# Decoding Natural Variation in Chloroplast Size

Chloroplasts, like their cyanobacterial ancestors, use binary fission to divide and generate new organelles. The origins of the division machinery stem from both the original eukaryotic host cell and the cyanobacterial endosymbiont (Chen et al., 2018). During chloroplast division, a contractile ring assembles at each of the outer and inner chloroplast membranes and together coordinate constriction and pinching off of both membranes to create two daughter chloroplasts (Fig. 1A). Mutation of individual components of the chloroplast division machinery typically results in larger chloroplasts, with fewer chloroplasts per cell (Chen et al., 2018). Defects in chloroplast division have been linked to abnormal chloroplast movement, effects on photosynthesis, and reduced mesophyll conductance (Austin and Webber, 2005; Dutta et al., 2017). In nature, chloroplast number or size can vary between species (Honda et al., 1971) and also between cell types of the same species (Ahmadabadi and Bock, 2012); however, the genes/alleles responsible for this variation are unknown. In this issue of *Plant Physiology*, Kadirjan-Kalbach et al. (2019) explored the molecular mechanisms underlying natural variation in chloroplast size using different accessions of Arabidopsis (*Arabidopsis thaliana*).

At the start of chloroplast division, the division machinery assembles at the organelle midzone on the surface of the inner membrane, forming the Z-ring. Correspondingly, the outer envelope plastid division ring, or PD-ring, assembles on the surface of the chloroplast (Fig. 1A). DYNAMIN-RELATED PROTEIN5 (DRP5; also known as ACCUMULATION AND REPLICATION OF CHLOROPLASTS5 [ARC5]) is recruited from the cytosol to the division site and initiates dimerization of the outer membrane proteins PLASTID DIVISION1 (PDV1) and PDV2 (Chen et al., 2018; Fig. 1A). In a chain reaction that follows, dimerization of the inner membrane protein ARC6 via interactions with PDV1 and PDV2 in the intermembrane space induces Z-ring condensation (Fig. 1A). The Z-ring is composed of the tubulin-like GTPase ring proteins FILAMENTING TEMPERATURE-SENSITIVE MUTANT Z1 (FtsZ1) and FtsZ2. FtsZ2 is important for Z-ring structure, while FtsZ1 is a more dynamic component, allowing for tightening of the Z-ring during division (Fig. 1A; Chen et al., 2018). In Arabidopsis, FtsZ1 is encoded by a single gene (*FtsZ1-1*), while two versions of FtsZ2 exist, encoded by *FtsZ2-1* and *FtsZ2-2*. FtsZ2-1 and FtsZ2-2 appear to be functionally interchangeable: it is the overall level of FtsZ2 protein that is critical for proper Z-ring function (Schmitz et al., 2009).

Kadirjan-Kalbach et al. (2019) began by taking advantage of existing near-isogenic lines (NILs) originating from parents that exhibit notable differences in chloroplast size. In an initial screen, the chloroplast area per mesophyll cell was measured in 22 Arabidopsis accessions, with the largest difference in chloroplast size occurring between Cvi-1 (large) and L*er*-2 or L*er*-0 (small). Existing NILs (generated by crossing L*er*-0 and Cvi-1 parents: LCNs; Keurentjes et al., 2007) were then analyzed for chloroplast size, revealing 18 LCNs with enlarged chloroplasts similar to Cvi-1 (Fig. 1B). These all had Cvi-1 introgressions in chromosome 3, and fine-mapping identified two possible responsible loci, one of which encoded FtsZ2-2 (Kadirjan-Kalbach et al., 2019). Given its known role in chloroplast division in Col-0, the *FtsZ2-2* gene from Cvi-1 was sequenced and compared with that of L*er*-0. Within Cvi-1, many polymorphisms were identified that distinguished Cvi-1 *FtsZ2-2* from that of L*er*-0. However, sequencing of several NILs with large chloroplasts pointed toward a single-nucleotide polymorphism in the last exon of *FtsZ2-2* that produced a premature stop codon, predicted to encode a protein 18 amino acids shorter than that of L*er*-0 FtsZ2-2. A truncated FtsZ2-2 protein was detected in all NILs with large chloroplasts using immunoblotting, which was not due to alternative splicing of the FtsZ2-2 mRNA (Kadirjan-Kalbach et al., 2019).

