TESTING THE MODEL FOR ENDOPLASMIC RETICULUM MORPHOLOGY AND FUNCTION IN THE PROCESS OF ROOT TIP GROWTH

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Testing the model for endoplasmic reticulum morphology and function in the process of root tip growth

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The goal of this project is to analyze the effect that the components of the cytoskeleton have the on the movement, morphology, and function of the endoplasmic reticulum (ER) and subsequent tip growth in *Arabidopsis thaliana* (*A. thaliana*). In particular the effects of the RHD3 protein will be observed to determine its connection to these components. In order to determine how RHD3 relates to the cytoskeleton, the effects of RHD3 loss of function will be analyzed in comparison to the effects of treatment with the herbicide oryzalin. Oryzalin has a well understood effect on the depolymerization of plant microtubules in root hair cells, and expresses a similar phenotype to the one shown in RHD3 mutants. The combination of these two conditions is pertinent to determining the pathway that RHD3 operates on to affect root tip growth in conjunction with the recently developed ER scaffold model.

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NOMENCLATURE

A. thaliana	*Arabidopsis thaliana*
ER	Endoplasmic Reticulum
RHD3	Root Hair Defective 3
MS media	Murashige and Skoog media
WT	Wild type

SECTION I

INTRODUCTION

Polarized root tip growth in *Arabidopsis thaliana*

Cell polarity is a characteristic feature found in eukaryotic cells; it is of particular interest to scientists when it comes to determining how polarity is achieved and maintained (Zheng and Chen, 2011). In order to develop an understanding of how this works, a common model organism to use is *Arabidopsis thaliana. A. thaliana* has become the typical model organism in studies based around higher plants. With its full genome published, the advancements in molecular experimentation are moving forward at a quick pace. Root hairs in particular are a beneficial cell type for examining the dynamics of polarized root tip growth. These tubular, root epidermal cells are important for nutrient and water uptake for the plant, as well as interaction with the *Rhizobium* (Foreman and Dolan, 2001). They are characterized by the localized growth at a small dome at their tip, and their simple and consistent structure makes any unusual phenotypes easy to observe (Zheng and Chen, 2011). The study of polarized tip growth is important to understand using this model system since it has been shown to be similar, both morphologically and sub-cellularly, to the process of neuronal outgrowth in animals (Baluska, 2010).

The endoplasmic reticulum scaffold model

The endoplasmic reticulum scaffold model is a model that is currently being developed for the organization and function of the ER within the growing root hair. The idea behind this model is that the ER acts as a tether, pulling along other organelles in the growing cell with the assistance

of the cytoskeletal network. This differs from previous ideas that suggest that the organelles are moved solely by the cytoskeleton and its motors. The ER is an extremely dynamic organelle that interacts with both the actin and microtubule networks, but the exact mechanisms by which it does so still remain unclear. There are three hypothesized methods by which the ER-organelle-driven motion may control ER movement shown in Figure 1. The first is that the organelles are associated with molecular motors that move them along the cytoskeleton, and the ER is pulled along with them through a tether. The second is that the ER is associated with the cytoskeletal motor and drags the tethered organelles with it, and the third is that both the ER and its tethered organelles move along with specific molecular motors that operate in conjunction with one another. The ER scaffold model also proposes that the ball of ER characteristically found at the tip is associated with the cytoplasmic microtubules and this ER-microtubule scaffold controls the process of tip growth outward from the site of polarization. The scaffold is also postulated to selectively tether to the plasma membrane at specific anchor sites enriched with ER-localized proteins (Griffing et al., 2016).

