DETERMINING THE ORIGIN OF A CA^{2+} WAVE RELEASED IN ARABIDOPSIS THALIANA UPON PHOTOSTIMULATION OF THE ER-CHLOROPLAST NEXUS

An Undergraduate Research Scholars Thesis

by

SARA MAYNARD

Submitted to the Undergraduate Research Scholars program at
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by Research Advisor: Dr. Lawrence Griffing

May 2017

Major: Molecular and Cell Biology

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ABSTRACT

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Sara Maynard
Department of Biology
Texas A&M University

Research Advisor: Dr. Lawrence Griffing
Department of Biology
Texas A&M University

The endoplasmic reticulum (ER) in *Arabidopsis thaliana* is the source of the calcium signal produced by high-fluence blue light sensed by the junction between the ER and chloroplast, the ER-chloroplast nexus. Photostimulation of this nexus results in an observable and quantifiable cytosolic calcium wave in *Arabidopsis* seedlings: using FRET analysis, we were able to see both an increase in cytosolic calcium and a decrease in ER luminal calcium. Treatment with cylcopiazonic acid (CPA), an inhibitor of SERCA, an ER Ca^{2+}-ATPase in animals, and ECA1, a homolog in *Arabidopsis*, caused an initial increase and delayed decrease in cytosolic calcium concentration of the wave. Lower concentrations of CPA resulted in higher magnitudes of this increase and decrease in of $[Ca^{2+}]_{cyt}$ than higher concentrations. Treatment with thapsigargin, an inhibitor of SERCA had less effect, causing a slight increase and decrease in the cytosolic calcium concentration of the observed wave similar to CPA but less extreme. CPA likely inhibits ER calcium regulation and resequestration, allowing more to initially be released and continuously depleting its store of calcium available for release over time.

ACKNOWLEDGEMENTS

I would like to thank Dr. Lawrence Griffing for all of his guidance and aid in this research project, along with the Department of Biology of Texas A&M University.

Thanks also goes to all of my fellow lab members, especially Meghan Connolly who worked with me in my experiments and the analysis of the data.

I would like to thank Dr. Ping He, Dr. Libo Shan, and Xiyu Ma of the Biochemistry department of Texas A&M for giving me so much guidance in research and advice about my future plans and goals.

Thank you to the LAUNCH program of Texas A&M University for allowing me the opportunity to be an Undergraduate Research Scholar.

Finally, I would like to thank all of my friends and family who supported me and encouraged me throughout this process, especially Brennen Taylor, my boyfriend and a fellow Undergraduate Research Scholar.

NOMENCLATURE

ER	Endoplasmic Reticulum
CPA	Cyclopiazonic Acid

CHAPTER I

INTRODUCTION

The ER-chloroplast nexus

In recent years, the junction between the endoplasmic reticulum (ER) and chloroplast, hereafter referred to as the ER-chloroplast nexus, has been shown to be more than just a location in the cell. Griffing (2011) showed that photostimulation of this nexus in tobacco plants produced ER protein bolus aggregation. There has also been shown to be lipid exchange between the two organelles. Precursors to lipids found in the thylakoid membrane are synthesized in the ER and therefore must somehow be transported to the chloroplast (Benning, 2009). Mutating a biosynthesis pathway of a nonpolar lipid only found in *Arabidopsis* chloroplasts affects fatty acid distribution in the ER (Mehrshahi et al., 2013). To explain this exchange of a nonpolar molecule when there are few known nonpolar molecule transporters, they proposed a hypothesis of hemifusion between the two organelle's membranes, explaining the large amounts of lipid exchange (Mehrshahi et al., 2013). While this specific hypothesis does not yet have much supportive evidence, there has been shown to be physical association between the two membranes, resisting a physical force up to 400 pN (Sandelius et al., 2007).

Calcium is involved in the stress response of plants

Following similar methods in Griffing (2011), photostimulation of this nexus in *Arabidopsis* seedlings has produced a cell-wide cytosolic calcium wave. Calcium is one of the most common second messengers in plants and a cytosolic calcium wave is seen in response to many stresses from drought to pollution (McAinsh and Pittman, 2009). There are unique calcium

signatures depending on the kind of stress to which the plant is exposed that engender response specificity (McAinsh and Pittman, 2009; Monshausen, 2012). Specificity can be due to variations in $[Ca^{2+}]_{cyt}$ oscillations and waves, the source of the calcium, and the channel through which the calcium is released (McAinsh and Pittman, 2009).

The ER is likely an important player in $[Ca^{2+}]_{cyt}$ regulation (Monshausen, 2012) and contains a high affinity Ca^{2+}-ATPase ECA1 that could be involved in the regulation of $[Ca^{2+}]_{cyt}$ (McAinsh and Pittman, 2009). ECA1 is structurally and functionally similar to the sarco-/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) in animals, a regulator of $[Ca^{2+}]_{cyt}$ after muscle contraction. Two known inhibitors of SERCA, cyclopiazonic acid (CPA) and thapsigargin, the ones we use in our experiment, have been tested on ECA1 expressed in yeast; CPA inhibited ECA1 while thapsigargin had no effect (Johnson et al., 2009; Liang and Sze, 1998). However, in both of these experiments *ECA1* was heterologously expressed in yeast, not in a plant where it naturally occurs.

