EFFECT OF HYPEROSMOTIC STRESS ON THE NETWORK MORPHOLOGY AND TRANSPORT FUNCTION OF THE ENDOPLASMIC RETICULUM IN TOBACCO

A Senior Scholars Thesis

by

OPEYEMI SAMSON ADENIJI

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2011

Major: Biology

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Approved by:

Research Advisor: Lawrence R. Griffing
Director for Honors and Undergraduate Research: Sumana Datta

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ABSTRACT

Effect of Hyperosmotic Stress on the Network Morphology and Transport Function of the Endoplasmic Reticulum in Tobacco. (April 2011)

Opeyemi Samson Adeniji
Department of Biology
Texas A&M University

Research Advisor: Dr. Lawrence Griffing
Department of Biology

To study the effect of hyperosmolarity on the dynamic morphology of the endoplasmic reticulum (ER) form change, *Nicotiana benthamiana* seedlings with ER-labeled GFP-HDEL were put in a culture chamber and gradually infused with high D-sorbitol concentrations. The ER was observed by time lapsed confocal microscopy. Under high osmotic stress, the dense reticulated network morphology of the ER reduced to a web or thread-like sparse connections. Further observational changes were decreased ER activity and increased cisternal ER domains. ER Recovery after replacing the culture chamber with MES buffer was rapid. FRAP analysis revealed that ER recovery after photobleaching in cisternae was more rapid than recovery in tubules. In addition, flow of molecules within ER post-treatment with high osmotic concentration was diffusional rather than directional. These results are consistent with many of the physiological stress response mechanisms that have been investigated.

DEDICATION

To whom has given humanity the ability to unravel mysteries

ACKNOWLEDGMENTS

I would like to thank my research advisor, Dr. Griffing, for his guidance and professional assistance through the course of this research. His support and encouragement are invaluable.

In addition, I appreciate the assistance of my colleague, Betsy Lewis, with experiments in the laboratory.

Finally, words cannot describe how grateful I am to my parents, the Goens, and the Board of the “A Samson Project” who without their love and support I would not have had this opportunity.

NOMENCLATURE

2D	Two Dimensional
3D	Three Dimensional
ER	Endoplasmic Reticulum
FRAP	Fluorescence Recovery After Photobleaching
GFP	Green Fluorescence Protein
MES	2-(*N*-morpholino)ethanesulfonic Acid
min	Minutes
MS agar	Murashige & Skoog Agar Medium
PM	Plasma Membrane
rt	Room Temperature
s	Seconds

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CHAPTER I

INTRODUCTION

The ER (endoplasmic reticulum) is the port of entry into the secretory pathway in cells and forms a dynamic web of membrane tubules and cisternae that pervade the cytoplasm. Its basic underlying organization is polygonal network of tubules connecting to and anastomizing into, sheet-like cisternae (Sparkes, et al. 2009a). It has been shown that the ER form undergoes rapid remodeling in an actin dependent manner and that the basic idea underlying its dynamic morphology is that the network arises as a result of tubule cycles of growth and shrinkage (Griffing, 2010; Sparkes, et al. 2009b). The ER has an organizing persistent structure of tubules and punctae upon which variations are displayed. This tubular network may provide an efficient delivery system by reduced dimensionality thus facilitating directionality to the diffusion of proteins in a cell. Diffusion to a particular organelle occurs more rapidly if it occurs within a 2D or near 2D cisternae and even more rapidly along a 1D or near 1D tubule (Griffing, 2010; Mirny, 2008).

In addition, studies have revealed that hyperosmotic stress reduces diffusional flow of misfolded glycoproteins (Nagaya, et al. 2008). On the other hand, depolymerization of actin and expression of truncated tail domain of myosin XI-K cause cisternalization and

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This thesis follows the style of *The Plant Cell*.

change from directional flow, but does not decrease the rate of diffusional flow within the ER. Will directionality of transport within the ER change under hyperosmotic conditions? Will ER morphology change? Will diffusional flow itself be diminished under conditions of hyperosmolarity. These are the questions this study addresses.

