d) PROJECT DESCRIPTION

It has long been appreciated that plant growth and development is regulated by touch, gravity, osmotic pressure, and other mechanical signals (reviewed in (1)). The nature of the molecules that directly perceive these forces in plants has been much discussed (2-7), but none has yet been definitively identified. To begin to shed light on the molecular mechanism by which plants perceive mechanical stimuli, we are characterizing a family of mechanosensitive (MS) ion channels found in the model flowering plant *Arabidopsis thaliana*. This family of proteins is related to the well-studied bacterial mechanoreceptor MscS, and our studies take advantage of a large body of knowledge regarding its structure and function. As MscS-Like (MSL) proteins are widespread in plants, but not found in animals, the MSL proteins of Arabidopsis provide a unique opportunity to investigate an intriguing class of MS channels in the context of a multicellular eukaryote.

We have shown that several MSL proteins function as MS channels (the first to be identified at the molecular level in land plants), making them excellent candidates for plant mechanoreceptors. Unexpectedly, the two channels about which we have the most functional information, MSL2 and MSL3, appear to mediate the perception of internal rather than external forces. MSL2 and MSL3 are localized to the poles of plastids, where they are fundamentally involved in controlling the size and shape of these plant-specific organelles. Organelle morphology control is both a novel function for MS channels, and is itself a little understood process. As MS channels are uniquely adapted to sense and respond to membrane bending, they may provide a general mechanism for controlling organelle and cell shape by transmitting mechanical information about membrane tension to the regulators of division and shape.

The primary goal of this proposal is to further our understanding of how the MS channels MSL2 and MSL3 control plastid size and shape. We test the hypothesis that MSL2 and MSL3 constitute a size-sensing apparatus at the pole of each plastid that communicates plastid size to the division machinery by altering ion flux in response to membrane tension. Electrophysiological characterization of the MSL2 and MSL3 channels is under way in collaboration with Drs. Doug Rees and Rob Phillips (both at California Institute of Technology), as part of a separate proposal. Also, as *MSL2* and *MSL3* are the only members of the MscS-Like gene family to exhibit a physiological phenotype when mutated, further investigation into the function of the other eight *MSL* genes is not proposed here, but will be pursued in the future.

BACKGROUND and SIGNIFICANCE

Mechanical Force in Biology. A basic question in biology is how proteins, cells, and organisms accomplish the task of force perception (8-10). Mechanotransduction, the process by which physical information is converted to a physiological signal, has been under investigation in plant systems for more than 100 years (11). Mechanical stimuli like gravity, temperature, and touch elicit a wide array of responses in plants, including organ movement, tropic growth, and alterations in developmental program, gene expression, and cell division plane. As sessile organisms, plants must develop specific strategies to deal with environmental stresses like wind and salt. In animal and bacterial systems, MS channels have been implicated in the perception of many mechanical stimuli (reviewed in (12)). Tension-responsive ion channel activities have been described in the plasma and vacuolar membranes of many plant species and plant cell types, suggesting that plants also use MS channels to sense and respond to force (see (13) for a summary). The pervasive nature of MS channels in plant membranes implies that they are important, and suggests that understanding their molecular and physiological function will provide important insights into plant biology in general. Furthermore, revealing the molecular nature of mechanotransduction in both plants and animals is essential if we wish to understand the full repertoire of strategies used by cells to sense mechanical stimuli.

MscS, a Canonical MS Channel. The only known mechanoreceptor for which plant homologues have been identified is MscS, the Mechanosensitive channel of Small conductance. MscS was first identified as an electrophysiological activity in the inner membrane of giant *E. coli* spheroplasts (14-17) and has been

well-studied at the functional, structural, and biophysical levels (reviewed in (18)). The crystal structure of MscS revealed a homoheptamer with three transmembrane (TM) domains and a large cytoplasmic domain (19). The molecular mechanism by which MscS functions is a topic of vigorous research, and it has been demonstrated that the MscS channel opens directly in response to changes in membrane tension, without any requirement for other proteins or cellular structures (20). MscS serves a critical function in bacteria, operating as an osmotic safety valve to protect cells from lysis upon severe hypoosmotic shock (21). It is thought that the increased internal pressure resulting from hypoosmotic shock causes a thinning of the lipid bilayer, and induces a rearrangement of the hydrophobic transmembrane helices so as to maintain their immersion in the plane of the membrane. Increased osmotic pressure also causes an increase in membrane curvature, which may alter the lipid-protein interactions at each leaflet of the bilayer (22).

Organelle Morphology Determination. In many organisms, the size, shape, and number of organelles varies according to cell type and growth conditions. Within a single cell, organelle morphology can be rapidly and dynamically altered during organelle movement, division, or trafficking. In plant systems, altered mitochondrial morphology is associated with de-differentiation and cell death (23, 24), and a specialized form of ER is found in the gravity-responsive cells of the tip (25). Recent interest in this area in mammalian and yeast systems has focused on the role played by membrane curvature in the production and alteration of organelle shape (26, 27). Membrane bending can be induced by altered lipid composition, the action of scaffolding proteins, or the intercalation of wedge-shaped proteins into the outer leaflet of an organelle envelope. The observed dynamic changes in membrane bending might activate MS ion channels, altering ion flux across the membrane and providing a mechanism by which organelle shape can be sensed.

Plastid Biology. Plastids are plant-specific endosymbiotic organelles, responsible for a variety of essential metabolic reactions that include photosynthesis, fatty acid synthesis, and amino acid synthesis (28). Though plastids retain multiple copies of a small, circular genome, they rely on the nuclear genome to direct much of their structural and regulatory protein production (29). During development, small proplastids present in the plant embryo become specialized, depending on the cell type and environmental conditions they are in (30). In this proposal, a distinction is made only between chloroplasts and all other "nongreen" plastids. Plastids are often referred to as "kidney" or "football"-shaped, but their shapes are highly variable and depend on species and cell type. One morphological feature common to many plastids is the stromule, a protrusion of the plastid envelope into the cytosol capable of transmitting small proteins (like GFP) from the stroma of one plastid to another (31-33). Plastid shape determination has not been directly studied. Several mutants isolated on the basis of their variegated leaves also have altered chloroplast morphology, in that they are underdeveloped (34, 35). Mutants with altered chloroplast size, (*accumulation and replication of chloroplasts*, or *arc* mutants), were directly identified through a genetic screen (36). Enlarged chloroplasts are also observed in mutants with defects in components of the plastid division machinery, several of which are homologous to bacterial fission proteins (reviewed in (37)). The effect of these mutations on plastid shape has not been reported. In general, the production of enlarged chloroplasts has been attributed to defects in the process of division rather than from defects in size control or perception.

Plastid Division and Bacterial Fission. Plastids are derived from an ancient cyanobacterial endosymbiont. They divide through binary fission, using proteins derived from their bacterial ancestor (38). Bacterial fission (reviewed in (39)) requires the formation of a contractile ring generated by FtsZ, a highly conserved tubulin-like GTPase. The correct placement of the FtsZ ring at midcell requires the action of MinE, MinD and MinC. In *E. coli*, MinC and MinD inhibit FtsZ function throughout the cell. In turn, MinE prevents MinCD function at midcell, allowing FtsZ ring formation at the correct location. Plant homologues of MinD and MinE have been identified and functionally characterized, and their function is consistent with their bacterial counterparts (37). Some differences from the bacterial system

exist; no MinC homologue has been identified in Arabidopsis, and there are two AtFtsZ proteins instead of one. In addition to the symbiont-derived plastid division machinery, operating from the plastid stroma, several proteins of eukaryotic origin (PDV1, PDV2 and ARC5) control constriction of the envelope from the cytosol (reviewed in (40).

PREVIOUS RESEARCH

1. Identification of MscS-Like Channels in Plants. MscS-related proteins were identified in *A. thaliana* and *S. pombe* by the Booth and Martinac groups (41, 42). We subsequently performed an extensive search for MscS family members in the eukaryotic lineage, and found multiple MscS family members in all plant genomes sequenced so far, in many fungal genomes, but not in animals (13). The portion of MscS that is conserved, referred to here as the MscS domain, forms the helix that lines the pore of the channel and an adjacent portion of the cytoplasmic chamber referred to as the middle β-domain (marked in red in Figure 1A and shown in Figure 1C). Outside of this stretch of approximately 100 amino acids, MscS family members are highly divergent, with predicted TM helix number ranging from 3 to 11, and heterogeneous extensions at both the N- and C-termini (43). There are ten MscS-Like (MSL) proteins in *Arabidopsis thaliana*; their predicted evolutionary relationship to each other and to MscS is shown in Figure 1B. As the MSL proteins are excellent candidates for mechanoreceptors in plants, with the potential to mediate the cellular response to mechanical stimuli in plants, we have initiated a complete analysis of their structure and function.