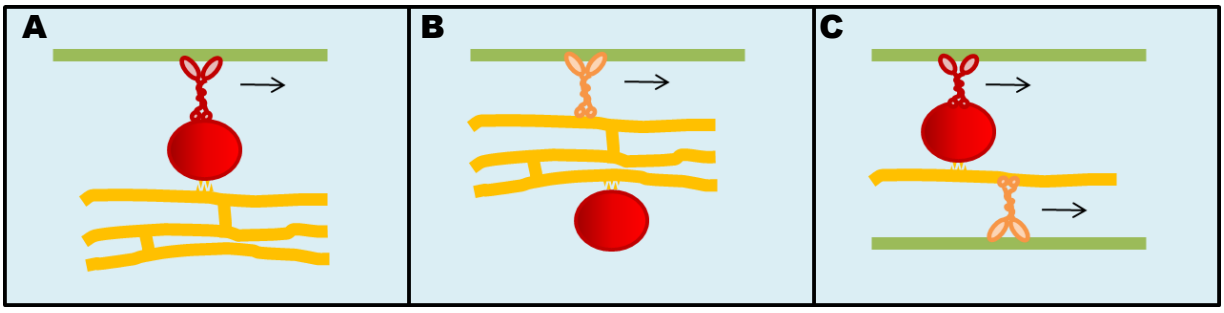

Figure 1. Representation of the three models for ER/cytoskeleton motion. A) shows the ER (yellow) tethered to an organelle (red) which is pulled along the cytoskeleton (green) by a molecular motor, B) shows the ER moving with a motor and pulling the organelle, and C) shows the organelle and ER moving with their own motors (Griffing et al., 2016).

RHD3 study in *A. thaliana*

The protein under investigation, RHD3 (Root Hair Defective 3), is a small GTPase involved in the generation of the tubular ER network in plant cells. This protein is the plant homolog of the atlastin protein found in mammals, although it is unclear exactly how much of the function of this protein is conserved in regards to the exact molecular mechanisms underlying the control of ER tubule morphology. In previous tests performed in *A. thaliana* RHD3 has been shown to localize to the endoplasmic reticulum where it does not interfere with the process of ER-to-Golgi protein trafficking, but rather is involved in shaping the tubular morphology of the ER (Chen et al., 2011). Loss-of-function of the RHD3 gene is phenotypically characterized by wavy and shortened root hair growth in *Arabidopsis;* this effect is most likely related to the disruption of the tubular ER network that occurs when the RHD3 gene has been knocked out (Wang et al., 1997). The study of the RHD3 protein will help to elucidate the unique morphological features and functions of the ER involved in tip growth of plants.

Interactions between RHD3 and the cytoskeleton

The RHD3 protein has been shown in previous tests to have interactions with both the actin and microtubule networks. Determining exactly where this protein fits in these pathways also lines up with determining its interaction with the ER, considering that it too can have interactions with both these networks during the process of polarized root tip growth (Chen et al., 2011). In particular the link between RHD3 and actin is strong as it relates to proper growth of the cell wall (Liu 2011). As a part of the cytoskeleton, actin plays an important role in root tip growth of *A. thaliana*. It is involved in the process of cytoplasmic streaming of organelles such as the ER, and as such, disruption of the actin network results in a decrease in the growth rate of root hairs

(Foreman and Dolan, 2001). In regards to the effect of RHD3 on microtubules, it has been previously shown to destabilize the microtubules; loss of function of the RHD3 gene stabilizes them (Gardiner and Mark, 2011).

The effects of oryzalin on growth polarity of root hairs

Oryzalin has been shown in previous experiments to disrupt the growth polarity of root hairs in *A. thaliana.* The wavy root hair phenotype expressed in RHD3 mutants is replicated in low concentrations of oryzalin, and in higher concentrations this effect is expanded to cause branching in root hair formation. The rate of growth of root hairs in oryzalin is not affected however, so it has been shown that the effect of oryzalin is limited to the growth polarity and stabilization of root hairs. These phenotypic characteristics are a result of the depolymerization of the microtubule network (Foreman and Dolan, 2001). Depolymerization of the microtubules using cytoskeleton-disrupting drugs typically leads to a loss of directionality of growth, and the formation of multiple growth points on a single root hair (Bibikova et al., 1999). At lower effective concentrations however, not all the microtubules are depolymerized, and there is little net disassembly. Microtubule dynamicity, a measure of the gain and loss of subunits at MT ends per unit time, is what is actually reduced. Treatment with lower concentrations of oryzalin therefore serves to destabilize the system rather than completely knock it out (Nakamura et al., 2004).

SECTION II

METHODS

Plant growth conditions

For the purposes of our experiments Col-0 WT (Lehle seeds, Austin Texas) and RHD3 knockout (Chris Hawes, Oxford Brookes University, Oxford UK) *Arabidopsis thaliana* seedlings were planted on MS 1/2 strength-Agar media for Plant Growth prepared to pH of 5.6-5.8 as described by Murashige and Skoog. Seeds were plated in a sterile hood after performing sterilization techniques. In order to sterilize the seeds they were soaked in 300-500µL of bleach solution for 3-5minutes. They were then washed with autoclaved water 4-5 times and planted onto petri dishes containing the appropriate media. The plates were wrapped with parafilm and incubated at room temperature under a light source. The plants were allowed to grow for 3 days before being transferred.

