RESEARCH PAPER

Variations in chloroplast movement and chlorophyll fluorescence among chloroplast division mutants under light stress

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Abstract

Chloroplasts divide to maintain consistent size, shape, and number in leaf mesophyll cells. Altered expression of chloroplast division proteins in Arabidopsis results in abnormal chloroplast morphology. To better understand the influence of chloroplast morphology on chloroplast movement and photosynthesis, we compared the chloroplast photorelocation and photosynthetic responses of a series of Arabidopsis chloroplast division mutants with a wide variety of chloroplast phenotypes. Chloroplast movement was monitored by red light reflectance imaging of whole plants under increasing intensities of white light. The accumulation and avoidance responses were differentially affected in different mutants and depended on both chloroplast number and morphological heterogeneity. Chlorophyll fluorescence measurements during 5 d light experiments demonstrated that mutants with large-chloroplast phenotypes generally exhibited greater PSII photodamage than those with intermediate phenotypes. No abnormalities in photorelocation efficiency or photosynthetic capacity were observed in plants with small-chloroplast phenotypes. Simultaneous measurement of chloroplast movement and chlorophyll fluorescence indicated that the energy-dependent (qE) and long-lived components of non-photothermal quenching that reflect photoinhibition are affected differentially in different division mutants exposed to high or fluctuating light intensities. We conclude that chloroplast division mutants with abnormal chloroplast morphologies differ markedly from the wild type in their light adaptation capabilities, which may decrease their relative fitness in nature.

Key words: Chloroplast division mutants, chlorophyll fluorescence, chloroplast movement, chloroplast size, light stress, photosynthesis.

Introduction

Chloroplasts are highly dynamic organelles that continuously regulate their size, shape, and numbers (Pyke, 2013). These dynamic processes play a critical role in cell physiology. Apart from being responsible for photosynthesis, chloroplasts provide a multifunction platform to the plant cell, contributing to the synthesis of lipids, amino acids, nucleotides, and...
various hormones, and to nitrogen and sulphur assimilation (Ohlrogge and Browse, 1995; Neuhaus and Emes, 2000; Lopez-Juez and Pyke, 2005). Because of their semi-autonomous nature (Timmis et al., 2004), all of these diverse functions are tightly regulated by both interchloroplast crosstalk and communication with other cell organelles (Raghavendra and Padmasree, 2003; Nott et al., 2006; Jarvis and Lopez-Juez, 2013; Bulychev and Komarova, 2015). Chloroplast continuity during cell division and their accumulation to high numbers in photosynthetic tissues are maintained by division of pre-existing chloroplasts (Osteryoung and Pyke, 2014).

Chloroplast division in Arabidopsis is mediated by mid-plastid-localized stromal and cytosolic contractile complexes, respectively designated the filamentating temperature-sensitive Z (FtsZ) ring and Accumulation and Replication of Chloroplasts 5 (ARC5)/Dynamin-Related Protein 5B (DRP5B) ring (Vitha et al., 2001; Gao et al., 2003; Hong et al., 2003). The FtsZ ring, consisting of the cytoskeletal GTPase proteins FtsZ1 and FtsZ2, is anchored to the stromal face of the inner envelope membrane by ARC6 (Pyke and Leech, 1994; Vitha et al., 2001, 2003; Olson et al., 2010; Smith et al., 2010; TerBush and Osteryoung, 2012; Yoshida et al., 2016). The midcell positioning of the FtsZ ring is regulated by a complex regulatory ‘Min’ system, comprising MinD1, MinE1, MinF, MinG, MinH, and MinE2 (MinD1 and MinE1 are required for MinD1 localization). The MinG/MinE1 complex recruits MinD1 to the division site by a signaling approach (Dutta et al., 2015). Somewhat surprisingly, we found that the photosynthetic phenotypes of these mutants were attributable largely to altered chloroplast size and shape rather than to diminished chloroplast movement capacity (Dutta et al., 2015). Our imaging platform also allowed us to tease apart the contributions of PSI quantum efficiency (ΦPSI) and non-photochemical quenching (NPQ) to the high-light susceptibility of these mutants. In the present study, we have extended our analysis to include a series of Arabidopsis genotypes that exhibit a wider array of chloroplast morphology phenotypes.

**Materials and methods**

*Plant materials and growth conditions*

Arabidopsis thaliana lines used in this study (Table 1) include: T-DNA insertion mutants arc5-2 (Miyagishima et al., 2006), arc6-5 (Crumpton-Taylor et al., 2012), pdv2-1 (Miyagishima et al., 2006), ftsZ1-1 (Yoder et al., 2007), ftsZ2-2 (McAndrew et al., 2008), arc3-2 (Shimada et al., 2004), parc6-1 (Glynn et al., 2009), and ftz (Gao et al., 2006) in the Col-0 background; the T-DNA insertion mutant minD1-1 in the Ws background (Zhang et al., 2013); ethyl methanesulfonate (EMS) mutants arc12 (Glynn et al., 2007), pdv1-1, and pdv1-1 pdv2-1 (Miyagishima et al., 2006) in the Col-0 background; the EMS mutant arc11 in the Ler background (Marrison et al., 1999); and a line overexpressing PDV1 and PDV2 in Col-0 (35S-PDV1 35S-PDV2) (Okazaki et al., 2009).

Seeds were sown on soil in individual pots and stratified at 4 °C for 48 h in the dark. Plants were germinated and grown in controlled-environment chambers at 20 °C and 60% humidity with a 16/8 h light/dark cycle in white light at 100 μmol photons m⁻² s⁻¹. Plants...
were transferred to the imaging chamber (photoperiod synchronized to the growth chamber) 1–2 d before an experiment for acclimation.

**Confocal microscopy**

For analysis of chloroplast arrangement, entire rosette leaves from 3-week-old plants were fixed and analyzed as described previously (Pyke and Leech, 1991; Dutta et al., 2015). Whole leaf samples were mounted, and mesophyll cells on the adaxial side of leaves were observed using an Olympus Fluoview 1000 Confocal microscope (Olympus Corporation of the Americas Inc., http://www.olympusamerica.com) excited with a 514 nm laser. Chlorophyll (Chl) was observed using an Olympus Fluoview 1000 Confocal microscope mounted, and mesophyll cells on the adaxial side of leaves were visualized using software described in Cruz et al. (2016).

