Running title: No independent division machinery in thylakoids

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Characterization of Thylakoid Division Using Chloroplast Dividing Mutants in *Arabidopsis*

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Summary: Chloroplasts unable to divide possess giant thylakoids suggesting that thylakoids do not possess independent division machinery

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Abstract:

Chloroplasts are double membrane bound organelles that are found in plants and algae. Their division requires a number of proteins to assemble into rings along the center of the organelle and to constrict in synchrony. Chloroplasts possess a third membrane system, the thylakoids, which house the majority of proteins responsible for the light-dependent reactions. The mechanism that allows chloroplasts to sort out and separate the intricate thylakoid membrane structures during organelle division remain unknown. By characterizing the sizes of thylakoids found in a number of different chloroplast division mutants in *Arabidopsis*, we show that thylakoids do not divide independently of the chloroplast division cycle. More specifically, we show that thylakoid division requires the formation of both the inner and the outer contractile rings of the chloroplast.

Introduction:

Chloroplasts are bound by outer and inner envelope membranes which enclose an additional compartment called the thylakoid. Specialized to capture the light energy available in sunlight and convert it into ATP and NADPH, thylakoids are responsible for the production of most of the oxygen in the Earth's atmosphere.

Structurally, thylakoids appear as an intricate network of columns of stacked discs, termed grana, interconnected by unstacked regions called stromal lamellae. This architectural complexity is further highlighted by the location of different proteins complexes within the different thylakoid regions. Photosystem II and the light-harvesting complex II trimer are found in the granal regions of the thylakoid, whereas the photosystem I - light harvesting complex I supercomplex and the ATP synthetase are found in the stromal lamellae; the cytochrome b6/f complex is located in both (Boardman et al., 1966; Boekema et al., 2000; van Roon et al., 2000; Daum et al., 2010). Spectroscopic studies , electron microscopy (Heslopharrison, 1963; Paolillo and Falk, 1966; Schoenknecht et al., 1990) and more recently, electron tomography (Shimoni et al., 2005; Daum et al., 2010; Austin and Staehelin, 2011) have shown that the thylakoid system within a single chloroplast consists of a single vesicle with its membrane intricately folded upon itself. A number of proteins, such as Thf1, VIPP1, FtsZ and FZL were shown to be essential for the development and maintenance of thylakoid structure (Kroll et al., 2001; Wang et al., 2004; Gao et al., 2006; El-Kafafi et al., 2008; Karamoko et al., 2011; Lo and Theg, 2012). The mechanism that allows chloroplasts to sort and separate the intricate structures of the thylakoid membrane during organelle division remain unknown.

Chloroplasts division requires a multitude of proteins to assemble into rings along the center of the organelle and to constrict in synchrony (Maple-Grodem and Raynaud, 2014;

Osteryoung and Pyke, 2014). The first ring that forms involves the stromal protein FtsZ interacting with Arc6, a protein found in the inner envelope membrane (McAndrew et al., 2001; Vitha et al., 2003). Arc6 has also been shown to interact with PDV2 in the outer envelope, which along with PDV1, recruits DRP5 or Arc 5, a dynamin-related protein that forms a contractile ring along the cytoplasmic side of the chloroplast (Yoshida et al., 2006; Glynn et al., 2008). FtsZ1 and isoforms of FtsZ2 in *Arabidopsis* have been found to associate with the thylakoid membrane in a developmentally dependent manner (El-Kafafi et al., 2008; Karamoko et al., 2011). While this is consistent with the involvement of these proteins with thylakoid division, this remains a matter of speculation.

The separation of thylakoids during chloroplast division has been captured in electron micrographs by a number of different groups (Leech et al., 1981; Oross and Possingham, 1989; Robertson et al., 1996). Images show thylakoids dispersed throughout the chloroplast in the early stages of plastid division. As division proceeds, the number of thylakoid grana and stromal lamellae that extend across the length of the original plastid dwindles until a single segment spans the isthmus of the constricted plastid. At the final stages of plastid division, the thylakoid membrane along with one of the daughter organelles appear to be twisted so that the thylakoid membrane loses its longitudinal orientation (Robertson et al., 1996). Thylakoid division appears to precede the separation of the daughter organelles (Whatley, 1980). The mechanism of thylakoid division, and its reliance on the chloroplast division cycle remain unknown. In this study we dissect the dependence of thylakoid division on the chloroplast division machinery by comparing the sizes of thylakoids found in chloroplast division mutants that are arrested at different states of plastid division.

Results:

Experimental theory and design

Wild type *Arabidopsis* plants possess ~ 120 chloroplasts per mesophyll cell, each with a single thylakoid membrane (Pyke and Leech, 1994). The composition of thylakoids from chloroplast mutants that are unable to divide is unknown. We first examined the effect of the lack of an inner contractile ring on the division of thylakoids. The chloroplast division mutant, *arc6*, has a premature stop codon near the amino-terminal region of the protein, thereby preventing the formation of an inner contractile ring and rendering the plastid incapable of division (Vitha et al., 2003). As a result, the mutant plant possess giant-sized chloroplasts (Pyke et al., 1994). We reasoned that there are two possible scenarios that can describe the fate of the thylakoids in *arc6* mutants. In the first, we assume that the thylakoids in the *arc6* chloroplasts are still capable of undergoing division independent of the chloroplast division cycle. As a result, the number and size of the thylakoids within the giant *arc6* chloroplasts will likely be similar to those found in wild-type cells. In the second scenario, we assume that the *arc6* thylakoids cannot divide independently of the chloroplast division cycle. Accordingly, the *arc6* chloroplasts would possess a single giant-sized thylakoid.