Plant transfer procedure

After at least 3 days of growth, one half of the seedlings were transferred to another MS 1/2 strength-Agar media plate while the other half was transferred to a MS 1/2 strength-Agar media plate containing a 100nM concentration of oryzalin (Sigma Aldrich). Transfer took place in a sterile hood and sterile techniques were used to transplant the seedlings. Following transfer, the petri dishes were wrapped with parafilm and incubated at room temperature under a light source.

Microscopy and Image Processing

After 2-3 days of growth following transfer, plants were observed using the Olympus FV1000 confocal microscope. In order to clearly view the morphology of the root hairs, the plants were stained with a few drops of a 1:100 dilution of 1mg/ml Propidium Iodide (Thermo-Fisher) stock prior to observation. ImageJ version 1.49g was used to process photos, and the plugin, LOCI, was used to import .oib files obtained from the confocal microscope. The 3D Project plugin was used to compile 3D images using the following settings: lower transparency bound of 1, upper bound of 255, surface opacity of 0, surface depth cueing of 100, interior depth cueing of 50, and no interpolation. The confocal microscope runs in conjunction with Fluoview software for image capture.

Data analysis

Root hairs were measured using the measure tool provided on ImageJ and values were exported to Microsoft Excel 2010. Statistical significance was determined by comparing average root hair length using the Analysis ToolPack Add-in t-test function with two sample unequal variance in Excel. Matlab R2015a was used to compile data and create the box and whisker plot.

SECTION III

RESULTS

The qualitative effects of oryzalin on root hair growth

The effects of oryzalin can be observed at the microscopic level following the confocal imaging of the seedlings. As shown in Figure 2, beyond the wavy phenotype an unusual branched phenotype was observed in WT seedlings. Branching is seen far more frequently, and to an even further extent than is ever seen in control conditions in MS media. Figure 2B-2D show a few examples of just how many branches formed and how extreme the observed branching was in the oryzalin treated plants. The root hairs branched more than once, and often formed branches off of branches.

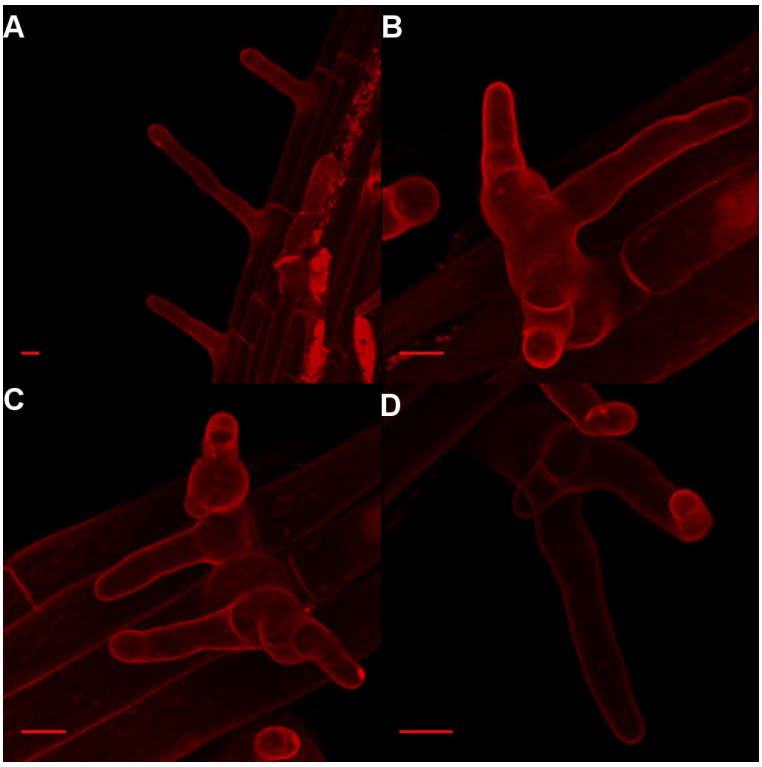

Figure 2. 3D composites of WT plants grown in MS and MS + oryzalin media. A) root hairs seen in MS media, B-D) branched root hairs seen in MS + Oryzalin media, scale bar = 10µm.

