FINAL PROGRESS REPORT
INTRACELLULAR DYNAMICS OF ENERGY-TRANSDUCING ORGANELLES
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INTRODUCTION AND OVERVIEW

Our initial rediscovery of long tubular stroma-filled plastid extensions (stromules) occurred with the use of GFP targeted to plastids by a transit sequence. We investigated whether two actual plastid protein complexes, RUBISCO and the enzyme aspartate aminotransferase, also traffic through stromules. In Kwok and Hanson (2004a) we showed that fluorescent protein-labeled small subunit of RUBISCO assembled into the RUBISCO complex and aspartate aminotransferase fusions dimerized into active enzyme. Both labeled complexes could be visualized within stromules, and through photobleaching experiments, both could be shown to traffic through stromules.

Stromules can enter into grooves and channels within the plant cell nucleus (Kwok and Hanson, 2004b). Two-photon microscopy visualized the channels through serial cross-sections. These observations suggest that stromules may facilitate communication between nucleus and plastids, perhaps reducing diffusion distance for signaling molecules. We also observed that stromules appeared to closely contact other organelles and the plasma membrane. Possible intercellular contacts were detected as well, through putative plasmodesmata.

Stromules in wild-type plants were visualized by electron microscopy, clearly indicating the envelope membrane surrounding stroma and lacking thylakoid membranes (Holzinger et al., 2006). With a device allowing control of specimen temperature, it could be shown that small protrusions from the main plastid body become evident with increasing temperature. In Holzinger et al. (2008) we assessed the effect of ARC gene mutations on plastids in nongreen tissue and stromules. We found that *arc6* mutants exhibit longer stromules in most tissue types, but the *arc5* mutation had little effect on stromules. Long twisted stromules were documented in *arc3* mutants by electron microscopy.

An Arabidopsis line transformed with talin-GFP was used to visualize microfilaments and stromules simultaneously (Kwok and Hanson, 2004c). Stromules were observed to directly interact with microfilaments. Mitochondria, plastids bodies, and stromules sometimes interacted with the same microfilament. These observations stimulated our interest in working with Arabidopsis myosins in order to identify which myosins are responsible for moving particular intracellular organelles.

A cytochrome P450 was labeled with GFP and found to be localized to the tonoplast, rather than the chloroplast or mitochondrial location expected from previous analysis of this gene family. The encoded protein is likely to be an allene oxide synthase active on 9-hydroperoxides. Three days after pollination triggers petal senescence, transcripts of this cytochrome P450 gene are 40 times more abundant than in other tissues, but its role in senescence is unknown, though its

upregulation suggests that fatty acid metabolism is important in petal senescence (Xu et al., 2006)

Proteins encoded by six myosin XI genes have been analyzed by transient expression of YFP-myosin tail fusions. The six myosins all appear to localize to vesicles; one also labels peroxisomes. Because they lack a motor domain, the expressed proteins would be expected to cause a dominant-negative phenotype; however, movement of labeled organelles occurred in many cells, implying that other motors may be responsible for moving the same organelle in plants. Deletion constructs defined the amount of the tail region required for organelle labeling (Reisen and Hanson, 2007).

Domains of nine additional plant myosin XI tail regions were fused to YFP to assess whether specific localization can be mediated by regions homologous to those known to be important in cargo binding in yeast. The YFP-myosin domain fusions labeled Golgi, peroxisomes, mitochondria, or plastids as well as vesicles of undetermined identity. Using virus-induced gene silencing, we determined that myosin silencing affects both mitochondrial and chloroplast movement and positioning. Myosin VIGs-induced impairment also caused yellowing and wilting of leaves. (Sattarzadeh and Hanson, unpublished).

Publications With DOE Support Since 2004:

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Ishida H, K. Yoshimoto, M. Izumi, D. Reisen, Y. Yano, A. Makino, Y Ohsumi, M.R. Hanson, T. Mae. 2008. Mobilization of Rubisco and stromal-localized fluorescent proteins of chloroplasts to the vacuole by an ATG gene-dependent autophagic process. Plant Phys 148:142-55.

Holzinger, A., E. Y. Kwok and M. R. Hanson. 2008. Effects of *ARC3, ARC5,* and *ARC6* mutations on plastid morphology and stromule formation in green and nongreen tissues of *Arabidopsis thaliana.* Photochemistry and Photobiology 84: 1324-35.

