

## SHORT REPORT

# The endoplasmic reticulum exerts control over organelle streaming during cell expansion

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## ABSTRACT

Cytoplasmic streaming is crucial for cell homeostasis and expansion but the precise driving forces are largely unknown. In plants, partial loss of cytoplasmic streaming due to chemical and genetic ablation of myosins supports the existence of yet-unknown motors for organelle movement. Here we tested a role of the endoplasmic reticulum (ER) as propelling force for cytoplasmic streaming during cell expansion. Through quantitative live-cell analyses in wild-type *Arabidopsis thaliana* cells and mutants with compromised ER structure and streaming, we demonstrate that cytoplasmic streaming undergoes profound changes during cell expansion and that it depends on motor forces co-exerted by the ER and the cytoskeleton.

**KEY WORDS:** ER, Cytoplasmic streaming, *Arabidopsis thaliana*

## INTRODUCTION

The rapid movement of organelles, commonly referred to as cytoplasmic streaming, is one of the most crucial and yet least understood feature of the crowded environment of eukaryotic cells. In animal cells microtubule-based organelle motility with dynein and kinesin as motor proteins is well established. In plant cells organelle movement is believed to depend on actin with myosins as motors (Brandizzi et al., 2002a; Brandizzi and Wasteneys, 2013; Esseling-Ozdoba et al., 2008; Mathur et al., 2002; Nebenführ et al., 1999; Shimmen and Yokota, 2004; Sparkes et al., 2009b; Verchot-Lubicz and Goldstein, 2010; Vick and Nebenführ, 2012). Nonetheless, quantitative confocal microscopy analyses of the streaming of the endoplasmic reticulum (ER), Golgi, peroxisomes and mitochondria have demonstrated that myosin inhibitors, as well as loss of single and multiple myosins, cause substantial reduction but not complete loss of organelle streaming (Avisar et al., 2009; Prokhnevsky et al., 2008; Ueda et al., 2010). These observations define a role for myosins as primary motors of cytoplasmic streaming but also underscore that additional unidentified forces contribute to organelle movement in plant cells. It is possible that organelles with a large membrane extension stir other organelles embedded in the cytosol. Indeed a causative relationship between the streaming of the ER and the cytosol has been suggested, based on a similar streaming pattern (Ueda et al., 2010). Proving that the ER movement has a bearing on the dynamics and positioning of the other cellular organelles

would identify a missing piece to solve the puzzle of the nature of the motors that, in addition to the actin–myosin cytoskeleton, are responsible for the unique intracellular motility in plants.

Remarkably little is known about cytoplasmic streaming patterns in plant cells. Cell expansion defects in *Arabidopsis thaliana* myosin mutants support the hypothesis that cytoplasmic streaming is crucial for plant cell growth (Peremyslov et al., 2008; Peremyslov et al., 2010; Tominaga et al., 2013). However, because cytoplasmic streaming analyses in plants have been conducted on fully expanded cells (Peremyslov et al., 2010; Prokhnevsky et al., 2008; Ueda et al., 2010), it is yet unknown whether the movement pattern of plant organelles is uniform during cell growth (Prokhnevsky et al., 2008).

In this work, we aimed to identify the forces that cooperate with the cytoskeleton to maintain efficient organelle streaming during cell expansion. Our underlying hypothesis was that large organelles, such as the ER, function as propelling forces of cytoplasmic streaming during cell expansion. We demonstrate that the shape and dynamics of the ER, as well as the streaming of the other organelles, increase as cells expand, and thus highlight a new parallel between cell expansion and organelle streaming. Furthermore, by extending our studies to cells defective in ER architecture and streaming because of a mutation in the conserved ER-shaping dynamin-like RHD3, we show that aberrant ER structure and streaming negatively affect the motility of the other organelles. Our analyses provide new perspectives into the existing model for cytoplasmic streaming by offering experimental support that during plant cell expansion organelle streaming depends on the combined forces exerted by the cytoskeleton and the ER.