Arabidopsis Col-0 *ftsZ2-2* mutants have large chloroplasts, with fewer chloroplasts per cell (Schmitz et al., 2009). To confirm that the Cvi-1 *FtsZ2-2* allele results in larger chloroplasts, it was introduced into the *ftsZ2-2* knockout background. Chloroplast size was similar in the *ftsZ2-2* mutant with or without the addition of *Cvi-FtsZ2-2*, while expression of L*er*-0 FtsZ2-2 in the *ftsz2-2* background restored chloroplast size to that of Col-0. Immunofluorescent labeling of FtsZ2-1 and FtsZ2-2 in both L*er*-0 and Cvi-1 showed that in Cvi-1, both Z-ring proteins were found not only at the chloroplast midzone but also in punctate and short filament structures that were absent from L*er*-0 (Kadirjan-Kalbach et al., 2019). This suggested that larger chloroplasts in Cvi-1 may be due to differences in Z-ring organization during plastid division.

The discovery of a unique *FtsZ2-2* allele controlling chloroplast size in Arabidopsis Cvi-1 prompted the authors to investigate additional *FtsZ2-2* polymorphisms in other Arabidopsis accessions. Using the 1001 Genomes database, the FtsZ2-2 amino acid sequence obtained from 1,135 accessions was compared with that of Col-0. Three accessions had amino acid deletions in FtsZ2-2, including Cvi-0, which was identical in sequence to Cvi-1, and TAD-04, which had a premature stop codon, effectively resulting in a null *FtsZ2-2* allele. Both of these accessions had large chloroplasts. A third

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www.plantphysiol.org/cgi/doi/10.1104/pp.19.01159

 

