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SUMMARY

During leaf growth and development, chloroplasts numbers increase as cells expand to maximize energy capture and photosynthetic capacity. Chloroplasts proliferate by dividing in the middle, producing a large population of small organelles of similar size and shape. This process is orchestrated by macromolecular complexes located both inside and outside the chloroplast whose assembly and contractile activity across the two envelope membranes must be coordinated and spatially regulated. Through research supported by our DOE funding, and by exploiting the powerful model system *Arabidopsis thaliana*, we have significantly advanced our knowledge of how coordination of the division complexes is achieved and how division-site placement is spatially restricted to the middle of the chloroplast. Using chloroplast division mutants, we gained new understanding of how chloroplast size and shape influence chloroplast movement and photosynthetic performance. We identified a gene contributing to natural variation in chloroplast size. Major findings are highlighted.

ANALYSIS OF THE CHLOROPLAST DIVISION MACHINERY

Prior to initiating our DOE-funded research, we and others had shown that chloroplast division requires the concerted action of two dynamic contractile structures: the FtsZ ring (Z ring) on the stromal side of the inner envelope membrane (IEM), and the DRP5B (ARC5) ring on the cytosolic side of the outer envelope membrane (OEM) (4,11) (Fig. 1). The Z ring is positioned at the division site by the chloroplast Min system (described below) and tethered to the IEM through interaction of FtsZ2 with the IEM protein ARC6. DRP5B is recruited to the chloroplast by the OEM proteins PDV1 and PDV2. Work under our DOE support significantly expanded our knowledge of the chloroplast division machinery.

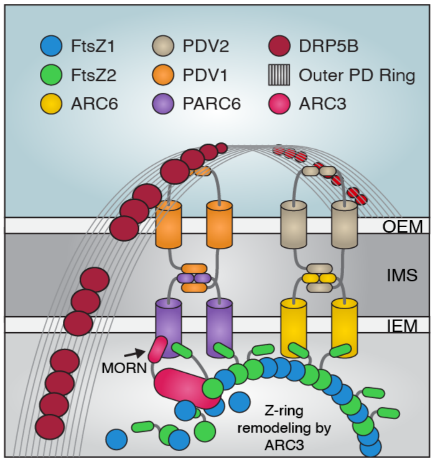

Fig. 1. Model of the chloroplast division machinery. Adapted from ref 4.

Role of ARC6

We discovered that ARC6 plays a critical role in coordinating the assembly of the FtsZ and DRP5B rings across the envelope membranes (Fig 1). Key experiments contributing to this conclusion showed that PDV2 is localized to the chloroplast division site and oriented with its N-terminus facing the cytosol and C-terminus facing the intermembrane space (IMS). Yeast two-hybrid and pull-down assays revealed that the IMS regions of ARC6 and PDV2 interact directly. Analysis with mutants and transgenic plants showed that ARC6 is required for localization of PDV2 to the division site as well as DRP5B recruitment from the cytosol to the chloroplast, and that *arc6* mutants expressing ARC6 lacking its IMS region phenocopy *pdv2* mutants. However, we found that the IMS region of ARC6 is not required for localization of ARC6 to the division site, which is likely established by its interaction with FtsZ2 inside the stroma. Collectively, our findings suggested a model in which interaction between ARC6 and PDV2 in the IMS relays positional information from the Z ring inside the stroma to PDV2 in the OEM, in turn resulting in DRP5B recruitment to the division site (Fig. 1). These activities help coordinate both the assembly of the division machinery and constriction of the two envelope membranes (21).

The ARC6 IMS region contains a domain of unknown function, DUF4101, that is highly conserved in ARC6 proteins throughout plants and green algae, and in its cyanobacterial cell division orthologs Ftn2/ZipN. In a collaboration with Edward Yu at Iowa State University, we determined the crystal structure of the ARC6 IMS region, the first for DUF4101, and showed it forms an α/β barrel that is open near the OEM and closed near the IEM. Key residues important for the formation of this structure are identical or similar to those at equivalent positions in most ARC6 and Ftn2/ZipN proteins, suggesting their significance for both chloroplast and cyanobacterial cell division (8).