**Chlorophyll fluorescence measurements**

Chl fluorescence imaging of intact plants was performed in a Percival AR41L2 chamber (Geneva Scientific, Fontana, WI, USA) refitted as a dynamic environment photosynthesis imager (DEPI) (Cruz et al., 2016). The initial fluorescence, \( F_0 \), was recorded by turning on a weak measuring light. Then, the plants were exposed to a 0.3 s saturation flash of \( \sim 10,000 \text{ mol m}^{-2} \text{s}^{-1} \), to obtain the maximal fluorescence, \( F_M \). The images were processed using software described in Cruz et al. (2016). The quantum yield of PSII (\( \Phi_{PSII} \)) was calculated as \( (F_{M}' - F_0)/F_M' \), where \( F_0 \) is the steady-state fluorescence and \( F_M' \) is the fluorescence maximum at steady state (Baker, 2008). NPQ was estimated using the equation \( (F_M'' - F_M')/F_M'' \) (Baker and Oxborough, 2004). The components of NPQ, specifically energy-dependent quenching (\( q_{E SV} \)) and ‘irreversible’ quenching (\( q_I \)), were calculated as \( F_M (F_M' - F_M'')/F_M' \) and \( (F_M' - F_M'')/F_M' \), respectively, where \( F_M'' \) is the post-illumination fluorescence maximum (Krause and Jahns, 2003).

Heat maps were generated with OLIVER software (Dutta et al., 2015).

**Chloroplast relocation assay using red light reflectance imaging**

Chloroplast movement was measured by monitoring white light-dependent changes in red light reflectance from whole plants as described (Dutta et al., 2015). Briefly, a DEPI prototype with a CCD camera (KP-FD145GV monochrome, Hitachi Kokusai Electric Inc., Tokyo, Japan) fitted with a 650BP100 band-pass filter (Omega Optical Inc., Brattleboro, VT, USA) was used to collect reflected red light (625 nm) from LEDs also used to excite Chl fluorescence. For each measurement, a 50 µs pulse of the red measuring light was triggered and 30 images were collected over 1.8 s (16.7 Hz) for averaging. Reflectance images were processed using ImageJ (Schneider et al., 2012). Reflectance from whole plants was quantified as described previously (Dutta et al., 2015). Relative reflectance was calculated as the difference between reflectance during illumination (\( R_i \)) and the last reflectance value recorded during an initial dark period (\( R_0 \)), normalized to \( R_0 \). Data were analyzed and visualized using software described in Cruz et al. (2016) and the Origin visualization package (OriginLab, Northampton, MA, USA).

**Dual imaging and analysis for non-photochemical quenching**

Simultaneous imaging of chloroplast movement and fluorescence was performed in the DEPI refitted with a second camera configured to collect reflectance. NPQ values were corrected for interference from chloroplast movement (NPQ\(_{corr} \)) using the protocol described in Dutta et al. (2015), with the following equations:

\[
\text{NPQ}_{corr} = c' \frac{F_M}{F_M'} - 1, \quad q_{E SV,corr} = c' \frac{F_M}{F_M} - c'' \frac{F_M}{F_M}, \quad \text{and} \quad q_{I,corr} = c'' \frac{F_M}{F_M} - 1,
\]

where \( c' \) and \( c'' \) are correction factors to account for changes in

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**Table 1. Properties of Arabidopsis genotypes with abnormal chloroplast morphologies used for this study**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Locus</th>
<th>Parental line</th>
<th>Chloroplast phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large chloroplasts</td>
<td>arc5-2</td>
<td>At3g19720 Col-0</td>
<td>3–6 giant chloroplasts, centrally constricted</td>
<td>Miyagishima et al. (2006)</td>
</tr>
<tr>
<td>arc6-5</td>
<td>At5g42480 Col-0</td>
<td>1–2 giant chloroplasts</td>
<td>Crumpton-Taylor et al. (2012)</td>
<td></td>
</tr>
<tr>
<td>arc12</td>
<td>At1g69390 Col-0</td>
<td>1–2 giant chloroplasts</td>
<td>Glynn et al. (2007)</td>
<td></td>
</tr>
<tr>
<td>pdv1-1</td>
<td>At5g53280 Col-0</td>
<td>2–5 giant chloroplasts, with constrictions</td>
<td>Miyagishima et al. (2006)</td>
<td></td>
</tr>
<tr>
<td>pdv2-1</td>
<td>At2g16070 Col-0</td>
<td>3–6 giant chloroplasts, with constrictions</td>
<td>Miyagishima et al. (2006)</td>
<td></td>
</tr>
<tr>
<td>pdv1-1 pdv2-1</td>
<td>At5g53280, At2g16070 Col-0</td>
<td>1–2 giant chloroplasts, with central constriction</td>
<td>Miyagishima et al. (2006)</td>
<td></td>
</tr>
<tr>
<td>Intermediate chloroplasts</td>
<td>ftsZ1-1</td>
<td>At5g55280 Col-0</td>
<td>Heterogenous, enlarged chloroplasts with some small chloroplasts</td>
<td>Yoder et al. (2007)</td>
</tr>
<tr>
<td>ftsZ2-2</td>
<td>At3g52750 Col-0</td>
<td>Fewer, slightly enlarged, uniform size</td>
<td>McAndrew et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>arc3-2</td>
<td>At1g75010 Col-0</td>
<td>~11 irregularly globular, large chloroplasts</td>
<td>Shimada et al. (2004)</td>
<td></td>
</tr>
<tr>
<td>parc6-1</td>
<td>At3g19180 Col-0</td>
<td>~11 irregularly globular, large chloroplasts</td>
<td>Miyagishima et al. (2004)</td>
<td></td>
</tr>
<tr>
<td>arc11/minD1</td>
<td>At5g24020 Ler</td>
<td>Heterogenous, giant chloroplasts with some small chloroplasts</td>
<td>Marrison et al. (1999)</td>
<td></td>
</tr>
<tr>
<td>minD1-1</td>
<td>At5g24020 Ws</td>
<td>Heterogenous, giant chloroplasts with some small chloroplasts</td>
<td>Zhang et al. (2013)</td>
<td></td>
</tr>
<tr>
<td>td</td>
<td>At1g03160 Col-0</td>
<td>Fewer, larger chloroplasts, heterogenous in distribution</td>
<td>Gao et al. (2006)</td>
<td></td>
</tr>
<tr>
<td>Small chloroplasts</td>
<td>35S PDV1 35S -PDV2</td>
<td>At5g53280, At2g16070 Col-0</td>
<td>More, smaller chloroplasts</td>
<td>Okazaki et al. (2009)</td>
</tr>
</tbody>
</table>
light reception caused by chloroplast movements. The values of $c'$ and $c''$ were estimated from reflectance images using the equations:

$$c' = 1 + m \frac{R' - R_0}{R_0}$$  and  $$c'' = 1 + m \frac{R'' - R_0}{R_0},$$

where $R_0$ is the reflectance at time zero, $R'$ and $R''$ are the reflectance values measured at the times $F_M'$ and $F_M''$ were taken, and $m$ is the slope of the relationship between fluorescence yield and changes in reflectance. As reported previously (Dutta et al., 2015), the value of $m$ for Arabidopsis was determined empirically to be ~1.

Pigment estimation

Chl and carotenoid contents in leaves of untreated or high-light-treated plants were estimated from $N,N'$-dimethylformamide leaf extracts as described by Porra (2002) and Wellburn (1994), respectively.

Results

Characterization of chloroplast photorelocation responses

Recent findings showed that mutants with 1–2 large chloroplasts failed to attain complete face or profile positioning when exposed to low- or high-light illuminations, respectively (Dutta et al., 2015). To explore further the dependence of the capacity for photorelocation on chloroplast size, we assayed mutant lines with a range of chloroplast morphologies (Table 1; Supplementary Fig. S1) by imaging Chl autofluorescence in fixed leaf tissue using confocal microscopy. Figure 1 shows chloroplast positioning observed in palisade cells of leaves after 1 h of dark adaptation or after 1 h of exposure to low-intensity (10 $\mu$mol m$^{-2}$ s$^{-1}$) or high-intensity (200 $\mu$mol m$^{-2}$ s$^{-1}$) white light. As reported earlier (Königer and Bollinger, 2012; Dutta et al., 2015), chloroplasts in the WT parental lines, Col-0, Ler, and Ws, accumulated along the periclinal walls in low light and along the anticlinal walls in high light. No specific distribution pattern was observed in dark-adapted leaves. Light-dependent photorelocation was less distinguishable in the large-chloroplast mutants (Fig. 1, left panels; Table 1). Low-light-treated leaves showed an uneven repositioning of chloroplasts, with chloroplasts occupying both face and profile positions within the same mesophyll cell. High-light-treated leaves showed a higher proportion of chloroplasts occupying the profile position, with some still exhibiting a face position. Overall the distribution patterns were similar among all the large-chloroplast mutants studied. In contrast, the distribution patterns were quite diverse among the intermediate-chloroplast mutants (Fig. 1, right panels except bottom; Table 1). The ftsZ2-2 and arc11 mutants showed chloroplast arrangements resembling those in their respective Col-0 and Ler WTs in both low and

![Fig. 1. Confocal images showing chloroplast arrangement in mesophyll cells of the indicated genotypes exposed to different light levels. Dark-adapted plants were kept in 60 min of darkness or exposed to 60 min of low or high white light illumination. Leaf samples were then harvested for imaging. The red signal shows Chl autofluorescence and reveals the shapes of the chloroplasts. The numbers at the top indicate the light intensity in $\mu$mol photons m$^{-2}$ s$^{-1}$. Scale bars=50 $\mu$m.](https://academic.oup.com/jxb/article-abstract/68/13/3541/3883524)
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high light. Chloroplast distribution patterns in the remaining intermediate chloroplast mutants were more similar to those of the large-chloroplast mutants. The chloroplast distribution in the small-chloroplast genotype, 35S-PDV1 35S-PDV2 (Fig. 1, right bottom panels; Table 1), was indistinguishable from that of the Col-0 parent under all light regimes.

To better quantify the effect of chloroplast morphology on photorelocation, dark-adapted plants were assayed for accumulation and avoidance responses by measuring changes in the absorption of red light using reflectance imaging (Dutta et al., 2015) in response to increasing intensities of white light (Fig. 2). The values and statistical analysis of the change in reflectance after 120 min at 10 µmol m⁻² s⁻¹ (maximum accumulation response) or 600 min at 500 µmol m⁻² s⁻¹ (maximum avoidance response) are shown in Table 2 and Supplementary Fig. S2.

All genotypes except minD1-1 and 35S-PDV1 35S-PDV2 were attenuated in their photorelocation responses. Many of the division mutants with impaired movement had similar accumulation efficiencies (Fig. 2A, B; Table 2), with average reflectance changes ranging from ~74% to 85% of the change in the WT. arc6-5, arc12, pdv1-1 pdv2-1, and parc6-1 were the exceptions, where the accumulation efficiency was ~56–65% that of the WT. In contrast, the maximum high-light avoidance efficiency differed more among the different groups of mutants. The large-chloroplast mutants showed severe impairment in avoidance responses, with most genotypes (arc6-5, arc12, pdv1-1, and pdv1-1 pdv2-1) showing reflectance changes approximating only 25–35% of the WT (Fig. 2A; Table 2; Supplementary Fig. S2). The other two mutants in this group (arc5-2 and pdv2-1) showed ~50% attenuation in maximum avoidance responses. The results indicate that large-chloroplast genotypes impacted the high-light avoidance response more severely than the low-light accumulation response. Among the intermediate chloroplast mutants, ftsZ1-1 and parc6-1 had avoidance responses similar to those of most of the large-chloroplast mutants (55–60% of the WT). Interestingly, the ftsZ2-2 and arc11 mutants, which had impaired accumulation responses, showed avoidance efficiencies similar to those of their respective WTs, suggesting they may be more capable of avoiding excess photodamage under high-light conditions. A partial attenuation in avoidance response was observed in the remaining two members of this group (arc3-2 and fzl), with reflectance changes averaging ~70–80% of that in the WT.