CHAPTER II

RESULTS

Hyperosmotic treatment causes a shift from tubular network to cisternal network

When cells are treated with osmoticum (0.75 M D-sorbitol in 10 mM MES buffer) in a perfusion culture chamber, the ER change in form can be monitored over time. The change in the general morphology of the ER when exposed to a hypersomotic medium is shown in Figure 1. Figure 1A shows untreated, control tobacco petiole cells expressing the fluorescent marker GFP-HDEL. Frames 1 -49 (interval of two frames) of a time-lapse image series show a representative region of the cell cortex rich in dynamic ER, and showing typical tubular morphology and active streaming. A representative image series of the plant petiole reveals that increased cisternalization occurs post-treatment with D-sorbitol, with the first noticeable change at about 25min showing. During the early stages of the D-sorbitol treatment, e.g. 5min, the dynamic activity of primarily tubular ER is observed. However, by 20min following treatment, streaming slowed. At 24min time point, cells begin to plasmolyze, with the protoplast shrinking away from the outer wall, out of plane of focus in some cells. ER movement almost comes to a complete halt late in treatment (about 59min) and its dense reticular nature becomes reduced to a sparse web that is highly cisternalized. Similar cisternalization has been seen after treatments that disrupt the actin cytoskeletal system, but not the microtubule cytoskeleton system (Sparkes et al. 2009a). Cisternalization also occurs after treatment with permanent acids e.g. acetic acid 5mM, pH4 (Quader et al. 1989).

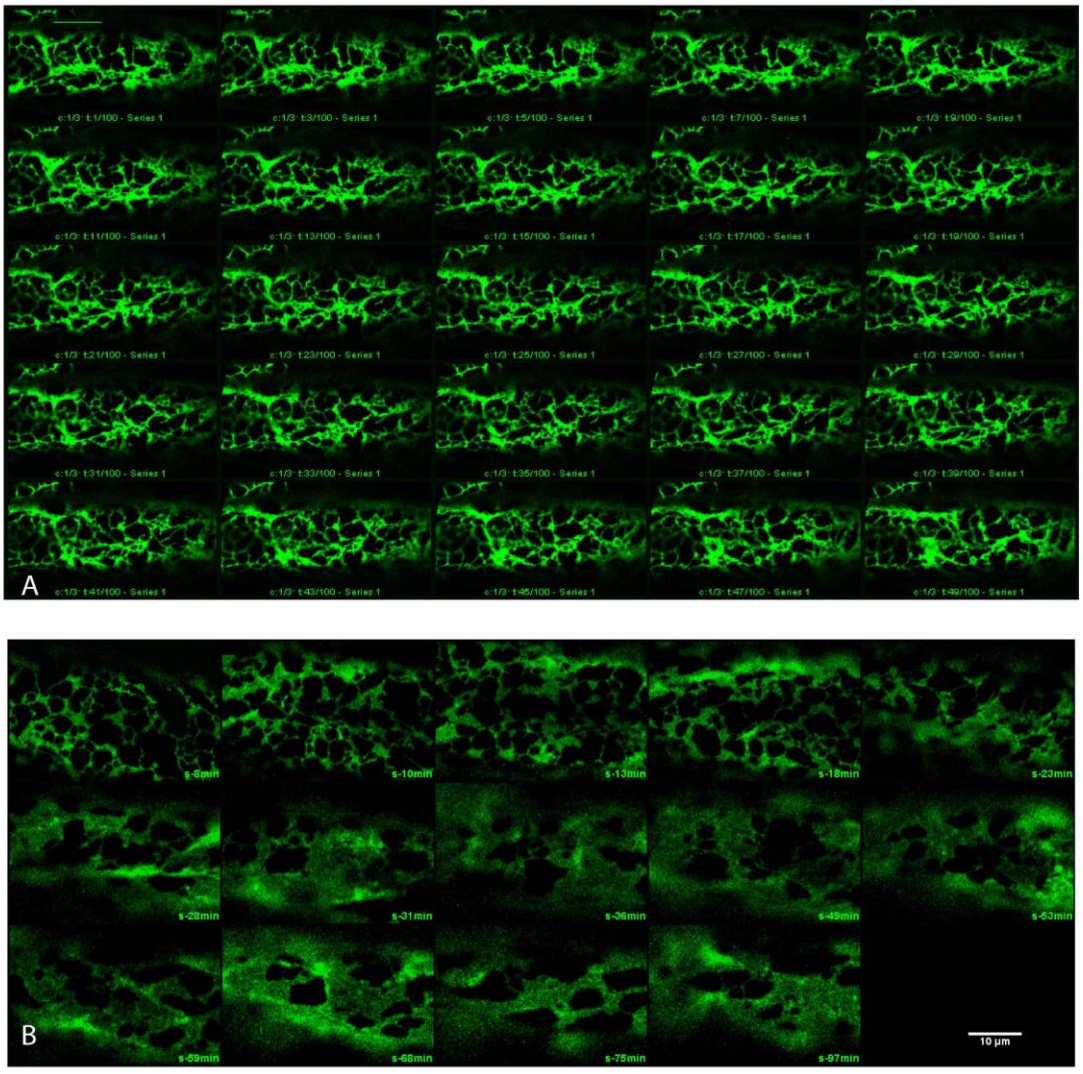


Figure 1. Form of the ER Changes with D-sorbitol Treatment.