In comparison with the wild type root hairs shown in Figure 2A, RHD3 loss of function mutant root hairs show a wavy and deformed phenotype that can be seen in Figure 3. The extent of their curved nature and length tends to vary for different regions of the root, but these results demonstrate the typical shortened, wavy phenotype that characterizes loss of function of RHD3.

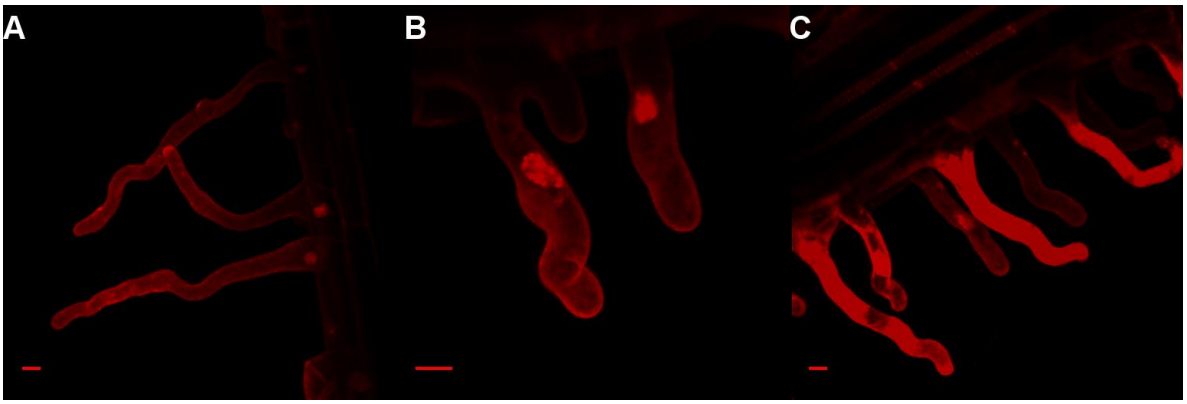

Figure 3. 3D composites of RHD3 Mutants grown in MS media. Three separate sections of typical root hairs of the RHD3 mutant seedlings, A) upper region, B&C) lower region, scale bar = 10µm.

When treated with oryzalin an almost opposite effect to the one observed in the wild type seedlings is observed in the RHD3 mutant plants. Figure 4A shows a region of the root that was allowed to grow in MS media while Figures 4B and 4C show regions grown in MS + oryzalin only. Buds and extremely short roots are characteristic of the oryzalin treated RHD3 mutant seedlings.

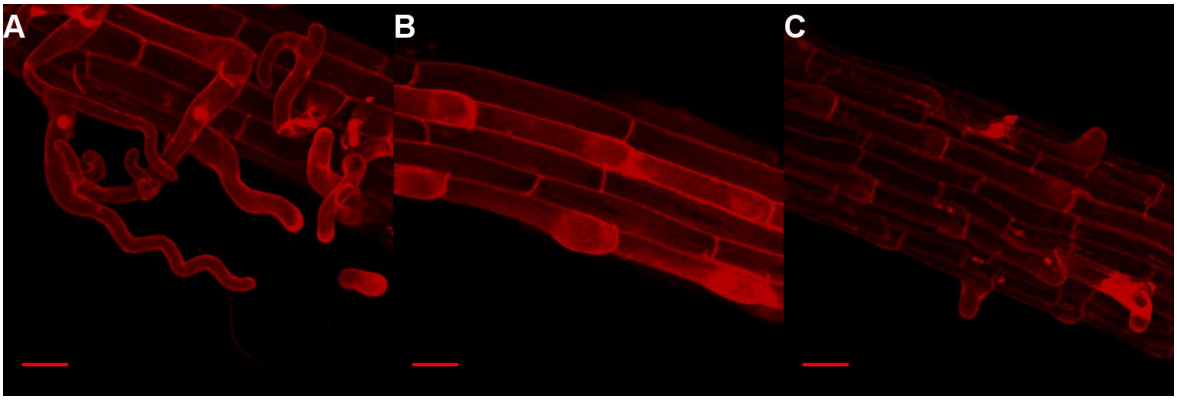

Figure 4. 3D composites of RHD3 Mutants grown in MS + oryzalin media. Three separate sections of typical root hairs of the RHD3 mutant seedlings, A) upper region grown in MS, B&C) lower region grown only in MS + oryzalin, scale bar = 25µm.