Based on the confocal and reflectance studies, we conclude that the accumulation and avoidance responses are differentially influenced by alterations in chloroplast size and shape, but that there is no simple relationship between chloroplast morphology and photorelocation phenotypes.

Fig. 2. Chloroplast movement measurements in 18-day-old Arabidopsis plants of the indicated genotypes based on red light reflectance. Change in reflectance intensity versus time is shown during alternating 60 min periods of darkness (gray bars) or white light illumination (white bars) at the intensities indicated at the top of the graph (10–500 µmol m⁻² s⁻¹). For all data points, n=4–6 and error bars represent SDs. (A) Large-chloroplast mutants and the corresponding Col-0 wild type. (B) Intermediate-chloroplast mutants and the corresponding Col-0 wild type. (C) Intermediate-chloroplast mutants, minD1-1 and arc11 and their corresponding parental WTs, Ws and Ler, respectively. (D) 35S-PDV1 35S-PDV2 and the corresponding Col-0 wild type.
Table 2. Change in reflectance values recorded at 120 min (accumulation response) and 600 min (avoidance response) in Arabidopsis plants of the indicated genotypes as described in Fig. 2.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Change in reflectance value ±SD (percentage of wild type)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>120 min (accumulation response)</td>
</tr>
<tr>
<td>Large chloroplasts</td>
<td></td>
</tr>
<tr>
<td>Col-0</td>
<td>-0.074 ± 0.007 (100)</td>
</tr>
<tr>
<td>arc6-5</td>
<td>-0.046 ± 0.006* (62.4)</td>
</tr>
<tr>
<td>arc5-2</td>
<td>-0.063 ± 0.009* (84.6)</td>
</tr>
<tr>
<td>arc12</td>
<td>-0.043 ± 0.007* (88.1)</td>
</tr>
<tr>
<td>pdv1-1</td>
<td>-0.055 ± 0.007* (74.7)</td>
</tr>
<tr>
<td>pdv2-1</td>
<td>-0.056 ± 0.008* (74.9)</td>
</tr>
<tr>
<td>pdv1-1 pdv2-1</td>
<td>-0.042 ± 0.007* (56.3)</td>
</tr>
<tr>
<td>Intermediate chloroplasts</td>
<td></td>
</tr>
<tr>
<td>Col-0</td>
<td>-0.076 ± 0.005 (100)</td>
</tr>
<tr>
<td>ftsz1-1</td>
<td>-0.056 ± 0.004* (74.1)</td>
</tr>
<tr>
<td>fts22-2</td>
<td>-0.065 ± 0.009* (86.1)</td>
</tr>
<tr>
<td>parc6-1</td>
<td>-0.049 ± 0.011* (65.6)</td>
</tr>
<tr>
<td>arc3-2</td>
<td>-0.061 ± 0.004* (80.1)</td>
</tr>
<tr>
<td>id</td>
<td>-0.066 ± 0.017* (87.0)</td>
</tr>
<tr>
<td>Ws</td>
<td>-0.070 ± 0.014 (100)</td>
</tr>
<tr>
<td>minD1-1</td>
<td>-0.075 ± 0.006 (105.9)</td>
</tr>
<tr>
<td>Lar</td>
<td>-0.071 ± 0.005 (100)</td>
</tr>
<tr>
<td>arc11</td>
<td>-0.056 ± 0.005* (76.8)</td>
</tr>
<tr>
<td>Small chloroplasts</td>
<td></td>
</tr>
<tr>
<td>Col-0</td>
<td>-0.075 ± 0.018 (100)</td>
</tr>
<tr>
<td>35S-PDV1 35S-PDV2</td>
<td>-0.081 ± 0.015 (107.5)</td>
</tr>
</tbody>
</table>

For all data points, n=4–6 and error represents ±SD. Values marked with asterisks are significantly different from those in the relevant WT control. Raw, replicated data and statistical analyses for this study are presented in Supplementary Figs S3–S7. Overall, loss of ΦHI was correlated with the degree to which the chloroplasts deviated from normal size and number and was most pronounced under fluctuating light (Fig. 3). Plants with large chloroplasts (Table 1) showed the most severe ΦHI phenotypes. These can be categorized into (i) severe (arc6-5, arc12, and pdv1-1 pdv2-1), where the ΦHI phenotype accumulated to a significant level by the end of Day 3 and persisted on Days 4 and 5; (ii) moderately severe (pdv1-1, arc3-2), where the ΦHI phenotype recovered to some extent after Day 3 but increased gradually on Day 5; and (iii) less severe (pdv2-1), where a gradual increase in the ΦHI phenotype occurred over the 5 d light treatment.

Most mutants with intermediate-chloroplast phenotypes (ftsZ1-1, ftsZ2-2, fzl, minD1-1, and arc11) exhibited ΦHI phenotypes similar to those of the WT throughout the 5 d regime (Fig. 3; Supplementary Fig. S6). In arc3-2, ΦHI decreased on Day 5, implying that photoprotection was reduced in this mutant and might be further so if light treatments continued. In contrast to the other intermediate mutants, parc6-1 showed a ΦHI phenotype resembling that of the most severe large-chloroplast mutants. No significant difference in ΦHI was recorded between the 35S-PDV1 35S-PDV2 line with small chloroplasts and the WT throughout the 5 d treatment (Fig. 3; Supplementary Fig. S6).