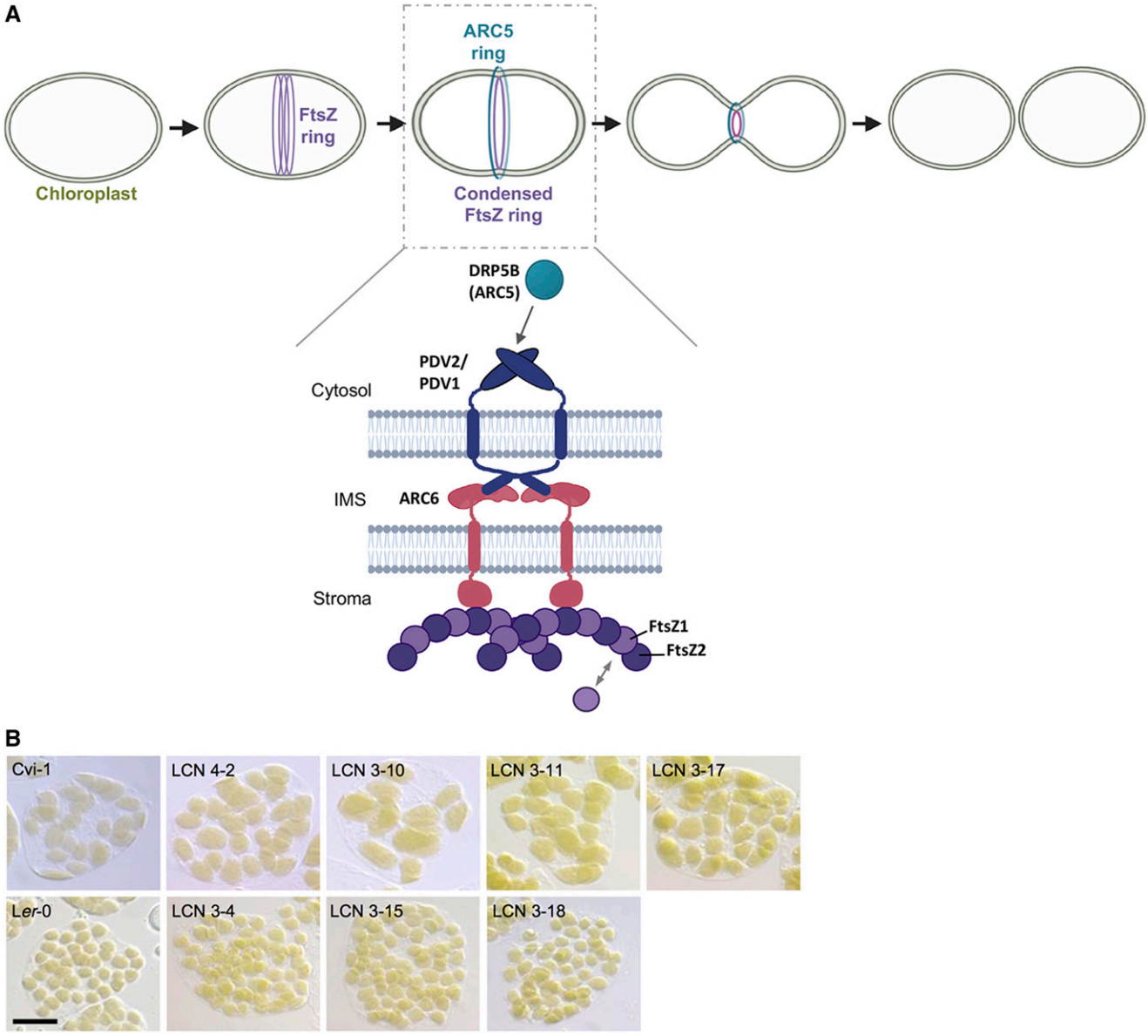


**Figure 1.** General model of chloroplast division and natural variation in NILs derived from Cvi-1 and L*er*-0 parents. A, The Z-ring is assembled at the midzone on the stromal surface of the inner chloroplast membrane and is tethered to the inner membrane via ARC6. Recruitment of DRP5B/ARC5 to the outer PD-ring by PDV1/2 stimulates condensation of the Z-ring by an interaction between DRP5B/ARC5 and ARC6 in the intermembrane space (IMS). Condensation of the Z-ring via remodeling of heterotypic interactions between FtsZ1 and FtsZ2 results in ring constriction and pinching off of the inner and outer chloroplast membranes, giving rise to two daughter chloroplasts (Miyagishima, 2017; Chen et al., 2018). The diagram was created with BioRender.com. B, Chloroplast size was examined in mesophyll cells of parental accessions L*er*-0 and Cvi-1 as well as NILs derived from their cross (LCNs). Several NILs with both enlarged and smaller chloroplast phenotypes are shown. Bar = 20 $\mu$m. (*Original micrographs reprinted from figure 3C of Kadirjan-Kalbach et al. [2019].*)

accession, ANH-1, had an amino acid deletion within the chloroplast targeting signal of FtsZ2-2, which had no effect on the localization of FtsZ2-2 or chloroplast size relative to L*er*-0 (Kadirjan-Kalbach et al., 2019). Thirteen accessions contained one or more amino acid polymorphisms in *FtsZ2-2*. Of these 13, two accessions (Vdm-0 and Sac-0) had unique amino acid substitutions and a quantitative increase in chloroplast size. However, FtsZ2-2 protein levels were much lower in these two accessions compared with Cvi-1 and L*er*-0, without differences in transcript levels, suggesting instability of the FtsZ2-2 protein (Kadirjan-Kalbach et al., 2019).

The presence of several unique *FtsZ2-2* alleles in different Arabidopsis accessions raised the question of whether they may be under evolutionary selection.

Using data available in the 1001 Genomes database, the authors calculated the number of substitutions per nonsynonymous site (amino acid change; dN) and the number of substitutions per synonymous site (no amino acid change; dS) for *FtsZ1*, *FtsZ2-1*, and *FtsZ2-2* in available Arabidopsis accessions. dN/dS gives information about the strength and mode of natural variation for a particular gene and can provide insight into its evolutionary history (Jeffares et al., 2015). A low dN/dS (<1) indicates that mutations that cause amino acid changes are not retained, as they may be deleterious to protein folding and/or function (Jeffares et al., 2015). Most protein-coding genes fall into this category. *FtsZ1*, *FtsZ2-1*, and *FtsZ2-2* all had dN/dS < 1, suggesting negative selection against nonsynonymous polymorphisms (Kadirjan-Kalbach et al., 2019). However, the authors point out that the relatively high dN/dS of *FtsZ2-2* compared with that of *FtsZ1* and *FtsZ2-1* may indicate a more relaxed tolerance of *FtsZ2-2* for variation.

This study nicely shows the utility of mining natural variation to understand fundamental cell biology processes and builds upon foundational work identifying the critical players involved in chloroplast division in Arabidopsis. Given our extensive use of Arabidopsis as a model plant, especially for cell and molecular biology, similar approaches will undoubtedly be useful in understanding more aspects of plant cell biology. This approach may also help inform our understanding of how chloroplast size is determined in species other than Arabidopsis, including those that show cell type-specific variation in chloroplast size (Ahmadabadi and Bock, 2012).

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