A) Persistency of ER morphology in control tobacco petiole cells expressing GFP-HDEL. A 100-frame movie with an interval of two frames depicting the dynamic and rich ER network- MES buffer pre-treatment.
B) Hyperosmotic treatment causes a shift from tubular network to cisternal network. 's', indicates D-sorbitol treatment.
Scale bar = 1 micrometer.

FRAP analysis of hyperosmotic stress

FRAP involves directing a strong pulse of laser which bleaches, or extinguishes the fluorescence of tagged GFP. By selectively photobleaching a defined tubule region, recovery can be monitored over time, as the remaining fluorescent molecules move into the bleached area. Three parameters can be measured during FRAP of ER tubules.

1) The amount of fluorescence that returns relative to the amount of fluorescence that was there before photobleaching. This is calculated as percent recovery and is a measure of the mobile fraction of the fluorescent probe.

2) The speed at which fluorescent molecules migrate back into the photobleached area. This is a measurement of the "diffusional mobility".

3) The directionality of movement within the ER. A ratioed image series Over a time course will reveal directionality of molecules over that time period.

The following figures illustrate the effect of osmotic stress on each of these parameters. Figure 2 illustrates parameter (1) and (2), while Figures 3 and 4 illustrate parameter (3).

Diffusional mobility and half-time recovery

Figure 2 (A) and (B) are ER of two different tobacco epidermal cells of the same plant. Figure 2A shows the ER network in an untreated control cell perfused with 10 mM MES alone, while Figure 2B shows a cell 30min post-treatment with 0.75 M D-sorbitol. As can be seen, the fluorescence rapidly recovers within eleven frames (0.3 seconds/frame,

or 3.3 seconds) in the control, Figure 2A, but recovery is delayed several frames longer in the treated sample, Figure 2B.