We also analyzed NPQ and its rapidly reversible, ΔPH- or energy-dependent component, qE (calculated as qEsv) (Krause and Jahns, 2003), and a longer lived photoinhibitory component, predominantly associated with photoinhibition, qI (Yamamoto and Kamite, 1972; Horton et al., 1996; Li et al., 2004; Murchie and Niyogi, 2011; Horton, 2012). Because chloroplast photorelocation efficiency influences NPQ measurements (Cazzaniga et al., 2013; Dutta et al., 2015), in these experiments the uncorrected fluorescence data (see below) could only be used to compare NPQ data for the set of genotypes in which photorelocation efficiencies (avoidance responses) were similar to that in the WT. Namely ftsZ2-2, minD1-1, arc11, and 35S-PDV1 35S-PDV2 (Fig. 2; Table 2). Figure 4 displays heat map representations of NPQ, qEsv, and qI values for these genotypes. The corresponding raw data and statistical analyses are shown in Supplementary Figs S8–S11. Few significant differences in NPQ, qEsv, or qI were observed between ftsZ2-2, minD1-1, or 35S-PDV1.

**Table 2.** Change in reflectance values recorded at 120 min (accumulation response) and 600 min (avoidance response) in Arabidopsis plants of the indicated genotypes as described in Fig. 2.

**For all data points, n=4–6 and error represents ±SD. Values marked with asterisks are significantly different from those in the relevant WT control.** Raw, replicated data and statistical analyses for this study are presented in Supplementary Figs S3–S7. Overall, loss of ΦHI was correlated with the degree to which the chloroplasts deviated from normal size and number and was most pronounced under fluctuating light (Fig. 3). Plants with large chloroplasts (Table 1) showed the most severe ΦHI phenotypes. These can be categorized into (i) severe (arc6-5, arc12, and pdv1-1 pdv2-1), where the ΦHI phenotype accumulated to a significant level by the end of Day 3 and persisted on Days 4 and 5; (ii) moderately severe (pdv1-1, arc3-2), where the ΦHI phenotype recovered to some extent after Day 3 but increased gradually on Day 5; and (iii) less severe (pdv2-1), where a gradual increase in the ΦHI phenotype occurred over the 5 d light treatment.

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Light adaptation capacity in chloroplast division mutants

The arc11 mutant, which like minD1-1 bears a mutation in MinD1 and shares a similar chloroplast phenotype but in the Ler background (Zhang et al., 2013) (Supplementary Fig. S1B), showed somewhat lower NPQ and qE values compared with the WT on the first two high-light days (Days 2 and 3) (Fig. 4B, C; Supplementary Figs S9, S11B, C). On Day 4, arc11 showed slightly higher NPQ values that could be attributed to a slight increase in qI, which nevertheless completely recovered by the end of the day (Fig. 4B, D; Supplementary Figs S9, S11B, D). These differences did not persist on Day 5, suggesting efficient acclimation of this mutant to high-light exposure.

Chl content was measured in rosette leaves prior to and following the 5 d light treatments. The WT exhibited the expected high-light responses, with a decreased Chl content and an increased Chl a/b ratio compared with untreated controls (Table 3), probably indicating preferential loss of antenna complexes following treatment (Bailey et al., 2001; Phee et al., 2004). These trends were also seen in the mutants, but with quantitative differences that fell into three categories. Prior to the 5 d experiment, the three large-chloroplast mutants (arc6-5, arc5-2, and arc12) already showed reduced Chl content (~82–87% that of Col-0), but this ratio remained about the same after the 5 d treatment. In contrast, one of the large-chloroplast mutants (pdv1-1 pdv2-1)

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and three intermediate-chloroplast mutants (ftsZ1-1, arc3-2, and parc6-1) had Chl contents close to that of the WT prior to the light treatment (99, 96, 94, and 92% of the WT for pdv1-1 pdv2-1, ftsZ1-1, arc3-2, and parc6-1, respectively), but displayed larger treatment-induced decreases (to 92, 88, 83, and 83% of the WT, respectively). Only two mutants (arc6-5 and parc6-1) exhibited significantly elevated Chl \( \alpha/b \) ratios (~40% higher than in the WT) after the 5 d treatment. These mutants and a few other intermediate-chloroplast mutants also showed small reductions (≤10%) in their Chl/carotenoid ratios (Table 3).

**Analysis of chloroplast movement and photosynthesis in chloroplast division mutants with impaired photorelocation**

Previous studies have shown that NPQ measurements are influenced by chloroplast movement (Cazzaniga et al., 2013; Dutta et al., 2015). Therefore, to disentangle the effects of chloroplast movement on NPQ, we employed dual imaging of red light reflectance and Chl fluorescence for simultaneous measurement of chloroplast movement and photosynthetic efficiency (Dutta et al., 2015). This allowed us to estimate...
NPQ and its qE and qI components in genotypes with reduced chloroplast mobility, specifically all the large-chloroplast mutants and the intermediate mutants ftsZ1-1, parc6-1, arc3-2, and fzl (Fig. 2; Table 2). Based on the pronounced effect on ΦII observed on Days 3 and 5 of the 5 d regime (Fig. 3), we chose the 16 h fluctuating light conditions for the single-day dual imaging experiments.

The time-courses of leaf reflectance changes are shown in Fig. 5A. The Col-0 WT showed a robust increase in reflectance after the start of the illumination period, saturating at ~6–8 h (maximum value ~0.4) and rapidly declining during the final few hours of illumination. In all the mutants studied, the onset kinetics of reflectance were slow and final recovery at the end of the illumination was significantly less than in the WT (Fig. 5A; Supplementary Table S2). The avoidance of NPQ and its components during the initial few hours of illumination was generally more pronounced in most of the mutants, with arc6-5 showing the most severe decrease in ΦII (Fig. 5B; Supplementary Figs S1A, S14). This may be because plants had not received any prior high-light exposure in the single-day experiment. However, in pdvl-1 pdv2-1, ΦII showed less susceptibility to fluctuating light in the single-day experiment, where the decrease in ΦII was much less pronounced at the end of the day than on Day 3 of the 5 d experiment (Fig. 3B). parc6-1 was also less susceptible to the single-day treatment and exhibited a slow recovery in ΦII after mid-day. pdvl-1 also showed a slight recovery. There was no such recovery in ΦII in either parc6-1 or pdvl-1 on Day 3 of the 5 d experiment (Fig. 3B).

