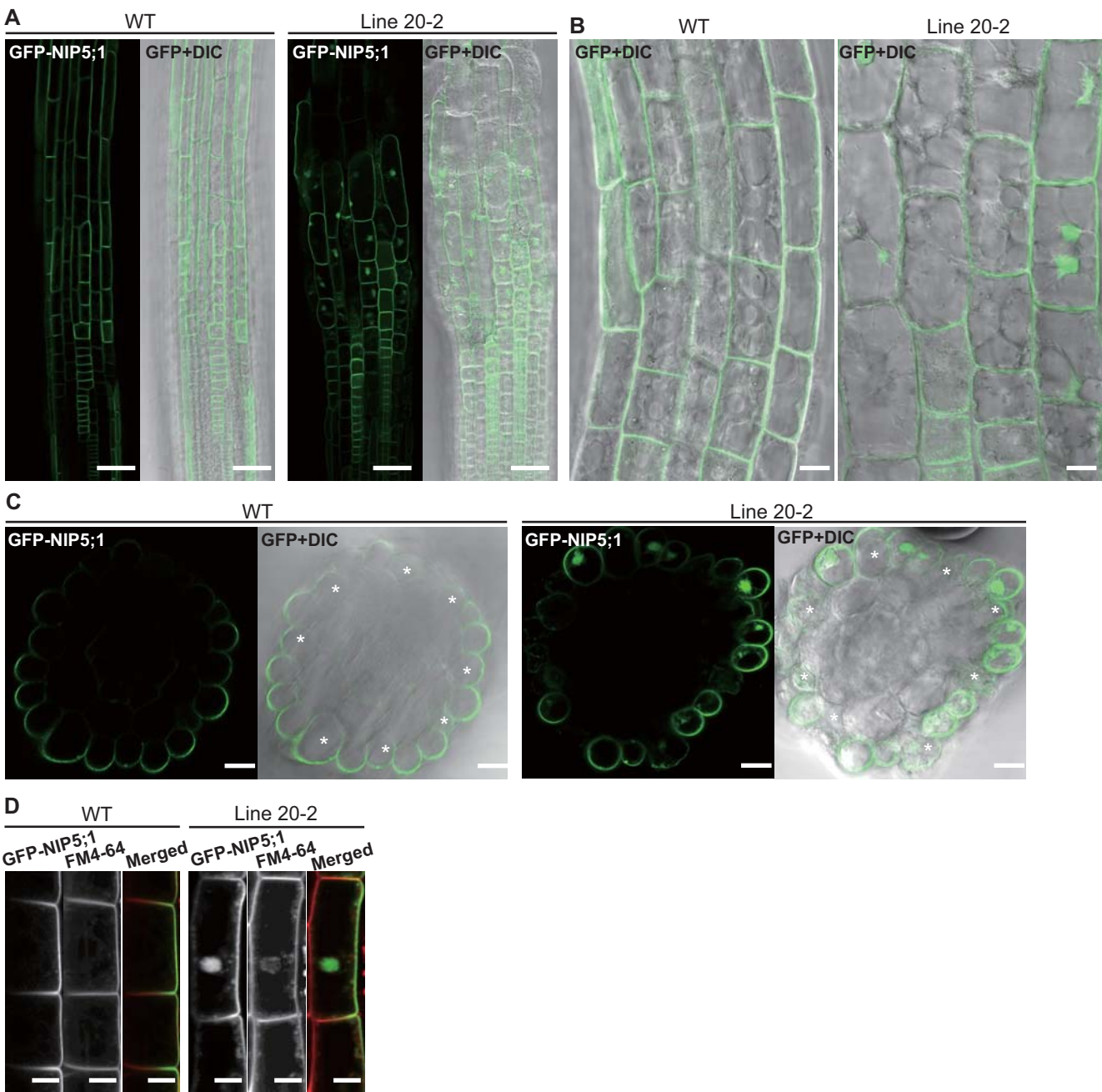
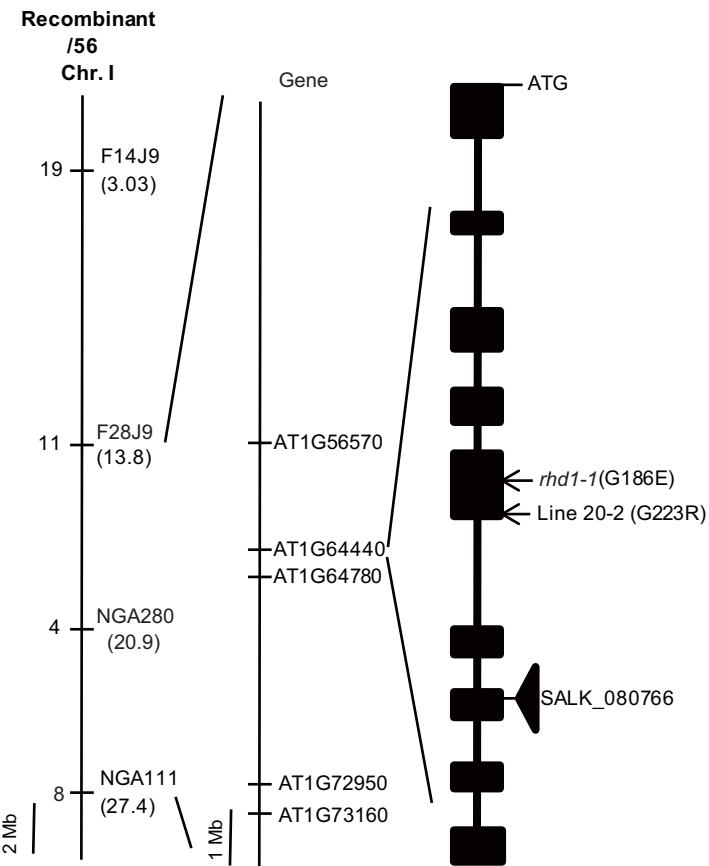
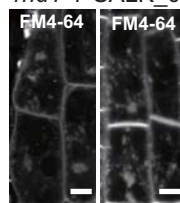
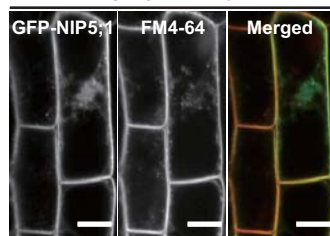