We sought to determine the relative sizes of the thylakoids in *arc6* and wild type chloroplasts by measuring the sensitivity of the ionic conductivity of the thylakoid membrane to the pore-forming ionophore gramicidin. This experiment, originally performed by (Schoenknecht et al., 1990), is based on the ability of a small number of gramicidin pores to short circuit the capacitance of a thylakoid membrane vesicle. Titration of the membrane capacitance with gramicidin will report on the size of the thylakoid electrical unit, with larger thylakoids being more readily short-circuited by a given concentration of ionophore than smaller

thylakoids. The conductivity of the thylakoid membrane can be conveniently and non-invasively monitored via the well-characterized decay of the carotenoid electrochromic shift at 520 nm induced by a short pulse of light (Junge and Witt, 1968; Witt, 1979; Bailleul et al., 2011). By this technique it was determined that the size of the thylakoid electrical unit within chloroplasts corresponds to all the photosynthetically active membranes within each plastid; that is, there is essentially one thylakoid per chloroplast (Schoenknecht et al. 1990). This conclusion has been confirmed by TEM micrographs of serially sectioned plastids (Heslopharrison, 1963; Paolillo and Falk, 1966; Mustardy and Janossy, 1979), and more recently, by electron tomography (Nierzwicki-Bauer et al., 1983; Shimoni et al., 2005; Mustardy et al., 2008; Austin and Staehelin, 2011; Daum and Kuhlbrandt, 2011).

The electrical unit in *arc6* chloroplasts is larger than that in wild-type chloroplasts

It would be expected that large thylakoid vesicles potentially formed in giant chloroplasts might be susceptible to disruption by shear forces applied during isolation. In order to minimize this possibility, protoplasts were made from both wild type and *arc6* plants and chloroplasts were subsequently isolated therefrom. Figure 1 shows that despite the expectation that the giant single chloroplasts present in the *arc6* mutants would be even more fragile than those in the wild type, they could be isolated intact from protoplasts by incubation in a carbonate-containing buffer. This is, to our knowledge, the first successful isolation of these giant chloroplasts with their envelopes intact.

We then probed the size of the thylakoid electrical units via the light-induced carotenoid electrochromic signal at 520 nm. After a 9 ms light pulse, the $\Delta\psi$-indicating ΔA_{520} nm signal exhibited biphasic relaxation kinetics in both wild type and *arc6* plastids. *Arc6* chloroplasts

displayed a faster $\Delta\psi$ relaxation rate than the wild type even in the absence of ionophore (Figure 2A-B). The addition of gramicidin to the chloroplasts resulted in an accelerated relaxation rate of the ΔA_{520} nm signal in both wild type and *arc6* samples (Figure 2A-B), with increasing concentrations resulting in faster decays. This was also conveniently manifested in the initial point recorded as the 9 ms actinic pulse was turned off. Since a 9 ms pulse is not a single turnover flash, the magnitude of the electric field measured at 9 ms results from competition between field generation by multiple reaction center excitations and decay by ion counter movement, the latter of which is accelerated by gramicidin. Accordingly, thylakoid membranes with increased ion permeability display a lower magnitude of the ΔA_{520} nm signal at 9 ms. Acceleration of the electrochromic shift decay can be clearly seen by plotting the magnitude of the 9 ms ΔA_{520} nm absorbance as a function of the logarithm of the gramicidin concentration (Figure 2C). This plot reveals the increased sensitivity of the thylakoid conductance to gramicidin exhibited by *arc6* chloroplasts over those from wild-type plants, and indicates that the thylakoids are larger in the *arc6* mutant.

***Arc6* chloroplasts possess giant thylakoids**

When isolated protoplasts are placed in very low osmotic conditions, the plasma membrane and the chloroplast outer and inner envelope membranes break, resulting in the release of the thylakoids (Mercer, 1954; Weier et al., 1965; Hinnah and Wagner, 1998). However, thylakoid membranes are more resilient to osmotic pressure than the rest of the cell's membranes, and thylakoids swell to form blebs. We used this property of thylakoids to provide an independent measurement of the size of wild type and *arc6* thylakoids. In the confocal microscope, chlorophyll autofluorescence serves as a marker for thylakoid membranes (Figure

3). Figure 3B shows a representative cross section image of a bleb formed from *arc6* thylakoids, and it is apparent that it is significantly larger than those formed from wild-type thylakoids (Figure 3A). The diameter of the wild type bleb (Figure 3A) is ~ 12.8 ± 3.9 µm, whereas the *arc6* bleb (Figure 3B) diameter averages 21.7 ± 11.2 µm. While the diameter of a spherical object such as a bleb observed in a single cross section could be mistakenly underestimated by examining an image off of the equatorial plane, those images in Fig. 3 were produced after scanning back and forth through the z-plane to find the maximum observed diameters. Representative z-projections, in which all z-plane images are stacked one upon each other, confirm the dramatic difference in sizes between *arc6* and wild type blebs (Figure 4A-B).

The values in Table 4.1 report the average diameters of 1000 bleb samples. There was, however, a remarkable variation in the sizes of the blebs formed from these (and other) plants, and this is seen clearly in the diameter distribution histograms in Figure 5A. The possible origins of this variation are examined in the Discussion.

Together with the measurements of the electrical unit size above, the confocal microscopy images in Figures 3 and 4 show that *arc6* chloroplasts possess giant thylakoids. This suggests that thylakoid division is dependent on chloroplast division.

Incomplete formation of contractile rings result in giant thylakoids

To test the dependence of thylakoid division on the constriction of the outer contractile ring, we examined two chloroplast division mutants, *pdv1* and *pdv2*, which exhibit 2-6 gigantic chloroplasts that possess a dumbbell-like structure. These proteins reside in the outer envelope membrane and act as a functional pair to interact with Arc 5 and form the outer contractile ring. The *pdv* mutant chloroplasts are still able to form the inner contractile ring, however without the outer ring the plastids never divide (Miyagishima et al., 2006). We tested two alleles of each *pdv*

mutant, one that contained a mutation close to the N-terminus and another to the C- terminus which we labeled -1 and -2, respectively. On average, *pdv1-1*, *pdv1-2*, *pdv2-1*, and *pdv2-2* blebs (Figure 3C-F) were much larger than wild-type blebs. The blebs found in these mutants were slightly smaller than those found in the *arc6* mutant (~20 µm vs ~22 µm, Table 1). Representative z-projections show the *pdv* blebs in their entirety and provide further evidence that the blebs are much larger than those found in wild type (Figure 4C-F). The variations observed in bleb sizes for the wild type and *arc6* plants were evident in these mutants as well (Figure 5B).