Overall, the results indicate that there is substantial variation among chloroplast division mutants in the susceptibility of ΦII to short-term (Fig. 5B) or prolonged (Fig. 3) high-light stress, with large-chloroplast mutants and parc6-1 generally exhibiting more pronounced ΦII phenotypes.

To compare NPQ and its components qE and qI in plants with different chloroplast movement deficiencies, we corrected the calculation of NPQ for interference from...
chloroplast movement using the method described in Dutta et al. (2015). Figure 6 shows heat maps of the corrected values for non-photochemical quenching (NPQcorr), energy-dependent quenching (qE SVcorr), and long-lived NPQ that predominantly reflects photoinhibition (qI corr), expressed as log-fold changes compared with Col-0. Raw results (for both apparent and corrected values) and statistical analyses are presented in Supplementary Figs S12B–D, S13, and S15. All mutants had increased NPQcorr compared with the WT during both ambient and fluctuating light (Fig. 6; Supplementary Fig. S15). The contribution of qI corr to the overall NPQcorr was higher than that of qE SVcorr at mid-day when light intensities were higher, suggesting that photoinhibition was more pronounced at those intensities. Except for arc6-5, arc12, and parc6-1, all other mutants recovered by the end of the day (Fig. 6A; Supplementary Fig. S15A). arc6-5 and arc12 showed the highest NPQcorr among all the large-chloroplast mutants. parc6-1 was the only mutant in the intermediate-chloroplast group that showed significantly higher NPQcorr than the WT, similar to arc6-5 and arc12. All other intermediate mutants (arc3-2, ftsZ1-1, and fzl) showed moderately increased NPQcorr compared with the WT, particularly at mid-day, which was primarily due to increased qI corr (Fig. 6B, C; Supplementary Figs S13, S15). arc5-2 and pdv2-1 had the lowest NPQcorr among the mutants studied. Our data indicate that although NPQ was affected in all the genotypes with impaired photorelocation, the distribution of NPQ and its components differed among mutants.

Discussion

In this study, we investigated the influence of chloroplast morphology on high-light adaptation in Arabidopsis. Previous studies have shown that severe chloroplast division mutants display impaired chloroplast movement responses, leading to the hypothesis that their high-light-induced photosynthetic defects were due predominantly to their diminished chloroplast movement capacity (Jeong et al., 2002; Königer et al., 2008). However, we recently developed a non-invasive, whole-plant imaging approach to measure chloroplast photorelocation and Chl fluorescence parameters simultaneously (Dutta et al., 2015). Our initial studies on chloroplast division mutants with only 1–2 drastically enlarged chloroplasts indicated that chloroplast size not only affected photorelocation but had additional effects on photosynthesis independent of the chloroplast movement defects (Dutta et al., 2015). Here, we have extended our analysis to plants with a wider array of chloroplast morphology phenotypes (Table 1).

Consistent with a previous study (Königer et al., 2008), we found a trend (Fig. 2) in which mutants with large or intermediate chloroplast sizes were defective in both the high-light avoidance response and low-light accumulation responses, but that the avoidance response was more strongly impacted. However, there were a few exceptions to these trends. Despite having somewhat larger chloroplasts than the Col-0 WT, the ftsZ2-2 mutant, whose chloroplasts are fairly uniform in size and shape (McAndrew et al. 2008;
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Schmitz et al., 2009) (Supplementary Fig. 1SB), was only impaired in its accumulation response (Table 2). Similarly, parc6-1, despite having intermediate chloroplast sizes on average (though with some large chloroplasts; Glynn et al., 2009) exhibited stronger defects in the accumulation response than some large-chloroplast mutants (Table 2). In addition, minD1-1, which is in the Ws background, was unaffected in its accumulation response whereas arc11, which is in the Ler background and has a chloroplast phenotype indistinguishable from that of minD1-1 (Zhang et al., 2013) (Supplementary Fig. 1SB), displayed an impaired accumulation response (Table 2). The latter results suggest that changes in chloroplast morphology may have distinct effects in different Arabidopsis accessions, which have been shown to exhibit differences in photorelocation responses (Königer et al., 2008).

The large-chloroplast mutants showed fairly comparable defects in their avoidance responses, while the responses varied more among intermediate mutants with chloroplasts of more variable morphology (Table 2). One possible explanation could be related to the spatial distribution of components associated with the avoidance response. The avoidance response is thought to be mediated primarily by a pool of the blue light photoreceptor phot2 that is localized to the chloroplast outer envelope membrane (OEM) (Kong et al., 2013b). Perception of strong blue light by OEM-localized phot2 results in the reorganization of short chloroplast-associated actin filaments (cp-actin); cp-actin disappears from the side of the chloroplast closest to the light, then accumulates on the distal side (Kong et al., 2013a). The chloroplasts move in the direction of greater cp-actin accumulation (i.e. away from high light; Kadota et al., 2009). The heterogeneity in chloroplast morphology in the intermediate division mutants may result in an altered distribution of phot2 in the OEM and/or more disorganized redistribution of cp-actin, thereby resulting in a more variable avoidance response. Study of cp-actin reorganization and localization of other chloroplast movement proteins in division mutants with chloroplasts of different sizes and shapes could provide mechanistic insight into the influence of chloroplast morphology on photorelocation efficiency.