The ER is a versatile organelle; it connects many organelles, is involved in lipid and protein transport and has been proposed to be involved in cell-wide communication (Griffing, 2010; Griffing et al., 2017). Due to its importance and probability of it being involved in regulation of $[Ca^{2+}]_{cyt}$, we hypothesize that the origin of the novel wave produced upon photostimulation of the ER-chloroplast in *Arabidopsis* seedlings is the ER.

CHAPTER II

METHODS

Transgenic plants

Arabidopsis thaliana seedlings expressing YCnano65 were gratefully received from Simon Gilroy of the Botany Department at the University of Wisconsin, Madison, WI. *Arabidopsis* seedlings expressing D1-ER were gratefully received from Gabriele Monshausen of the Biology Department of Penn State, State College, PA. Both were grown on half-strength MS medium under 24 hour-light. No additional nutrients were added. Five- to seven-day old seedlings were used.

Photostimulation

Photostimulation was done using an Olympus Fluoview 1000 confocal microscope with a 405nm SIM laser provided by the Microscopy Center at Texas A&M University, the same microscope used by Griffing (2011). Photostimulation was done with a 405 nm laser with a dwell time of 10 microseconds per pixel. Each video was recorded over an area of 200x200 pixels, or 6855.84 μm^2. Videos of the CFP, YFP, autofluorescence and light channels were recorded for 20.8 seconds with a scanning speed of 4.0 μm per second.

The site of photostimulation was limited to the same area on the seedlings: surface hypocotyl cells approximately five cells up from the root-shoot junction; chloroplasts near the center of the cells to avoid any interaction with the plasma membrane; and the edge of the chloroplast centered to ensure stimulation of the junction. High voltage values of each channel

were initially adjusted to display an image with optimal fluorescence of a control plant; these same values were used for all experiments. Seedlings were first observed in 10 mM MES buffer to check for YCnano65 expression levels before photostimulated.

Inhibitors

Over the course of our experiments, seedlings were treated with 250 μM, 125 μM, 62.5 μM, and 31 μM CPA and 1.0 μM thapsigargin. 250 μM CPA and 1.0 μM thapsigargin were used specifically based on data obtained by Johnson et al. (2009). Our buffer used was 10 mM MES buffer. A 100 mM MES buffer was created by adding potassium salt to ddH_2O and adjusting the pH to 5.8 using HCl. A 1:10 dilution was done to get 10 mM MES buffer. CPA solutions were made by adding DMSO to powdered CPA obtained from Sigma Aldrich to get a stock solution of 100 mM. A 1:400 dilution was done using 10 mM MES buffer to get a final concentration of 250 μM. Three 1:2 dilutions were then done using 10 mM MES to make the 125 μM, 62.5 μM, and 31 μM solutions. The 1.0 μM thapsigargin solution was made by adding 100% ethanol to thapsigargin obtained from Sigma Aldrich to make a stock solution of 1 mM. A 1:1000 dilution was done with 10 mM MES buffer to get the desired concentration of 1.0 μM.

Data analysis

All videos were taken using the confocal microscope; all videos were processed using ImageJ; all images were created using ImageJ and all graphs were created using Microsoft Excel.

Videos were taken of each photostimulation for 20.8 seconds and processed in ImageJ. Using FRET analysis, the ratio of fluorescence in the presence of Ca^{2+} (an increase in YFP

fluorescence) to lack of Ca^{2+} was analyzed by dividing the YFP channel by the CFP channel. Background videos of the microscope without samples were subtracted from the videos, a radius=1 median filter was applied and color was added for visualization. Green represents a high ratio, therefore high $[Ca^{2+}]_{cyt}$, and blue represents a low ratio, therefore low $[Ca^{2+}]_{cyt}$. Images were rotated so that the cell was vertical; 5.8μm (14 pixel) wide slices were taken of each frame of the video with the site of photostimulation centered and put together to create the montage. A kymograph representing the values of the ratios over the distance of the montage was created, graphing the average ratio value of every 0.414 pixels (0.171 μm) of the montage. Data points from this kymograph were analyzed in Microsoft Excel. The average ratio of every four slices (23.2μm, 56 pixels) was plotted against time, each slice representing 0.4 seconds, therefore four representing 1.6 seconds. This same method was used to analyze all videos recorded.

CHAPTER III

RESULTS

Quantification of the calcium wave

Photostimulation of surface hypocotyl cells of five- to seven-day old *Arabidopsis* seedlings was done using an Olympus Fluoview 1000 confocal microscope, as described in the materials and methods section. Photostimulation of this junction resulted in a cytosolic calcium wave that spanned the whole cell. Figure 1 shows a surface hypocotyl cell before photostimulation (Fig. 1A) and after photostimulation (Fig. 1B) under control conditions (10 mM MES buffer).