Fig. 2**A****B***rhdl-1* SALK_080766**C**Line 20-2 x *rhdl-1*

Line 20-2 x SALK_080766

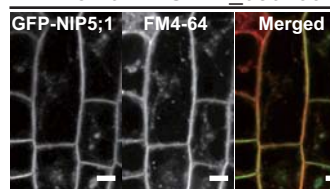


Fig. 3

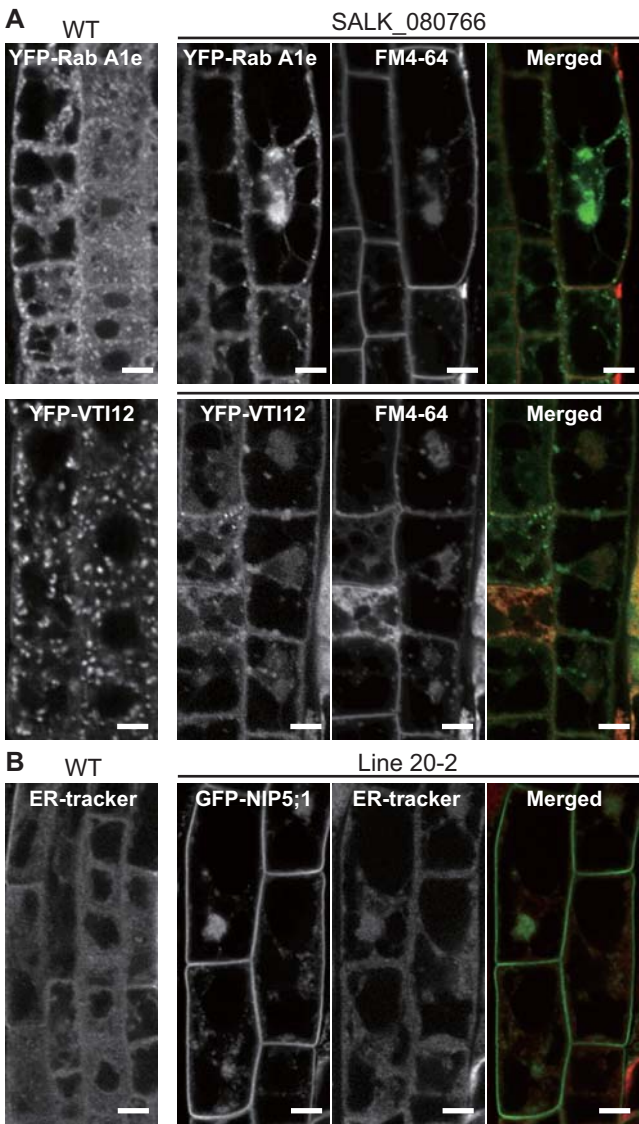