## RESULTS AND DISCUSSION

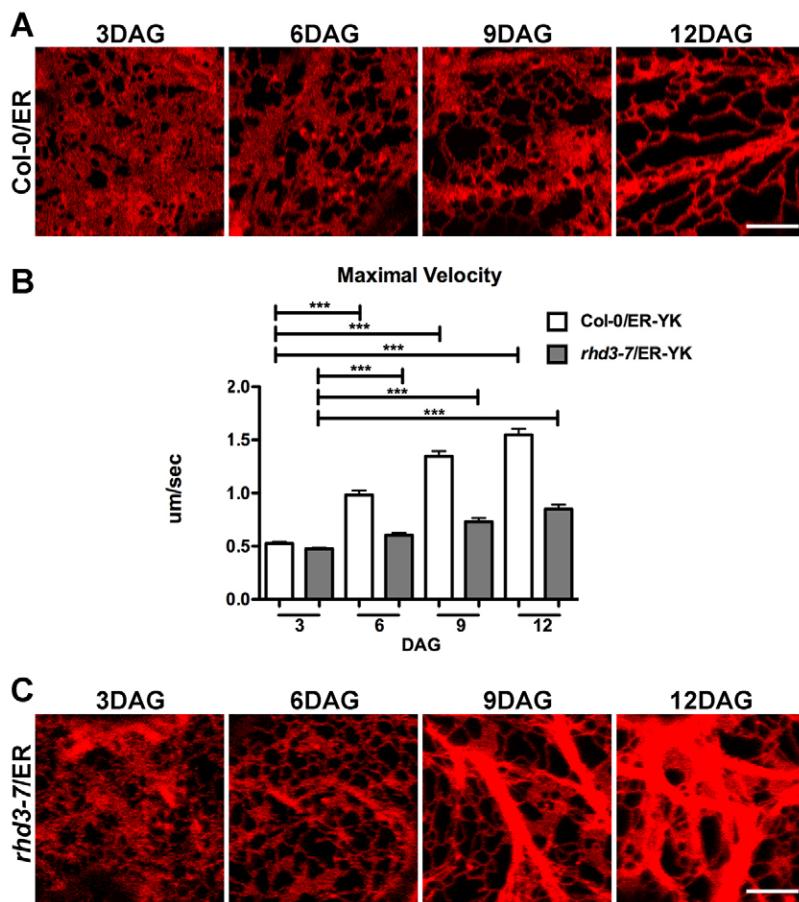
### The ER architecture undergoes substantial changes in morphology and dynamics as cells expand

We first aimed to establish if architecture and streaming are constant features of the ER during cell expansion. To do so, we performed quantitative live-cell confocal microscopy analyses on cotyledon epidermal pavement cells for which expansion phases have been well defined (Zhang et al., 2011). To visualize the ER, we used seedlings stably expressing the inert ER lumen marker ER-YK (Nelson et al., 2007), and analyzed epidermal cells in the apical one-third region of cotyledons at early [3 days after germination (DAG)], rapidly expanding (6 and 9 DAG), and fully expanded cell phases (12 DAG) (Qiu et al., 2002; Zhang et al., 2011). We found that at 3 DAG the ER morphology was characterized predominantly by enlarged perforated sheets of large cisternae (Col-0/ER; Fig. 1A). Such organization radically changed during cell expansion, with a predominant presence of ER tubules and strands at 12 DAG (Fig. 1A; supplementary material Movie 1). Combined with earlier observations that the ER morphology undergoes changes during hypocotyl cell expansion (Ridge et al., 1999), these data support a broad model

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**Fig. 1.** The architecture and dynamics of the plant ER are correlated to cell expansion in an RHD3-dependent manner. (A,C) Confocal images of cortical ER of cotyledon epidermal cells at different phases of cell expansion in wild-type (Col-0/ER; A) and a *rhd3* knockout mutant (*rhd3-7/ER*; C) cells. Scale bars: 5  $\mu$ m. (B) Maximal streaming velocity of ER in wild-type (Col-0) and *rhd3-7* cotyledons expressing the ER lumen marker ER-YK.

that the shape of the ER network can undergo profound changes during cell growth.

To assay for the occurrence of ER streaming variations during cell expansion, we measured the maximal velocity of the ER using live-cell imaging approaches successfully employed previously (Stefano et al., 2012; Ueda et al., 2010). We found that at 3 DAG the ER streaming was characterized by a low velocity (Col-0/ER; Fig. 1B; supplementary material Fig. S1; Movie 1) but that it progressively increased through the transition to rapidly expanding cells (6 and 9 DAG) and in fully expanded cells (12 DAG; Fig. 1B; supplementary material Fig. S1; Movie 1). These data provide evidence that in addition to architecture changes, the ER undergoes significant ( $P<0.001$ ) increases in streaming as cells expand.