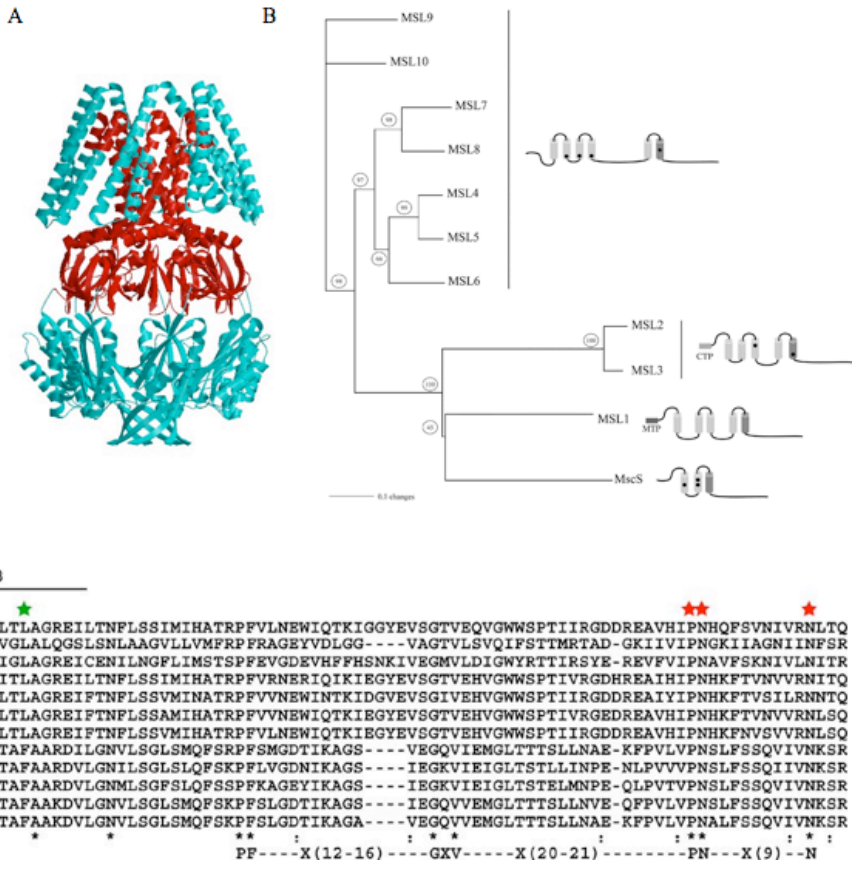

Figure 1. MscS Family Conserved Domain. A) The structure of E. coli MscS (after Bass, et al., 2002). The region marked in red is conserved among MscS family members, comprising TM helix 3 and a portion of the cytoplasmic domain known as the middle β domain. B) Phylogenetic tree of the MSL protein family, generated by CLUSTALX and PAUP. Predicted topologies are shown to the right of the tree. C) Alignment of residues in the conserved domain from MscS family members in *E. coli* (MscS), Arabidopsis (MSL3, MSL1), Chlamydomonas (MSC4), and other plant genomes. A consensus sequence is shown below the alignment. Hydrophobic seal residues are marked with green stars and a highly conserved motif is marked with red stars.

2. MSL Proteins are Mechanosensitive Channels. Like their bacterial counterpart MscS, the MSL channels are gated by membrane tension. We have shown that MSL1 and MSL3 can rescue the osmotic shock sensitivity of an *E. coli* MS channel mutant ((44), unpublished data). Furthermore, in collaboration

with the Barbier-Brygoo group at the INRS in Paris, we have used complementary electrophysiological and molecular genetic approaches to demonstrate that *MSL9* and *MSL10* are directly required for an MS channel activity in the plasma membrane of the Arabidopsis root (45). The ability to form a MS channel may be a conserved feature of the larger MscS family, as it was recently shown that MSC1, a MscS family member from the green alga *Chlamydomonas reinhardtii*, provides a MS channel activity when expressed in *E. coli* spheroplasts (46).

3. MSL Protein Expression. Tissue-specific expression of reporter transgenes and the intracellular localization of GFP-fusion proteins suggest that MSL proteins function in many aspects of plant biology. They are found in discrete cellular compartments: three of the ten proteins (MSL1, MSL2, and MSL3) localize to mitochondria or to chloroplasts, while several of the other seven (MSL4-MSL10) have been localized to the plasma membrane ((44, 45), unpublished data). Many of the *MSL* genes are expressed in a cell-type specific manner, particularly in cells that undergo large changes in turgor. These include the cells of the vasculature (transport water and solutes throughout the plant), guard cells (regulate the opening and closing of stomata on the leaf surface), cells undergoing elongation, and the stigma cells of the carpel (exude water upon interaction with pollen during fertilization) (13).

4. MSL Channel Localization. The MSL proteins show a distinctive localization to sub-domains within the membrane. MSL2 and MSL3-GFP fusion proteins are observed at the poles of the plastid envelope (Figure 2). MSL9 and MSL10, two MSL channels expressed in the root, localize to the cell plate during cell division (45). MSC1 is found in punctate spots in the cytoplasm and the chloroplast of algae (46), and MscS aggregates in the membrane, though a specific localization to the cell poles has not been observed (unpublished data, (47)). Clustering may be a common feature of MS channel complexes, as there is evidence for clustering of MEC-4 and MEC-10, two mechanosensors involved in touch perception in *C. elegans*, and MS channels in plants and bacteria show bursts of activity under the patch pipette that is consistent with combinatorial activation (48, 49). In the case of MSL2 and MSL3, our preliminary data suggest that localization to plastid poles is correlated with proper channel function (see below). The experiments proposed here will further elucidate the mechanism and the role of polar localization of these channels. The localization of MSL2 and MSL3 clusters to the plastid poles, where membrane curvature is highest, may be important in this regard.

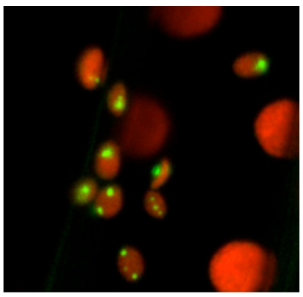

Figure 2. Localization of MSL3. Chloroplasts (red) in an epidermal cell of the Arabidopsis stem. MLS3~GFP (green) is localized to one or both poles of the plastid.

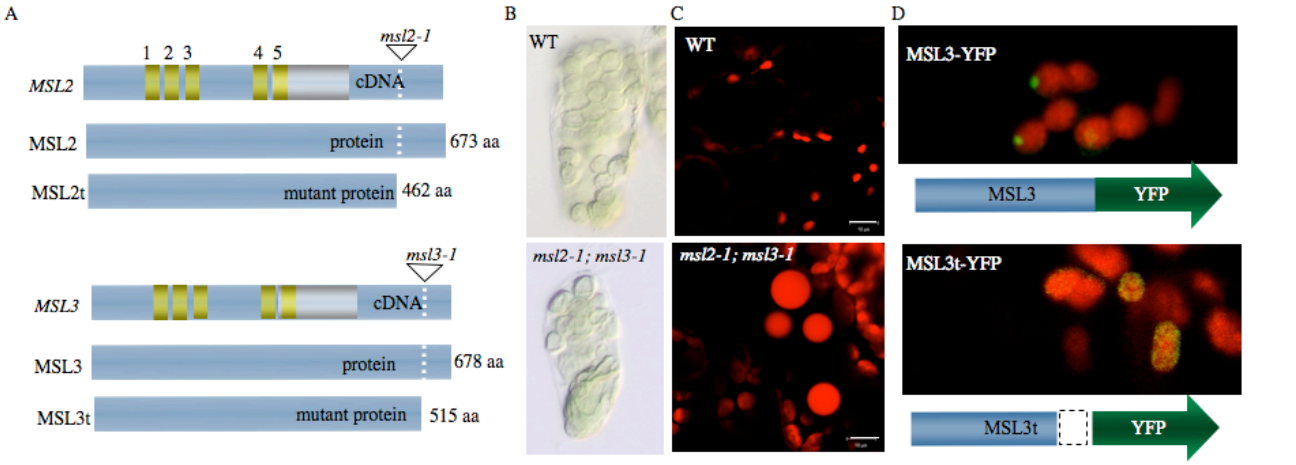

Figure 3. MSL2 and MSL3 play a role in plastid morphology. A) Schematic of *msl2-1* and *msl3-1* alleles. TM helices are shown in gold and the conserved MscS domain in silver. B) Light microscopy of a fixed mesophyll cell from each genotype. C) Non-green plastids in the epidermis. Plastids are visualized with RecARed, a reporter gene encoding dsRED targeted to plastids with the RecA plastid transit sequence. D) MSL3 fused to YFP localizes to the poles of plastids, while MSL3t~YFP is distributed throughout the plastid envelope. The dotted box indicates the C-terminal domain which is deleted in the *msl3-1* allele. Both fusion proteins were expressed in a wild-type background, under the control of the endogenous MSL3 promoter. Red is chlorophyll and green is YFP.