Fig. 4

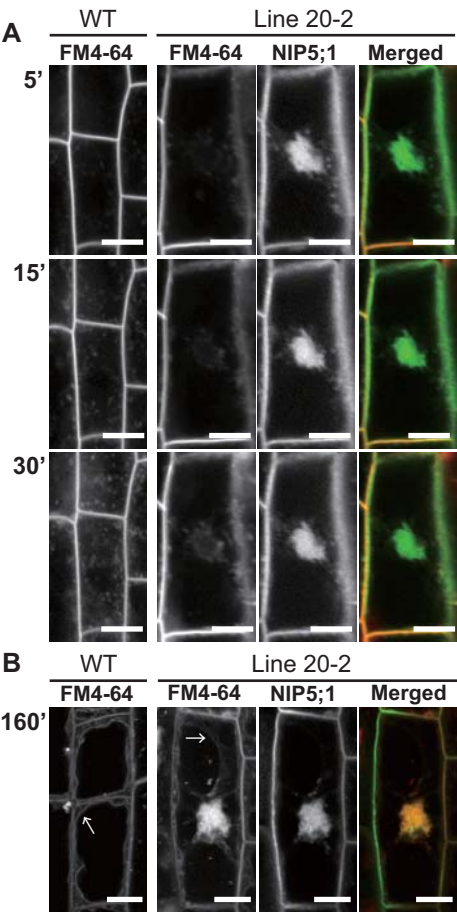


Fig. 5

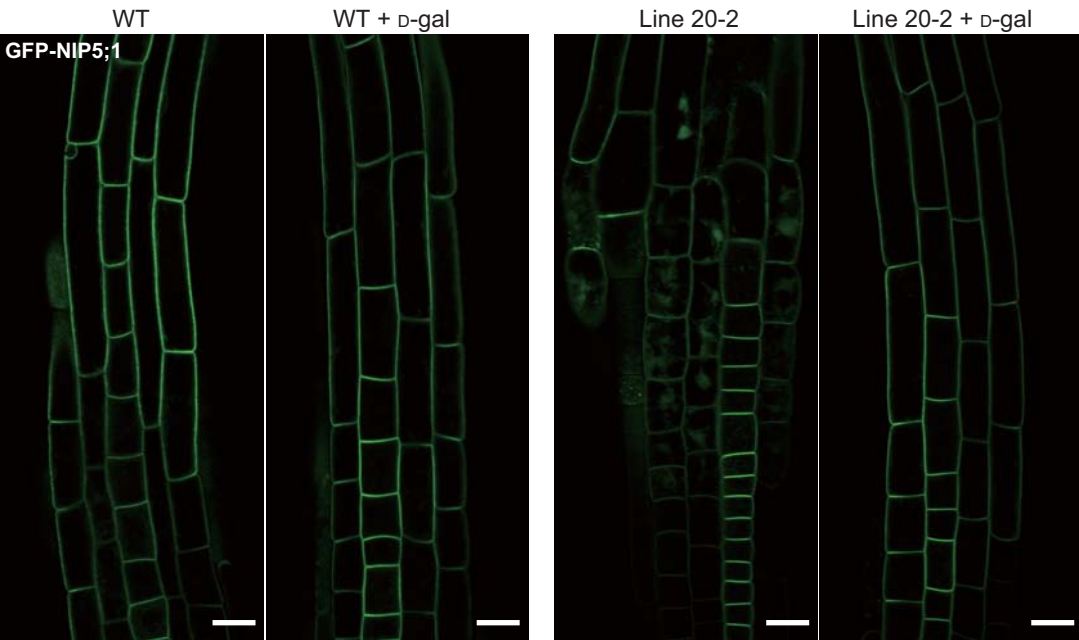
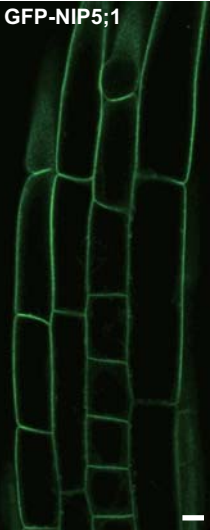