To examine the behavior of thylakoids in a mutant that undergoes asymmetric plastid division we measured bleb sizes from an *arc3* mutant (Figure 3G) in which the inner contractile ring is misplaced (Pyke and Leech, 1994; Zhang et al., 2013). The *arc3* mutant plastids possess ~ 15 chloroplasts per cell (Burch-Smith et al., 2007). Here we expected the bleb sizes to average closer to those of the wild-type blebs, but with a larger variation, as an asymmetric division should result in one larger-than-wild-type and one smaller-than-wild-type chloroplast. As per these expectations, the *arc3* bleb sizes averaged 15.6 µm, somewhat larger than the wild-type blebs, and displayed a standard deviation of ± 6.5 µm (Table 1, Figure 5C). While we obtained a similar large standard deviation of bleb sizes from the giant chloroplast mutants, we note that blebs formed from the arc3 mutants are only 22% larger than those from wild type plants, but display a 67% increase in the diameter standard deviation.

These results in aggregate reveal that thylakoids do not possess an independent division machinery and cannot divide if the chloroplasts cannot divide.

Discussion:

Thylakoid division is dependent on the chloroplast division machinery

Thylakoids represent a membrane system essential to shaping and maintaining the biosphere. Yet, the reproduction and partitioning of thylakoids, which must occur multiple times in green plants during each cell cycle, has so far escaped much scrutiny. In this work we have examined the question of whether thylakoids possess their own division machinery that operates independently of the chloroplast reproduction events.

In wild type plants it has been shown that each chloroplast possess a single thylakoid (Heslopharrison, 1963; Paolillo and Falk, 1966; Nierzwicki-Bauer et al., 1983; Schoenknecht et al., 1990; Shimoni et al., 2005; Mustardy et al., 2008; Austin and Staehelin, 2011; Daum and Kuhlbrandt, 2011). We reasoned that there are two possibilities for the structure of thylakoids found in plastid-division mutants. If the thylakoids are capable of dividing independently of chloroplast division, then the thylakoids in the plastid division mutants should be the same size as in wild-type chloroplasts. If, on the other hand, thylakoid division is dependent upon chloroplast division, then we would expect larger-than-wild-type thylakoids. We used two completely independent and unrelated methods to show that larger thylakoids are found in mutants that make giant chloroplasts. Changes in the conductivity of the thylakoid membrane in response to gramicidin addition can be used as a measure of relative thylakoid size (Schoenknecht et al., 1990). At any given ionophore concentration, it is expected that a larger vesicle would incorporate more gramicidin pores per vesicle than would a smaller one. Experimentally, this would be manifested as an increased sensitivity of the decay of the membrane potential to gramicidin. Using the amplitude of the carotenoid electrochromic signal at the end of a 9 ms illumination pulse as an indicator of the membrane conductivity, we found

that the *arc6* thylakoids were indeed considerably more sensitive to gramicidin addition in the manner suggested. From this we conclude that thylakoid vesicles in the *arc6* mutant are larger than those in the wild type. This would imply that the thylakoids cannot divide independently of plastid division.

It should also be noted that the ratio of gramicidin/chlorophyll required to accelerate the rate of electric field dissipation is higher in our studies than what was previously reported by (Schoenknecht et al., 1990). This discrepancy can be accounted for as a result of differences in thylakoid preparations. The thylakoids utilized in our studies were prepared by lysing intact chloroplasts within the same chamber that was carried out for taking the ΔA_{520} nm measurements. As a result, the thylakoid samples contained additional chloroplast envelope membranes which absorbed gramicidin and increased the working gramicidin/chlorophyll ratio up to 50 fold (Nishio and Whitmarsh, 1991). Thus the higher gramicidin concentrations used in this study are to be expected. Additionally, differences in gramicidin's dimerization constants in thylakoids from different plant species can also contribute to this discrepancy (Schoenknecht et al., 1992). The difference in membrane permeability between the *arc*6 and wild type thylakoids exhibited in Figure 2A-B may result from *arc6* thylakoids possessing naturally more ionically conductive membranes. It is also likely that the chloroplast solution used for the ECS measurements contained intact and broken chloroplasts which may have resulted in a slightly accelerated ΔA_{520} nm decay due to the damage that the thylakoids may have sustained during the event that caused the lysis of the intact chloroplast. However, the sensitivity of the thylakoids towards increasing concentrations of gramicidin would not be expected to be due to the presence of lysed chloroplasts. Accordingly, our observation that the *arc6* thylakoids are more sensitive

towards increasing amounts of gramicidin indicates that the *arc6* thylakoids are larger than their wild type counter parts.

The sizes of osmotically lysed thylakoids from the different chloroplast division mutants provided an independent measurement of thylakoid structure. It is apparent that the thylakoids formed from *arc6* chloroplasts were considerably larger than wild-type blebs. The average diameter of the wild-type bleb was 12.8 µm, whereas that of *arc6* blebs was 21.7 µm (Table 1). Our similar findings with the *pdv1* and *pdv2* mutants suggests that thylakoid division is closely tied to the chloroplast division process (Table 1). The *arc6* chloroplasts are unable to form contractile rings, whereas the *pdv1* and *pdv2* mutants are capable of forming an inner contractile ring but only a partial outer ring. Our results suggest that separation of thylakoid membranes depends on the constriction forces applied by the chloroplast division machinery. The sizes of the *arc3* blebs show that thylakoid division is closely tied with the final stages of plastid division since the aberrant placement of the plastid ring leads to a rather large spread in thylakoid bleb sizes. Thus, our results from the bleb studies agree with the previously published electron microscopy images showing that thylakoid division occurs at the late stages of plastid division (Whatley, 1980; Robertson et al., 1996).