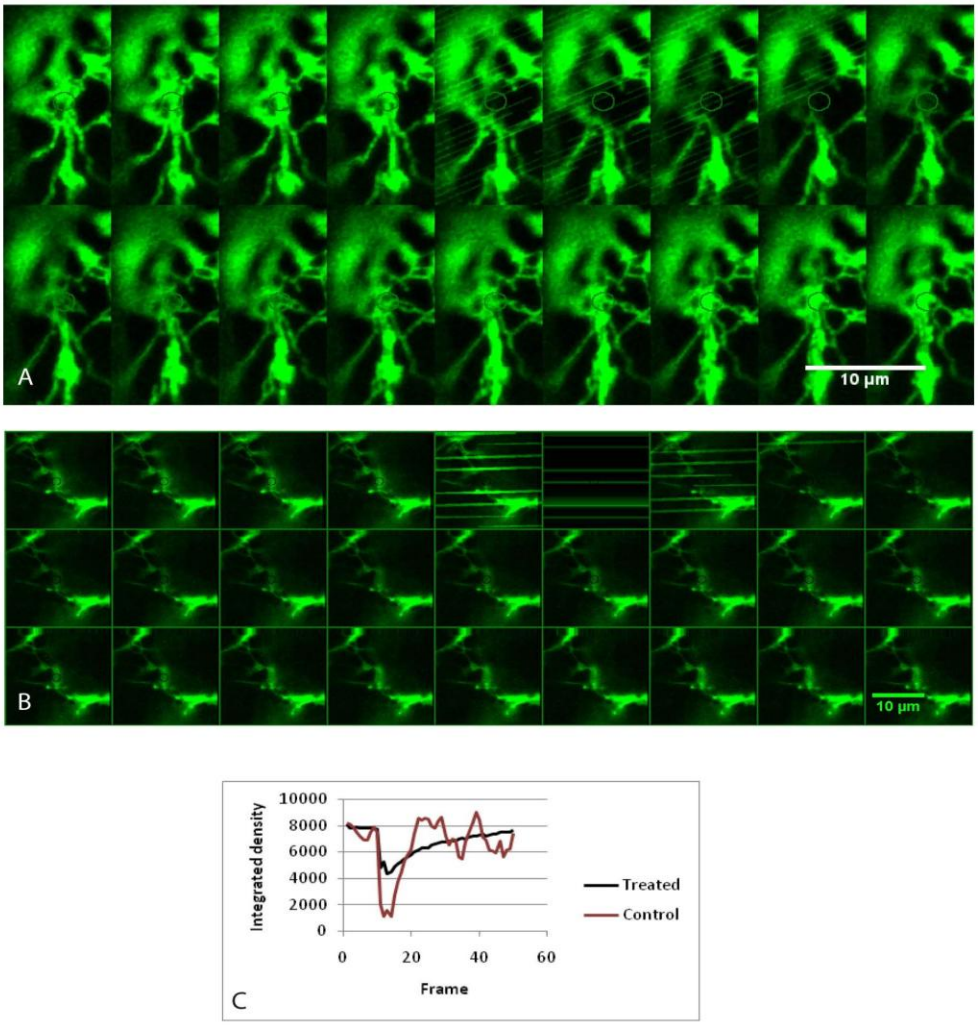


Figure 2. Hyperosmotic Treatment Changes the Speed of Movement in the ER Network, but not the Mobile Fraction.

A) Pretreated control cells showing ER morphology without osmotic treatment. In this figure, the mobility of molecules into photobleached area is rapid. (Circle indicates photobleached spot).
B) ER 30min post-treatment with 0.75M D-sorbitol. The mobility of molecules after photobleaching is slower than in control (A). (Circle indicates photobleached spot).
C) Fluorescence recovery in the photobleached spot in A (red line, control) and B (black line, treated).

Mobile fraction and diffusional mobility gradually change with time of treatment

Figure 3 shows a time course of the FRAP of a representative group of cells that were monitored over time. Figures 3A and 3B are FRAP of leaf epidermal cells without treatment. The recovery is high as indicated on the graph. In Figures 3C-E, the recovery is not as high when compared with control cells as early as 19 min post treatment. This effect correlates well with the transition to a cisternal form, as shown in Figure 1. It also corresponds to similar work on animal cells, which shows that diffusion diminishes in the ER after hyperosmotic shock (Nagaya et al., 2008).

Directionality of diffusion of molecules within the ER

It has been sugggested that directional diffusion within the connected ER network may provide an efficient delivery system. (Griffing, 2010). Directional diffusion has been demonstrated in plants, but not in animal cells (Sparkes et al. 2009a, Runions et al. 2006). Does hyperosmotic treatment affect directionality of flow within the ER? To approach this question we have quantified and imaged directional flow using ratio imaging and FRAP recovery graphs of different regions along a tubule. FRAP recovery graphs of different region along a tubule in time can reveal directional motion. The recovery of molecules downstream of FRAP site should reveal a characteristic FRAP recovery graph relative to the "origin" of flow. If flow is directional, Figure 4A depicts the hypothesized graph of ER recovery.