5. Genetic Analyses of MSL Channel Function. Insertion alleles of all *MSL* genes have been isolated, and combinations of up to five mutant alleles made (45). To date, the only *msl* mutants for which a physiological defect has been identified are plants with lesions in *MSL2* and *MSL3*. *MSL2* and *MSL3* encode two highly similar channels with a chloroplast transit peptide, 5 TM helices, and the conserved MscS domain (see Figure 3A). Analysis of several mutant lines indicates that *MSL2* and *MSL3* are involved in the control of plastid morphology.

We first isolated and characterized homozygous lines harboring T-DNA insertions in the last exon of *MSL2* (*msl2*-1) and *MSL3* (*msl3*-1) (Figure 3, (44)). Neither single mutant is distinguishable from the wild-type, but *msl2-1; msl3-1* double mutant plants have variegated leaves (not shown), enlarged chloroplasts and enlarged, spherical nongreen plastids (Figure 3B, C). RT-PCR experiments confirm that the T-DNA insertions do not affect *MSL2* or *MSL3* transcript levels, suggesting that truncated versions of MSL2 and MSL3 (referred to here as MSL2t and MSL3t) are produced from each allele. Functional analysis of these truncated proteins has proven informative. MSL3t can rescue the osmotic shock sensitivity of an *E. coli* MS channel mutant (data not shown), suggesting that the truncated versions are not defective in MS channel function *per se*. However, MSL3t- and MSL2t-YFP fusion proteins have aberrant intraplastidic localization, appearing dispersed around the periphery of the plastid rather than clustered at the poles. (Figure 3D) These results suggest that the polar localization of MSL2 and MSL3 may be important for their function, and that the C-terminus of each protein is critical for both polar localization and for plastid size control.

We have recently obtained an allele of *MSL2* (*msl2*-3) that encodes a stop codon at amino acid 98 ((50), data not shown). As this insertion is predicted to produce a peptide of only 24 amino acids, *msl2-3* is likely to be a null allele. (No candidate null allele of *MSL3* is yet available). Homozygous *msl2-3* plants produce two to three deformed, variegated leaves early in development, but later leaves appear wild-type (Figure 4A). A preliminary analysis of chloroplast morphology in the early leaves of *msl2-3* mutants by

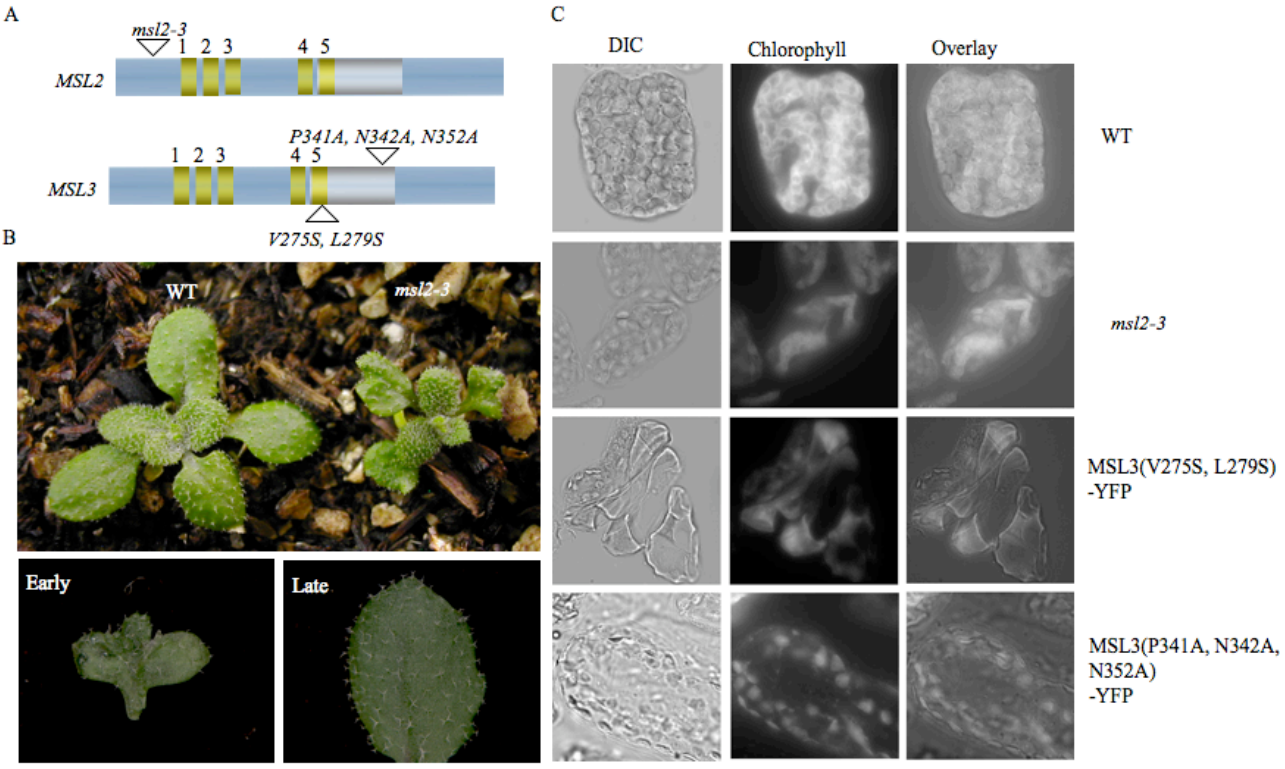

Figure 4. MSL2 and MSL3 Play a Role in Plastid Morphology. A) Schematic of *msl2-3* and *MSL3* point mutants. TM helices are in gold and the conserved MscS domain is in silver. B) Leaf morphology phenotype of the *msl2-3* mutant. C) Chloroplast morphology in mesophyll cells from wild-type leaves, *msl2-3* mutant leaves, and from plants overexpressing MSL3(V275S,L279S)-YFP, or MSL3(P341A,N342A, N352A)-YFP.

light and fluorescence microscopy revealed profoundly malformed and enlarged chloroplasts (Figure 4B). These data support our hypothesis that the control of plastid morphology is an integral function of MSL2 and MSL3, and given their proposed function as MS channels, suggests that the maintenance of organelle shape and size requires the perception of mechanical events.

A role in cell or organelle morphology may have developed early in the eukaryotic lineage, as defects in cell shape have not been reported for bacterial MscS mutants, but a MscS-Like protein is required for normal chloroplast morphology in the unicellular alga Chlamydomonas (46). This relatively new function still requires the conserved MscS domain. Preliminary data show that mutating two highly conserved features of the MSL family found in MSL3 results in altered plastid morphology. In the crystal structure of MscS, L105 and L109 lie within the conserved membrane spanning region and form a constriction at the cytoplasmic end of the pore called the hydrophobic seal ((19), see Figure 1 and 4A). Replacing L109 in MscS with serine causes a strong gain-of-function (GOF) phenotype in *E. coli*, primarily characterized by inhibited growth (51). Another conserved motif, with unknown function, is found at the C-terminal end of the MscS domain (Figure 1C). Mutant versions of MSL3-YFP containing mutations in the hydrophobic seal residues (V275S, L279S) and in the conserved motif (P341A, N342A, N352A) were generated and introduced into wild-type plants under the control of a strong, constitutive promoter (Figure 4A). Plants expressing MSL3 (V275S, L279S)-YFP and MSL3 (P341A, N342A, N352A)-YFP exhibited extreme variegation and grossly enlarged chloroplasts, and small rounded chloroplasts, respectively (Figure 4C). Neither of these phenotypes was seen in cells or plants expressing the wild-type MSL3-YFP. The fact that alteration of two residues in the predicted permeation pore of MSL3 causes significant defects in plastid shape and size suggests that the transduction of ions through MSL3 is critical for plastid shape control.