Role of ARC3

In bacteria, placement of the Z ring and cell division site is controlled by the Min system, which inhibits FtsZ self-assembly everywhere except the division site. Chloroplasts have retained two key components of this system called MinD and MinE, both of which function in positioning of the chloroplast division site, but lost MinC, the primary FtsZ assembly inhibitor. We showed that the plant-specific chloroplast division protein ARC3 evolved an analogous function. In work leading to this conclusion, we discovered that the chloroplast division mutant *arc3* has multiple Z rings in its chloroplasts (22). We further found that ARC3 is required for MinD and MinE function and that ARC3 overexpression disrupts both FtsZ assembly and chloroplast division *in vivo.* We demonstrated that a truncated form of ARC3 lacking a region near its C-terminus called the MORN domain interacts directly with FtsZ2 whereas full-length ARC3 does not (described further below). Our studies revealed that ARC3 replaced MinC as the primary negative regulator of Z-ring assembly in the chloroplast Min system (12).

Role of PARC6

In our functional studies of ARC6, we discovered that ARC6 is required for PDV1 as well as PDV2 localization to the division site but that the IMS regions of ARC6 and PDV1 do not interact. These results suggested that another factor acts downstream of ARC6 to position PDV1 (21). We hypothesized this factor was PARC6 (Paralogue of ARC6), which we identified based on its sequence similarity to ARC6. In studies to begin testing this hypothesis and investigating the function of PARC6, we established that PARC6 is required for chloroplast division and localized at the division site. We showed it is targeted inside the chloroplast and resides in the IEM. We generated *arc6 parc6* double mutants and confirmed that PARC6 acts genetically downstream of ARC6. We demonstrated that PARC6 is required for PDV1 localization to the division site (19). We later showed that PARC6 has a topology similar to that of ARC6 in the IEM, that the IMS region of PARC6 interacts with the IMS region of PDV1 but not PDV2, and that the stromal region of PARC6 interacts with FtsZ2 and colocalizes with FtsZ2 filaments assembled in yeast cells. These findings provided strong support of a role for PARC6 in coordinating the internal and external components of the division complex downstream of ARC6 via direct interaction with PDV1 (7) (Fig. 1).

We discoverd that *parc6* mutants have multiple Z rings and spirals in their chloroplasts, and that PARC6 overexpression inhibited Z-ring assembly. These phenotypes indicated that PARC6 behaves as a negative regulator of Z-ring assembly. They also somewhat resembled those of ARC3 mutants and overexpressors, prompting us to test for PARC6-ARC3 interaction. We found that the stromal region of PARC6 interacts with ARC3, and that the ARC3 MORN domain is both required and sufficient for this interaction (7, 19). We probed the significance of this interaction in several ways. We showed that ARC3 exhibits two localization patterns in chloroplasts, one dispersed throughout the stroma that likely functions to prevent ectopic Z-ring assembly and hence division-site misplacement, and a more concentrated pool at the division site. We demonstrated that PARC6 recruits ARC3 to the division site. Using yeast three-hybrid assays we found that PARC6-ARC3 interaction via the MORN domain enables FtsZ to bind to full-length ARC3. We further discovered that interaction of ARC3 with PARC6 promotes the dynamic turnover FtsZ rings reconstituted in a heterologous yeast system. Taken together, our results suggested a model wherein interaction between ARC3 and PARC6 via the MORN domain alters the conformation of ARC3 at the division site, which allows it to interact with FtsZ proteins in the Z ring. The latter interaction enhances subunit exchange from the Z ring, facilitating Z-ring remodeling during chloroplast constriction (Fig. 1). These studies also revealed a novel function for a MORN domain in the regulation of protein-protein interactions within a macromolecular complex (3).

Role of CLMP1

We discovered that an Arabidopsis T-DNA mutant had chloroplasts that were clustered together and named the affected gene *CLUMPED CHLOROPLASTS I (CLMP1)*. We showed that chloroplasts in *clmp1* proceed through all known stages of division but remained attached by thin membranous connections, suggesting that CLMP1 functions in the very final stages of chloroplast separation. CLMP1 is cytosolic and associated with the plasma membrane and with chloroplasts. We speculate the CLMP1 may help "pull" thin membranous connections between deeply constricted chloroplasts apart via attachment to the plasma membrane to complete division (14).