PROGRESS REPORT

Completion of an earlier DOE project

The DOE grant provided supply funds for research of a sabbatical visitor from Japan, Hiroyuki Ishida. His arrival allowed completion of a project initiated by former graduate student Yan Xu who had graduated and joined a biotechnology company. Her thesis work was supported by our DOE grant between 1998 and 2000. Yan Xu had identified a unique cytochrome P450 associated with petal senescence and done considerable characterization of its sequence and expression. Dr. Ishida undertook a project to fractionate transgenic plants carrying a P450-GFP fusion, and found that the protein was localized to the tonoplast membrane. Dr. Daniel Reisen performed some confocal microscopy analysis of the same plants. These data allow me to rewrite, update, and submit the manuscript, which is published in *BMC Plant Biology*.

Descriptive analysis of stromules

A collaboration was initiated with an Austrian group that had observed stromules in alpine plants by electron microscopy. We provided an Arabidopsis line carrying GFP-labelled plastids as well as some information about our prior experiments. Andreas Holzinger, a skilled electron microscopist, was able to obtain excellent images of stromules and their locations within the cell, often in proximity to mitochondria and the nucleus. With a device allowing control of specimen temperature, it could be shown that stromules are not evident at low temperature. Small protrusions become evident with increasing temperature. This work is now published in *Protoplasma*.

In 2007-2008, we continued our collaboration with Andreas Holzinger, who came to my lab for a three-week in August 2007 to perform confocal microscopy. We had constructed Arabidopsis lines carrying 3 different *ARC* mutations and a chloroplast GFP markers. Mutants in *ARC* genes affect chloroplast division, resulting in plants with reduced numbers of chloroplasts in mesophyll cells. By introducing the chloroplast GFP marker gene in the *arc* mutant background, we could assess the morphology of *arc* mutant chloroplasts in nongreen tissues and determine the effect of the mutations on stromules. This work is published *Photochemistry and Photobiology*.

Two review articles were written to highlight tissue-specific stromule formation, trafficking of molecules, and possible functions. The first was published in *J. Microscopy* in 2004 and the second in *Plant Cell and Environment* in 2008. The P.I. has given 8 invited conference lectures and 3 invited research seminars on the DOE project during the project period.

Probing the function of stromules in leaf senescence

During his one-year sabbatical in my lab Dr. Hiro Ishida produced Arabidopsis lines useful for studying how chloroplasts decrease in protein content during short periods of light or sugar limitation. He obtained Arabidopsis lines labeled with dsRed in the chloroplast and analyzed our existing chloroplast GFP lines. He found that small autophagic vesicles containing stroma (so-called Rubisco-containing Bodies, or RCBs) exit chloroplasts from leaves held in darkness overnight and enter the vacuole for destruction. A likely mechanism for creation of RCBs is formation and breakage of stromules followed by their conversion into autophagosomes. To determine the role of the autophagic system in chloroplast senescence, Arabidopsis lines were

constructed that contained *atg* (autophagy) mutations and fluorescent protein-labeled chloroplasts. In *atg* mutants, RCBs do not form. Furthermore, in lines containing ATG-GFP fusion proteins and the chloroplast dsRed marker, both GFP and dsRed label RCBs, indicating they are autophagosomes. This work demonstrates that chloroplast proteins are not merely destroyed within the chloroplast during periods of nutrient limitation, but also that stromal contents are recycled by shuttling to the vacuole. This work is published in *Plant Physiology*.

Role of plant myosin XI in mobility of mitochondria and plastids/stromules

Early inhibitor studies we performed sparked our interest in taking our project in a new direction: analysis of the role of myosins in formation of stromules and in organelle movement.
In Kwok and Hanson (2003), we showed that the presence of the actin-inhibited drugs cytochalasin D (CD) or latrunculin B (LatB), plastid and stromule movement ceased and average plastid length decreased. Stromule number and length decreased. Five hours after inhibitor was removed, plastids resumed movement though stromules had not yet reformed, indicating stromules are not required for plastid movement. Treatment with tubulin inhibitors gave contrasting results. While the number of stromules descreased somewhat, long, thin stromules remained and their motility increased, as if the microtubular network was constricting movement of stromules. Washout of inhibitor results in reduction of stromule movement to initial values after 5 hours (Kwok and Hanson, 2003)

Subsequently, in order to visualize interaction of microfilaments with plastids and stromules, we obtained an Arabidopsis line carrying talin-GFP and visualized stromules and plastids by DIC in hypocotyl cells. Stromules and plastids sliding along actin microfilaments were evident, and stromule tips sometimes appeared to be waving freely, then "grabbed" by a microfilament. Movies illustrating these actin/stromule interactions were published in 2004 in *BMC Plant Biology*. We have also examined the effect of myosin inhibitors N-ethyl maleimide (NEM) and butanedione monoxime (BDM). Treatment with either inhibitor resulted in cessation of cytoplasmic streaming and immobilization of stromules (Kwok and Hanson, unpublished).

