Final Technical Report Cover Page

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DISCLAIMER

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1.0 EXECUTIVE SUMMARY

Although it can be broken down into its molecular and protein components, photosynthesis is a process that exists in living cells that deal with contrasting environments. Because photosynthesis is one of the most highly reactive processes to exist in biology, cells have evolved complex regulatory mechanisms that allow chloroplasts and their photosystems to self-assemble, self-repair, and self-disassemble. Understanding of these processes is only beginning, but we now have an excellent system to dissect controlled chloroplast repair and disassembly. By studying how these processes work in nature and in the cell, we can gain fundamental knowledge that will allow us to manipulate energy capture in crops or help us in designing synthetic photosynthesis machines that can cope with light damage.

The biogenesis, repair, and breakdown of chloroplasts and their photosynthetic machinery and membranes is controlled by a network of regulatory factors. Expression and regulation of the proteins involved is regulated developmentally and environmentally by signals from the chloroplast, hormones, sugars, and the circadian clock in a cell-type-specific manner. As such, an improved understanding of chloroplast biogenesis, maintenance, and quality control mechanisms will require continued genetic analysis to define the regulatory networks. Completion of this part of the project* provided an increased understanding of the proteomic and metabolomic changes in cells responding to chloroplast damage, how these changes are turned into signals, and how these signals are propagated. These studies aided our abilities to manipulate plant growth and development, which is crucial to our quest for an abundant food supply and cheap, dependable sources of energy.

***Award #DE-SC0019573 at the University of Arizona (Dr. Jesse Woodson, PI) will be used for the majority of the work going forward and all three aims. Award #DE-SC0018024 at the Salk Institute (Dr. Joanne Chory, PI) was used to support Aim 1 with sequencing and metabolic analyses.**

2.0 BACKGROUND

As the Principal Investigator, I have spent more than 30 years using *Arabidopsis thaliana*, a small flowering mustard plant as a model for plant growth. I have pioneered the use of molecular genetics to study how plants alter their size, shape and form to optimize growth and photosynthesis for particular environments. Utilizing plant genetics, coupled with biochemical studies has allowed me and my team to determine one of the most complex signaling networks that controls growth and development in response to environmental change.

I am the co-founder of the Harnessing Plants Initiative here at the Salk Institute, using an innovative, scalable approach to fight climate change by optimizing a plants natural ability to capture and store carbon and adapt to diverse climate conditions. We aim to help plants grow bigger, more robust root systems that can absorb larger amounts of carbon by burying it in the ground in the form of suberin, a naturally occurring carbon-rich substance.

The Harnessing Plants Initiative group is located here in La Jolla, CA., with a state of the art greenhouse in Encinitas, 15 miles north of Salk. Our scientific team consists of Professors, staff scientist, postdocs, graduate students and lab personnel from all over the world and diverse backgrounds.

3.0 PROJECT OBJECTIVES

Although it can be broken down into its molecular and protein components, photosynthesis is a process that exists in living cells that deal with contrasting environments. Because photosynthesis is one of the most highly reactive processes to exist in biology, cells have evolved complex regulatory mechanisms that allow chloroplasts and their photosystems to self-assemble, self-repair, and self-disassemble. Understanding of these processes is only beginning, but we now have an excellent system to dissect controlled chloroplast repair and disassembly. By studying how these processes work in nature and in the cell, we can gain fundamental knowledge that will allow us to manipulate energy capture in crops or help us in designing synthetic photosynthesis machines that can cope with light damage.

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4.0 DESCRIPTION OF ACTIVITIES PERFORMED

The major goal of the project was to define the signal(s) and signaling pathways from chloroplasts that regulate selective chloroplast degradation. The studies integrated genetic, molecular, and biochemical approaches in *Arabidopsis*.

(1) Understand the mechanism(s) by which singlet oxygen triggers chloroplast degradation and signaling

The mechanisms that trigger chloroplast ubiquitination and degradation are not understood, but accumulation of $^1O_2$ is associated with initiating the response. To this end, we completed our genetic screen of *ferrochelatase 2 suppressor* (*fts*) mutants. One mutant, *fts39*, mapped to a point mutation in *At3g12670* (*CTPS2*), one of five *Arabidopsis* genes encoding cytidine triphosphate synthase (CTP) enzymes. We showed that the *fts39* mutation affects proper splicing of the *CTPS2* mRNA transcript and that the suppressor phenotype can be completed with a wild type copy of *CTPS2*. Interestingly, we have also shown that the *fts39* suppressor phenotype can be complemented by *CTP1*, *4*, and *5*, but not by *CTPS3*. These constructs included a YFP tag that allowed us to assess expression levels and cellular localization. Although all proteins were expressed at equivalent levels, they also appeared to localize to different parts of the cell. As such, we separated pools of CTP may operate differently, with some pools being important for chloroplast function and/or signaling.

We have also revisited the unsequenced mutants isolated from our original screen and chose three for further study (*fts11*, *fts44*, and *fts45*). Based on their normal tetrapyrrole synthesis levels, we expected them to be true signaling mutants like *pub4-6*. Also, none of these mutants were allelic to *pub4-6* according to our reciprocal crosses and likely represented new loci. Interestingly, *fts11* has elongated hypocotyls and was insensitive to blue light. We sequenced the *CRY1*, *CRY2*, *ROC1*, *BIT1*, and *PP7* genes known to be involved in blue-light inhibition of hypocotyl elongation, but we found no mutation in these genes' coding regions. Thus, *fts11* may represent a new gene involved in blue light signaling and provide a link between light signaling and chloroplast degradation. We back-crossed each of these mutants to the *fc2* parent line and generated a mappings population for sequencing and "SHOREmap" analysis.