Fig. 6

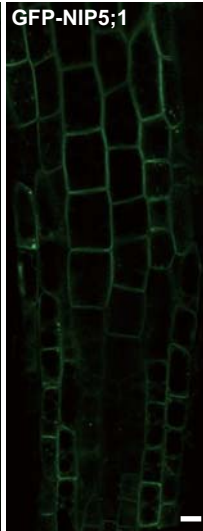
A Control

GFP-NIP5;1



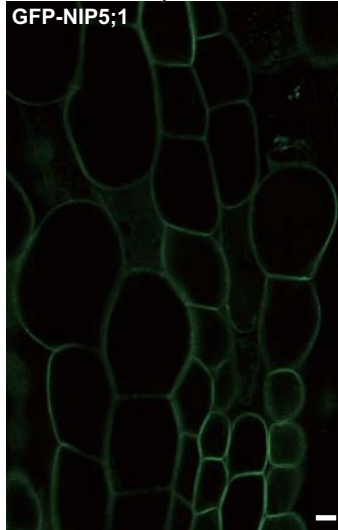
B Latrunculin B

GFP-NIP5;1



C Oryzalin

GFP-NIP5;1

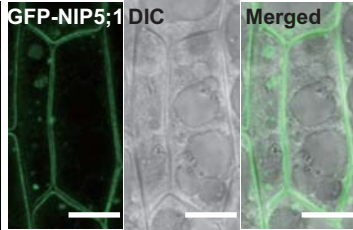