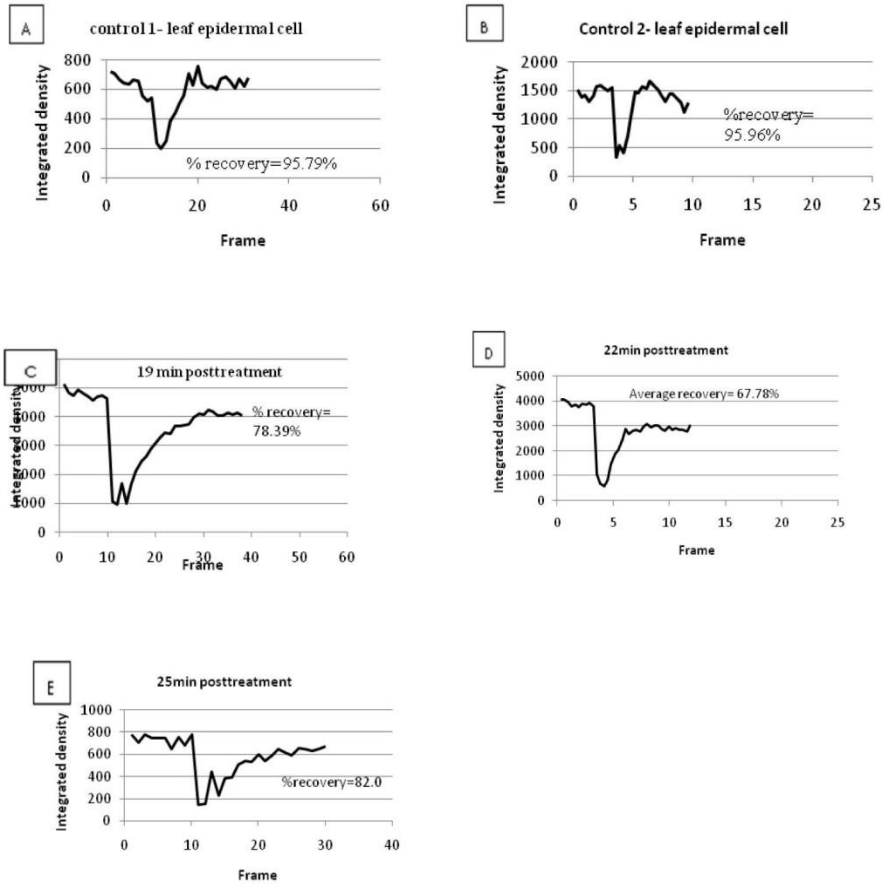


Figure 3. The Percent Recovery of ER in Epidermal Cells at Different Timepoints vs Controls.

A & B) Control recovery of photobleaching.
C-E) FRAP after sorbitol treatment for C: 19 minutes, D: 22 minutes, and E: 25 minutes.

Figures 4B, 4C and 4E shows the images and the graph of a FRAP time course in a sorbitol-treated cell. As evident in Figure 4C, there is uniform photobleaching around all the tubules leading into the photobleached area, indicating non-directional diffusion. Another way to image directional recovery is with our new technique of ratio-imaging FRAP. By dividing the post-bleach image (minus background) by the pre-bleach image (also minus background), the recovery and loss of fluorescence across the whole image can be seen, Figure 4D. In this case, the initial photobleaching is causing a uniform decrease in fluorescence in the region surrounding the original photobleached spot, as would be expected from the localized analysis shown in Figures 4B and C.

Figure 5 shows the ratioed image series of controls (A) with the quantitated regions (B) and graph of FRAP. Unlike treated ER, directionality of molecules within the ER is observed as depicted in the figure below.

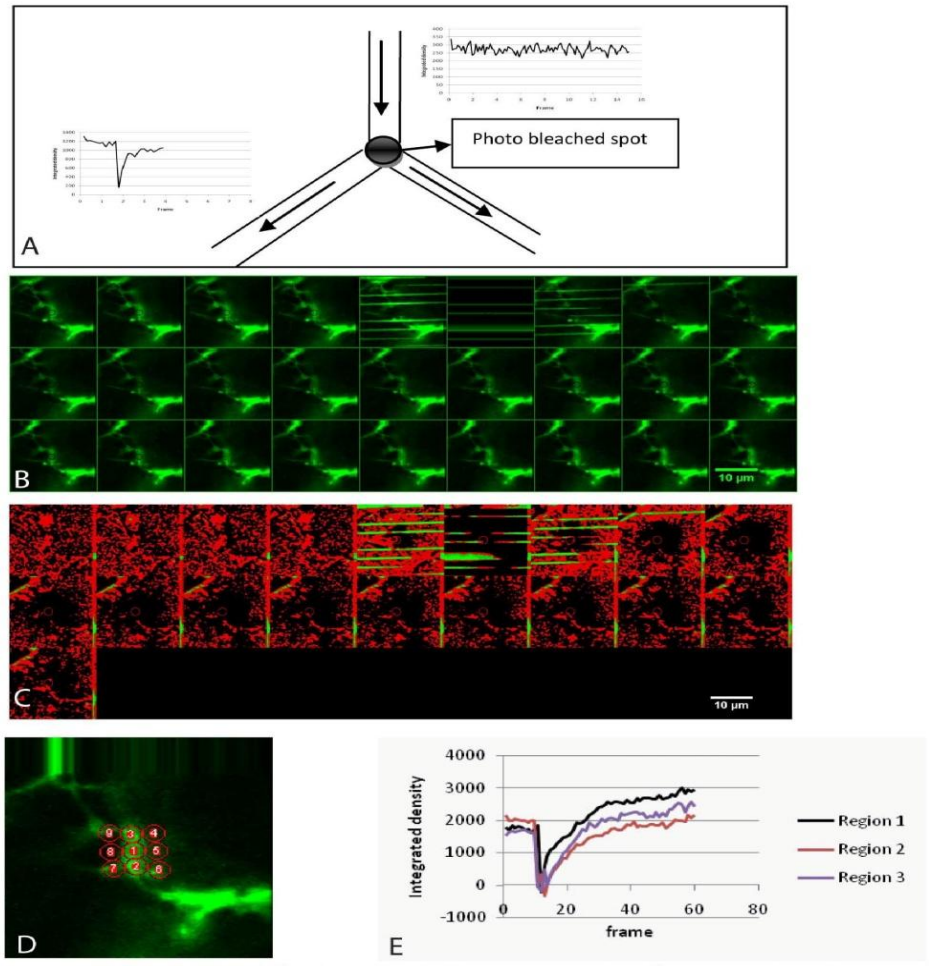
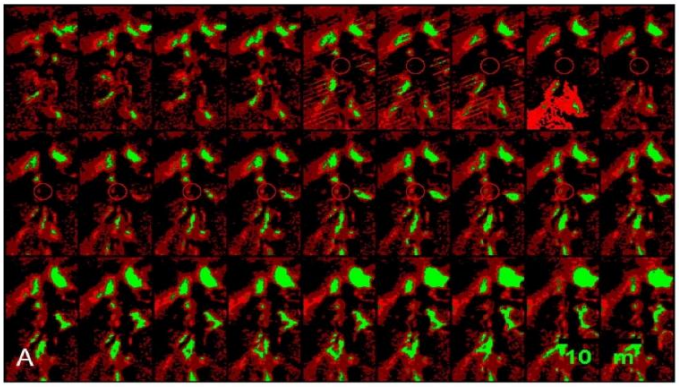
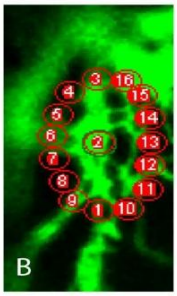