It is noteworthy that we observe a distribution of bleb sizes in our experiments (Figure 5), even in the wild type plants, the reasons for which may be manifold. First, the plastid division mutants we examined do not display giant chloroplasts in every cell type. Measurements of chloroplasts size found in the *arc6* guard cells made by (Pyke et al., 1994) and (Robertson et al., 1995) show that the *arc6* chloroplasts in the guard cells are smaller than the ones observed in mesophyll cells. Similarly, guard cells in *pdv* mutants also have smaller plastids compared to mesophyll cells (our observation). All cell types would have been in our preparations. Upon

reflection one sees that a small number of cells with a normal allotment of chloroplasts mixed in with a preponderance of cells containing one and or a few giant chloroplasts would lead to a skewed bleb size distribution, as seen in our experiments. Second, it is clear from the patchiness of the observed chlorophyll fluorescence that thylakoids do not unstack and unravel completely during bleb formation, and incompletely expanded thylakoids would form smaller blebs. This incomplete unfolding was not a consequence of short incubations in water, as neither did the observed patchiness decrease (not shown), nor did the bleb diameters increase with longer incubation times (Figure S1). Third, while we made every effort to be gentle with the samples, we cannot be sure that we did not cause some breakage of the blebs during handling. We would expect osmotically swollen vesicles to be more fragile than normal thylakoids, and any such breakage would necessarily result in the formation of smaller blebs. Fourth, budding as an alternative plastid division mechanism has been observed in the tomato suffulta mutant (Chen et al., 2009), and *Bryophyllum pinatum* (Kulandaivelu and Gnanam, 1985). In a study that targeted GFP to the stroma of *arc6* mutant chloroplasts, small vesicular bodies that contained GFP were suggested to have budded off of the giant chloroplasts (Forth and Pyke, 2006). Such buds would be expected to contain smaller thylakoid vesicles. Finally, there is inherent variability in our samples. Wild type Arabidopsis cells do not always contain 100 chloroplasts, *arc6* mutant cells do not always contain one chloroplast, and *pdv1* mutant cells do not always contain three chloroplasts. Instead, these are averages. Thus we would expect some variability in bleb sizes even if the other factors mentioned above were not in play. We hold that the fact that we see any giant blebs at all in giant chloroplast mutants provides strong evidence that thylakoid division and chloroplast replication are not independent and uncoupled events.

292 **Thylakoid division mechanism**

293 We propose that the constriction of the envelope membrane by the chloroplast division
294 machinery acts to partition portions of the thylakoid into the two different poles of the dividing
295 organelle. To reach this stage wherein a small portion of the thylakoid vesicle is trapped in the
296 central isthmus of the dividing chloroplast, proteins that are known to be involved in thylakoid
297 formation and remodeling, such as Vipp1, Thf1, FtsZ and FZL (Kroll et al., 2001; Wang et al.,
298 2004; Gao et al., 2006; El-Kafafi et al., 2008; Lo and Theg, 2012), might be brought to bear on
299 the thylakoid structure. These proteins have been proposed to govern the fission and fusion
300 events accompanying stacking and unstacking of the thylakoid membrane in response to
301 different light conditions (Chuartzman et al., 2008), and are thus candidates for those that might
302 be involved in the final separation of the thylakoid vesicle into the two daughter plastids.
303 Mesophyll cells in which the thylakoid protein FtsZ1, FtsZ2-2 and FZL has been knocked out
304 possess fewer and larger chloroplasts compared to wild type cells (Gao et al., 2006; El-Kafafi et
305 al., 2008; Karamoko et al., 2011), which suggests the involvement of thylakoid remodeling
306 proteins during thylakoid division and also highlights the interdependence of both thylakoid and
307 chloroplast division machineries for the complete fission of the daughter organelle. Our findings
308 do not negate the possibility that other specific thylakoid division proteins are activated during a
309 late stage of chloroplast division, but those proteins have yet to be identified. It appears more
310 likely that the forces generated by the chloroplast envelope contractile rings are those that are
311 responsible for dividing the thylakoid membrane as well. It will surely be interesting to elucidate
312 through future studies the mechanism through which these forces generated on one membrane
313 system are transduced to the other.
314

Materials and Methods:

Plant material, protoplast and chloroplast isolation

The Col-0 ecotype of *Arabidopsis thaliana* was used as the wild type. The chloroplast division mutants, *arc6* (SAIL_693_G04), *pdv1-1*, *pdv1-2*, *pdv2-1*(SALK_059656), and *pdv2-2* (SAIL_875E10) were kindly provided by Dr. KW Osteryoung. Landsberg Erecta *arc3* (CS264) seeds were obtained from ABRC. All plants were genotyped and only homozygous lines were used (see supplemental materials for further detail). All plants were grown for 5 weeks on Murashige and Skoog (Phytotechnologies, Santa Cruz, CA) agar before harvesting the tissue for chloroplasts (Theg and Tom, 2011); growth chamber conditions were 20^{o}C with 16 hrs light cycle of 100 µmol photons/m^{2}/sec at 60% humidity. Plant tissue was harvested following the procedure described previously (Fitzpatrick and Keegstra, 2001) with some minor modifications. Briefly, plants were minced in a petri dish and washed with digestion buffer (400 mM sorbitol, 0.5mM $CaCl_2$, 20 mM Mes-KOH, pH 5.2). The minced plant tissue was then incubated with 0.05 g/mL cellulase 'onozuka' R-10 and 0.01 g/mL macerozyme R-10 (Yakult Pharmaceutical Ind, Tokyo, Japan) for 3 hrs at room temperature with gentle rocking. Digested samples were passed through 4 layers of cheese cloth, and then centrifuged at 370 x *g* for 5 minutes at 4^{o}C. The pellet was gently resuspended in resuspension buffer (400 mM sorbitol, 0.5mM $CaCl_2$, 20 mM Mes-KOH, pH 6.0). Samples were centrifuged again at 660 x *g* for 5 minutes at 4^{o}C, and if blebs were to be formed, the pellet was resuspended in a minimal volume of resuspension buffer and kept on ice in the dark until further use.

For wild-type chloroplast isolation, the protoplast pellet from the wild-type plants was resuspended in breakage buffer (330 mM Sorbitol, 5 mM EDTA, 5 mM EGTA, 10 mM $NaHCO_3$, 0.1% BSA, 20 mM Tris-KOH, pH 8.4) and passed through 20 µm and 10 µm mesh

through a syringe 4 times. For *arc6* chloroplast isolation, the protoplast pellet from the *arc6* tissues was resuspended in breakage buffer and incubated for 5 minutes on ice in the dark. Both samples were then passed through a Percoll gradient containing half volume of Percoll and half volume of grinding buffer (330 mM Sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.1% BSA, 50 mM Hepes-KOH, pH 7.3) at 8035 x *g* at 4^{o}C. Intact chloroplasts were removed from the bottom band, and washed with a storage buffer (330 mM Sorbitol, 50 mM Hepes-KOH, pH 8). Chloroplasts were centrifuged at 1475 x *g* at 4^{o}C for 5 min. This step was repeated again to remove the remaining Percoll. After chlorophyll determination (Arnon, 1949), isolated chloroplasts were stored in the storage buffer on ice in the dark until used.