Fusion of fluorescent proteins with plant myosin XI genes

Myosins all have three domains: a "head" motor domain, which binds ATP and interacts with ATP, a neck domain comprised of the so-called IQ motif, which binds calmodulin or other proteins, and a tail domain (Reddy, 2001). Plant myosins of class XI, like myosin Vs of yeast and animals, have coiled-coil regions in the tail, likely indicating that the protein forms a homodimer (Fig. 1). The tail domain is the least conserved and may provide specificity in interaction with various cargoes, as appears to be the case in other systems (Reilein et al., 2001).

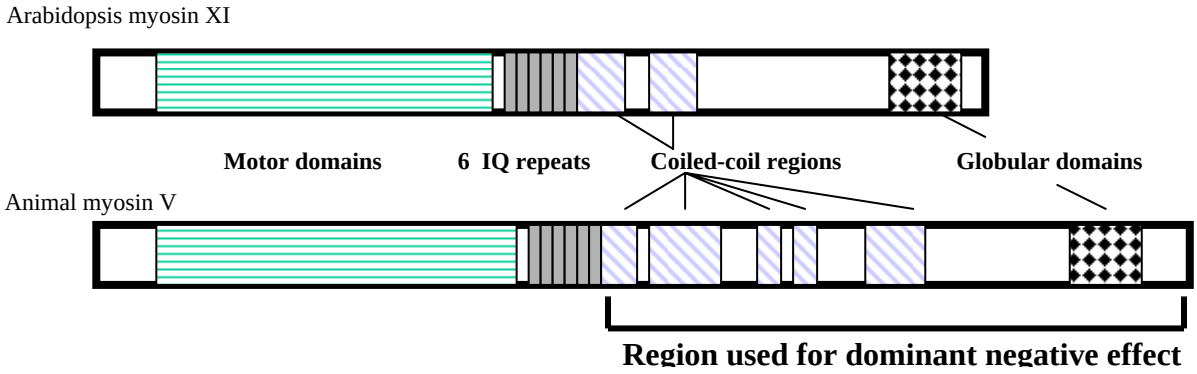

Fig. 1. Comparison of structure of one of the Arabidopsis myosin XIs and an animal myosin V. Data from Reddy and Day (2001) and Morimatsu et al. (2000). Expression of the C-terminal animal myosin V region without the motor domain results in cessation of movement by a dominant negative effect.

Seventeen Arabidopsis myosin genes, all in classes VIII AND XI, are detectable in the genome database (Reddy and Day, 2001) The 13 plant myosin genes in class XI are the most relevant in connection with organelle movement, because class XI myosins are related to the class V myosins, which are involved in organelle transport in vertebrates and yeast.

Postdoctoral associate Daniel Reisen fused various portions of the tail region of 6 myosin XI genes to YFP and assessed the fusion proteins' locations in transiently transformed plant cells. He found that the particular portion of the tail region used in the fusion affected the subcellular targeting, perhaps by changes in protein folding. One of the 6 myosins specifically labeled peroxisomes; constructs containing portions of all 6 myosins labeled vesicles of various sizes, though the size of the myosin fragment needed for vesicle labeling differed. One unexpected finding is that the fluorescently labeled organelles remained motile, though sometimes motility was reduced. In animal cells, constructs lacking the motor domain but carrying the coiled-coil region result in dominant-negative phenotypes, preventing movement of the labeled organelles. The lack of motility is thought to be caused by dimerization of the defective myosin with wild-type myosins, resulting in an ineffective motor (Rogers et al., 1999). We suspect that the continued motility results from (1) inadequate amounts of the transiently expressed defective myosin to bind to all myosins capable of moving a particular cargo, allowing continued movement mediated by normal myosins and/or from (2) motility of plant organelles mediated by more than one myosin, to which the defective myosin does not bind. This work was published in *BMC Plant Biology*.

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