5. Interactions between MSL2, MSL3, and the Plastid Division Machinery. Several pieces of preliminary data suggest that there is a functional relationship between MSL2 and MSL3 and the plastid division (PD) machinery. First, the disruption of many components of the PD machinery (including AtMinE, AtMinD, and AtFtsZ1) produces enlarged plastids similar to those observed in the *msl2-1; msl3-1* double and the *msl2-3* null mutant (38, 52, 53). Second, the characteristic polar localization of MSL2 and MSL3 closely resembles that described for AtMinD and AtMinE ((54), and Figure 5A), and we have further shown that MSL2 and MSL3 and AtMinE co-localize at the poles of plastids (44). Third, the intraplastidic localization of AtMinD-YFP is dramatically altered in *msl2-1; msl3-1* mutants. As shown in Figure 5B, both AtMinD-YFP and AtMinE-YFP are localized to the poles of root epidermal plastids in wild-type plants, but in the enlarged, spherical plastids of the *msl2-1; msl3-1* mutant AtMinD-YFP is primarily localized to the stroma. Fourth, we have uncovered a genetic interaction between *MSL2*, *MSL3*, and *AtMinD*. When *msl2-1; msl3-1* double mutants were crossed to the *arc11* mutant (a loss-of-function allele of AtMinD (52)), only a small subset of the possible genotypes was recovered in the F2 generation. An *arc11-/-; msl2-1 +/-; msl3-1 +/-* individual was identified, selfed, and its progeny genotyped; no triple mutants were identified in extensive screening of over 95 individuals. The interaction between *msl2-1;msl3-1* and *arc11* appears to be specific, as triple *arc3; msl2-1*, *msl3-1* mutants were easily obtained by this same crossing strategy (data not shown).

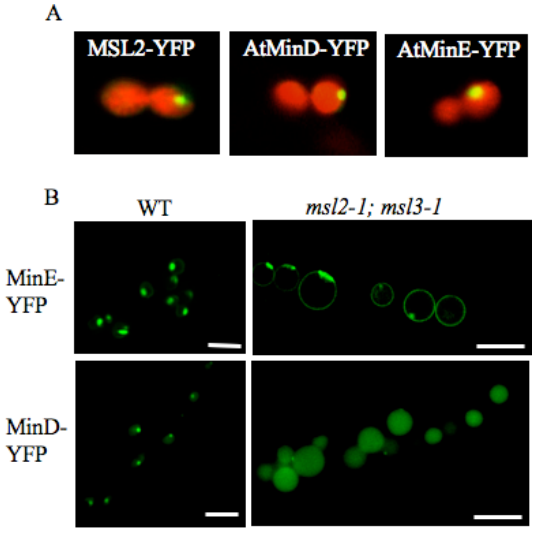

Figure 5. A) MSL2, MinD and MinE localization to the poles of dividing plastids. B) MinD-YFP and MinE-YFP localization in wild-type and mutant plastids of the root epidermis. Red is chlorophyll; green is YFP signal. Bars are 5 µm.

PROPOSED RESEARCH

Taken together, our preliminary data support a working model wherein mechanosensitive channels in *Arabidopsis thaliana* function to inform plastid size through interaction with the PD machinery at the plastid poles. The experiments proposed here build on this preliminary data, and have the following goals:

- Understand how and why MSL2 and MSL3 localize to the plastid poles.
- Further investigate genetic and physical interactions between MSL2, MSL3, and the plastid division machinery.
- Explore the role played by MscS-Like proteins in cyanobacteria (prokaryotic cousins of the chloroplast), incorporating the contributions of a high school teacher-intern.
- Develop nongreen plastids in Arabidopsis as a model system for the study of organelle morphology, a small-scale project to be undertaken by undergraduate researchers.

Funding to address the relationship between specific structures and molecular functions of MSL2 and MSL3 (including a collaborative effort at electrophysiological analysis of these channels) and to study the other eight proteins in the MSL family is requested from other sources.

I. MSL2 and MSL3 Localization to the Plastid Poles

The localization of MSL2 and MSL3 to the poles of plastids probably has functional significance, as aberrant localization of MSL2 and MSL3 is correlated with abnormal plastid morphology in the *msl2-1; msl3-1* double mutant. We therefore wish to determine how and why this localization takes place. MSL2 and MSL3 might be localized to the plastid poles through interaction with other proteins already found there (AtMinE and AtMinD are prime candidates), or through partitioning to specialized lipid domains (such as the cardiolipin domains of *E. coli* (55)). The former explanation is most likely, as topology analyses predict that the C-termini of MSL2 and MSL3, which are required for polar localization of both proteins, are in the stroma rather than in the membrane. We therefore propose a number of experiments designed to further delineate the localization domain of MSL2 and MSL3 and to identify proteins that bind to their C-termini (as well as to the full-length proteins). We also propose to characterize temporal and dynamic aspects of the polar localization of MSL2 and MSL3, and begin to address the functional relevance of this phenomenon. Expressing MSL2- and MSL3-GFP in the *msl2-1; msl3-1* double mutant rescues the variegation phenotype of this mutant, suggesting that these fusion proteins are functional. However, an important tool for many of the experiments described below will be antibodies directed against MSL2 and MSL3. MSL2- and MSL3-specific antibodies will allow us to determine the localization of endogenous and transgenic proteins without the artifacts associated with variable expression levels of a transgene or the potential functional consequences of fusion to a tag.

1. Identify the cis-acting sequences involved in polar localization. As shown in Figure 3D, full-length MSL2 and MSL3 localize to the poles of plastids, while MSL2t and MSL3t (proteins truncated as predicted in the *msl2-1; msl3-1* mutant) do not. We will perform a straightforward deletion analysis of the C-termini of MSL2 and MSL3, to identify the minimal sequence required for localization to the plastid poles. If localization is important for function, it is likely conserved; a highly conserved 24 amino acid sequence found at the C-terminus of all chloroplast-targeted MscS homologues is an obvious candidate to mediate the localization (Figure 6A). This sequence is not found in non-plant MscS-Like proteins, nor in any other plant proteins, suggesting that it is specific to those MS channels found in chloroplasts. We have made several MSL3-YFP fusion constructs designed to test the relevance of this sequence, referred to as the putative localization domain, or PLD (Figure 6B). These constructs will first be screened for defects in polar localization by transient expression in onion cells. Selected constructs will then be put into plants for stable expression under the control of their own promoter, as high expression of even the

wild-type protein can result in signal around the entire plastid envelope. We will also determine if the PLD alone is sufficient to localize just the transmembrane domain of MSL2 or MSL3 to the plastid poles.