Electrochromic shift measurements

All electrochromic shift measurements contained 0.02 µg/µL chlorophyll concentration, 1 mM methyl viologen, and various amounts of gramicidin D (Sigma-Aldrich, St. Louis, Missouri); measurements were performed in 1.2 mL of storage buffer. Gramicidin stock solutions were prepared in ethanol; the total percentage of ethanol did not exceed 0.03% for all titrations. The measurements typically began with the addition of chloroplasts into a 2.5 ml polystyrene cuvette (Fisher Scientific, Houston, Texas) containing a master mix of methyl viologen, storage buffer and gramicidin. Samples were mixed with a stir bar for 2 minutes at room temperature, and then transferred into a 1.5 mL Polystyrene cuvette (Fisher Scientific, Houston, Texas) with a 10 mm path length. The absorbance readings were performed using a JTS-10 LED pump-probe spectrometer (Bio-Logic SAS, Claix, France). All 520 nm absorption measurements consisted of a 1 sec dark baseline followed by a 9 ms actinic pulse; the relaxation kinetics were followed out to 4 sec.

Thylakoid bleb formation

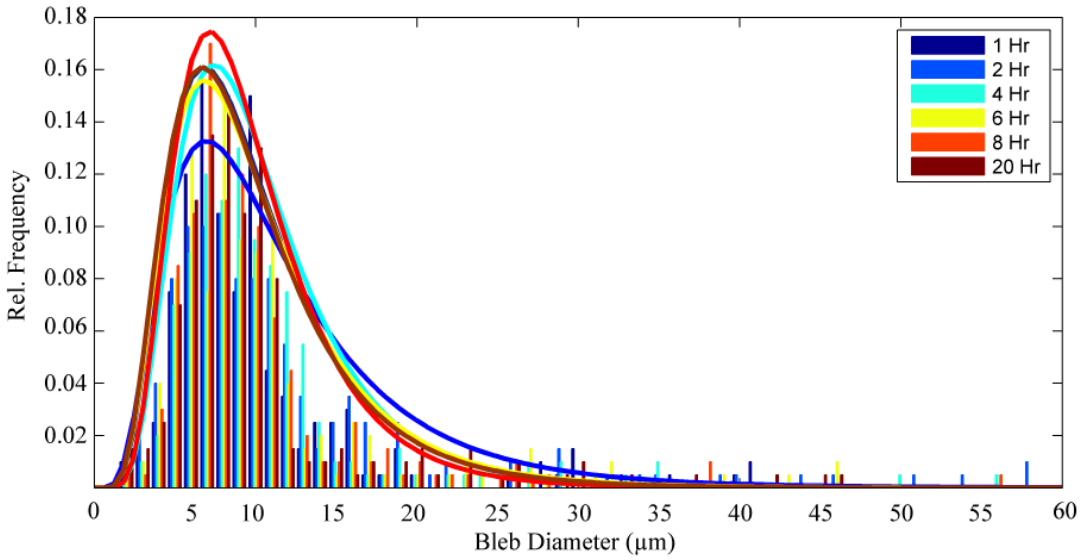
Blebs were formed by diluting isolated protoplasts containing 2µg of chlorophyll into 2 mL of doubly distilled water stored at 4^{o} C, and incubated on ice in the dark on ice for 1 hr before images were taken. Equal volumes of sample were loaded into a homemade perfusion chamber consisting of 1.8 mm x 100 mm x 1.1 mm piece of polycarbonate and a 50 x 22 mm coverslip (Fisher Scientific Houston, TX) which had been coated with poly-L-lysine (Sigma-Aldrich St. Louis, MO). Samples were centrifuged for 15 minutes at 60 x *g* using a GS-6KR swinging bucket centrifuge (Beckman Coulter Inc., Brea, CA) at 10ºC and imaged.

Microscopy

Phase contrast microscopy was performed with a Zeiss Standard 25 ICS microscope. Images at 160x magnification were taken by placing a camera, Canon powershot A620 (Canon, Melville, New York), into the ocular eyepiece. Fluorescence microscopy was performed using a Zeiss LSM 710 confocal microscope (Zeiss, Oberkochen, Germany). Image compilation and analysis were performed using Fiji software. Bleb diameters were measured from populations of isolated blebs taken from protoplast isolation preparations. The reported bleb diameter size were determined by scanning through the z-stack and finding the maximal diameter of the bleb. All reported values for bleb diameters were determined as being the largest distance from one end of the membrane to the other throughout the entire z-axis per bleb.

Supplemental Material

384 **Figure S1.** Thylakoid blebbing time course



385

386 **Figure S1.** Thylakoid blebbing time course

387 A histogram reporting the frequency of bleb diameter sizes from a mixture of *arc6* and wild type

388 samples over the course of 20 hours. (n=200 for all time points) The plots were fit with a log-

389 normal distribution function.

390

Table S1. Table displaying the primers utilized to genotype plants from the T-DNA insertion lines, *arc6, and pdv2 -1*, *pdv2-2*.

Plants from the T-DNA insertion lines, *arc6, and pdv2 -1*, *pdv2-2*, were genotyped using the following primers:

Genotype	5' - 3' Primer Sequence
arc6	FP: ATCAGCAACGGACATTTCAAC
arc6	RP: TAAATGGTTTAAGCGGTGTGC
arc6	SAIL LB1: GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC
pdv2-1	FP: AATTACTTGTCTCTCTTCTCTTCCCCACCA
pdv2-1	RP: ATACTACTCAAATGAATCTCTCCCTCATCCA
pdv2-1	SALK LB1.3: ATTTTGCCGATTTCGGAAC
pdv2-2	FP: CAGCTTGCTTCTTTACAGGTTTGAACCAAA
pdv2-2	RP: ATACTACTCAAATGAATCTCTCCCTCATCCA
pdv2-2	SAIL LB1: GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC

Table S2. Table displaying the primers utilized to genotype *Pdv1-1*, *pdv1-I*, and *arc3* plants by detecting single nucleotide polymorphisms.