The recently established role of the ER-membrane-associated dynamin-like protein ROOT HAIR DEFECTIVE 3 (RHD3) in ER network organization (Chen et al., 2011; Stefano et al., 2012) prompted us to test the involvement of this protein in ER architecture and streaming during cell expansion. Consistent with published reports (Chen et al., 2011), live-cell confocal microscopy analyses of the ER established the occurrence of long and unbranched ER tubules in cells of an *rhd3* null allele transformed with ER-YK [*rhd3-7/ER* (Stefano et al., 2012); Fig. 1C]. Interestingly, we found that the ER defects were only progressively visible as cells underwent rapid expansion (Fig. 1C). We also verified that ER streaming was comparable

between wild type and *rhd3-7* in early phases of cell expansion; however, in fully expanded cells where ER streaming is maximal in wild type, ER streaming was significantly ( $P<0.001$ ) reduced in *rhd3-7* compared with wild-type cells (Fig. 1B; supplementary material Fig. S1; Movie 1). These data support the findings that RHD3 is required in the transition from cisternal to tubular ER architecture. Furthermore, in light of the published evidence for the role of myosin XI-K in ER streaming (Ueda et al., 2010) and our results that loss of RHD3 prevents ER streaming during cell expansion (Fig. 1B), our data also suggest that cytoskeletal and ER-associated proteins co-exert control of ER streaming as cells expand.

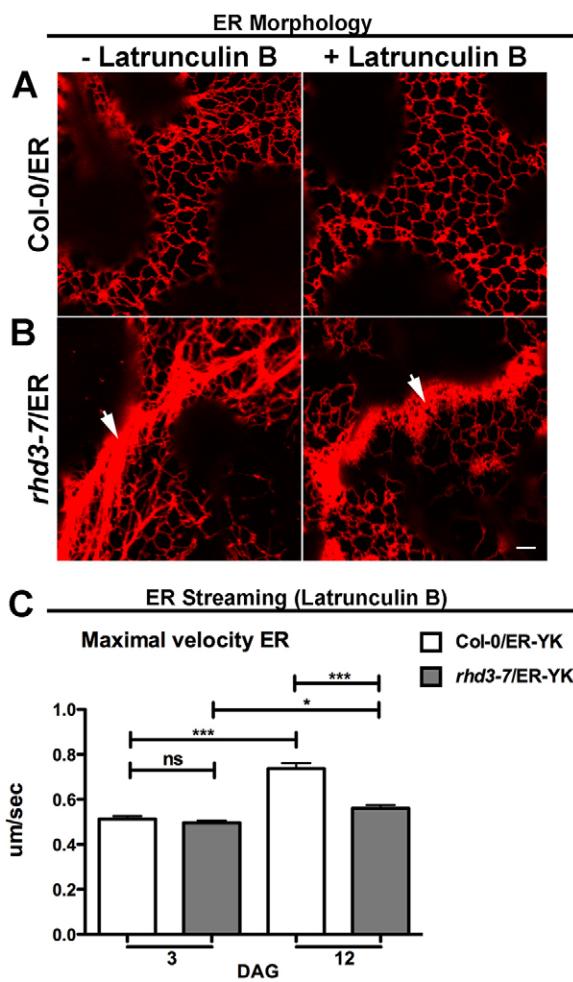
#### RHD3 has a role in ER network integrity that is independent from the actin cytoskeleton

Because defects in actin organization have been seen in an RHD3 loss-of-function mutant (Hu et al., 2003), we aimed to test whether the compromised ER network organization in *rhd3-7* (Fig. 1C) is linked to defects in the actin-myosin cytoskeleton. If this is the case, then depletion of actin in the *rhd3* null background would result in restoration of a wild-type ER phenotype. To test this we treated *rhd3-7/ER-YK* cells with latrunculin B (25  $\mu$ M for 1 hour), which disrupts the integrity of the actin-myosin cytoskeleton through actin depolymerization (Boevink et al., 1998) and found that the ER defects of the RHD3 mutant largely persisted (Fig. 2A,B). These results establish that

RHD3 can influence the ER network structure independently from the cytoskeleton.