The blue light-insensitive phenotype of *fts11* is intriguing, as the CRY1 blue light signaling protein has been implicated in $^1O_2$ signaling in the *flu* mutant that also overproduces $^1O_2$ in the chloroplast. This $^1O_2$ signal also lead to cellular degradation. We tested the possibility that CRY1 may also be involved in chloroplast degradation in the *fc2* mutant by generating *fc2*/*cry1* double mutants. The *cry1* mutation failed to suppress *fc2* suggesting that $^1O_2$ may be acting differently in the *fc2* and *flu* backgrounds. This is supported by our other observations that the *flu* suppressors *ex1* and *ex2* also failed to rescue the *fc2* phenotype. In addition, *pub4-6*, which suppresses *fc2,* failed to suppress the *flu* phenotype. Instead, *pub4-6* was able to suppress cellular degradation in two additional chloroplast $^1O_2$ producing mutants, *Accelerated Death 2* (*acd2*) and *chlorina 1* (*ch1*). Together, this data suggested that multiple $^1O_2$ signaling pathways exist in plants, PUB4 may be involved in responding to more than one source of $^1O_2$, and that $^1O_2$ signaling was more complicated than previously thought.

(2) Explore the roles of ubiquitination in chloroplast quality control

Our studies showed that chloroplast proteins in *fc2* mutants became ubiquitinated after $^1O_2$ stress. To understand this role of chloroplast ubiquitination, we used a proteomics approach and identified three potential $^1O_2$ stress-induced ubiquitination targets. One candidate is PEPC2, a phosphoenolpyruvate (PEP) carboxylase involved in primary carbon metabolism that catalyzes the addition of bicarbonate to PEP to form oxaloacetate and inorganic phosphate. We also observed that it associates with chloroplasts in stressed *fc2* mutants and that ubiquitination (mono- or poly-) may be regulating this change in localization.

We aimed to confirm if PEPC2 plays a role in chloroplast degradation or protection. We h generated *PEPC2* overexpression using the constitutive *35S* promoter, but these constructs were unstable and prone to silencing. Thus, we tried using alternative promoters (*PEPC2*, *RBCS2b*) and generating new overexpression lines that have lower (and stable) expression. However, we were able to obtain preliminary results using the T2 generation of overexpression lines. A careful analysis allowed us to test the effect of increased PEPC2 abundance on biomass, chlorophyll accumulation, chloroplast ultrastructure, and chloroplast degradation. Surprisingly, overexpression of *PEPC2* in wt or *fc2* backgrounds led to increased biomass (on average, a 19% increase in wt and a 26% increase in *fc2*) and increased chlorophyll accumulation (on average, a 25% increase in wt and a 22% increase in *fc2*). This appeared to be due to increased chloroplast size and membrane composition as shown by preliminary transmission electron microscopy data. On average, *PEPC2* overexpression increased chloroplast area by 15% and 41%, grana stacking by 58% and 57%, and starch granule area by 79% and 450% in wild type and *fc2*, respectively. Moreover, overexpression of *PEP2* in *fc2* led to a mild *fts* phenotype (survival in light cycling conditions) suggested that it affects the chloroplast degradation pathway.

Although very striking, these results were the opposite of what we expected. If a ubiquitinated protein promotes chloroplast degradation, then its overexpression would be expected to increase chloroplast degradation. Thus, some of these ubiquitinated proteins may be a signal to protect chloroplasts from ROS damage. Together, we are very excited about these results as it points to PEPC2 having a surprising role in chloroplast function and stress.

(3) Determine the role of chloroplast quality control under natural light stress

We tested our model of $^1O_2$–induced chloroplast quality control under natural excess light (EL) stress. We defined the stress conditions that lead to chloroplast degradation. First, plants were grown under mild light conditions (~100 uMoles min-1 sec-1). Then at various stages of plant development, they were treated with EL (~1,300-1,500 uMoles min^{-1} sec^{-1}) for various lengths of time using our high light growth chamber (Conviron Model E8), which we have used to study EL stress previously. Using the same tools as before, we monitored $^1O_2$-production (with the fluorescent probe Singlet Oxygen Sensor Green, Molecular Probes), chloroplast ubiquitination (anti-ubiquitin immunoblots), cell death (visual inspection of tissue lesions and trypan blue staining), growth yield (weighing dried tissue) expression of marker genes identified in our microarray experiment, and chloroplast ultrastructure (via TEM). This allowed us to

determine if and when chloroplasts are being stressed and/or degraded, and the sequence of events. A role for PUB4 in EL stressdetermined by monitoring its expression by qPCR and immunoblot (using our stable *Arabidopsis* lines expressing PUB4-YFP with its native promoter). As PUB4 may be recruited to damaged chloroplasts, its localization was followed by confocal microscopy before and after EL stress. We performed the above EL stress experiments with our *pub4* mutants to determine which steps require a functional PUB4 protein. Together, this work defined the role of the chloroplast quality control pathway under natural light stress conditions.

5.0 CONCLUSIONS AND RECOMMENDATIONS:

At the time we proposed this project it was a basic biological scientific endeavor and did not specifically contribute to the Tribe/Tribal community's energy vision.

6.0 LESSONS LEARNED:

The biggest challenge towards the end of the project was coping with COVID 19. This slowed down the continuation of the project that we were collaborating with Dr. Woodson, as we were doing samples for him to send to Arizona. Salk then had to come up with protocols in order for the labs to continue their work. It was a daunting task, and slowed things considerably, but we managed through it, as many scientific labs throughout the country did.