D Latrunculin B

GFP-NIP5;1

DIC

Merged



E Oryzalin

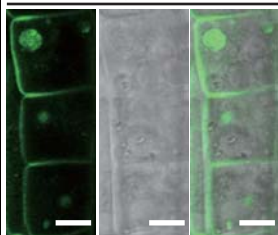
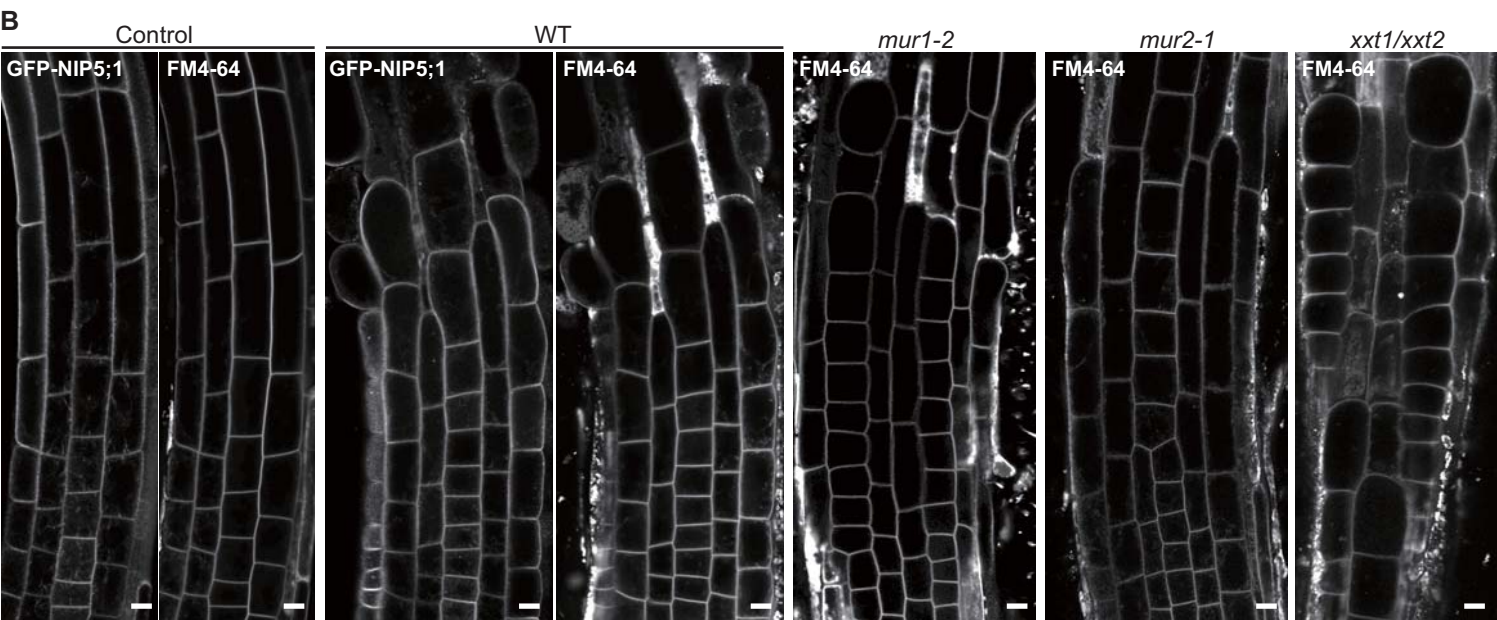
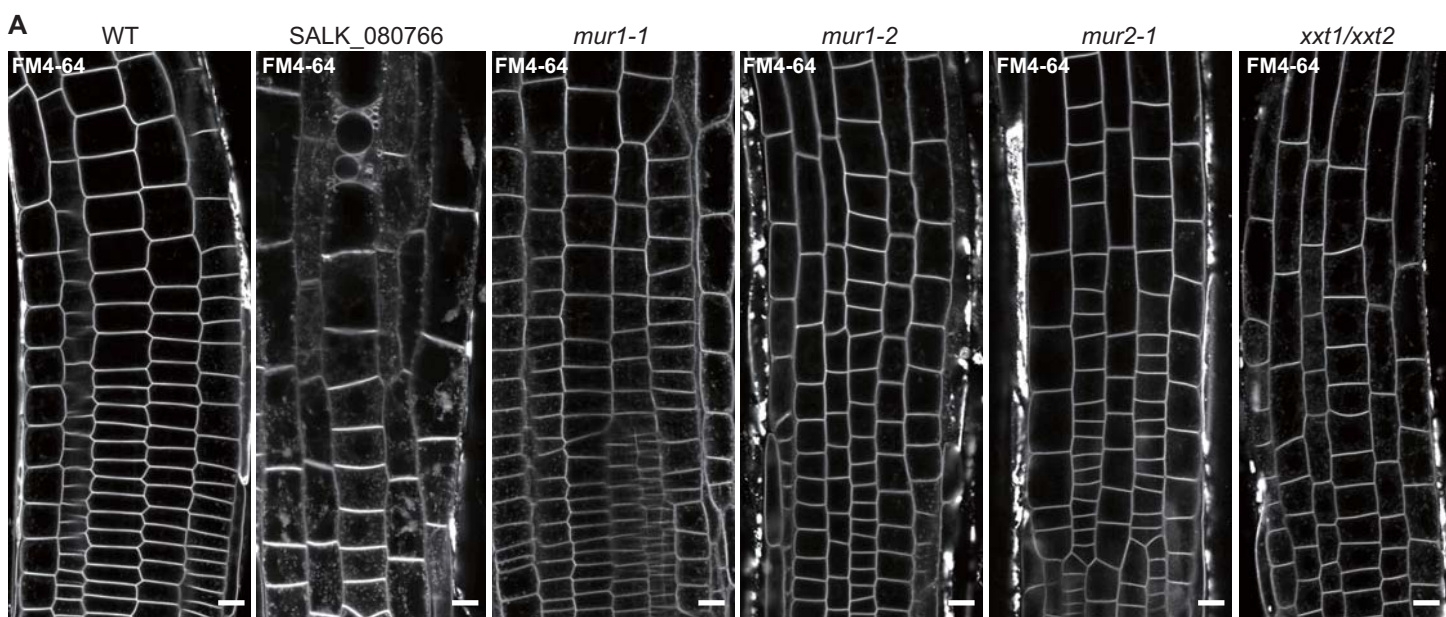
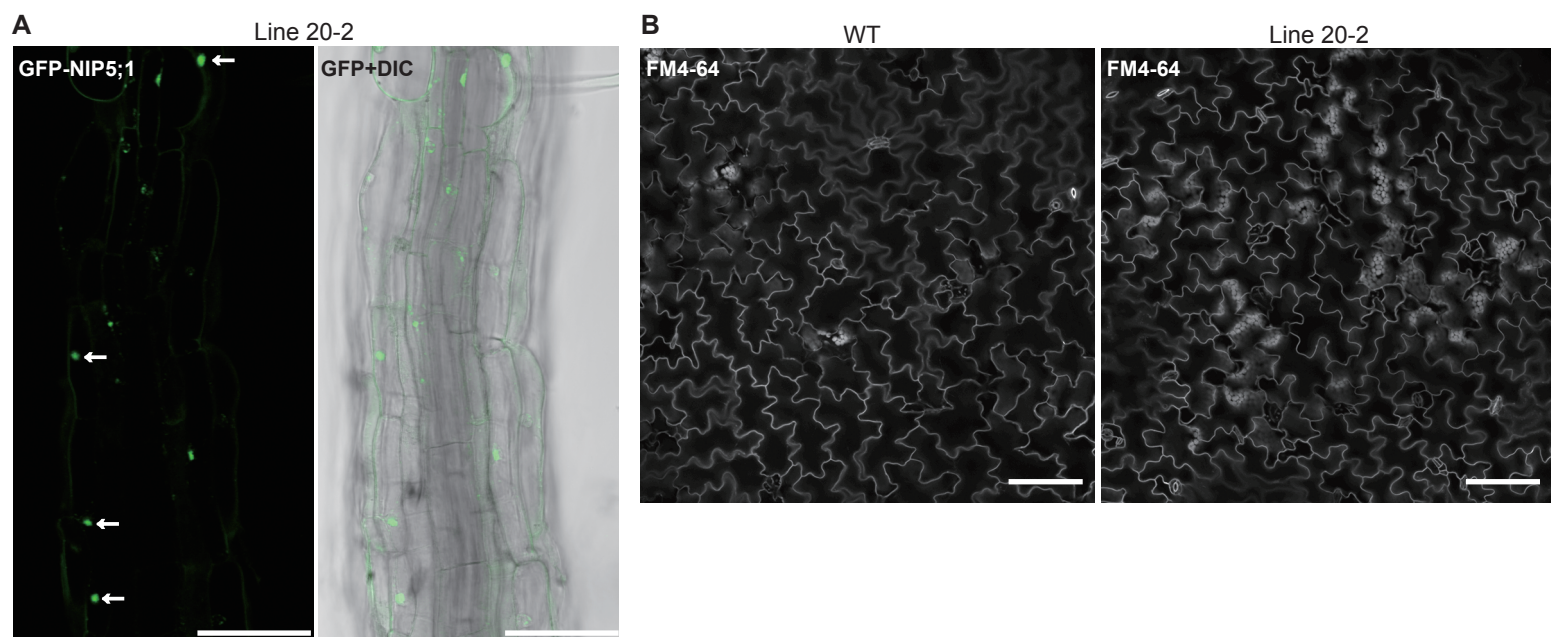