#### RHD3 has a cytoskeleton-independent role in ER dynamics

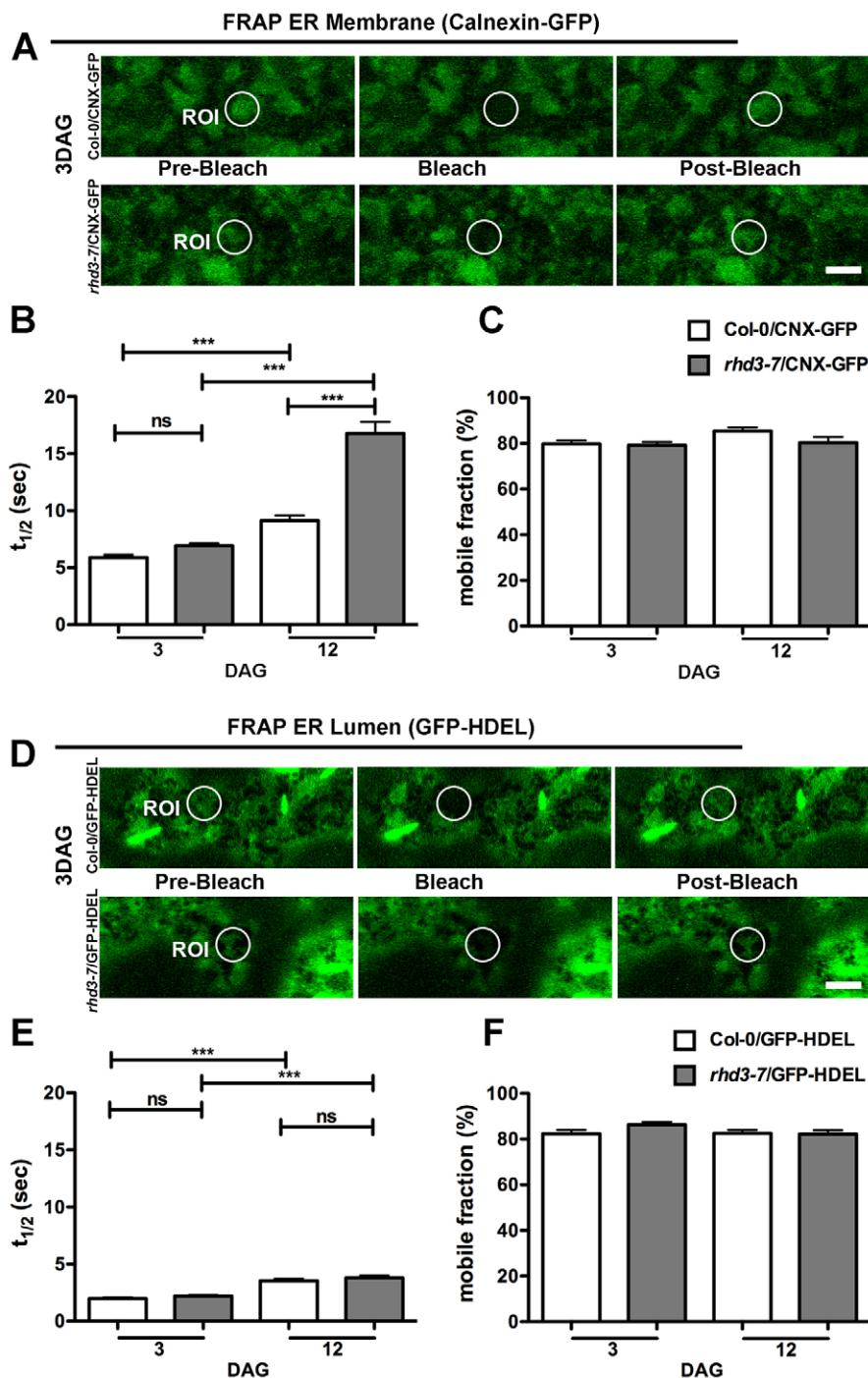
A causative correlation between expression of myosin XI-K, a member of the plant-specific myosin XI class, and ER streaming has been shown in plant cells (Ueda et al., 2010). However, the evidence that chemical disruption of myosins as well as deletion of XI-K alone or in combination with other myosins do not completely abolish ER streaming (Ueda et al., 2010), and that ER streaming changes as cells expand (Fig. 1B) suggested to us that myosins may not be the sole components responsible for the variations in ER streaming during cell expansion (Fig. 1B). In light of the evidence that loss of *rhd3* reduces ER streaming during cell expansion (Fig. 1B) and influences the ER network organization independently from the cytoskeleton (Fig. 2B), we hypothesized that RHD3 has a role in ER streaming that is independent from the cytoskeleton. To test this, we conducted ER streaming measurements in latrunculin B-treated cells and found



**Fig. 2. Defects in ER network integrity due to loss of RHD3 persist in the absence of the actin cytoskeleton.** (A,B) Confocal images of the cortical ER in wild-type and *rhd3-7* knockout mutant cotyledon epidermal cells expressing the ER marker ER-YK, at 12 DAG. The ER structure is shown before and after 1 hour of latrunculin B treatment. In the mutant the compromised ER structure (arrows) persists after 1 hour treatment with latrunculin B. (C) Maximal velocity of the ER in latrunculin B-treated cells in the Col-0/ER and *rhd3-7*/ER-YK background. Scale bar: 5 μm.

that *rhd3* loss resulted in a reduction of ER streaming velocity compared with that of the wild type at 12 DAG (Fig. 2C), when the differences in ER streaming velocities between wild type and the *rhd3* mutant are markedly different (Fig. 1B). The maximal streaming velocity of the ER in fully expanded *rhd3* cells treated with latrunculin B is 0.56 μm/second (Fig. 2C), which is lower than in untreated wild-type (1.55 μm/second) and untreated *rhd3* cells (0.85 μm/second) (Fig. 1B), consistent with an additive role of RHD3 and acto-myosin motors on ER streaming. These data support the underlying hypothesis that myosins may not be the sole ER streaming motors and attribute a role to RHD3 in ER streaming.