Figure 4. Theoretical Result of FRAP Analysis if Movement is Directional and Non-directional Movement After D-sorbitol Treatment.

A) After photobleaching, the photobleached GFP-HDEL moves out of the photobleached spot exclusively in the direction of the branches. Under that condition of directionality, the source tubule from which the flow occurs, does not photobleach (top graph insert), but supplies the photobleached spot with unbleached GFP (bottom graph insert).
B) FRAP time series of D-sorbitol treated tobacco epidermal cells.
C) Ratio image of B: ER 30min post-treatment with D-sorbitol. Ratio imaging reveals FRAP recovery of the whole image. Because there is movement of the ER contents, showing recovery as a ratio through the whole image can tell diffusional vs. directional movement. Ratioed images at 30min post-treatment with 0.75M D-sorbitol showing that movement of molecules within ER is diffusional (the absence of green color along tubule). Circle indicates photobleached spot.
D) Regions taken to make graph in 4E. Region 1 is the photobleached spot. The background density (region 4-9) was averaged and subtracted from the density along tubule (1-3) and graphed against the frame slices.
E) Comparison of FRAP in tubules surrounding photobleached site in (B). Both regions 2 and 3 show movement of photobleached GFP-HDEL from site 1, the photobleached spot.





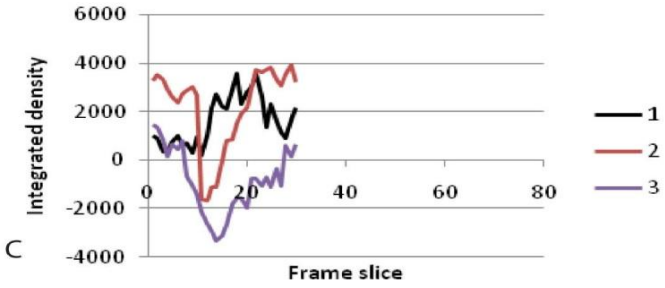


Figure 5. Directionality of Diffusion in Untreated Cells.

A) Ratio image of recovery in control. Ratio images showing FRAP recovery of the whole image. Increased green color intensity below photobleached spot (circle) reveals that ER contents recovered and that movement of contents is in the direction above photobleached spot (circle).
B) Regions taken to make graph in 4E. Region 2 is the photobleached spot. The background density (region 4-16) was averaged and subtracted from the density along tubule (1-3) and graphed against the frame slices. Region 2 is the photobleached spot.
C) Graph of regions depicted in along tubule 5C revealing that movement of molecules after photobleaching was directional, in the direction of region 3. The difference in the graph of region 2 and 3 shows that, in time the bleached molecules moved from region 2 to 3. Region 1 increasing in density in time also support the observation that molecules move from region 1 to 3. Region2 is the photbleached spot.