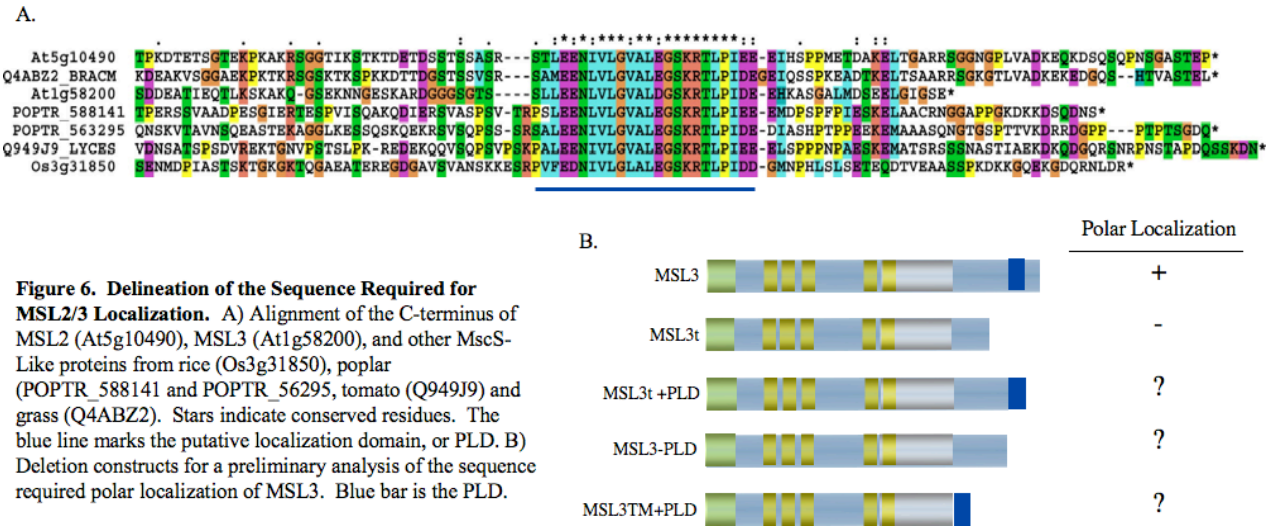

Figure 6. Delineation of the Sequence Required for MSL2/3 Localization. A) Alignment of the C-terminus of MSL2 (At5g10490), MSL3 (At1g58200), and other MscS-Like proteins from rice (Os3g31850), poplar (POPTR_588141 and POPTR_56295, tomato (Q949J9) and grass (Q4ABZ2). Stars indicate conserved residues. The blue line marks the putative localization domain, or PLD. B) Deletion constructs for a preliminary analysis of the sequence required polar localization of MSL3. Blue bar is the PLD.

2. Identify proteins that bind to the C-termini of MSL2 and MSL3 (and to the full-length proteins). To identify proteins that might mediate the polar localization of MSL2 and MSL3, and for clues to their function, we aim to find binding partners for MSL2 and MSL3. We began with a yeast two-hybrid approach, and tested for direct interactions between the soluble domains of MSL2 and MSL3 and the plastid division proteins AtMinD, AtMinE, AtFtsZ1, and AtFtsZ2. These experiments were not successful (alternative approaches to directly test for interactions between MSL2, MSL3 and plastid division proteins are described below in Section II.3). It is possible that the soluble domains used in our experiments do not have the proper structure for interaction with binding partners. However, the C-terminus (soluble domain) of PamA, a MscS homologue from *Synechocystis*, does interact with the signaling protein PII in a yeast two-hybrid experiment. This interaction was confirmed in vitro, and PamA appears to play a vital role in PII signaling (56). As these results confirm that the soluble domains of MscS-like proteins are capable of making relevant protein-protein interactions, we will perform a yeast two-hybrid screen for proteins that interact with the soluble domain of MSL2 and MSL3. Though all interacting proteins will be pursued, interactors that bind specifically to full-length MSL2 and MSL3, and not to MSL2t or MSL3t will be candidates for proteins that mediate polar localization.

Though it is accessible, easy to perform, and has been successfully applied in a related system, the yeast two hybrid approach has several drawbacks: both bait and prey must be soluble, and it is restricted to proteins that are functional and present in the prey library (for example, we might miss any proteins that are tissue-specific or expressed at low levels). We are therefore also preparing an unbiased biochemical approach to identify chloroplast proteins that interact *in vivo* with affinity-tagged versions of MSL2 and MSL3. FLAG- and GFP-tagged versions of MSL2 and MSL3 are efficiently solubilized in 1% octyl β-glucoside, a non-ionic detergent (data not shown). Chloroplasts will be purified according to the method of Somerville et al. (57) as previously described (44). Chloroplast extracts are considerably less complex than whole cell extracts—the chloroplast proteome is estimated at approximately 2400 proteins, 10-fold less than in the entire cell (58)—and may significantly reduce background signals or false positives among candidate interactors. We will use 2-D gel electrophoresis, spot-picking, and mass spectrometry to identify proteins that co-immunoprecipitate with affinity-tagged versions of MSL2 and MSL3. We will do these experiments in collaboration with Dr. Leslie Hicks at the Donald Danforth Proteomics and Mass Spectrometry Facility, as she specializes in proteomic approaches with plant proteins (see collaboration letter in Special Information and Supplementary Documentation, Section j). Again, any protein that interacts with MSL2 and MSL3 will be interesting, but we will first pursue any

interactors that are specific to full-length MSL2/MSL3, failing to interact with MSL3t or MSL2t. A useful approach may be to compare the protein interaction profiles of full-length and truncated proteins (by 1-D or 2-D electrophoresis of eluted proteins), and focus on isolating bands or spots that specifically associate with the full-length protein.

Once potential interactors are identified and validated by the above approaches, we will evaluate their contribution to MSL2 and MSL3 localization, as well as to any processes in which MSL2 and MSL3 are implicated. T-DNA insertions in the genes that encode the interacting proteins will be obtained, if possible, from publicly available seed stocks (50, 59, 60). If not, the expression of artificial microRNAs or double-stranded RNA silencing can be used to reduce the expression of genes in Arabidopsis (61, 62). MSL2- and MSL3-GFP fusion proteins will be expressed in these mutant or reduced-expression lines, and their localization assayed by fluorescence microscopy. We will also characterize any alterations in chloroplast and nongreen plastid morphology present in the mutants, and look for any genetic interactions with *msl2* or *msl3* mutant alleles.

3. Characterize the temporal and dynamic nature of MSL2 and MSL3 localization in wild-type and mutant plastids. Live-imaging approaches will be used to follow the localization of MSL2 and MSL3 during plastid development and division. The Haswell laboratory has a laser scanning confocal microscope, and the PI has previous experience with live imaging approaches in Arabidopsis from her postdoctoral work with Elliot Meyerowitz. We will characterize the temporal and dynamic behavior of MSL2-YFP and MSL3-YFP when expressed under the control of their endogenous promoters in live plants. We will investigate expression of these fusion proteins in the undeveloped pro-plastids of the embryo and meristem, and in the differentiated plastids of the leaves, shoot and root at the seedling, vegetative, and flowering stages. MSL2- and MSL3-YFP localization will also be monitored during live plastid division in the stems and petioles of young, rapidly growing seedlings (1 week old).

We will correlate any dynamic and developmental changes in localization of fluorescently tagged versions of MSL2 and MSL3 with that of critical components of the PD machinery. Live imaging of AtMinE or AtMinD has not yet been reported, and they may exhibit oscillatory behavior, as in *E. coli* (63, 64), or stable polar localization, as in *B. subtilis* (65). We will determine the extent of the association between MSL2 and MSL3 and AtMinE during the plastid division cycle, and to look for transient co-localization with AtMinD, AtFtsZ1, or AtFtsZ2. AtMinE-, AtMinD-, AtFtsZ1-, and AtFtsZ2-CFP fusion constructs were generated and introduced into plants. CFP (cyan fluorescent protein) fluorescence is distinguishable from YFP and GFP, but signal can be faint. If the CFP fusion proteins prove difficult to detect, we will instead use mCherry, a much brighter tag (66). To determine if mislocalization of MSL2 and MSL3 can affect the intraplastidic localization of PD machinery proteins, we will characterize co-localization of AtMinD, AtMinE, AtFtsZ1, and AtFtsZ2 with MSL2t- or MSL3t-YFP fusion proteins (these truncated versions of MSL2 and MSL3 do not localize to the plastid poles (Figure 3D)). We will also assess plastid division protein localization patterns and behavior in *msl2-1; msl3-1* and *msl2-3* mutant plastids (these experiments are described more fully in Section II.1).

An important control will be to test fusion protein transgenes for complementation of mutant alleles (if mutant alleles are available: *msl2*-3, *arc11*, *arc12* and *SALK_073878* are loss-of function mutations in *MSL2*, *AtMinD*, *AtMinE*, and *AtFtsZ1*, respectively). A possible pitfall in the imaging of plastid division is that the bright light generated by the confocal laser might affect the division of the chloroplasts, causing arrest. This may be avoided by imaging non-green plastids of the root, which are likely to be less photosensitive. Non-green plastids can be visualized with the RecARed reporter gene (see Figure 3C).