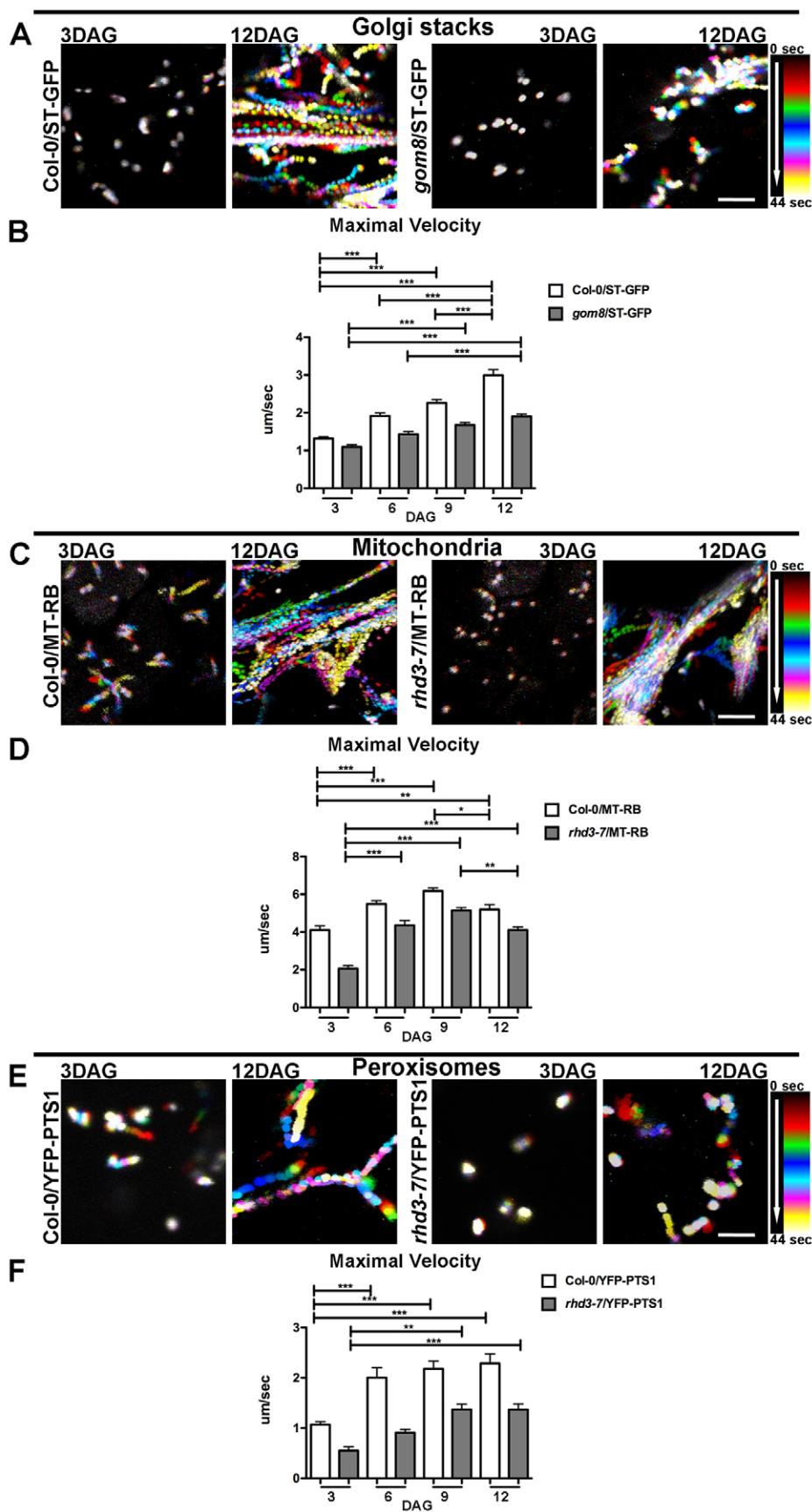
To test whether RHD3 could also have a cytoskeleton-independent role to ensure the flow of membrane and lumen of the ER, we performed fluorescence recovery after photobleaching (FRAP) experiments in wild-type and *rhd3* cells upon latrunculin B treatment. Analyses of calnexin-GFP (CNX-GFP), an ER membrane marker consisting of a GFP fusion to the transmembrane domain and cytosolic tail of an ER lectin (Irons et al., 2003), established that membrane protein mobility was similar between *rhd3-7* and wild-type cells at 3 DAG (Fig. 3A; supplementary material Fig. S2), when the ER streaming is similar in the two backgrounds (Fig. 1B). However, in fully expanded cells where ER streaming is higher in the wild type than in the *RHD3* mutant (Fig. 1B), we found that the mobility of CNX-GFP was greatly reduced in the *RHD3* knockout (Stefano et al., 2012) (Fig. 3A,B). Conversely, FRAP analyses verified a slight but significant ( $P<0.001$ ) increase in the flow of the ER lumen marker GFP-HDEL (Brandizzi et al., 2003) in fully expanded cells compared with cells in early stages of expansion in both the wild-type and *rhd3* knockout cells, but no significant differences between the wild type and the mutant at the same stages of cell expansion (Figs 3D,E; supplementary material Fig. S2). The differences in membrane flow and the absence of significant differences in the flow of ER lumen content between wild-type and *rhd3* mutant cells indicate that loss of RHD3 specifically affects the flow of the ER membrane. RHD3 is an ER-shaping protein with membrane fusogenic ability (Chen et al., 2011; McNew et al., 2013; Stefano et al., 2012; Zhang et al., 2013) with a dominant function compared with the other *Arabidopsis* isoforms RHD3-Like 1 (At1g72960; RL1) and RHD3-Like 2 (At5g45160; RL2). This is supported by the evidence that, unlike *rhd3*, depletion of these isoforms alone does not cause obvious vegetative phenotypes (Chen et al., 2011) and that overexpression of RHD3-Like 2 GTPase mutants does not disrupt ER morphology and Golgi movement (Lee et al., 2013). It has been shown that the C-terminal region of RHD3 that encompasses a membrane-inserted hairpin domain is necessary for the ER-shaping role of RHD3 (Stefano et al., 2012). It is possible that this region influences the organization of the ER lipid bilayer; therefore its absence could cause the observed membrane flow phenotype. It is also possible that a loss of RHD3 compromises the balance required for the functional homeostasis of other ER-shaping properties, such as reticulons. This is supported by the observation that the RHD3 isoform RHD3-Like 2 affects ER morphology only when co-expressed with reticulon RTNLB13 (Lee et al., 2013). Independently of the effect on reduced membrane flow in the *rhd3* mutant, our results indicate that RHD3 is necessary to maintain optimal ER membrane diffusion in fully expanded cells independently from the cytoskeleton. We speculate that the reduced ER membrane fluidity linked to the loss of RHD3 negatively affects the overall ability of the ER to move, and thus compromises ER streaming independently from the cytoskeleton.



