ER stress signaling requires RHD3,
a functionally conserved ER-shaping GTPase

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ABSTRACT

Whether structure and function are correlated features of organelles is a fundamental question in cell biology. Here we have queried the ability of mutants with defective structure of the endoplasmic reticulum (ER) to invoke the unfolded protein response (UPR), an essential ER signaling pathway. Through molecular and genetic approaches we show that loss of the ER-shaping GTPase Root Hair Defective 3 (RHD3) specifically disrupts UPR by interfering with the mRNA splicing function of the master regulator IRE1. These findings establish a novel role for RHD3 in the ER and support specificity of the effects of ER-shaping mutations on ER function.
INTRODUCTION

The unique reticulated architecture of the endoplasmic reticulum (ER) relies on a dynamic remodeling of interconnected sheets and tubules, as well as tubule initiation, growth and fusion (Chen et al., 2013a; Sparkes et al., 2011). The extent to which disruption of ER architecture affects the function of this essential organelle is largely unknown.

Optimal ER architecture depends on its lipid composition as well as ER-structuring proteins. For example, genetic disruption of the ER phospholipid biosynthetic pathway in Arabidopsis leads to a drastic modification of the ER shape with replacement of tubules by sheets (Eastmond et al., 2010). Furthermore, ER tubule-forming proteins (reticulons and DP1/Yop1p) and dynamin-like GTPases named atlastins (ATLs) have been found to have critical roles in ER structure. While reticulons are required and sufficient to form an ER tubular network, most likely by stabilizing the high curvature of the tubules (Hu et al., 2008; Park et al., 2010; Voeltz et al., 2006), ATLs mediate the process of ER tubule fusion in metazoans (Hu et al., 2009; McNew et al., 2013; Orso et al., 2009). Yeast and plants do not have sequence homologs of ATLs, but they have proteins called Sey1p in *S. cerevisiae* and Root Hair Defective 3 (RHD3) in *A. thaliana* (Hu et al., 2012; Hu et al., 2009), with similar protein signature motifs of ATLs. Similarly to ATLs and Sey1p, RHD3 has a role in ER architecture (Chen et al., 2011; Stefano et al., 2012) and facilitates membrane fusion (Zhang et al., 2013).

In animals and plants, defects in ER structure due to loss of atlastins or RHD3 have been implicated in severe growth and developmental phenotypes, including abnormal tissue growth and embryonic lethality (Audhya et al., 2007; Chen et al., 2011; Park et al., 2010), underscoring that maintenance of an optimal architecture of the ER due to the presence of these proteins has important implications for the life of the organism. Nonetheless, how a loss of function of ER-shaping proteins translates into growth defects at cell and tissue levels is unknown. The smaller size of the aerial and root tissues in *RHD3* loss-of-function mutants compared to wild type (Chen et al., 2011; Hu et al., 2003; Stefano et al., 2012; Wang et al., 1997) is associated to reduced cell elongation (Wang et al., 1997). The evidence that ER export of membrane and soluble fluorescent protein markers is not affected in *RHD3* loss-of-function mutants (Chen et al., 2011) implies that the plant phenotype of *rhd3* is linked to yet-to-be discovered causes that are unrelated to defects in bulk flow from the ER.
Intriguingly, loss of the reticulon Rnt1 in Drosophila has been shown to cause elevated levels of ER stress (O’Sullivan et al., 2012), which is a condition that cells generally experience when the ability of the ER to balance protein synthesis demand and capacity is compromised. In conditions of ER stress, a largely conserved cytoprotective signaling pathway, known as the unfolded protein response (UPR), is activated (Liu and Howell, 2010; Ron and Walter, 2007). Compromised UPR leads to serious conditions and even death in animals and plants (Chen and Brandizzi, 2012; Chen and Brandizzi, 2013; Deng et al., 2011; Iwawaki et al., 2009). The evidence that loss of an ER-shaping protein in Drosophila activates the UPR suggests that the homeostasis of ER-shaping proteins influences not only ER morphology but also a critical cellular response that is related to the function of this organelle.