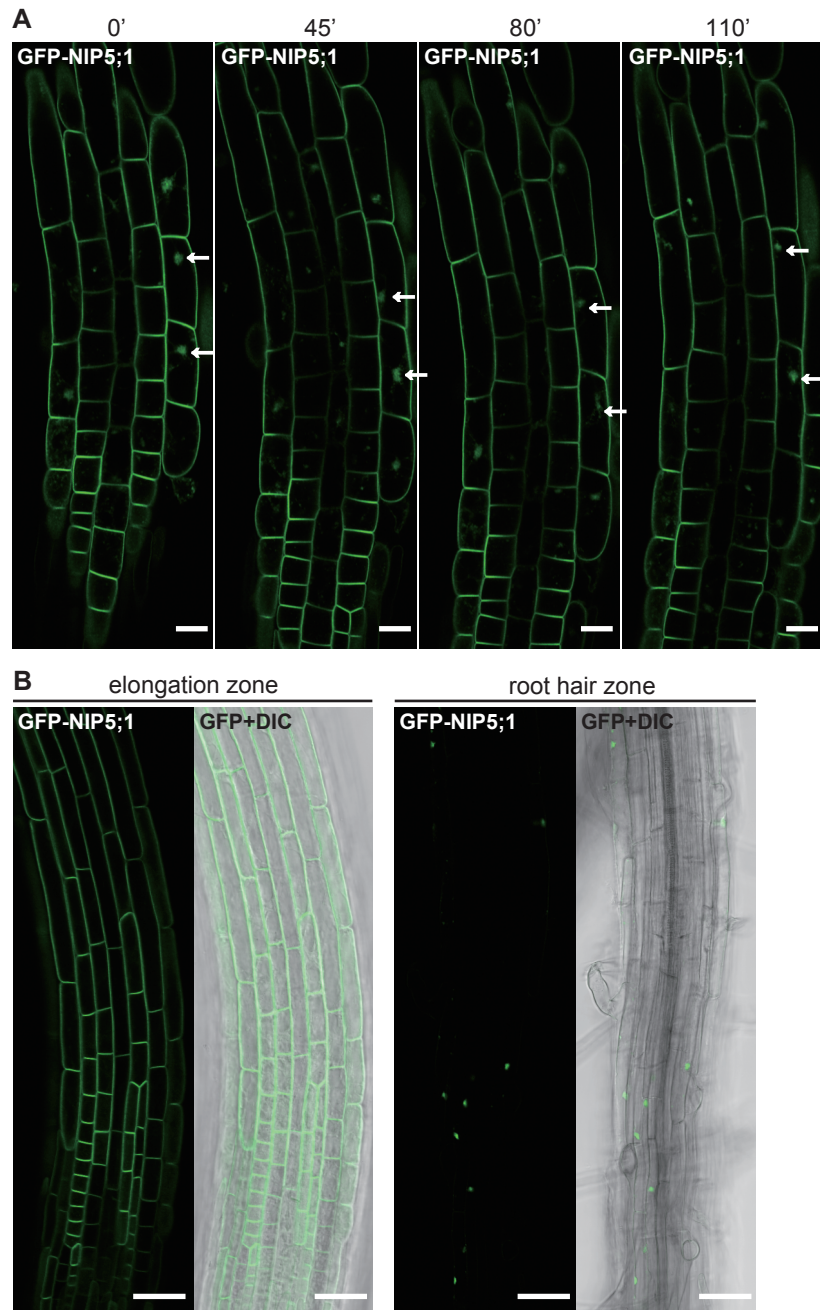
Fig. 7





Supplementary Fig. S1

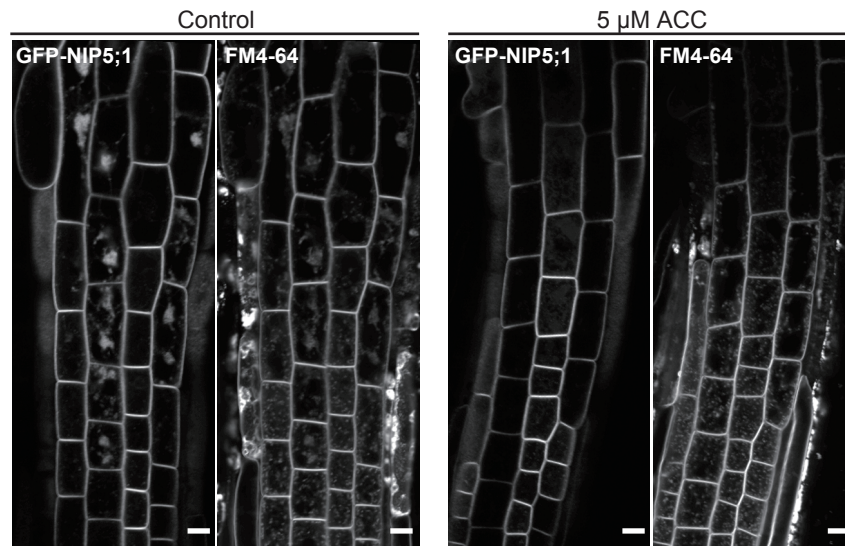
Aggregates were observed in epidermal cells in the root hair zone, but not in laeves, of Line 20-2. A) In epidermal cells of the root hair zone, GFP-NIP5;1 was localized in the aggregates. Note that the signal of GFP-NIP5;1 on the PM was low in this area. White arrows indicate the