Genotype	5' - 3' Primer Sequence	Digestion Enzyme
pdv1-1	FP: AATCTCATCGCTAAGCTTGTCATGTAGAGC	SacI
pdv1-1	RP: TTTTTTGTTGTGTCAATGAAATGGGAGAAA	SacI
pdv1-2	FP: ACGTGTAGACAAGTTCGATCTCTGGTGTTAA	HpaI
pdv1-2	RP: GCAGAAGAAAGAAGACAGATTCTACAGAAGCC	HpaI
arc3	FP: AACAAAACTTGTTCATTTGTCTTGCAGGGGTG	HphI
arc3	RP: GGCCTTCGCCACTGGCTTTTCCTTTCCAGA	HphI

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Table 1. Bleb Dimensions

Genotype	Diameter (µm)
wild type	*12.8 ± 3.9*
arc6	*21.7 ± 11.2*
pdv1-1*	*19.5 ± 8.6*
pdv1-2*	*20.1 ± 8.8*
pdv2-1*	*18.9 ± 6.9*
pdv2-2*	*19.8 ± 7.8*
arc3	*15.6 ± 6.5*

For all samples n= 1,000

Table 1. Bleb Dimensions
A table reporting the average bleb diameters and standard deviations for all genotypes. All values reported were statistically significant ($P<0.05$) from a student's t-test other than those annotated with an (*). Samples annotated with (*) are not statistically significant from each other ($P>0.05$).

Ho et al. Figure 1

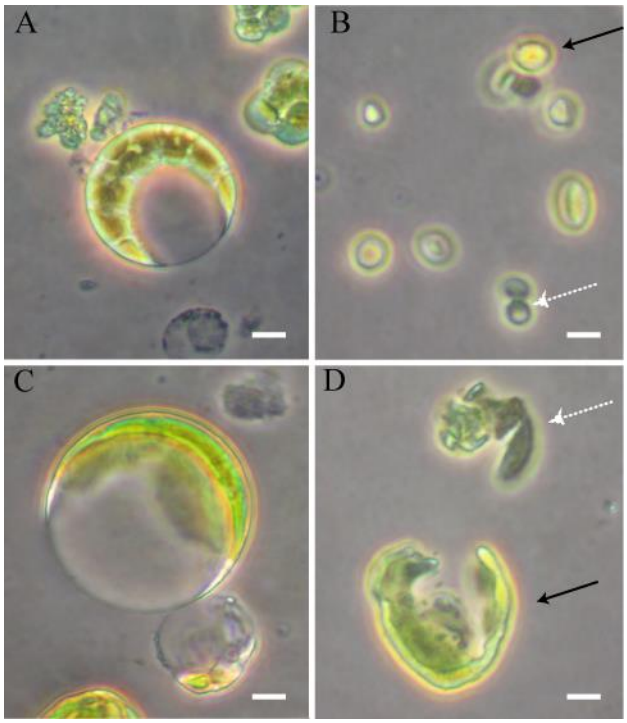
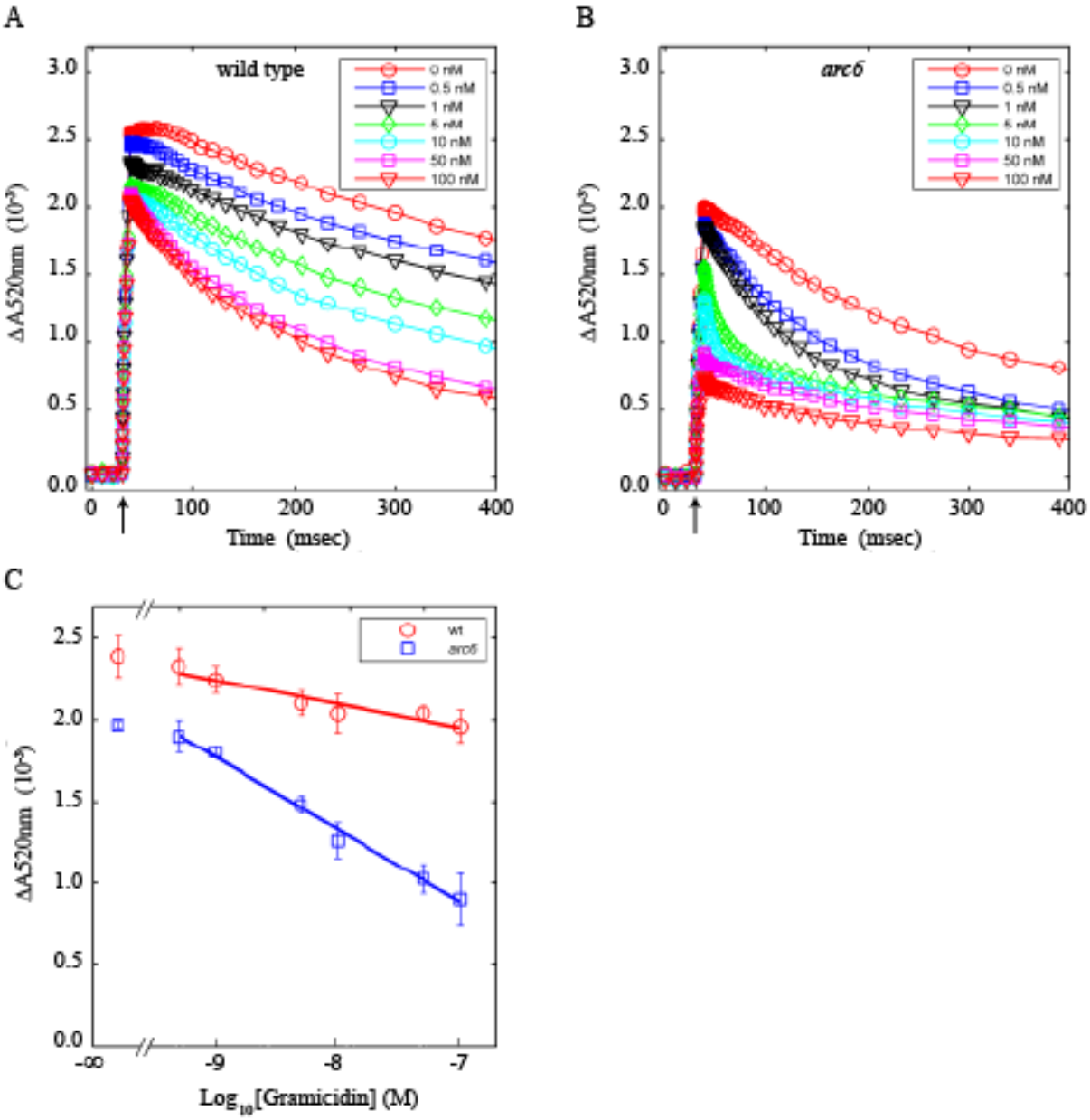


Figure 1. Isolation of intact chloroplasts.