405nm excitation causes release of PM/chloroplast region from the wall and eliminates recovery

The ER associates with other organelles within a cell including the chloroplast. After hyperosmotic treatment, when an ER tubule near a chloroplast is photobleached, Figure 6A, the ER/plasma membrane detaches form the chloroplast, fluorescence is lost, and it does not recover, Figure 6C. The laser photobleaching seems to have a mechanical effect on the integrity of the network. This does not occur, however, when the ER is photobleached in regions far from chloroplasts in sorbitol-treated cells, Figure 6B. It not clear what causes this disruption in the network.

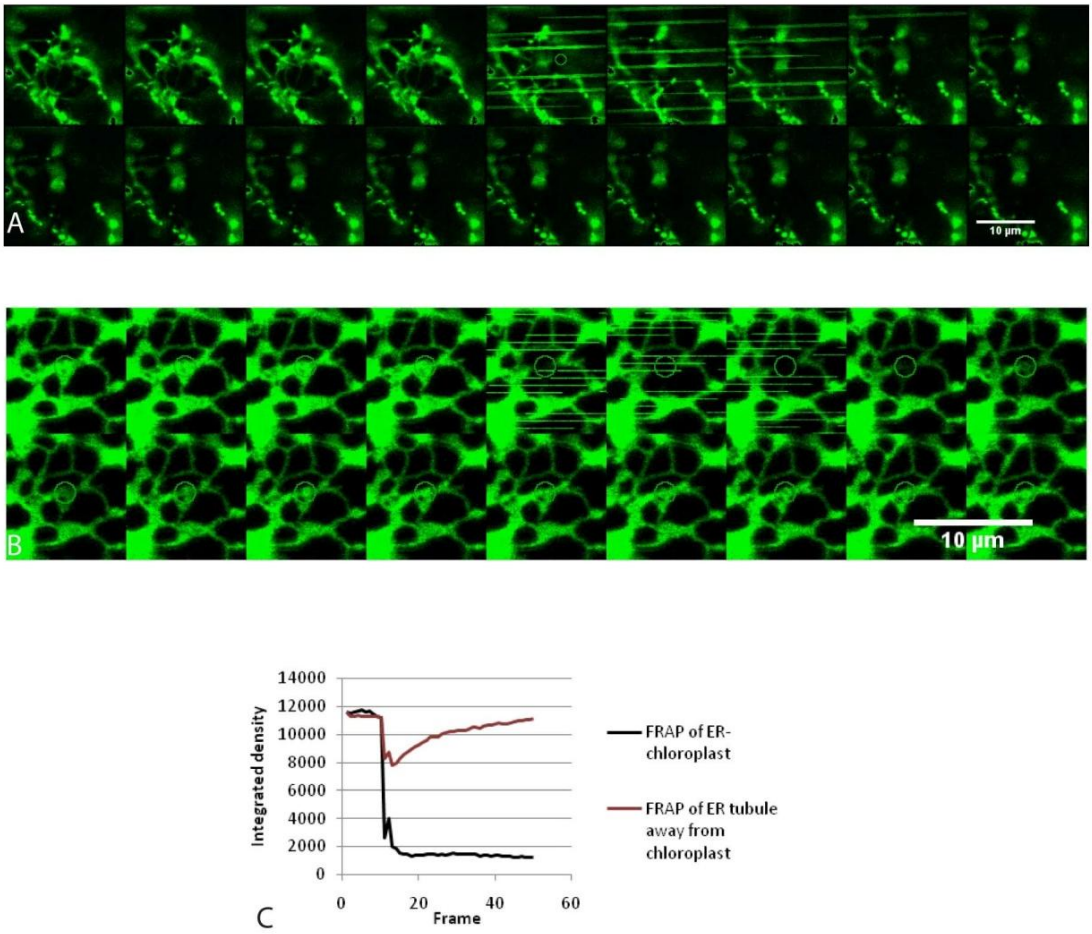


Figure 6. Presence of the Chloroplast has an Effect on FRAP in Sorbitol-treated Cells.

A) 405nm excitation causes release of PM/chloroplast region from the wall and eliminates recovery. Photobleaching near the chloroplast causes the ER near the wall to detach and fragment. It is not clear whether this is due to a mechanical action of the laser or if there is some blue photoreceptor in the chloroplast that feeds back on the network cytoskeleton and disrupts it.
B) Photobleaching ER away from chloroplast under similar conditions does not lead to ER detaching from wall. 405nm excitation does not result in release of PM/ER region from the wall and the fluorescence recovers.
C) FRAP analysis of recovery. ER photobleached near chloroplast does not recover.