In this work we aimed to establish whether loss of proper shape affects functional aspects of organelles by querying the ability of the ER to respond to stress in genetic backgrounds that have defects in ER network integrity. Amongst the mutants tested, we found that loss of RHD3 negatively affects the UPR activation arm mediated by the major ER stress sensor, IRE1. Our data demonstrate a novel requirement of RHD3 in cell physiology besides a known role in ER architecture (McNew et al., 2013; Stefano et al., 2012; Zhang et al., 2013), and show that ER network integrity can be correlated to a function of the ER although the phenotype is linked to specific ER architecture mutations.

RESULTS AND DISCUSSION

ER architecture defects affect UPR activation in a background-specific manner

To establish whether defects in the structure could affect the ER ability to evoke the UPR, we adopted mutant backgrounds with marked ER architecture defects (Supplemental Figure 1). Specifically, we used pah1 pah2 (herein referred as to pah1/2), a double knock out of two phosphohydrolase genes involved in the biosynthesis of phospholipids, in which the ER tubules are converted into sheets (Eastmond et al., 2010). We also used mutants with deformed ER network in which ER tubules are intertwined into large globular structures: gold36/MVP1/ERMO3 (herein referred as to gold36), which is linked to a loss of function mutant of a pseudo-lipase (Agee et al., 2010; Marti et al., 2010; Nakano et al., 2012), and g92/ERMO2
(herein referred as to \( g92 \)), which is a partial loss of function of the COPII coat component AtSEC24A (Faso et al., 2009; Nakano et al., 2009). In addition, we adopted mutants of RHD3, specifically a null allele (\( rhd3-7 \)) (herein referred as to \( rhd3 \)) as well as a mutant bearing a non-silent missense mutation (\( gom8 \)) (Stefano et al., 2012), which have long unbranched ER tubular structures similar to mutations linked in atlastins and Sey1p in metazoans and yeast, respectively (McNew et al., 2013; Orso et al., 2009; Zhang et al., 2013).

To test whether these mutants have defects in the UPR, we analyzed variations in mRNA abundance of well-established UPR molecular markers in the absence or presence of the ER-stress inducer tunicamycin (Tm) using quantitative RT-PCR (qRT-PCR) (Chen et al., 2013b; Chen and Brandizzi, 2012). In marked contrast to all the other backgrounds, in the \( rhd3 \) mutants the induction levels of UPR genes were significantly lower compared to wild type in conditions of ER stress (Figure 1B). Nonetheless, compared to wild type, in \( rhd3 \) the basal levels of UPR gene transcripts as well as the transcript levels of genes encoding either secretory or cytosolic proteins were similar (Supplemental Figure 2). Together these findings show that \( RHD3 \) is required for the expected increase of UPR gene transcripts in conditions of induced ER stress and that this phenotype is not a general feature of mutants with abnormal ER morphology.

Next, we analyzed the expression of UPR indicators in loss-of-function backgrounds of the other RHD3 isoforms \( RHD3-L1 \) and \( RHD3-L2 \) (Chen et al., 2011) and found that \( BiP3 \) transcript levels were unaffected in the knock-outs compared to wild type (Supplemental Figure 3). Although we cannot exclude that RHD3-like proteins and RHD3 may share partially overlapping roles in the UPR, these data indicate that \( RHD3 \) loss has a predominant impact on the plant’s ability to respond to ER stress compared to the other \( RHD3-like \) genes. The pathway components of the UPR are essential: when the cell’s adaptive responses to stress are insufficient, cells enter apoptotic death. Since in the absence of RHD3 cells fail to actuate the UPR properly, we suggest that the reported lethality of higher order mutations within the RHD3 family (Zhang et al., 2013) may be at least partially linked to the inability of cells to evoke the UPR efficiently during growth, which imposes physiological stress on the ER.
RHD3 is required for efficient splicing of the mRNA of the AtIRE1-ER stress target, bZIP60