4. Characterize MSL2 and MSL3 localization in chloroplast division mutants. The observed co-localization between MSL2, MSL3, and MinE suggests that plastid division proteins may be required for proper intraplastidic localization of MSL2 and MSL3. To begin to test this idea, we will characterize the intraplastidic localization of MSL2- and MSL3-YFP fusion proteins in the many available plastid division mutants. The *accumulation and replication of chloroplast (arc)* mutants have enlarged chloroplasts and several have been shown to harbor lesions in critical components of the known PD machinery (*arc11* is

an allele of AtMinD, while *arc12* is an allele of AtMinE) (38, 52). Also available are several mutations in AtFtsZ1 (67). If any of these mutants are required for normal MSL2 and MSL3 localization, we will assess genetic and biochemical interactions between the proteins as described below in Section II.

5. Address the functional role of polar localization of MSL2 and MSL3. Evidence that the polar localization of MSL2 and MSL3 is important for their function is correlative; the *msl2-1; msl3-1* mutant has enlarged plastids, and the truncated proteins produced in this mutant are distributed throughout the plastid envelope (Figure 3D). The tools generated in the experiments described above should help us further address this issue. We will test the ability of each of the MSL2 and MSL3 protein deletion mutants (shown in Figure 6B) to complement the leaf morphology and plastid phenotype of the *msl2*-3 mutant (and other null mutants as they are identified). If MSL2 and MSL3 localization is important for their function, there will be a correlation between a defect in localization and an inability to complement mutants. Similarly, any mutant lines that do not localize MSL2 and MSL3 properly due to loss of a protein that interacts with MSL2 and MSL3 (identified based on protein-protein interactions with MSL2 and MSL3) will be assayed for defects in plastid morphology by light, confocal, and EM microscopy. Future experiments will be necessary to establish a causal relationship between plastid morphology changes and mislocalization of MSL2 and MSL3. These may involve using inducible RNAi (68) to reduce the expression of genes that affect MSL2 and MSL3 localization in a wild-type background, and then monitoring any subsequent alterations in plastid morphology.

II. Interactions between MSL2, MSL3, and the Plastid Division Machinery

Several of our previous results suggest a functional interaction between the PD apparatus and MS ion channels: co-localization of AtMinE, MSL2 and MSL3, mislocalization of AtMinD in an *msl2-1; msl3-1* mutant; and genetic interactions between MinD, MSL2 and MSL3. Below we propose several experiments designed to further investigate the genetic and physical interactions between MSL2 and MSL3 and plastid division proteins. Future experiments will explore the possibility that MSL2 and MSL3 affect plastidic Ca^{2+} levels, thereby affecting plastid division protein function. These experiments have the potential to uncover a new role for MS ion channels and a new mechanism for control of organelle morphology.

1. Determine if MSL2 and MSL3 are involved in the proper localization of plastid division proteins.

To test for an interaction between plastid division proteins and MSL2 and MSL3, we will determine if the localization of plastid division proteins requires the normal function of MSL2 and MSL3. Both AtMinD and AtMinE are localized to the poles of chloroplasts in wild-type plants, while AtFtsZ1 forms a ring around the chloroplast that generates the contractile ring during plastid division. We have constructed fluorescent protein fusions of AtMinD, AtMinE, and AtFtsZ, and begun to characterize their localization in wild-type and *msl2-1; msl3-1* mutant plastids. These experiments will also be performed in the early leaves of *msl2-3* mutants, and in other mutants as they become available. Preliminary results showed that AtMinD-YFP was mislocalized in *msl2-1; msl3-1* mutant plastids, while AtMinE-YFP localization was unchanged (Figure 5B). We are now constructing the lines that will allow us to repeat this experiment with the same AtMinD-YFP transgene in either a wild-type or *msl2-1; msl3-1* double mutant background (to control for the effects of transgene insertion location). Lines with low levels of expression will be isolated, as they are least likely to exhibit overexpression phenotypes. As a positive control for altered localization, we will construct versions of AtMinD-YFP carrying the A296G or K72A mutations, both of which show aberrant localization within the plastid (52, 69). The same plant lines expressing wild-type and mutant versions of AtMinD-YFP will be used in subcellular fractionation, a complimentary method to test for the effect of MSL2 and MSL3 on the localization of AtMinD. Chloroplasts will be purified and separated into soluble and membrane fractions as performed previously (44). Western blotting against the YFP tag in each fraction will allow us to quantify the amount of AtMinD in the stroma and in the

membrane, and to compare these values in mutant and wild-type plastids. The large subunit of Rubisco, a soluble stromal protein, will be used as a fractionation control.

We will also characterize the intraplastidic localization of AtFtsZ in wild-type and mutant plastids. AtMinD and AtMinE regulate AtFtsZ ring formation. If MSL2 and MSL3 alter plastid shape through interactions with AtMinE or AtMinD, AtFtsZ ring assembly should be affected in an *msl2-1; msl3-1* mutant. To visualize AtFtsZ rings, we are using immunofluorescence of whole-mounted, fixed chloroplasts, according to a protocol described in Strawn et al. (70). As a preliminary step, we have established that antibodies raised to *B. subtilis* FtsZ by Petra Levin (also at Washington University) can recognize Arabidopsis FtsZ1. Anti-BsFtsZ antibodies robustly recognize AtFtsZ1-YFP in overexpression lines on a Western blot (Figure 7A), and preliminary immunofluorescence experiments show clear rings of fluorescent signal (arrow, Figure 7B) that closely resemble those observed by Katherine Osteryoung's group using antibodies raised to Arabidopsis FtsZ1 and FtsZ2 (71, 72). After optimization of the conditions, we will compare the AtFtsZ rings present in wild-type with those in *msl2-1; msl3-1* and *msl2-3* mutant plants. As a negative control, we have obtained a previously described AtFtsZ1 null mutant (*SALK_073878, (67)*). This line will also allow us to distinguish between signal from AtFtsZ1 and AtFtsZ2. A letter of collaboration from Dr. Levin is included in Section j.

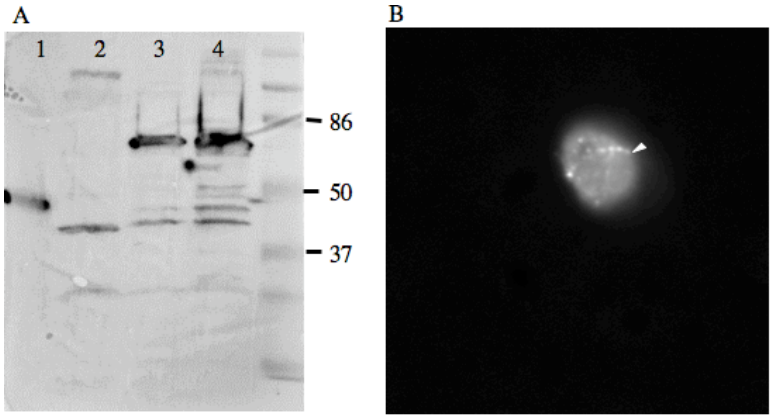

Figure 8. Antibodies raised to B. *subtilis* FtsZ recognize Arabidopsis FtsZ1. A) Western blot. Lane1: *B. subtilis* extract; lane2: *A. thaliana* extract; lanes 3 and 4: *A. thaliana* extract from lines overexpressing an AtFtsZ1-YFP fusion protein. B) Immunofluorescence showing AtFtsZ localization in a ring around a wild-type chloroplast. A FITC-labeled secondary antibody was used for detection.

Immunofluorescence has the advantage of avoiding the phenotypic effects of overexpression from a transgene, and it has been documented that overexpression of AtFtsZ can lead to alterations in plastid size (72). However, it is a time-consuming procedure and is performed on fixed tissue. As an alternative approach to visualizing AtFtsZ in mutant plastids, and to provide tools for the live-imaging experiments described in Section I.3, we have constructed AtFtsZ1-YFP fusion lines and will select low-expressing lines for analysis in wild-type, *msl2-1; msl3-1*, and *msl2-3* mutant backgrounds. We will also construct and analyze AtFtsZ2-YFP expression lines.