A) and C), Isolated protoplasts from wild type and *arc6 Arabidopsis* under a phase contrast microscope. B) and D), Wild type and *arc6* isolated chloroplasts. Intact chloroplasts possess a halo and are highlighted by the black arrow; broken chloroplasts are indicated by the white arrow. Scale bar corresponds to 10 µm.

439 **Ho et al Figure 2**

440



441

442 **Figure 2.** Thylakoid membrane conductivity measured by the electrochromic shift of

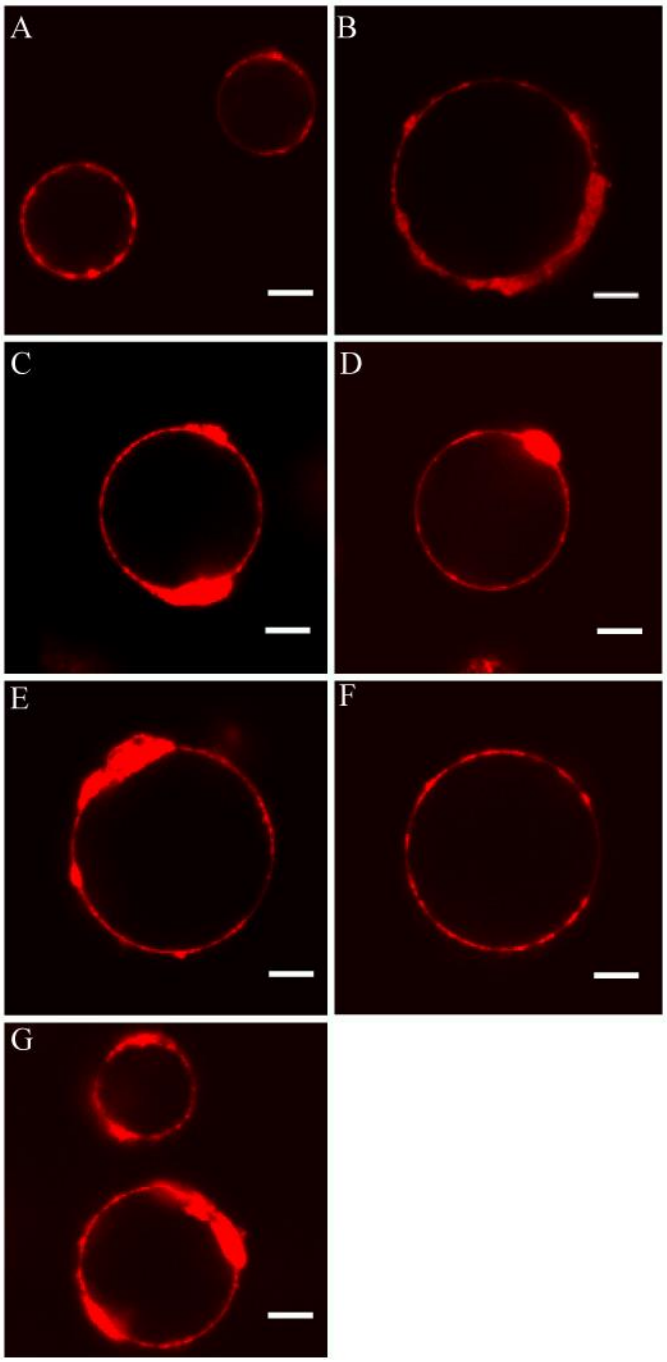
443 carotenoids.

444 Representative ΔA_{520} nm measurements of A) wild-type and B) *arc6* chloroplasts with

445 increasing amounts of gramicidin; the arrow indicates the initiation of the 9 ms actinic flash. C)

446 Plot of the first point of the ΔA_{520} nm signal after the 9 ms actinic flash vs. the log of gramicidin
447 concentration. Bars indicate standard deviation of n=9 for each data point. The wild-type (red
448 circles) and *arc6* (blue squares) data points fit by linear regression; the ΔA_{520} nm values for 0 nM
449 gramicidin values are plotted to the left of the axis break and not utilized in the fit.
450
451