In animals and plants, IRE1, an ER-associated protein kinase and ribonuclease, functions as a major ER stress sensor and transducer (Chen and Brandizzi, 2012; Tirasophon et al., 2000; Urano et al., 2000). The Arabidopsis genome encodes two sequence homologues of IRE1, AtIRE1A and AtIRE1B (Chen and Brandizzi, 2012; Koizumi et al., 2001; Noh et al., 2002). Unlike RHD3 loss, AtIRE1 loss does not affect ER structure in optimal conditions of growth and under ER stress induction (Supplemental Figure 1B). To understand how RHD3 could affect the UPR, we tested whether RHD3 loss could compromise AtIRE1 signaling in ER stress using a genetic approach. We generated a triple mutant, hereby named ire1/rhd3, by crossing atire1a atire1b double mutant (Chen et al., 2013b; Chen and Brandizzi, 2012) with rhd3, and tested its sensitivity to a Tm concentration in the medium that inhibits growth of ire1 for comparison to wild type, rhd3 and ire1. We found that rhd3 grew similar to wild type, while the triple mutant showed sensitivity to Tm similar to ire1 mutant (Figure 2A). Next, we measured the induction levels of UPR genes in conditions of ER stress in wild type, rhd3, ire1 and ire1/rhd3 mutants (Figure 2B). As expected, we found a reduction of UPR transcript genes in ire1 and in rhd3; notably however, in ire1/rhd3 the reduction of transcript levels of UPR genes was similar to ire1 (Figure 2B). The oversensitive phenotype of ire1 to Tm treatment compared to wild type and rhd3 highlights that, contrarily to RHD3, AtIRE1 is essential to respond to ER stress. Together with the evidence that the induction of UPR genes in the ire1/rhd3 mutant exposed to Tm is comparable to ire1 and significantly different from rhd3, the results also support that RHD3 acts upstream AtIRE1 in ER stress responses.

In all eukaryotes, the IRE1 RNase domain initiates splicing of mRNAs encoding bZIP transcription factors, namely XBP1 in mammalian cells, HAC1 in yeast and bZIP60 in plants (Cox et al., 1997; Deng et al., 2011; Kawahara et al., 1997; Moreno et al., 2012; Plongthongkum et al., 2007; Shen et al., 2001; Sidrauski and Walter, 1997). Similarly to the effect of RHD3 deletion on the UPR, loss of bZIP60 splicing interferes with UPR activation (Moreno et al., 2012). Although bZIP60 is downstream AtIRE1 in the UPR signaling, a bZIP60 loss-of-function mutant does not show the ER stress oversensitive phenotype that is typical of ire1 (Chen and Brandizzi, 2012; Deng et al., 2013) (Figure 3A), indicating that in addition to the splicing of
bZIP60 mRNA, AtIRE1 has other roles that are essential to cope with ER stress. Therefore, a lack of a ER stress oversensitive phenotype of RHD3 and bZIP60 mutants [Figures 3, (Deng et al., 2013)], and the evidence of a genetic interaction between RHD3 and AtIRE1 for UPR induction (Figure 2) suggested that RHD3 loss could affect the AtIRE1-splicing of bZIP60 mRNA. To test this, we measured the abundance of spliced bZIP60 (sBZIP60) mRNA as a readout of AtIRE1 activity in the UPR in wild type and rhd3 alleles in normal conditions of growth as well as in the presence of Tm. We found that in the rhd3 alleles, sBZIP60 transcript was detectable but at significantly reduced levels compared to wild type (Figure 3B). These data support that the defects in UPR gene induction in RHD3 loss-of-function backgrounds during ER stress is linked to interference with the AtIRE1-mediated splicing of bZIP60 mRNA. Upon UPR activation in yeast and in mammalian cells, IRE1, which is distributed over the ER network in physiological conditions of growth, localizes into dynamic clusters in the ER, which are supposed to function as specialized molecular microenvironments for IRE1 signaling in the UPR (Li et al., 2010). Whether AtIRE1 also undergoes dynamic clustering in the UPR is unknown but it is likely given the conservation of IRE1 (Chen and Brandizzi, 2013). We speculate that the disruption of optimal membrane rearrangements due to loss of RHD3 may reduce the ability of AtIRE1 either to traffic through the ER membrane to form signaling clusters or interact efficiently with its bZIP60 mRNA substrate.

The involvement of RHD3 in ER stress responses and in physiological growth is uncoupled