2. Characterize genetic interactions between MSL2 and MSL3 and plastid division proteins. To test for genetic interactions between *MSL2*, *MSL3* and genes that encode plastid division proteins, we have crossed the *msl2-1; msl3-1* mutant to the seven *arc* (*accumulation and replication of chloroplast*s) mutants available from a public database (60). Our preliminary results, wherein no viable triple mutants were recovered from a cross between *msl2-1; msl3-1* and *arc11,* point to a strong and specific genetic interaction between MS channels and the PD machinery (see Previous Results). We would like to follow up on this result through characterization of the terminal phenotype of the triple mutant, as it may give insight into the critical functions shared by these genes. To determine if the missing genotypes from this cross can be attributed to arrested embryogenesis, siliques from control and test lines (wild-type, *msl2-1; msl3-1*, *arc11*, and *msl2-1; msl3-1* x *arc11* F1) will be collected, cleared in Hoyer's solution, and embryo morphology inspected by DIC imaging. We will also stain fertilized carpels with aniline blue to look at pollen tube elongation in *msl2-1; msl3-1* carpels that are either self-fertilized or fertilized with *arc11* pollen. In this case and in any other interactions we detect, it will be important to test other alleles of the genes in question. For example, we will now analyze the phenotype of *arc11; msl2-3* double mutants. We will also continue our analysis of potential genetic interactions between the *msl2-1; msl3-1* double and

other *arc* mutants, and begin to assess interactions with other plastid division mutants, such as *pdv1pdv2* (73).

3. Protein-protein interactions between MSL2 and MSL3 and plastid division proteins. One way for MSL2 and MSL3 to affect the function of the PD apparatus is through direct protein-protein interactions. We have tested for *in vivo* interactions between MSL2, MSL3, and several plastid division proteins by the yeast two-hybrid, as described above in Section I.2, but no interactions were detected. An alternative approach would be co-immunoprecipitation of full length, affinity-tagged proteins. As a first approach, we will use FLAG-tagged versions of MSL2 and MSL3, which are currently being expressed in *E. coli* for antigen production. We will determine if FtsZ-, MinD- or MinE-YFP in plant extracts will interact with recombinant MSL2-FLAG or MSL3-FLAG using anti-FLAG agarose beads. Another attractive option is to use FRET (fluorescence resonance energy transfer) between YFP and CFP-fused proteins in vivo. This approach to testing for direct interactions has been used successfully to characterize the dimerization of AtMinD and its interaction with AtMinE (74). A deconvolution microscope appropriate for this experiment is available in the Biology Department Microscopy Facility.

4. Test the predictions of our model. In the long term, it will be important to test the predictions of any model we develop for the interaction between MSL2 and MSL3 and the PD machinery. For example, if our data suggest that MSL2 and MSL3 negatively regulate the association of AtMinD with the plastid envelope, we will determine if overexpression of AtMinD (using a strong, constitutive promoter to drive AtMinD expression *in planta*) will rescue the enlarged plastids in the *msl2-1; msl3-1* and *msl2-3* mutants. In *E. coli*, MinE controls the membrane association of MinD (75). If the plastid system is analogous, reducing AtMinE function will allow more MinD to associate with the membrane. To test this, we will determine if *arc12* (a lesion in the *AtMinE* gene (38)) suppresses the *msl2-1; msl3-1* plastid phenotype. We will also begin to consider specific mechanisms by which MSL2 and MSL3 might affect AtMinD function. For example, AtMinD's association with the plastid envelope is regulated by calcium ions (69), opening up the possibility that MSL2 and MSL3 affect AtMinD membrane association (and thereby plastid division) indirectly by altering the concentration of plastidic Ca^{2+}. Future experiments may include the use of *in vivo* calcium imaging to determine Ca^{2+} concentrations in wild-type and *msl2-1; msl3-1* double mutant and *msl2*-3 null mutant plastids. A chloroplast-targeted version of aequorin, a Ca^{2+}-selective photoprotein, has been used to detect Ca^{2+} fluxes in response to light conditions (76). Alternatively, a Ca^{2+}-sensitive YFP, referred to as Yellow Cameleon 2.1 (77), has been used to image Ca^{2+} transients in Arabidopsis roots and guard cells at the single-cell level (5, 78).

5. Compare the mechanical properties of wild-type and mutant chloroplasts. The possibility that the *msl2-1; msl3-1* mutant plastids are enlarged due to osmotic pressure is consistent with the known function of the bacterial channel MscS, and provides an explanation for the size and shape changes that are observed in the nongreen plastids of mutant plants (this explanation is not mutually exclusive with a defect in plastid division control). To test this hypothesis, we will purify chloroplasts from wild-type and mutant plants and subject them to increasing levels of osmotic shock. If the *msl2-1; msl3*-1 mutant chloroplasts are indeed under pressure, they will lyse after a smaller downshock than wild-type chloroplasts. As chloroplasts are routinely purified in 400 mM sorbitol (57), purified wild-type and mutant chloroplasts will be serially diluted into buffer without sorbitol, and the percentage of intact chloroplasts at each dilution will be quantified via ferricyanide reduction (79). Another approach to the same question will involve subjecting purified wild-type and mutant chloroplasts to mechanical testing with a rheometer, a laboratory device designed to measure the response of a liquid or slurry to shear stress. This experiment will be performed in collaboration with Professor Amy Shen in the Mechanical, Aerospace, and Structural Engineering Department at Washington University. Dr. Shen will provide a rheometer and expertise in its use (see collaboration letter in Section j). This particular experiment thus has a broader impact in that it promotes the formation of networks and partnerships between Biology and Engineering.

Another test of this hypothesis would be to target a heterologous MS channel to the envelope of a *msl2-1; msl3-1* mutant plastid, with the expectation that MS channel activity there would allow exit of solutes in response to membrane tension, thereby rescuing the large plastid phenotype. We will generate a transgene expressing bacterial MscS fused to the RecA plastid transit sequence, and determine if it rescues the large-plastid phenotype of the *msl2-1; msl3-1* mutant. *MSL2* and *MSL3* should complement when their own plastid transit sequences are replaced by that from RecA. In this experiment, failure to complement will have a number of trivial explanations, but if complementation occurs, it will have important implications for future studies. In this case, it will be important to show that the MS channel activity of MscS is required, and an inactive version of MscS, I48D/S49P (51), does not complement.

III. Determine the Functional Role of MscS-Like channels in Cyanobacteria.

Our data point to a functional relationship between MS channels in the plastid envelope and the shape and size of those plastids. It is therefore of interest to determine how recently in evolutionary history this novel function for MS channels developed. *E. coli* MscS mutants have normal size and shape when grown in standard media (unpublished data), suggesting that a role for MscS-Like channels in size and shape control evolved relatively recently. Since chloroplasts are thought to have descended from an ancestral cyanobacterium, we propose to elucidate the function of MscS-like proteins in *Synechocystis* sp. PCC6803, a well-characterized cyanobacterium with a sequenced genome and a wealth of genomic and molecular genetic tools. There are eight MscS-like proteins in *Synechocystis*, one of which (PamA) has been briefly characterized by virtue of its interaction with the signaling protein PII (56). Focusing first on the proteins most similar to MSL2 and MSL3 in the MscS domain (these are encoded by the *slr0109* and *sll0590* genes), we will delete each gene through homologous recombination (80). We will use markerless deletion constructs (81), so that multiple mutations can be combined in case of redundant function. We will then determine if these genes are required for osmotic shock resistance in *Synechocystis*, as they are in *E. coli* and *B. subtilis* (21, 82) or if they are involved in controlling or monitoring cell/organelle morphology as in Arabidopsis. We will assay wild-type and mutant strains for osmotic shock sensitivity as performed previously (44), and will characterize cell morphology using DIC light microscopy.

These experiments will be performed in collaboration with our colleague at Washington University Biology, Dr. Himadri Pakrasi, who has extensive experience with the molecular genetics of *Synechocystis* sp. PCC6803 (see letter of collaboration in Section j). These experiments will also have a broader impact, as we will incorporate the efforts of high school teachers in collaboration with Washington University's Science Outreach. In this program, a teacher intern works in a research lab for several weeks during the summer and uses the experience to develop innovative curricula for high school science education. Teachers are selected from schools in the St. Louis school district that serve groups traditionally underrepresented in the sciences. In our project, the teacher will integrate into our research program, and explore ways to communicate the life history of chloroplasts, their identity as endosymbionts and their evolutionary relationship to cyanobacteria, to high school students (see full description of the Outreach program in Facilities, Equipment and Other Resources, Section i, and letter of collaboration in Section j).

IV. The Undergraduate Plastid Project: Undergraduates Developing Nongreen Plastids in Arabidopsis as a Model System for the Study of Organelle Morphology.