Our previous studies have shown that the susceptibility of ΦII to high-light stress in severe chloroplast division mutants was due more to increased chloroplast size itself than to the accompanying chloroplast movement defects (Dutta et al., 2015). Using Arabidopsis plants with widely diverse chloroplast morphologies, we have now found that ΦII is variably affected by changes in chloroplast size and shape, but is generally correlated with the severity of the morphology phenotype. For example, in the 5 d light regime experiment (Fig. 3), the mutants with 1–2 drastically enlarged chloroplasts (arc6-5, arc12, and pdv1-1 pdv2-1) showed a pronounced loss of ΦII by the end of Day 3 as well as on Days 4 and 5, indicating long-term effects, whereas pdv1-1 and pdv2-1, which have slightly less severe chloroplast phenotypes (Miyagishima et al., 2006), showed overall milder ΦII phenotypes. However, on Days 1 and 3, ΦII was more affected in pdv1-1 than in pdv2-1 even though their chloroplast morphology phenotypes are very similar (Table 1; Supplementary Fig. S1B).
The nature of the photosynthetic phenotypes also differed between mutants. For example, in the 5 d treatment, arc11 showed statistically significant decreases in qE compared with the WT on the high-light days (Days 2, 3, and 5), whereas qE was mostly unaffected on the same days in ftsZZ-2 and minD1-1 (Fig. 4C; Supplementary Figs S8, S9, S11C). Interestingly, the decreased qE in arc11 was not accompanied by a significantly reduced PSII efficiency (Fig. 3B; Supplementary Figs S4, S6). This suggests the possibility of decreased capacity for the formation of a proton motive force through increased ATP synthase activity or reduced cyclic electron flow in this mutant (Kanazawa and Kramer, 2002; Munekage et al., 2004), or alterations in other qE components, such as a decrease in xanthophyll cycle activity or reduced abundance of PsbS (Müller et al., 2001; Li et al., 2004; Kiss et al., 2008).

Alternatively, these differences may reflect qE responses specific to Ler, the arc11 parent, as natural variation in NPQ and other photosynthetic responses has been observed (Jung and Niyogi, 2009; Yin et al., 2012).

While most of the mutants with intermediate-chloroplast phenotypes had \( \Phi_H \) responses comparable with those in the WT throughout the 5 d treatment, parc-6-1 was a notable exception, showing a greater loss of \( \Phi_H \) on Days 3–5 (Fig. 3; Supplementary Fig. S6). This may be partly because parc-6-1 has overall fewer and larger chloroplasts than other mutants categorized as intermediate, consistent with the greater severity of its chloroplast movement defects (Fig. 2; Table 2). However, all the large-chloroplast mutants except arc-5-6 had less pronounced \( \Phi_H \) phenotypes than parc-6-1 (Fig. 3), suggesting that additional factors impacted overall photosynthetic efficiency in parc-6-1 and arc-5-6. As ARC6 and PARC6 are paralogous proteins (Glynn et al., 2009), it is possible that some related aspect of their functions contributed to the similarity of their mutant phenotypes despite their distinct functions in chloroplast division (Vitha et al., 2003; Glynn et al., 2008, 2009; Zhang et al., 2016). Both parc-6-1 and arc-5-6 had drastically increased Chl \( \text{chl} \) ratios (~40% higher than that of the WT) after the 5 d treatment (Table 3). This may suggest that changes in the components of the reaction center–antenna complex and antenna size might have contributed to the overall reduction of photosynthetic efficiency in these mutants (Tyystjärvi et al., 1991; Falbel et al., 1996).

Overall, the \( \Phi_H \) data from the 5 d experiment suggest that plants with moderately oversized chloroplasts in their mesophyll cells are more capable of adjusting to high-light stress than plants with drastically enlarged chloroplasts. Further, the generally elevated susceptibility of \( \Phi_H \) observed in the single-day fluctuating light regime compared with that on Day 3 of the 5 d experiment (Figs 3, 5B) suggests that plants with increased chloroplast size may be more affected by short-term high-light fluctuations that frequently occur under natural conditions.

As in the 5 d experiment, the kinetics and distribution of the components of NPQ in the single-day experiment were distinct in the mutants (Fig. 6; Supplementary Figs S13, S15). Photoinhibition (\( q_{I_{corr}} \)) was the main contributor to elevated NPQ\( _{corr} \) at mid-day, whereas \( q_{ESV_{corr}} \) was dominant in the early and later phases of the photoperiod. This result suggests that, although pH-regulated energy dissipation in the antenna of PSII (\( q_E \)) is affected at the beginning and end of the periods of fluctuating light, photodamage of PSII outstripped repair at the saturating light intensities experienced at mid-day by these mutants. Accumulation of zeaxanthin associated with qE is also suggested to induce an additional, longer lived form of photoprotective quenching, termed qZ (Nilkens et al., 2010). Therefore, the zeaxanthin level and hence elevated qZ may also have contributed to the NPQ\( _{corr} \) at mid-day in the division mutants. In contrast to the minimal \( \Phi_H \) phenotype observed at mid-day in the intermediate-chloroplast mutants arc-3-2, ftsZZ-1, and \( fzs \) (Fig. 5B; Supplementary Figs S12, S14), q\( _{I_{corr}} \) was significantly higher in these mutants than in the WT at mid-day (Fig. 6C; Supplementary Figs S13C, S15), indicating that their photosynthetic apparatuses are more prone to photodamage under saturating high-light intensities. As increased qI in high light corresponded with the degree to which chloroplast movements were suppressed in our previous study (Dutta et al., 2015), the higher q\( _{I_{corr}} \) observed in these mutants may be due to impairment in their avoidance responses (Figs 5A, 6C; Table 2). The \( \Phi_H \) and NPQ responses of arc-5-2 and pdv2-1 (Figs 5B, 6; Supplementary Figs S14, S15) demonstrate that the losses in photosynthetic capacity in these mutants are predominantly due to impaired PSII operating efficiencies rather than being a consequence of photoinhibition under high light. The fact that we found few or no significant differences from the WT during the 5 d light regime in photosynthetic capacity in three of the mutants with intermediate-chloroplast phenotypes (ftsZZ-2, arc11, and minD1-1) (Figs 3, 4; Supplementary Figs S6, S8, S9, S11) suggests that other physiological functions of chloroplasts apart from photosynthesis may also be important for establishing the size, shape, and number of chloroplasts (Ohlrogge and Browse, 1995; Neuhaus and Emes, 2000; Lopez-Juez and Pyke, 2005; Bobik and Burch-Smith, 2015). The absence of any significant differences in chloroplast movement and photosynthetic efficiencies between 35S-PDV1 35S-PDV2 and Col-0 in our study raises further the fundamental question of why chloroplast division does not produce greater numbers of ‘small’ chloroplasts in mesophyll cells of Arabidopsis. It is also possible that the treatments used in this study were not sufficient to affect photosynthesis adversely in the 35S-PDV 35S-PDV2 line.