Mutants with defective expression of either RHD3 or AtIRE1 show an obvious phenotype in the elongation of primary root (Chen et al., 2011; Chen and Brandizzi, 2012; Hu et al., 2003; Stefano et al., 2012; Wang et al., 1997) (Figure 4A), but the underlying mechanisms are unknown. In order to investigate whether RHD3 and AtIRE1 interact in the control of organ growth, we analyzed the roots of the ire1/rhd3 mutant for comparison to wild type, rhd3 and ire1. The length of the primary root was shorter in ire1/rhd3 than in the respective rhd3 and ire1 mutants, supporting an addictive interaction in root growth (Figure 4). Further support to this observation was provided by quantitative semi-automated cell segmentation analyses (French et al., 2012) specifically designed for the unbiased identification of the transition zone, which marks the boundaries of the division zone and the elongation zone (Figure 4C,D). Consistent with the
length measurements of the primary root, in *rhd3* and *ire1* the division zone was shorter than in wild type and even shorter in the *ire1/rhd3* mutant (Figure 4C), further demonstrating that the triple mutant has additive phenotypic defects of *ire1* and *rhd3* mutations. Together these data indicate that even if the function of AtIRE1 in UPR signaling largely depends on the cellular availability of RHD3, AtIRE1 and RHD3 have independent roles in physiological organ growth. It cannot be excluded however, that RHD3 and AtIRE1 may share the control of some critical components in pathways necessary for root growth. During physiological growth, which requires enhanced production of the building blocks of the cell and cell wall, loss of AtIRE1 is likely to compromise protein synthesizing capacity of the ER. On the other hand, loss of RHD3 function is known to interfere with the subcellular positioning and movement of the Golgi apparatus (Chen et al., 2011; Stefano et al., 2014; Stefano et al., 2012). Therefore, AtIRE1 and RHD3 may be controlling convergent pathways in organ growth through functionally parallel routes whereby loss of AtIRE1 may disrupt the production of proteins necessary for organ growth, while disruption of RHD3 may lead to aberrant subcellular distribution of such proteins due to a spatial disorganization of the critical sorting organelles such as the Golgi.
MATERIALS AND METHODS

Tm treatment

Tm (Sigma, T7765; dissolved in DMSO) was directly added in the medium as follows: 0.5 µg/ml for UPR induction analyses and 0.05 µg/mL for testing sensitivity to ER stress. Mock control: Tm volume was replaced by the same volume of DMSO (Chen and Brandizzi, 2012).

qRT–PCR analysis

Total RNA extraction and qRT-PCR were performed in triplicate, as described earlier (Chen and Brandizzi, 2012). Relative expression levels were normalized to that of UBQ10. Values are representative averages from three technical replicates. Similar patterns of expression were observed in three independent biological replicates. Primers and AGI numbers are provided in Supplemental Table 1. Col-0 was used as wild-type reference genotype.

Root measurements

To establish the position of the transition zone, the Cell-o-Tape macro (open source ImageJ/Fiji) was used (French et al., 2012). The CellSeT software was used to build the root segmentation based on the propidium iodide staining (1 µg/ml), as described earlier (Pound et al., 2012).

Statistical analyses

Statistical analyses included either Student's two-tailed t-test, assuming equal variance or unpaired t-test (Figure 4).
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**FIGURE LEGENDS**

**Figure 1.** Unlike other ER mutants, *rhd3* shows compromised UPR

(A, B) 14-day-old seedlings of the indicated genotypes were transferred to growth medium containing Tm (0.5 µg/ml) for 1 day. Samples were analyzed by qRT-PCR for transcriptional levels of the UPR indicators *BIP3, PDIL* and *ERDJ3A*. Values are presented relative to indicated DMSO control, which was set to 1. Error bars represent SE of three replicates. Data significantly different from the corresponding controls are indicated (mutant versus wild type under treatment: *P < 0.05, **P < 0.01, NS, not significant).

**Figure 2.** RHD3 and IRE1 work dependently in UPR signaling

(A) To examine Tm sensitivity of Col-0, *ire1, rhd3, ire1/ rhd3*, seeds were sown on solid medium containing Tm (0.05 µg/mL). Images show the appearance of 14-day-old seedlings grown on control (DMSO) or Tm plates.

(B) qRT-PCR of different UPR indicators. cDNA was synthesized using 14-day-old treated with Tm (0.5 µg/mL) for 1 day. Values are presented relative to indicated DMSO control, which was set to 1. Error bars represent SE among three replicates. Data significantly different are indicated (*P < 0.05, ***P < 0.001, NS: not significant).

**Figure 3.** Loss of *RHD3* results in reduced splicing of *bzip60* mRNA under ER stress

(A) To examine Tm sensitivity of *bzip60, ire1, rhd3* and *gom8*, approximately 36 seeds of each genotype were sown on growth medium containing Tm (0.05 µg/mL). The images of representative seedlings shown in the figure were captured at 10 days after germination. Col-0 was used as control. Note that wild type, *bzip60, rhd3* and *gom8* did not show the strong Tm sensitivity of *ire1*. 