**Fig. 3. ER membrane fluidity is influenced by RHD3 availability in the ER membrane.** (A,D) FRAP experiment in cells expressing the ER membrane marker calnexin-GFP (CNX-GFP; A) or the ER lumen marker (GFP-HDEL; D) at 3 DAG. White circles indicate sites of spot photobleaching. Scale bar: 5  $\mu$ m. (B,C,E,F) Analysis of FRAP experiments showing the half time and mobile fraction of CNX-GFP (B,C) and GFP-HDEL (E,F) in wild-type and *rhd3-7* cells at 3 and 12 DAG.

**The ER has a crucial influence on the streaming of other organelles**  
 Evidence that chemical disruption of the actin-myosin cytoskeleton and genetic ablation of myosins do not completely abolish the movement of organelles such as Golgi, peroxisomes and mitochondria (Avisar et al., 2009; Brandizzi et al., 2002a; Peremyslov et al., 2008; Prokhnovsky et al., 2008; Ueda et al., 2010; Vick and Nebenführ, 2012) indicates that additional non-cytoskeletal forces are in place to propel these organelles. The ER

is arguably one of the organelles with the largest membrane extension and it is in close proximity to the other organelles (supplementary material Fig. S3). The evidence described above, that ER streaming does not entirely depend on the cytoskeleton (Fig. 2C), prompted us to test whether the ER network has a bearing on the motility of other organelles. To do so, we first analyzed the movement of Golgi, peroxisomes and mitochondria during cell expansion. We expected that if the ER streaming is