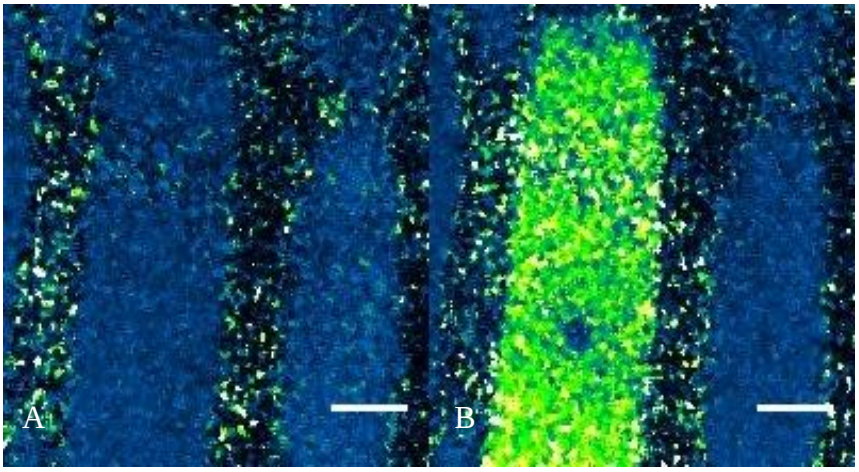

Figure 1. Calcium wave produced by photostimulation of the ER-chloroplast nexus. Surface hypocotyl cell of a 7-day old *Arabidopsis* seedling in 10 mM MES buffer. Scale bar = 15μm. A) Before photostimulation. B) 20 seconds after photostimulation. The dark spot in the middle of the cell is the chloroplast.

Further quantification of this wave as described in the materials and methods section resulted in a montage (Fig. 2A), kymograph (Fig. 2B) and excel graph showing a gradual increase and eventual plateauing of $[Ca^{2+}]_{cyt}$ (Fig. 2C). The calcium spread quickly throughout

the cell and concentration levels remain constant after approximately 12 seconds at a ratio value of about 4.5. Figure 2C is a good representation of the signature of the calcium wave and should be referred to when comparing effects of putative inhibitors. This data is the same as represented in Figure 1.