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14-day-old seedlings were transferred into liquid growth medium containing Tm (0.5 µg/mL) for 2 hrs. Abundance of spliced bZIP60 mRNA in two independent rhd3 alleles was established by qRT-PCR in non-treated samples (0 hrs) or in Tm-treated samples (2 hrs). Values are presented relative to indicated DMSO control, which was set to 1. Error bars represent SE. (**P<0.01).

**Figure. 4. Analyses of ire1/rhd3 support a synergistic interaction between RHD3 and IRE1 in the control of primary root elongation**

(A) 10-day-old seedlings of wild type, ire1, rhd3 and ire1/rhd3 were grown on solid growth medium on vertical plates.

(B) Measurements of the primary root length of wild-type, ire1, rhd3 and ire1/rhd3 seedlings (n=30-35/genotype). Error bars represent SE. (****P<0.0001, NS, not significant; Unpaired t-test).

(C-D) Landscape analyses of root defects. Images of primary roots stained with propidium iodide to counterstain the cell wall (C) were segmented using CellSeT software, and the TSZ was identified using the Cell-o-Tape macro (see methods section). In C, a dotted line marks the transition zone (TSZ) based on the coordinates established in panel D. From these analyses it appears that the division zone is shorter in the ire1/rhd3 mutant compared to rhd3 and ire1. EZ: elongation zone; DS: division zone; CS: cell size; CN: cell number.

**SUPPLEMENTAL TABLE**

**Supplemental Table 1.** List of primers and Arabidopsis gene identifiers (AGI).

**SUPPLEMENTAL FIGURE LEGENDS**

**Supplemental Figure 1.** ER phenotypes of Arabidopsis mutants with either defective ER structure or UPR signaling.
(A) Representative images of 12-day-old cotyledon epidermal cells with the following genotypes: wild type, *pah1/2, gold36, rhd3* and *g92*, either stained with Rhodamine-B (top panel) or expressing the ER lumen marker (ER-YK). Note that Rhodamine-B stains the ER network (arrows) and Golgi stacks (arrowheads). Inset indicates the inner region of the cell.

(B) Col-0 or *ire1* expressing the ER marker GFP-HDEL (lumenal marker) or calnexin-GFP (CNX-GFP; membrane marker) in control conditions (DMSO) and Tm (0.5 µg/ml) treatment for 1 day. No obvious differences were noted in the morphology of the ER in response to the treatment and in the two backgrounds. Scale bars= 5 mm.

**Supplemental Figure 2. Loss of RHD3 does not affect the basal levels of the UPR and the genes encoding secretory and cytosolic proteins in conditions of ER stress**

(A) qRT-PCR of UPR indicators. cDNA was synthesized using 14-day-old seedlings exposed to medium containing DMSO, which serves as ER stress treatment control, for 1 day. The experiment shows that the basal levels of UPR indicators in wild type and *rhd3* are low and similar.

(B, C) Transcriptional levels of secretory proteins such as the prevacuolar sorting receptor, BP80, the ER associated VAMP-like protein, PVA12, and the tonoplast intrinsic protein, TIP4, and AtIRE1 isoforms IRE1A, IRE1B, as well as cytosolic proteins actin (ACT3) and tubulin (TUB4). Error bars represent SE among three replicates. (NS, not significant).

**Supplemental Figure 3. The compromised UPR induction phenotype is specific to the loss of RHD3**

qRT-PCR of UPR indicators in wild type and *RHD3*-like mutants. cDNA was synthesized using 14-day-old seedlings treated with Tm (0.5 µg/ml) for 1 day. Values are presented relative to indicated DMSO control, which was set to 1. Error bars represent SE of three replicates. No significant differences were found from the corresponding controls (mutant versus wild type under treatment). (NS, not significant).
Figure 1

(A) Relative expression of BIP3, PDIL, and ERDJ3A in different genotypes and treatments. The graphs show the relative expression levels of BIP3, PDIL, and ERDJ3A in Col-0, pah1/2, gold36, and g92 genotypes under DMSO and Tm treatments. The expression levels are compared using NS (not significant) and statistical significance markers (*, **).