aggregates. B) No aggregate stained with FM4-64 in the epidermal cells of cotyledon in both the WT and Line 20-2. Plant leaves were stained with 4 μ M FM4-64 for 30 min. Bars = 100 μ m.



Supplementary Fig. S2

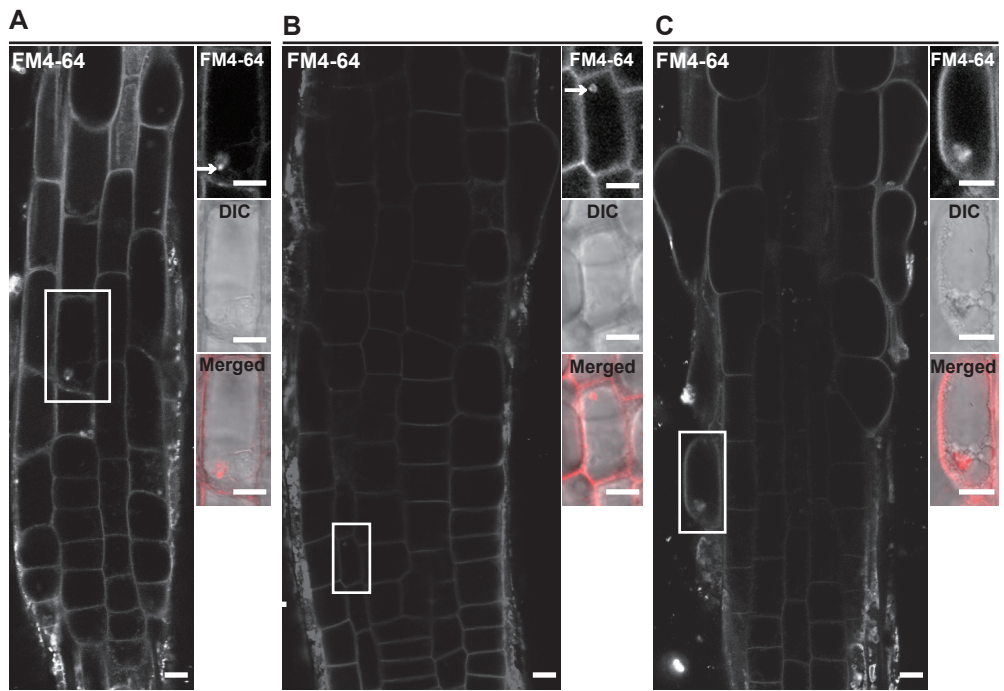
The presence of 10 mM D-galactose did not affect existing aggregates. (A) The aggregates (indicated by arrows) remained evident after 110 min.