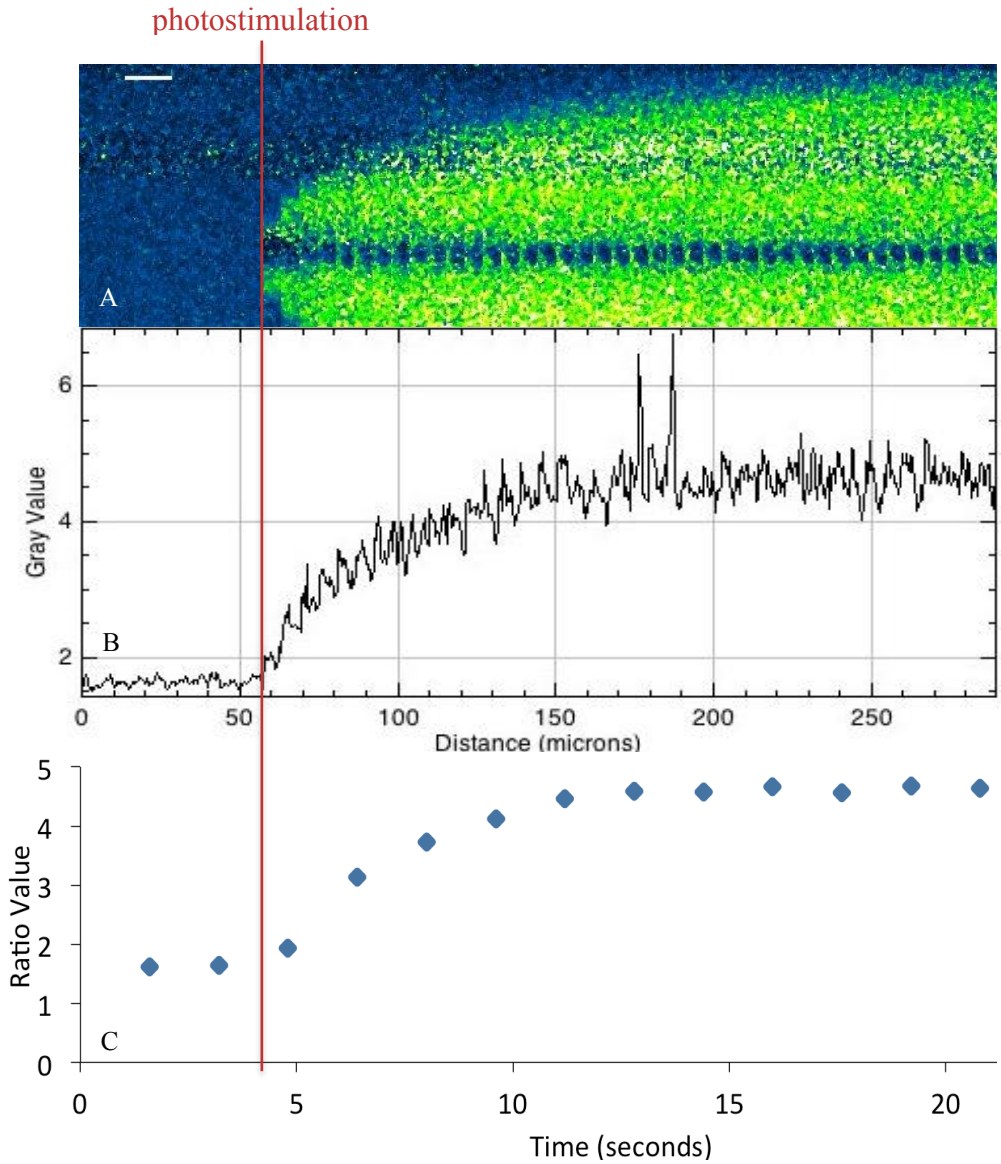

Figure 2. Time-lapse and quantification of the calcium wave. A) A montage of 5.8 µm-wide (14 pixel-wide) segments of the original video. The dark spot in the middle is the chloroplast. B) A kymograph of the montage in part A plotting the ratio value against the width of the montage. C) An excel graph plotting the average ratio value of every four segments against time. Each segment corresponds with 0.4 seconds.

Effect of cyclopiazonic acid

Four different concentrations of cyclopiazonic acid (CPA) were used to treat four different *Arabidopsis* seedlings to observe an effect of dosage. Each seedling was prepared on a microscope slide in 10 mM MES buffer and observed for YCnano65 expression. The CPA of concentration of choice was added to the slide, replacing a majority of the 10 mM MES buffer and incubated for approximately twenty minutes. After twenty minutes, the seedling was placed in the microscope and multiple ER-chloroplasts nexuses of the same seedling were photostimulated, with one to two minutes between photostimulation. The seedling incubated in 250 μM was photostimulated five times; 125 μM, seven times; 62.5 μM, nine times; and 31 μM, seven times. Values of each concentration were averaged and graphed in Figure 3.

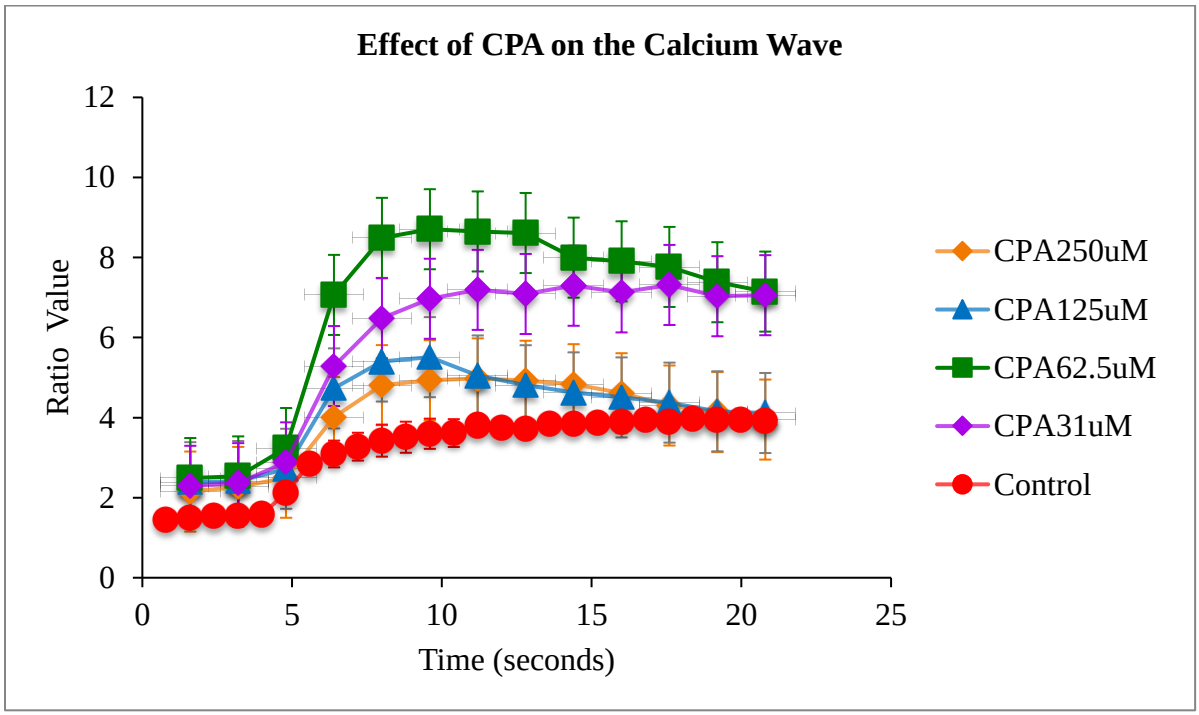

Figure 3. The effect of CPA on the calcium wave. Four different concentrations of CPA were used to treat four different *Arabidopsis* seedlings. Values were averaged and plotted above with standard deviation bars. Experiments were done on the same day with the same environment; all seedlings were obtained from the same batch and were therefore the same age.