A broader view of the scope of RHD3 loss of function root hairs that have been inhibited by treatment with oryzalin can be observed in Figure 5. Although patchiness is seen in both cases, the inhibition of growth of root hairs was characteristically found in long stretches of the RHD3 loss-of-function, oryzalin treated root.

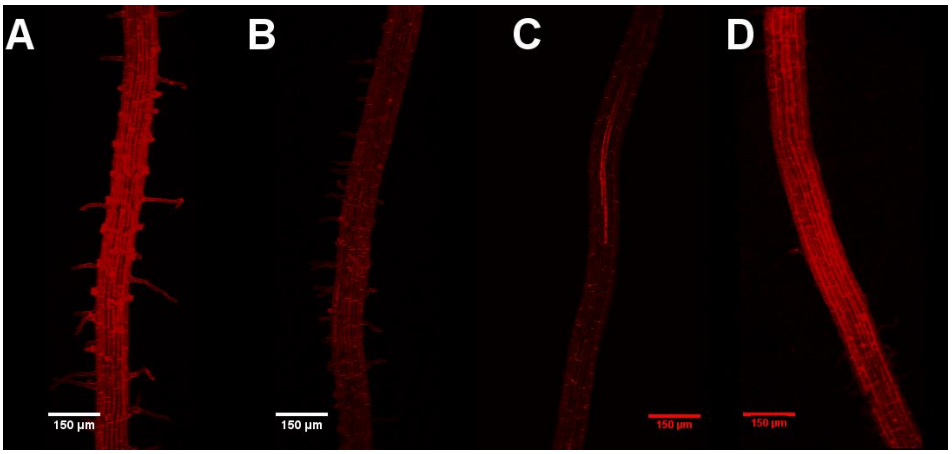

Figure 5. 3D composites of large sections of RHD3 Mutants grown in MS and MS + oryzalin media. Many root hairs are seen on A&B) RHD3 in MS media while few are seen on C&D) RHD3 in MS + Oryzalin media, scale bar = 150µm.

The quantitative effects of oryzalin on root hair growth

The effects of oryzalin on the RHD3 mutant can be quantified by measuring the lengths of the root hairs. Following the first round of collection for this data it was observed that there were patches of the root where root hairs were not able to form and only buds were observed. On that same root however there were patches of fully grown hairs. Thus, in order to compare the relative effects of oryzalin the overall results were compiled into a box and whiskers plot detailing the measured changes, as can be seen in Figure 6. Relative to the RHD3 mutants grown only in MS media the plants grown in MS + oryzalin had a shorter median length of 36.452µm compared to the median length for the MS plants of 91.081µm. The average length of the RHD3 mutant in MS + oryzalin, 36.336µm, was determined to be significantly lower than the average length of the RHD3 mutant in MS, 90.197µm, with a p-value of 1.831 E-39.