CHAPTER III

METHODS

Nicotiana benthamiana seeds were provided by Dr. Griffing. These seeds constitutively express GFP-HDEL under a cauliflower mosaic virus promoter. Before planting, seeds were sterilized in 2% chlorox solution for 2 minutes; washed 5 times with sterile water and then transferred to a half-strength MS (Caisson Inc., ID, USA) salts (no sucrose or hormones) in agar (Kodak Inc. NY, USA) medium to grow. Transferred plants were then placed under fluorescent light at room temperature.

The reagents (osmoticums) used was D-sorbitol solution with 10 mM MES (Sigma-Aldrich, MO, USA) buffer (pH= 6.5 at room temperature). 0.75M stock D-sorbitol solution was prepared and stored at -20^{o}C for treatment. In addition, 10 mM MES buffer stock solution was prepared as a pretreatment medium.

Plant seedlings no more than one and a half weeks old were used both for treatment and image acquisition. For treatment and image acquisition, seedlings were placed in a Dvorak-Stotler perfusion culture chamber (Nicholson Instruments, NY, USA), and MES buffer gradually infused with a tuberculin syringe initially. The osmoticum was then later introduced to displace the MES buffer solution in the chamber at treatment. The ER in cortical region of the cell in the petioles of the first primary leaf of the seedlings was imaged. Imaging was done using the 499nm band of the argon laser on an Olympus Fluoview 1000 confocal microscope using an Olympus IX81inverted microscope. Images were analyzed in Image J (version 1.44p).

For FRAP and photobleaching experiments, SIM scanner was used at high temporal resolution with a 405 nm laser, using 10 microseconds dwell time/pixel of the photobleaching laser and 2 microseconds dwell time/pixel of the imaging laser. FRAP ratio images were made by subtracting background from the prebleached image, then dividing into the post-bleach image with background subtracted. This ratio was multiplied by 100 to give percent recovery. A red-green color table was used to show that below 70% recovery, the ER remained red, and as it approached 100% recovery, it became dark to lighter green.

CHAPTER IV

CONCLUSION

Hyperosmotic shock causes the ER to change its morphology from primarily tubular in the cell cortex to primarily cisternal over the period of 15-20 minutes. The dynamics of transport of transport also change. Transport was measured by examining the movement of photobleached GFP-HDEL through the connected branches of the tubular network. As the network converted to a cisternal form, the mobile fraction of the GFP-HDEL diminishes, as indicated by a drop in the percent recovery of fluorescence in the photobleached region. The diffusion of the GFP-HDEL also decreases, and motion becomes non-directional. These results that the mobile fraction is changed and the diffusion becomes non-directional are similar to those caused by removal of the actin cytoskeleton with the drug latrunculin B, and with dominant-negative transient expression myosin XI-K truncated tail domains (Sparkes et al. 2009a). Hence, the possibility arises that hyperosmotic shock signals detachment of the ER from the actin cytoskeleton. The Griffing lab will further investigate these results in Arabidopsis that constitutively co-express CFP-HDEL and YFP-actin, a marker of the actin cytoskeleton. The observation that the diffusional mobility of the GFP-HDEL decreases is different from the results seen in Sparkes et al. (2009a). Hence, actin uncoupling is not an explanation for this aspect of these results. The actual mechanism of the diminished diffusional mobility under hypertonic conditions may be similar to that which underlies the reduced diffusion coefficient of misfolded glycoproteins reported by Nagaya et al.

2008. Low water potentials in the ER might influence flow by increase the local viscosity of the tubule. Viscosity measurements can be done with injected dextrans. There is a recent report, now in press, that the ER can be microinjected with dextrans. This will also be attempted in the Griffing lab to determine if local viscosity changes occur in the ER upon hypertonic treatment.

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APPENDIX

MS Agar

MS Media packet	Add 1600ml
NaH2PO4	10ml
Vitamins	2ml
pH	2.8
Agar	4.5g
Autoclave	20min

MES buffer (10mM)

2.33g in 100ml	FW=233.3g/mol
pH	6.5
Useful pH range	5.5-6.7

D-Sorbitol (0.75M)

Concentration	0.75M; 6.84g (FW= 182.2g/mol)
Add 5ml to 45ml D-sorbitol solution to make 50ml	

CONTACT INFORMATION

Name:	Opeyemi Samson Adeniji
Professional Address:	c/o Dr. Lawrence R. Griffing Department of Biology MS 3258 Texas A&M University College Station, TX 77843
Email Address:	sammyson247@tamu.edu
Education:	B.S. Biology, Texas A&M University, May 2011 Undergraduate Research Scholar Cum Laude Alpha Epsilon Delta