Cyclopiazonic acid had inhibitory effect of this wave by causing a gradual decrease in $[Ca^{2+}]_{cyt}$ (Fig. 3). When comparing the ratio values after photostimulation when they are on average the highest (at 9.6 seconds) to ratio values at the end of the data set (20.8 seconds), we find that 62.5 μM CPA caused the largest decrease, 1.558 ratio value units, and 31 μM caused the smallest decrease, 0.089 ratio value units (Table 1). Every concentration, except perhaps 31 μM, caused an observable decrease in the calcium over time. This can be seen in the graph in Figure 3: $[Ca^{2+}]_{cyt}$ goes down over time after treatment with CPA.

CPA also appears to have caused a larger increase of $[Ca^{2+}]_{cyt}$ immediately after photostimulation. When comparing the initial ratio values (at 1.6 seconds) to the ratio values after photostimulation (9.6 seconds) we find that 62.5 μM caused the highest increase, 6.216 ratio value units, 4.124 higher than the control, and 250 μM CPA caused the lowest increase, 4.673 ratio value units, 0.688 higher than the control (Table 1). The lower concentrations of CPA, 31 μM and 62.5 μM, caused higher initial concentration values than when treated with 125 μM or 250 μM CPA. This can also be seen in the graph of Figure 3A.

Table 1. Comparing the ratio values of treatments with different concentrations of CPA.

Ratio value at:	**250uM CPA**	**125uM CPA**	**62.5uM CPA**	**31uM CPA**	**Control**
1.6 seconds	2.153	2.386	2.490	2.296	1.505
9.6 seconds	4.933	5.510	8.706	6.969	3.597
20.8 seconds	3.951	4.115	7.148	7.059	3.908
Amount of $[Ca^{2+}]_{cyt}$ decrease	0.982	1.395	1.558	-0.089	-0.310
Amount of initial $[Ca^{2+}]_{cyt}$ increase	2.780	3.124	6.216	4.673	2.092

Note: The amount of $[Ca^{2+}]_{cyt}$ decrease was determined by subtracting the ratio values at 9.6 seconds by those at 20.8 seconds. The amount of initial $[Ca^{2+}]_{cyt}$ increase was determined by subtracting the ratio values at 9.6 seconds by those at 1.6 seconds. Yellow = largest magnitude of the four different concentrations; red = smallest magnitude.

The control plotted is an average of six different control photostimulation events and the ratio value is averaged over every two cropped video frames instead of four as with the other data. The control maintains the shape seen in Figure 2, indicating its accuracy.

Effect of thapsigargin compared to CPA

Two different seedlings were incubated in 1.0 μM thapsigargin and two different seedlings were incubated in 250 μM CPA to increase variability. Data obtained from these 250 μM CPA treatment is not repeated data from Figure 3. Seedlings were incubated in the inhibitors for varying amounts of time before photostimulation. Thapsigargin: One seedling was photostimulated after 18, 45, and 47.5 minutes and the other after 22.5, 52 and 55. CPA: One seedling was photostimulated after 8 and 40.5 minutes and the other after 14 and 38 minutes. All values were averaged and shown in Figure 4.