INTERRELATIONSHIP BETWEEN CHLOROPLAST MORPHOLOGY, CHLOROPLAST MOVEMENT AND PHOTOSYNTHESIS

We exploited our extensive collection of Arabidopsis chloroplast division mutants, which exhibit a wide array of distinct chloroplast-morphology phenotypes, to investigate the interrelationships between chloroplast morphology, chloroplast movement and various photosynthetic parameters. For this purpose we collaborated with David Kramer and coworkers at Michigan State University to develop a new high-throughput platform for simultaneous, non-invasive imaging of chlorophyll fluorescence and chloroplast movement in whole plants grown under white light. Chloroplast movement measurements were based on a new approach involving imaging of the reflectance of pulsed red light from leaf surfaces, which we showed accurately reports chloroplast movement. In our initial analysis, we compared the chloroplast movement and photosynthetic responses of Arabidopsis *arc6* (Fig. 2) and two *ftsZ* mutants, which bear 1-2 giant chloroplasts in their mesophyll cells, with those of a phototropin mutant, *phot1-5 phot2-1*, that exhibits impaired chloroplast movement but normal chloroplast morphology. We used heat-map approaches for data visualization, which allowed us to discern patterns in both photosynthetic responses (Fig. 2B) and statistical analyses. Surprisingly, we discovered that the photosynthetic phenotypes of the large-chloroplast mutants in response to fluctuating high-light stress were due predominantly to their altered chloroplast size and shape rather than to their diminished chloroplast movement capacity (9). We extended this analysis to include division mutants that exhibit a wider array of distinct chloroplast morphology phenotypes, including differences in size, shape and morphological heterogeneity. The results indicated that reduced PSII efficiency was generally correlated with the severity of the chloroplast morphology phenotype, but also revealed considerable variability in the susceptibility of PSII to high-light stress (5). In this work we also developed a new method using reflectance measurements to approximate and correct for the known but previously unquantified effects of chloroplast movement on the calculation of non-photochemical quenching (NPQ) and its qE qnd qI components (5, 9).

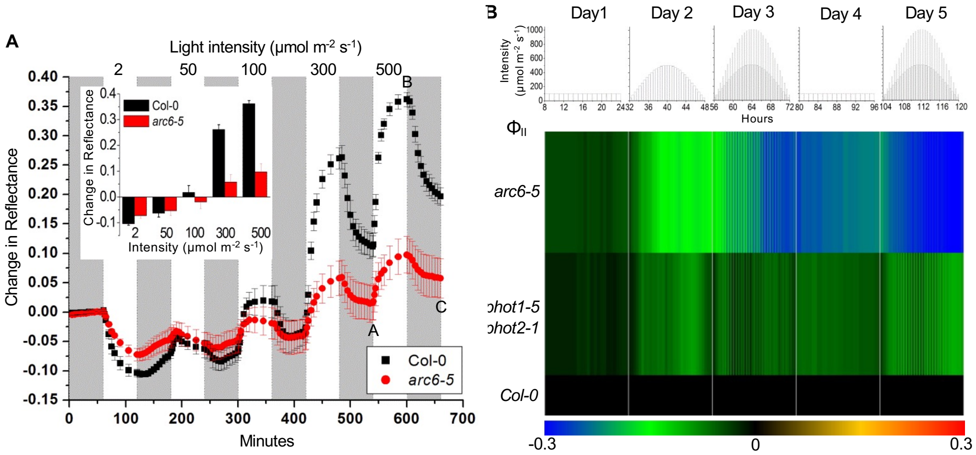

Figure 2. Chloroplast movement and photosynthesis measurements in Arabidopsis Col-0 and *arc6-5*. A, Kinetics of red-light reflectance changes from an exposed region of the leaf surface in Col-0 and *arc6-5* during alternating 1-hour periods of darkness (gray bars) or illumination (white bars) with white light at increasing intensities. The inset shows reflectance values at the end of each light period relative to those at time 0. Compared to Col-0, *arc6-5* displayed a greater defect in its avoidance than accumulation response, indicated by an increase and decrease in reflectance, respectively. B, Heat map of changes in PSII efficiency (Φ_{II}) measured over a 5-day period in *arc6-5* and *phot1-5 phot2-1*. The light regime is shown at the top. The bar at the bottom indicates the log-fold change in Φ_{II} values in the mutants normalized against those in Col-0. Adapted from ref. 9.