**Fig. 4. Intracellular streaming of organelles is correlated with cell expansion and ER network integrity.** Streaming of Golgi stacks (ST-GFP; A), mitochondria (MT-RB; C) and peroxisomes (YFP-PTS1; E). Colors represent the position of individual organelles over the course of 90 captured frames with dark red representing the position of individual organelles in initial frames and white indicating the position of the organelle in the last frame. Scale bar: 5  $\mu$ m. (B,D,F) Maximal streaming velocity of the organelles in wild-type and *RHD3* mutant cotyledon epidermal cells as described for A, C and E, respectively.

involved in the movement of other organelles, then streaming of Golgi, peroxisomes and mitochondria should parallel the increase in ER streaming observed during cell expansion (Fig. 1B). In accordance with our hypothesis, we verified that the streaming of Golgi, mitochondria and peroxisomes increased during cell expansion (Fig. 4) but was not completely abolished by disruption of actin cytoskeleton (supplementary material Fig. S4). To establish the influence of ER on organelle streaming, we carried the same analyses in *rhd3* loss-of-function mutants (Stefano et al., 2012). Indeed, we verified that organelle streaming was negatively affected in the *RHD3* mutants compared with the wild type (Fig. 4), especially at later stages of cell expansion when the presence of RHD3 has a major role in ER streaming (Fig. 1B). These data support that general organelle streaming is not constant during the growth of vegetative cells and that the presence of RHD3 is necessary to ensure streaming of other organelles besides the streaming of the ER.

Together our data suggest that the ER can influence the streaming of the other organelles. In land plants, the streaming of Golgi stacks, peroxisomes and mitochondria largely depends on the actin–myosin cytoskeleton (Boevink et al., 1998; Mathur et al., 2002; Nebenführ et al., 1999; Zheng et al., 2009). The measurements of ER streaming in this work feature ER protein flow although the analyses also include a component of translational movement of the ER tubules and cisternae. We found that streaming of non-ER organelles is lowest in cells at an early phase of expansion when the ER membrane is enlarged and when the ER is characterized by low levels of streaming and membrane protein movement. Conversely the streaming of non-ER organelles is highest in fully expanded cells when the ER assumes a typical reticulated appearance and the streaming of the ER and ER membrane protein movement peak. We also found that cytoplasmic streaming is altered in the absence of RHD3, and that loss of RHD3 compromises ER streaming and ER membrane fluidity. We propose therefore that cytoplasmic streaming depends on cooperative forces based on the actin–myosin cytoskeleton and movement of ER tubules and cisternae as well as ER membrane fluidity. In this model effective movement of the ER components may contribute to propel the other organelles in the cytosol; conversely, reduced movement of the ER would interfere with actin–myosin-driven streaming of non-ER organelles. The influence exerted by the ER on the motility of the other organelles may be through interactions that are either non-regulated or structured. Connections between the ER and other organelles are known to exist (English and Voeltz, 2013). In plant cells, ER–mitochondria attachment sites have been observed in electron microscopy analyses (Staehelin, 1997). Furthermore, attachment of ER to chloroplasts and Golgi stacks have been demonstrated through experiments based on laser optical tweezers showing that ER tubules follow chloroplasts or Golgi stacks that are pulled by the tweezers (Andersson et al., 2007; Hawes et al., 2010; Sparkes et al., 2009a). Although the nature of the proteins that enable the connections of the ER with other organelles is yet unknown, it has been hypothesized that the ER can contact chloroplasts through hemifused membrane domains (Mehrshahi et al., 2013). Therefore, it is possible that the ER contributes to the movement of the other organelles through heterotypic membrane connections that depend on contacts between proteins or lipid bilayers. Based on the evidence that the fluidity of ER membrane proteins depends on the availability of RHD3 independently of the cytoskeleton, we speculate that it is also possible that in the *rhd3* mutant the movement of the tethers that connect the ER to the

membranes of other organelles is compromised. Therefore, variations in ER membrane diffusion would in turn affect motility of the other organelles.

## MATERIALS AND METHODS

### Plant materials and growth conditions

Homozygous transgenic *Arabidopsis thaliana* lines (ecotype Col-0) expressing ER-YK were obtained through either floral crosses or the floral-dip method (Clough and Bent, 1998), and subsequent selection on Murashige and Skoog (MS) medium supplemented with Gamborg's B5 vitamins, 1% w/v sucrose, the appropriate antibiotics and 0.8% w/v agar. *Gom8* is linked to a missense mutation in the *At3g13870* locus (*RHD3*) (Stefano et al., 2012). *Rhd3-7* is a T-DNA insertion allele of *RHD3* (SALK\_106309) (Alonso et al., 2003; Stefano et al., 2012). Seeds were surface-sterilized and germinated at 21°C under 16 hour light/8 hour dark conditions.