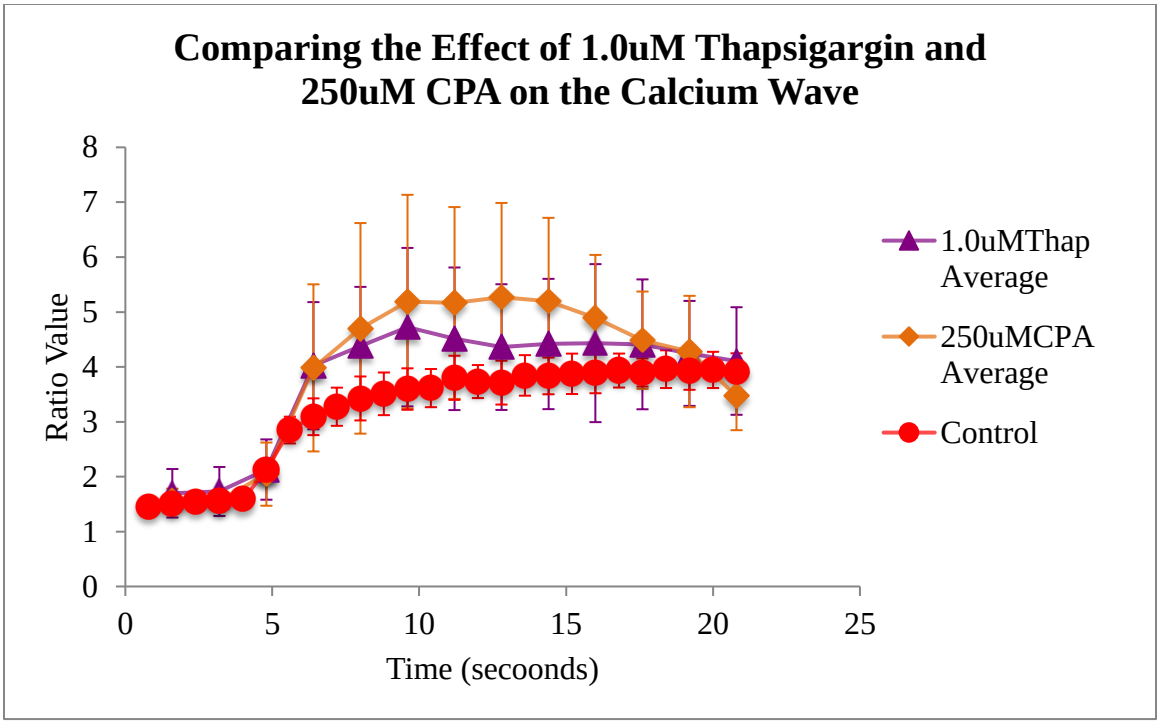

Figure 4. Comparing the effect of 1.0 µM thapsigargin to 250 µM CPA on the calcium wave. Two different seedlings per each inhibitor were photostimulated multiple times. Values were averaged and plotted above with standard deviation bars. Experiments were done on the same day with the same environment; all seedlings were obtained from the same batch and were therefore the same age. The control is the same values as used in Figure 3.

Thapsigargin had a slight inhibitory effect on the calcium wave by causing a decrease in $[Ca^{2+}]_{cyt}$ (Fig. 4). When comparing the ratio values after photostimulation when they are on average the highest (at 9.6 seconds), to ratio values at the end of the data set (20.8 seconds), we find that thapsigargin caused a decrease of 0.617 ratio value units, 0.307 higher than the control (Table 2). However, when compared to effects of 250 µM CPA, thapsigargin has less of an inhibitory effect. When comparing these values, we find that CPA caused a decrease of 1.102 ratio value units higher than thapsigargin (Table 2).

There also appears to be an increase in initial $[Ca^{2+}]_{cyt}$ after 1.0 µM thapsigargin treatment, however the differences are small and the standard deviation bars overlap the control

values. More data analysis needs to be done to determine significance; however, the graph shows a higher increased caused by 250 μM CPA than thapsigargin. The control values in Figure 4 are the same as those in Figure 3.

Table 2. Comparing the ratio values after 1.0 μM thapsigargin and 250 μM CPA treatment.

Ratio value at:	**1.0uM Thap Average**	**250uM CPA Average**	**Control**
1.6 seconds	1.698	1.566	1.505
9.6 seconds	4.724	5.190	3.597
20.8 seconds	4.108	3.472	3.908
Amount of $[Ca^{2+}]_{cyt}$ decrease	0.617	1.719	-0.310

Note: The amount of $[Ca^{2+}]_{cyt}$ decrease was determined by subtracting the ratio values at 9.6 seconds by those at 20.8 seconds. Yellow = largest magnitude; red = smallest magnitude.

Associated drop in luminal calcium

Arabidopsis seedlings expressing D1-ER, an ER lumen fluorescent probe, were used to observe the effect of this photostimulated on the concentration of calcium within the ER. The same methods were used to photostimulate the nexus and to analyze videos. Photostimulation of this nexus did indeed cause a decrease in luminal ER (Fig. 5). An immediate drop in $[Ca^{2+}]_{ER}$ is seen after photostimulation followed by partial, but incomplete recovery, resulting in an overall decrease of luminal calcium.