QTL MAPPING FOR GENES CONTROLLING CHLOROPLAST MORPHOLOGY

Chloroplast size

Chloroplast size differences between and within plant species have been associated with differences in light conditions and other environmental factors but the molecular determinants are unknown. To address this, we took a QTL mapping approach. We first measured chloroplast sizes in 22 *Arabidopsis thaliana* accessions representing the parents of 12 available recombinant inbred line (RIL) populations and one near-isogenic line (NIL) population under several sets of conditions. Chloroplast sizes varied among accessions but were always biggest in Cvi-1. The Cvi-1 and L*er*-2 RIL parents and related Cvi-1 and L*er*-0 NIL parents consistently exhibited greater differences in chloroplast size than any other combination of parental accessions. Therefore, we took advantage of a set of 92 NILs in which Cvi-1 genomic DNA is introgressed across all five chromosomes in the L*er*-0 background to map genomic regions associated with the increased chloroplast size in Cvi-1. We identified linkage with a region on chromosome 3, then developed markers and fine-mapped the Cvi-1-like phenotype to an interval spanning only two loci, one encoding the chloroplast division protein FtsZ2-2, one of two FtsZ2 proteins in Arabidopsis, and the other encoding a protein of unknown function. Because a knockout allele of *FtsZ2-2* in Col-0 had previously been shown to have enlarged chloroplasts resembling those in Cvi-1, we considered *FtsZ2-2* to be a strong candidate as the gene responsible for the Cvi-1 phenotype.

We found that an *FtsZ2-2* genomic fragment from Cvi-1 contained numerous polymorphisms compared with the Col-0 reference and Ler-0 *FtsZ2-2* sequences (which are identical). One was a single-nucleotide deletion that created a frameshift and produced a premature stop codon, yielding a predicted gene product 18 amino acids shorter than in L*er*-0. Consistent with the C-terminal truncation, immunoblotting showed that FtsZ2-2 migrated about 2 kD smaller in Cvi-1 and all NILs with the Cvi-1-like phenotype than in Ler-0 or NILs with smaller Ler-0-like chloroplasts, but that the FtsZ2-2 protein levels, as well as FtsZ2-1 and FtsZ1 levels were similar to those in Ler-0. Transformation of an *ftsZ2-2* null mutant with a Cvi-1 or Ler-0 *FtsZ2-2* genomic fragment confirmed that *FtsZ2-2* is causal for the large-chloroplast phenotype in Cvi-1. Immunofluorescence staining showed that Z rings are more disorganized in Cvi-1 than Ler-0, probably because the Cvi-1 truncation removes a C-terminal peptide required for FtsZ2 interaction with ARC6, which may weaken Z-ring anchoring to the IEM in Cvi-1.

Comparisons of the *FtsZ2-2* coding sequences in the 1,135 accessions available in the 1001 Genomes database uncovered 32 unique FtsZ2-2 protein variants, 21 of which occurred in only a single accession. We grew representative accessions with each sequence variant and identified four that had visibly larger chloroplasts. One was Cvi-0, which has the same *FtsZ2-2* allele as Cvi-1, one carried a natural null allele, and two had reduced levels of FtsZ2-2 protein, but not FtsZ2-1 or FtsZ1 protein. Quantitative RT-PCR showed the latter two accessions did not have reduced transcript levels, suggesting their phenotypes were due to reduced FtsZ2-2 stability. Comparison of the nonsynonymous to synonymous substitution rates revealed more relaxed genetic constraint on the *FtsZ2-2* than *FtsZ2-1* or *FtsZ1* coding sequences. Our results suggested that allelic variation in *FtsZ2-2* may contribute to fine-tuning chloroplast size in natural populations. They also demonstrated that natural variation in *Arabidopsis thaliana* can be used to uncover the genetic basis for differences in cell biological traits (2).

Chloroplast coverage

Previous analyses in numerous species have shown that, beyond an early stage of leaf growth, the proportion of the mesophyll cell surface occupied by chloroplasts, called chloroplast coverage, is tightly regulated, indicating stringent control over the ratio between total chloroplast compartment size and cell size. We measured chloroplast coverage in our RIL parent accessions and showed that *Arabidopsis* exhibits natural variation in this trait. The parents of one RIL population showed a difference in coverage of ~30%. We initiated QTL mapping in the RIL population to identify loci potentially involved in the regulation of chloroplast coverage. Typically, bright-field images of fixed leaf tissue have been used to measure both cell area and total chloroplast area per cell for coverage measurements, often by hand. In an effort to expedite the necessary phenotyping, we experimented with using merged bright-field and chlorophyll autofluorescence images to fully automate measurements of cell and chloroplast areas, respectively, but found that cell boundaries were not accurately predicted by the software. Therefore, cell areas were measured by hand in the merged images, though chloroplast area measurements based on autofluorescence and coverage measurements were automated using custom scripts. Chloroplast coverage measurements were obtained from the parent lines and each RIL in the population, with images from three plants and 12 cells per genotype. Rough-mapping for significant QTL was carried out using available genetic data for the RIL population. No significant QTL were identified in this first analysis.

PUBLICATIONS ACKNOWLEDGING DOE SUPPORT

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