### Confocal laser scanning microscopy

An inverted laser scanning confocal microscope Zeiss LSM510 META (<http://www.zeiss.com/>) and an Olympus Fluoview 1000 (<http://www.olympus.com>) were used for confocal analyses. The fluorescent proteins used in this work were GFP5 (Haseloff et al., 1997), mCherry and EYFP. The markers used in this study were: the ER luminal marker ER-YK (Nelson et al., 2007), which is composed of YFP fused to the signal peptide of AtWAK2 and retained in the ER by the tetrapeptide HDEL; the Golgi membrane marker ST-GFP, which is based on a GFP fusion to the transmembrane domain and cytosolic tail of a rat sialyl transferase (Boevink et al., 1998); the mitochondria marker MT-RB, which is composed of the first 29 amino acids of cytochrome *c* oxidase IV from yeast fused to mCherry; and the peroxisome marker YFP-PTS1, which is composed of a peroxisome-targeting type 1 signal, the tetrapeptide Ser, Lys and Leu, fused to YFP. Imaging settings for single fluorochromes or combinations were as described previously (Brandizzi et al., 2002b; Faso et al., 2009; Hanton et al., 2007). To analyze the velocity of the Golgi stacks, peroxisomes and mitochondria, 30 time-lapse sequences with 90 frames for each sequence in the cortical region (3.0–5.0 μm depth) of cotyledon pavement cells were recorded. Time-lapse frames at a 512×512 pixel resolution were captured using a 2 μm pinhole at low laser power (i.e. 10% of an argon 488 nm laser line) to avoid photobleaching, and 3× digital zoom using an EC Plan-Neofluar 40×/1.30 NA objective. Velocity was calculated post-acquisition using Image Pro-Plus 6 (Media Cybernetics). Velocity values were calculated by averaging the velocity of all the Golgi stacks in the 90 512×512 pixel frames in each time-lapse sequence; then values were calculated as the mean of the velocities estimated in the 50 time-lapse sequences. Maximal velocity values were estimated by averaging maximal values of each time-lapse sequence for each sample.

To evaluate ER streaming, 70–100 time-lapse sequences at 100 frames per sequence in the cortical ER (3.0–5.0 μm depth) were taken of cisternae and bundled tubule regions (see also supplementary material Fig. S1) for each sample at a 256×256 pixel resolution, using a 2 μm pinhole at low laser power (i.e. 5–10% of an argon 514 nm laser line) and 3× digital zoom using a Plan-Apochromat 60×/1.42 NA objective. ER streaming in ER-YK (control) and the *rhd3-7*/ER-YK mutant cells were analyzed using the KbiFlow plug-in available through ImageJ. Values for ER streaming were then calculated as the mean for ER streaming estimated in 75 time-lapse sequences. Maximal velocity values for ER streaming were estimated by averaging values of each time-lapse sequence for each sample.

Statistical analysis was based on one-way ANOVA analysis with Tukey's post tests for velocity measurement and for FRAP data. Asterisks indicate statistical analysis significance: \*\*\*P<0.001; \*\*0.001>P<0.01; \*0.01>P<0.05; n.s., not significant. Error bars indicate s.e.m. Photoshop (<http://www.adobe.com/>) was used for image handling.

### FRAP analyses

The ER in the cortical region of abaxial cotyledon epidermal pavement cells was imaged. Cotyledon cells at 3 and 12 DAG were incubated in

latrunculin B (25 μM) for 20 minutes prior to imaging. For FRAP experiments, ten prebleach scans were captured using settings for GFP with the 488-nm laser transmission set to 1–2% transmission prior to bleaching of a 3.6 μm<sup>2</sup> spot using 30 interactions of a 488/514-nm laser lines set to 100% transmission. The number of bleach events captured was 44, 60, 32 and 32 for Col-0 GFP-HDEL (3 DAG), *rhd3-7* GFP-HDEL (3 DAG), Col-0 GFP-HDEL (12 DAG) and *rhd3-7* GFP-HDEL (12 DAG), respectively. For Col-0 CNX-GFP (3 DAG), *rhd3-7* CNX-GFP (3 DAG), Col-0 CNX-GFP (12 DAG) and *rhd3-7* CNX-GFP (12 DAG) the number of bleaching events was 46, 62, 75 and 63, respectively. Normalized data were plotted using Prism 5 software (GraphPad Software), as described previously (Stefano et al., 2010).

### Competing interests

The authors declare no competing interests.

### Author contributions

G.S., L.R. and F.B. conceived, designed the experiments and wrote the manuscript.

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### Supplementary material

Supplementary material available online at

<http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.139907/-DC1>

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