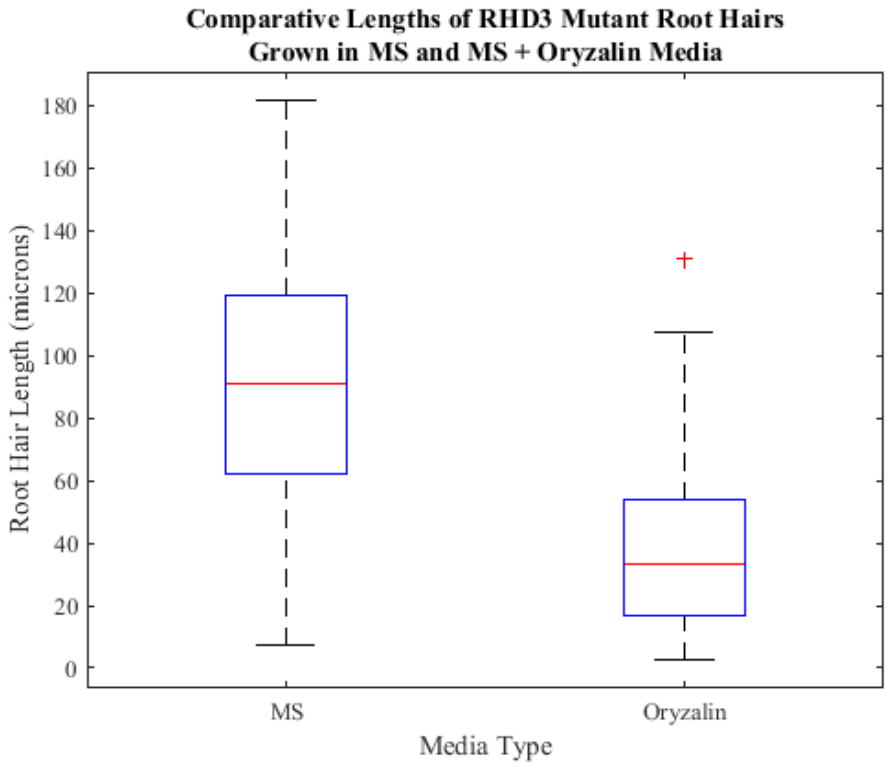

Figure 6. Comparative Lengths of RHD3 Mutant Root Hairs Grown in MS and MS + Oryzalin Media. MS media minimum = 7.455μm, maximum = 181.921μm, median = 91.081μm. and Oryzalin minimum = 2.845μm, maximum = 131.142μm, median = 36.452μm.

SECTION IV

DISCUSSION

The interaction between oryzalin and RHD3

When initially planning on the use of oryzalin, the idea was to use a known method of replicating the RHD3 phenotype in order to determine the way that RHD3 was interacting with the ER in conjunction with the cytoskeletal components of the growing cell. The effect however was completely unexpected and has led to developments in the way that we think about how RHD3, the cytoskeleton, and the ER interact to produce normal root tip growth. In regards to the pathways that RHD3 could be acting on, we hypothesized that in relation to the ER scaffold RHD3 could be involved in either helping the microtubules extend or the interactions between the ER and the plasma membrane. By combining the loss of function of RHD3 with the mild depolymerization of the microtubules using low concentrations of oryzalin we were able to observe whether or not they function in the same pathway. The effect of oryzalin on polarized tip growth can be shown through our replication of the unusual branching effects in wild type plants. This effect shows that when the microtubule network is destabilized it interferes with the establishment of branch points. When this same level of oryzalin treatment is combined with the RHD3 mutation however, the growth of the root hairs is blocked. Root hair buds will typically form, but the impairments to the microtubule network caused by oryzalin combined with the RHD3 mutation has prevented the growth that is usually seen with these two conditions on their own. These results lead us to believe that RHD3 and the microtubules are involved in two different pathways when it comes to the process of polarized root tip growth. If they were on the same pathway a more extreme version of the wavy phenotype likely would have been observed,

but since growth has been altogether inhibited they more likely act on two separate but equally important pathways. Since buds can still form it is clear that the impairment comes in the growth itself rather than the initiation. This corroborates the idea that these two pathways both contribute to the function of the ER scaffold.

Moving forward

While our results showed promise in figuring out the relationship between the ER, RHD3, and the microtubules, there were problems with reliability and consistency. Patchiness of root hair growth was observed in many of the plants, so we can't definitively support our results. In order to improve reliability we will be experimenting with new methods of growing the plants in order to eliminate the manner of the root digging in and out of the agar. This factor is the main one that we suspect as the cause of the patchiness. Combined with this technique we would also like to grow the plants directly onto the slides to prevent malformations of root hairs when transferring from agar plates to slides. Beyond improving reliability we would also like to create more fluorescent lines of *A. thaliana*. It would be beneficial to have fluorescent labels for the RHD3 protein in order to observe its dynamics in the living cell. Fluorescent labels for the ER and cytoskeletal components in RHD3 mutant lines should also be created and further testing should also be performed with long term live cell imaging overnight in order to monitor the growth of the seedlings and observe exactly what is happening to impair root hair growth. Preliminary experiments in the Griffing lab have indicated that RHD3 is bound to the plasma membrane as well as the ER in other plant cell types, so it may also have tethering capabilities. We would like to test this potential quality through colocalization with known ER-plasma membrane tethers such as VAP27.

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