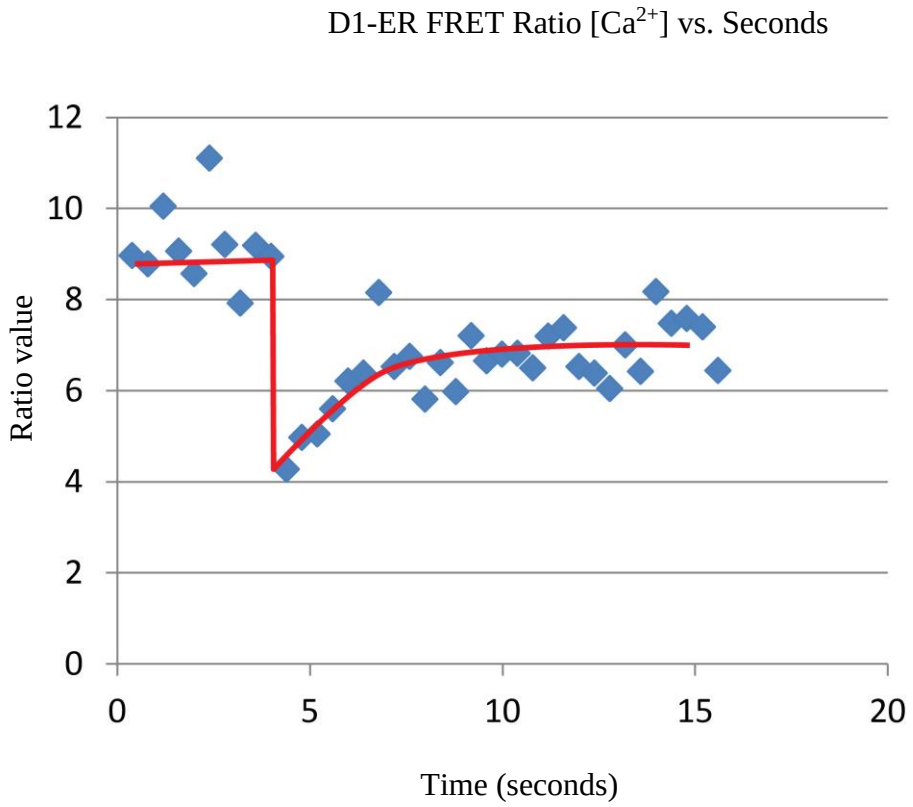

Figure 5. FRET analysis of calcium concentration in the ER after photostimulation. This shows a decrease of luminal calcium after photostimulation of the ER-chloroplast nexus.

Summary

In summary, our results show a cytosolic calcium wave induced upon photostimulation of the ER-chloroplast nexus in surface hypocotyl cells of *Arabidopsis thaliana*. This is shown in before and after pictures, a time-lapse montage of the increase of cytosolic calcium, a corresponding kymograph plotting the grey-scale value difference, indicating calcium, versus the length of the montage, and the resulting graph after an averaging of these values. Treatment with varying concentrations of cylcopiazonic acid resulted in a decrease of calcium in the wave after photostimulation along with a slight initial increase. Both thapsigargin and CPA had an inhibitory effect on the wave by causing a decrease in $[Ca^{2+}]_{cyt}$ over time. CPA had more of an

effect, causing a higher decrease in $[Ca^{2+}]_{cyt}$ than thapsigargin. In additional experiments, a drop in luminal calcium is also observed upon photostimulation of the nexus.

CHAPTER IV

CONCLUSION

Analysis of the calcium wave

The novelty and uniqueness of this wave must be noted. Its calcium signature, a definition explained by McAinsh and Pittman (2009), is different from most immediate responses; it lacks oscillations and remains at a constant level over time. However, in other experiments not reported in this paper, treatment with gadolinium, an inhibitor of another ER calcium release channel in plants (Klusener et al., 1995), caused a second "bump" in the $[Ca^{2+}]_{cyt}$, indicative of an oscillation pattern. This effect of apparently changing the calcium signature is of interest and should be studied further.

Origin of the calcium wave

When looking at our data, we concluded that the calcium making up the cytosolic wave induced upon photostimulation of the ER-chloroplast nexus in *Arabidopsis* seedlings is released from the endoplasmic reticulum. Inhibitory effects by ER Ca^{2+}-ATPase inhibitors cylcopiazonic acid (CPA) and thapsigargin point to the ER as being the source by causing a decrease in $[Ca^{2+}]_{cyt}$ over time. Perhaps the most supportive evidence of our hypothesis is the drop seen in $[Ca^{2+}]_{ER}$ after photostimulation. This calcium must obviously go somewhere, and where better to go than the cytosol? This drop in luminal calcium associated with an increase in cytosolic calcium is strong evidence for the ER being the source.

Inhibitory effect of cyclopiazonic acid and thapsigargin

Cyclopiazonic acid (CPA) had an inhibitory effect on the wave indicating that ECA1 is involved in this response. While thapsigargin has not been shown to inhibit ECA1, our results show inhibition of the wave due to thapsigargin, indicating that it may inhibit ECA1. Studies done by Johnson et al. (2009) and Liang and Sze (1998) expressed *ECA1* in mutant yeast to observe the effect of inhibitors. This heterologous expression may affect ECA1 in a way that prevents thapsigargin inhibition that normally occurs when ECA1 is expressed. Our study varies from the two aforementioned in that we test thapsigargin on live *Arabidopsis* seedlings where ECA is naturally expressed. Thapsigargin likely inhibits ECA1 in similar methods to CPA due to the similar results of both inhibitors, however with less effect. Future studies with thapsigargin and its effect on ECA1 and this calcium wave should be done.