452 **Ho et al. Figure 3**



453

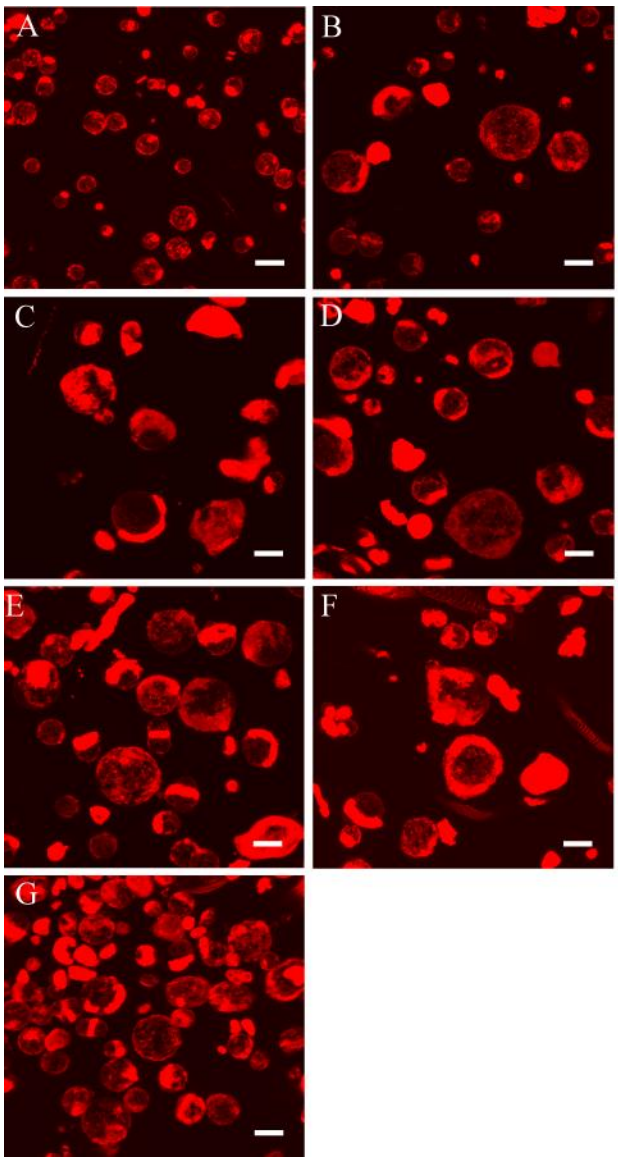
454

455 **Figure 3.** Chloroplast division mutants contain thylakoids that are much larger than those in

456 wild-type plants.

457 A-G) Representative cross-sections of osmotically swollen thylakoids from wild-type, *arc6*,
458 *pdv1-1*, *pdv1-2*, *pdv2-1*, *pdv2-2*, and *arc3* plants, respectively, were taken using a laser scanning
459 confocal microscope. Chlorophyll autofluorescence is used to track the presence of the thylakoid
460 membrane. The scale bar corresponds to 10 μm.
461
462

463 **Ho et al. Figure 4**



464

465

466 **Figure 4.** Z-projections of various thylakoid division mutants

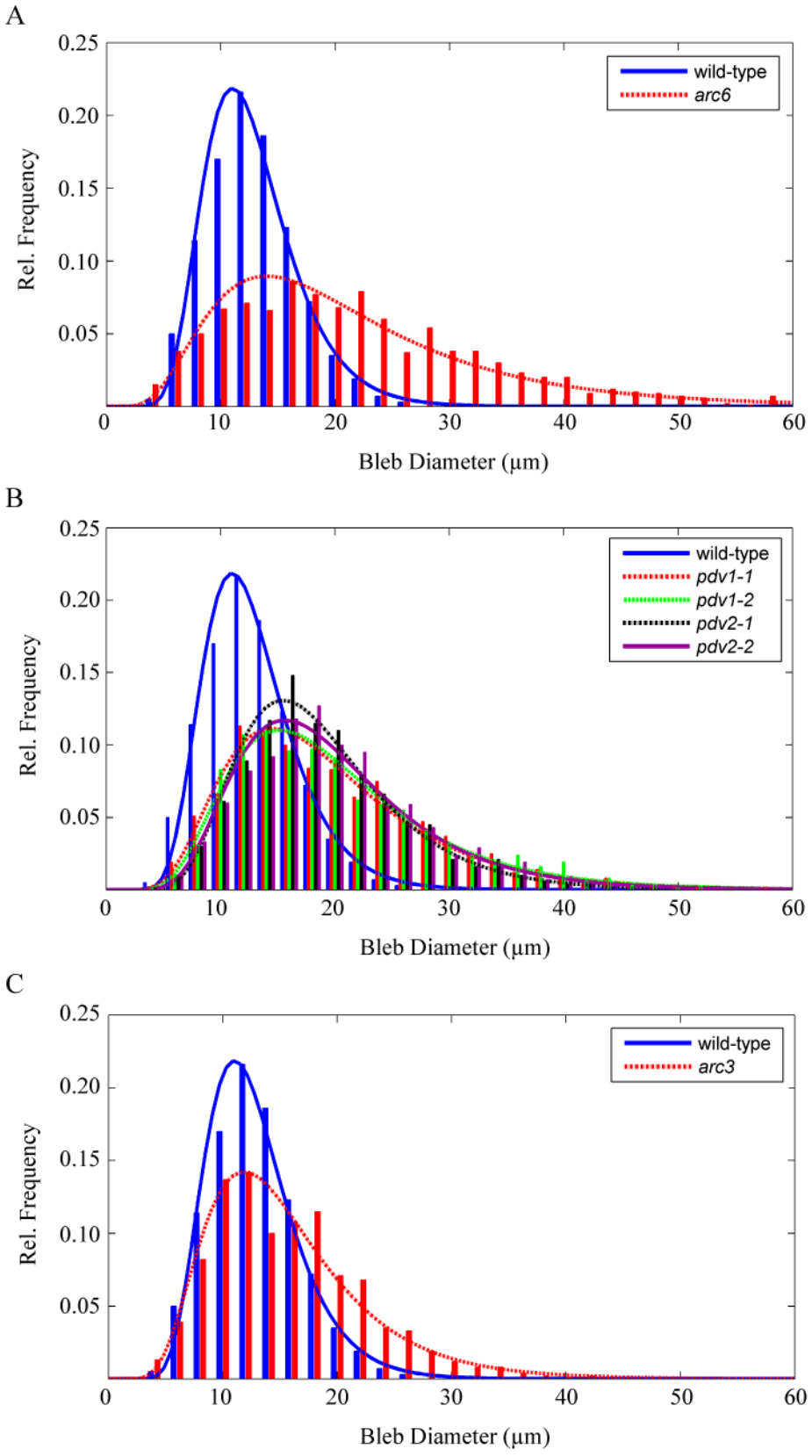
467 Representative z- projections of osmotically swollen thylakoids from A) wild-type, B) *arc6*, C)

468 *pdv1-1*, D) *pdv1-2*, E) *pdv2-1*, F) *pdv2-2*, and G) *arc3* plants were taken using a laser scanning

469 confocal microscope. The scale bar corresponds to 20 µm.

470

471 **Ho et al. Figure 5**



472

473

474 **Figure 5.** Thylakoid bleb population size distribution histogram

475 A) A histogram comparing the frequency of bleb diameter sizes between the wild type and A)

476 *arc6* , B) all *pdv* mutants, and C) *arc3* plants (n=1,000 for all genotypes). The plots were fit with

477 a log-normal distribution function.

478

479

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