(B) The aggregates remained evident in epidermal cells in root hair zone, while they were not observed in the root elongation zone after 19 h in the presence of D-galactose. Bars = (A) 20 μ m and (B) 50 μ m.



Supplementary Fig. S3

Ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) treatment rescued the aggregates. Five-day-old seedlings of Line 20-2 were transferred to solid medium containing 30 μM boric acid (Control) or 30 μM boric acid and 5 μM ACC for 2 days. Bars = 10 μm.



Supplementary Fig. S4

The abnormal structures were observed in some of the roots of the *xxt1/xxt2* mutant treated with Yariv reagent. The plants were treated with 30 μM β -D-glucosyl for 24 h and then stained with 4 μM FM4-64 for 30 min (A-C). Enlarged images with FM4-64 and DIC are shown in the right panels. In some of the abnormal structures, single spots with high intensity were observed (A and B, indicated by white arrows). Bars = 10 μm .

Supplementary Table S1: Primer sequences for rough mapping

Chromosome	Marker name	Location (Mb)	Forward (5'-3')	Reverse (5'-3')
1	F14J9	3.0	CGTGAACCCACTCGTTACATT	TGCATTTCAACTTTACCAACCA
	F28J9	13.8	ATGTTGGATTCAAGCACTTCC	AAGGTTCCGTCAGAGACGTG
	NGA 280	20.9	GGCTCCATAAAAAGTGCACC	CTGATCTCACGGACAATAGTGC
	NGA111	27.4	TGTTTTTTAGGACAAATGGCG	CTCCAGTTGGAAGCTAAAGGG
2	T12J2	3.5	ATCAACATCCGCAAAGTTCC	ACCTCCTTAGTCGCGTGAAA
	NGA168	16.3	GAGGACATGTATAGGAGCCTCG	TCGTCTACTGCACTGCCG
3	NGA162	4.6	CTCTGTCACTCTTTTCCTCTGG	CATGCAATTTGCATCTGAGG
	CIW4	18.9	GTTCAATAAACTTGCGTGTGT	TACGGTCAGATTGAGTGATTC
4	NGA8	5.6	TGGCTTTCGTTTATAAACATCC	GAGGGCAAATCTTTATTTTCGG
5	MBK20	2.5	CCAAGACCAAAACCAAAACC	CATGCAATAGGCTTCGGAGT
	K20J1	19.9	TGACAACTTTGGGCAATTAAGA	CGCATGATGCATAGCAAAGT

Supplementary Table S2: List of candidate genes

Gene	SNP	Mutation	Protein (reference to TAIR)
AT1G56570	gGa=>gAa	G=>E	a mitochondria-localized pentatricopeptide repeat protein PGN
AT1G61310	Cag=>Aag	Q=>K	LRR and NB-ARC domains-containing disease resistance protein
AT1G64440	Gga=>Aga	G=>R	UDP-glucose 4-epimerase (AtUGE4/AtRHD1/AtREB1)
AT1G64780	Cag=>Tag	Q=>Stop	ammonium transporter 1;2
AT1G70340	Aga=>Tga	R=>Stop	unknown function
AT1G70580	ttG=>ttA	L=>L	glyoxylate aminotransferase
AT1G72390	aaG=>aaA	K=>K	unknown function
AT1G72950	gGt=>gAt	G=>D	Disease resistance protein (TIR-NBS class)
AT1G73160	Gag=>Aag	E=>K	UDP-Glycosyltransferase superfamily protein

Red letters mean non-EMS mutation.