CPA, and to a lesser effect, thapsigargin, affect the calcium wave by causing an increase in the initial levels of calcium in the wave right after photostimulation and by causing a gradual decrease of this concentration over time. We propose that CPA affects the levels of calcium by inhibiting the regulation and resequestration of calcium in the ER through inhibiting ECA1. If ECA1 is the main regulator of calcium in the ER, inhibiting it would significantly affect luminal calcium concentration. In a non-photostimulated seedling, ECA1 may be responsible for the regulatory release of luminal calcium as other pumps uptake calcium. However, there may not be an affect on the action of these other pumps when ECA1 is inhibited. If this is the case, they will continue to uptake calcium while ECA1 is unable to release it, leading to the larger initial release of calcium from the ER. In a photostimulated seedling, ECA1 may be responsible for regulatory uptake of calcium when concentration in the cytosol is high. If the ER continuously releases

calcium after photostimulation, then inhibition of its resequestration would deplete its store of calcium, causing less to be released over time, causing the drop of $[Ca^{2+}]_{cyt}$ we see. Other organelles, mostly likely the mitochondria and plasma membrane, are likely involved in the regulation of this calcium wave after the initial release from the ER due to the tight coordination of calcium regulation between organelles (Monshausen, 2012). As the wave progresses, the sequestration mechanisms of these organelles likely kick in. Because the calcium is released from all of the ER, not just the site of photostimulation, calcium could be travelling to adjacent cells via the desmotubule, the ER that traverses the plasmodesmata, perhaps also adding to the observed decrease. In fact, in some of our experiments, we noticed a slight increase in cytosolic calcium of adjacent cells after photostimulation (data not shown), however this increase was not fully quantified and should be studied further.

Specificity of CPA concentrations

An interesting result of CPA treatment is the effects of different CPA concentration on the overall wave. Because different concentrations of CPA had varied effects, there must be an optimal concentration that elicits the strongest response. Our results indicate that a concentration around 62.5 μM is the most effective at inhibiting ECA1; it caused the highest initial increase and the largest decrease. This lower concentration of CPA may be more specific at inhibiting ECA1 than the higher concentrations of 250 μM and 125 μM. 31μM may be too low to elicit the same response. However, with an increase in CPA comes an increase in DMSO concentration, which is known to increase the leakiness of tonoplast membranes (Delmer, 1979). Therefore, the observed differences in the increase in initial calcium concentrations and the later decrease caused by different concentrations of CPA may also be due to this effect. Future experiments

should be done testing the effect of different concentrations of DMSO and how it affects the varied inhibition caused by the different concentrations of CPA.

Future research

Future research in this area should include further studying the specific effects of CPA and determining which concentration elicits the strongest response. Along with this, studies should be done looking into the effects of different concentrations of DMSO on the leakiness of the tonoplast membrane, therefore determining how much of the change in $[Ca^{2+}]_{cyt}$ is due to CPA versus DMSO. This will be done by keeping the concentration of CPA constant at 31 μM and varying the concentration of DMSO. The inhibitory functions of thapsigargin on this calcium wave should also be studied further. Series analyses should be done of all obtained data to determine the statistical significance of our results. Experiments using inhibitors of different pumps such as gadolinium, and ER calcium pump inhibitor, and lanthanum, a plasma membrane Ca^{2+}-ATPase inhibitor, should be done to determine if there are multiple sources or regulators of this response to photostimulation. Connolly and Griffing (unpublished) have done work with both, yet results were inconclusive. Expansion of this work would be interesting. Working with other inhibitors may also show involvement of other calcium pumps. Work done by Griffing (unpublished) has shown an associated increase in luminal pH upon photostimulation, pointing to the involvement of an antiporter. If the positive calcium ion is pumped out, the positive hydrogen ion may be pumped in to maintain charge, thereby increasing pH. There are also several things to keep in mind for future studies. During our experiments we photostimulated the same seedling multiple times. The first photostimulation may cause a plant-wide response, affecting the response to all following photostimulation events. The age of the seedling may

affect the expression of fluorescent probes or even the total response of the plant to this photostimulation. It would be interesting to observe the effects of this photostimulation of *Arabidopsis* plants of all ages. We also want to look more into the effect of photostimulation on adjacent cells and if the calcium wave can indeed be spread through multiple cells.

Future experiments are planned to observe the effect of CPA and thapsigargin on [Ca^{2+}] in the ER lumen by following the same methods as described in this paper, but using seedlings expressing D1-ER rather than YCnano65, and to observe the effect of the inhibitors on calcium levels in adjacent cells. We expect to see a lowered recovery of [Ca^{2+}] in the ER lumen when treated with CPA due to its inhibition of calcium resequestration and some kind of inhibitory response when treated with thapsigargin. We expect to see a decrease in calcium in adjacent cells due to inhibition of ECA1 in all of the ER, not just at the site of photostimulation.

Summary

By inhibiting ECA1, a plant ER Ca^{2+}-ATPase, with cyclopiazonic acid (CPA) and thapsigargin, we saw an initial increase and delayed decrease of calcium in a calcium wave induced upon photostimulation of the ER-chloroplast nexus. This along with an associated decrease of ER luminal calcium led us to the conclusion that the ER is the source of the calcium wave. We propose that CPA inhibits regulation and resequestration activities of ECA1, leading to the observed initial increase and delayed decrease. In future studies we plan to test the effect of the inhibitors on both luminal calcium concentration and on cytosolic calcium concentrations in adjacent cells.

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