Overall, the observed diversity of effects seen in the mutants implies that additional factors, beyond chloroplast size itself, contribute to decreased photosynthesis. It is not possible with the current data set to identify these factors unambiguously, but several possibilities are suggested by past work. In some of the mutants, asymmetric division results in heterogeneity in chloroplast shapes and sizes (Marrison et al., 1999; Colletti et al., 2000) (Table 1; Supplementary Fig. S1). In this case, the strength of the photosynthetic phenotype could be influenced by multiple factors including: (i) the size, shape, and distribution of the largest chloroplasts; (ii) competition between less efficient large chloroplasts and normal chloroplasts; and (iii) possible asymmetric distribution of internal components (e.g. thylakoids, Rubisco) among chloroplasts. In turn, these differences could directly or indirectly affect photosynthetic
capacity, control, and regulation. Earlier work showed that several chloroplast division mutants have altered thylakoid organization and in some cases low mesophyll conductance compared with the WT (Pyke et al., 1994; Austin and Webber, 2005; Weise et al., 2015). Variations in the degree of thylakoid stacking, which have been observed in some chloroplast division mutants (Austin and Webber, 2005), could influence the relative distribution of the major photosynthetic complexes and consequently redox poise of the donor side of the electron transfer chain and susceptibility to PSII photoinhibition. ARC5 (also called DRP5B) is required for peroxisome as well as chloroplast division (Zhang and Hu, 2010); thus alterations in peroxisome function could contribute to the photosynthetic phenotypes in arc5-2. In addition, differences in chloroplast shape (i.e. degree of folding, constriction, curvature, etc.) could influence the chloroplast surface-to-volume ratio, possibly limiting the rates of intracellular exchange of metabolites and leading to metabolic imbalances that, in turn, could limit the export of fixed carbon or alter the relative demands for ATP and NAPDH from the light reactions. We expect that any combination of these factors should affect the function of the light reactions by directly interfering with normal energy capture or by inducing feedback regulatory processes.

To conclude, our results suggest a number of possible explanations for the fact that plants with populations of enlarged or heterogeneous chloroplasts are rare or undescribed in nature. First, plant productivity may be affected by a reduction in optimum light absorption under low-light conditions (twilight, shade, overcast sky). Secondly, the reduction in PSII efficiency, particularly under fluctuating high light, may account for a significant loss of fitness in plants with larger or variably sized chloroplasts in a sunny environment. Thirdly, an increase in photoinhibition caused by suppression of the avoidance response may result in reduction in fitness in these plants. Taken together, this study demonstrates that it is necessary to maintain ‘normal’ chloroplast size and number in mesophyll cells of Arabidopsis for maximum photosynthetic performance under changing light conditions. Further functional and biochemical characterization under different stress conditions will provide additional insight into the effect of chloroplast morphology on plant performance.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Phenotypes of 30-day-old Arabidopsis plants used for this study.

Fig. S2. Heat maps showing the statistical significance of differences in reflectance values for data shown in Fig. 2 and Table 2.

Fig. S3. Raw PSII quantum yield (ΦII) data for plants with large-chloroplast phenotypes used to generate the results shown in Fig. 3.

Fig. S4. Raw PSII quantum yield (ΦII) data for plants with intermediate-chloroplast phenotypes used to generate the results shown in Fig. 3.

Fig. S5. Raw PSII quantum yield (ΦII) data for plants with small-chloroplast phenotypes used to generate the results shown in Fig. 3.

Fig. S6. Heat maps showing the statistical significance of differences in ΦII between the WT and the indicated genotypes at each measurement time point for the data shown in Fig. 3 and Supplementary Figs S3–S5.

Fig. S7. Heat maps comparing the statistical significance of differences between ΦII values in arc6-5 and other large-chloroplast mutants (upper panels), and between parc6-1 and other intermediate-chloroplast mutants (lower panels) at each time point for the data shown in Fig. 3.

Fig. S8. Raw NPQ (left), qEsv (middle), and qI (right) data for fisZ2-2 and its corresponding Col-0 wild type used to generate the results shown in Fig. 4.

Fig. S9. Raw NPQ (left), qEsv (middle), and qI (right) data for minD1-1, arc11, and their corresponding parental lines used to generate the results shown in Fig. 4.

Fig. S10. Raw NPQ (left), qEsv (middle), and qI (right) data for 35S-PDV1 35S-PDV-2 and its corresponding Col-0 wild type used to generate the results shown in Fig. 4.

Fig. S11. Heat maps showing the statistical significance of differences in photosynthetic parameters at each time point for the data shown in Fig. 4.

Fig. S12. Raw data for ΦII and ‘apparent’ (traditional) NPQ, qEsv, and qI values for the experiment shown in Figs 5B and 6A–C, but uncorrected for chloroplast movements.

Fig. S13. Raw NPQcorr, qEsvcorr, and qIcorr data for the results shown in Fig. 6A–C.

Fig. S14. Heat map showing the statistical significance of differences in ΦII between the WT and the indicated genotypes at each measurement time point for the data shown in Fig. 5B and Supplementary Fig. S12A.

Fig. S15. Heat maps showing the statistical significance of differences in photosynthetic parameters at each time point for the data shown in Fig. 6.

Table S1. Light conditions for the 5 d experiment shown in Fig. 3.

Table S2. Change in reflectance values from Fig. 5A recorded at the point of the maximum avoidance response and at the end of illumination in plants with large- and intermediate-chloroplast phenotypes.

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