Our interest in MSL2 and MSL3 and their unexpected function in nongreen plastid shape control has led us to ask more broadly how nongreen plastid morphology is determined. The study of nongreen plastids has been particularly neglected (especially compared to chloroplasts), despite their essential, nonphotosynthetic metabolic contributions to plant growth (28). Only a few genes other than *MSL2* and *MSL3* have yet been implicated in nongreen plastid morphology (one other example is found in (83)). We thus propose to screen previously isolated mutants for defects in nongreen plastid morphology. Nongreen plastids will provide an excellent model system for the general study of organelle morphology determination, for several reasons: 1) It is possible to propagate plants with strong defects in plastid

morphology; 2) Nongreen plastids may be particularly sensitive to morphological perturbation compared to chloroplasts (this is true in the *msl2-1; msl3-1* mutant); and 3) A previously described RecARed reporter gene allows robust visual analysis of nongreen plastid morphology in the plant epidermis (Figure 8A, (44, 84)).

This portion of our work has dual purposes: it is both the first directed characterization of the genetics of nongreen plastid morphology, and it is also designed to serve as a platform for undergraduate training in cell biology and imaging technologies. The Undergraduate Plastid Project (UPP) is to be undertaken in collaboration with the Washington University Undergraduate Imaging Sciences Pathway, a multidisciplinary program that provides students with extraordinary research opportunities in the imaging sciences (see a full description of the program in Section i, and a letter of collaboration in Section j). Students will undertake research during the summer, with stipends provided by the Pathway program.

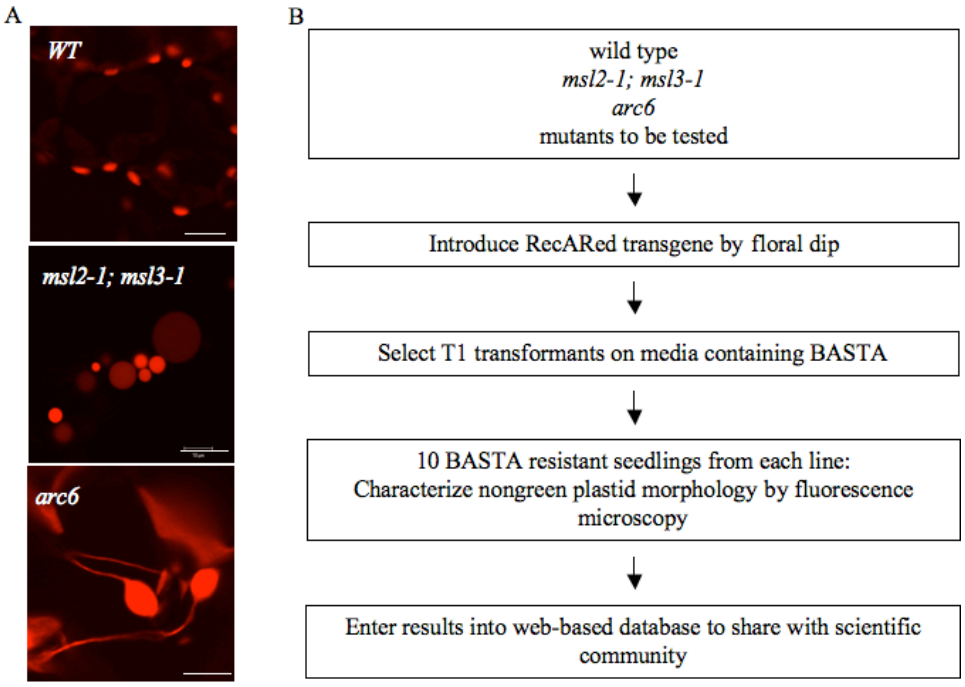

Figure 8. Undergradute Plastid Project. A) RecARED-based imaging of nongreen plastid morphology in wild-type and mutant leaves. B) The workflow plan for summer projects.

1. A turnkey system for visualizing nongreen plastids. There are several challenges to introducing young scientists to imaging technologies. One is the technical and expensive nature of microscopes. Another is the qualitative rather than quantitative nature of many imaging approaches. We propose to develop a platform, the Undergraduate Plastid Project, which will overcome these challenges. We have developed a fluorescent marker for nongreen plastids, the RecARed reporter. Nongreen plastids can be easily visualized in the epidermis of the leaf or root with this extremely bright fluorescent marker. As shown in Figure 8A, known mutants (*msl2-1; msl3-1*, and *arc6*) can be easily distinguished from the wild-type using confocal microscopy of an early rosette leaf. We propose to purchase an upgrade for our laboratory confocal that will motorize several aspects of fluorescent and bright field imaging (see additional information about this upgrade in Section j). Students will be able to access pre-set conditions, allowing them to image nongreen plastids in a straightforward and reproducible manner. In addition, the software upgrade will allow image quantitation, measuring the dimensions of each plastid, and even estimating volume through 3-D rendering. To start, the students will be trained by senior members of the laboratory. Then experienced students will be paired with novice students in a second round of analysis. Each pair of undergraduates will have 2 or 3 characterized mutant lines, along with wild-type controls and

the *arc6* and *msl2; msl3* mutants as positive controls for plastid morphology changes. The students will then be responsible for carrying these plants through the flowchart of experimentation shown in figure 8B. The last step of their project will be to enter their results into a database that will be maintained on a publicly available website, advertised in conference posters, and in email listservs to interested audiences (such as the Arab-gen mailing list). The UPP will thus achieve a broader impact by integrating research and education, advancing discovery while promoting teaching, training and learning.

2. Sources of mutants for nongreen plastid shape analysis. Based on our experience with the *msl2-1; msl3-1* and the *msl2-3* mutants, we hypothesize that other mutants with variegated leaves or altered chloroplast size may have aberrant nongreen plastid morphology. A large number of mutants with enlarged chloroplasts have been isolated, including the twelve *arc* mutants (36), several *ftsZ1* alleles (67), and the *pdv1pdv2* double mutant (73). Nongreen plastids have not been inspected in the variegation mutants *var1*, *var2*, *cue1*, *immutans*, and others (34). These mutants will be obtained from publicly available seed stocks or from the appropriate laboratory and will be entered into the UPP workflow.

A second source of mutants for the project will be M2 (the second generation after mutagenesis) lines from a screen already performed in the laboratory, designed to identify genes that interact with *msl2-1*. This screen relied on the fact that while *msl2-1; msl3-1* mutants have leaves that are variegated in color; single *msl2-1* and *msl3-1* mutants appear wild-type. In an *msl2-1* background, EMS-generated mutants with variegated leaves similar to those observed in the *msl2-1; msl3-1* mutant were identified (Figure 9). Preliminary analysis of the resulting 32 M2 lines indicates that several mutants have altered chloroplast morphology (data not shown). Mutants from this screen will be fed into the workflow for the UPP. For the purpose of this proposal, we will also determine if they require the *msl2-1* allele for the phenotype, and perform complementation testing and PCR screening to identify any mutants with lesions in *MSL3* (as no null alleles are yet available). Mapping and complete characterization of these mutants will be addressed in the future and are not proposed here.

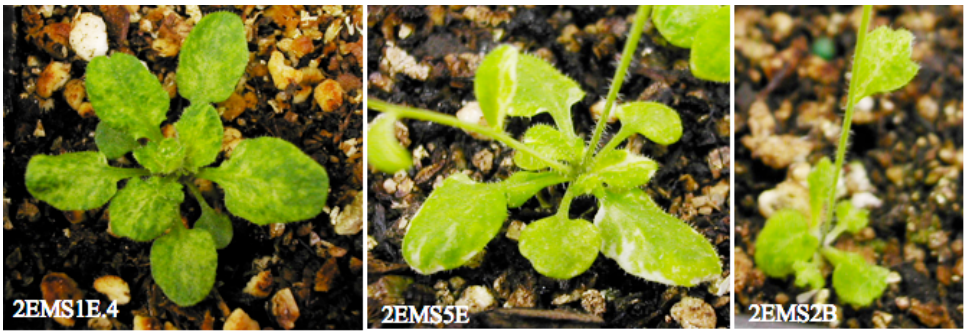

Figure 9. Enhancers of *msl2-1* are a source of nongreen plastid phenotypes. Three examples of M2 lines selected for further analysis.

RESULTS FROM PRIOR NSF SUPPORT

No prior support.

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