The PLASTID DIVISION1 and 2 Components of the Chloroplast Division Machinery Determine the Rate of Chloroplast Division in Land Plant Cell Differentiation

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In most algae, the chloroplast division rate is held constant to maintain the proper number of chloroplasts per cell. By contrast, land plants evolved cell and chloroplast differentiation systems in which the size and number of chloroplasts change along with their respective cellular function by regulation of the division rate. Here, we show that PLASTID DIVISION (PDV) proteins, land plant–specific components of the division apparatus, determine the rate of chloroplast division. Overexpression of PDV proteins in the angiosperm Arabidopsis thaliana and the moss Physcomitrella patens increased the number but decreased the size of chloroplasts; reduction of PDV levels resulted in the opposite effect. The level of PDV proteins, but not other division components, decreased during leaf development, during which the chloroplast division rate also decreased. Exogenous cytokinins or overexpression of the cytokinin-responsive transcription factor CYTOKININ RESPONSE FACTOR2 increased the chloroplast division rate, where PDV proteins, but not other components of the division apparatus, were upregulated. These results suggest that the integration of PDV proteins into the division machinery enabled land plant cells to change chloroplast size and number in accord with the fate of cell differentiation.

INTRODUCTION

Chloroplasts originally derived from a bacterium related to extant cyanobacteria, which was engulfed by a primary nonphotosynthetic eukaryotic host cell more than a billion years ago. Over time, the engulfed bacterial endosymbionts have been reduced to chloroplasts and vertically transmitted to subsequent generations (Reyes-Prieto et al., 2007; Gould et al., 2008). Reminiscent of their free-living ancestor, chloroplasts multiply by division (Possingham and Lawrence, 1983; Boffey and Lloyd, 1988; Kuroiwa et al., 1998). However, most of the genes once present in the engulfed bacterial endosymbiont have been lost or transferred to the host nuclear genome; those still used by the chloroplasts are translated by the host and targeted back into the chloroplasts to express their functions. Therefore, chloroplasts cannot divide by themselves, and the division is performed by nucleus-encoded proteins.

Chloroplast division is performed by ring structures at the division site, spanning both the inside and the outside of the two chloroplast envelope membranes. (Yoshida et al., 2006; Maple and Moller, 2007; Yang et al., 2008). The ring structures were identified by earlier electron microscopic studies (Kuroiwa et al., 1998; Miyagishima et al., 2001; Yoshida et al., 2006), and recent studies have identified several proteins that form a complex at the division site. Consistent with the endosymbiotic origin of chloroplasts, the division complex includes FtsZ, a self-assembling tubulin-like GTPase (Osteryoung and Vierling, 1995; Osteryoung et al., 1998; Mori et al., 2001; Vitha et al., 2001; Kuroiwa et al., 2002), and ACCUMULATION AND REPLICATION OF CHLOROPLASTS6 (ARC6), a J-domain containing protein, both of which are descended from the cell division machinery of the engulfed cyanobacterium (Vitha et al., 2003). In addition, the division complex includes DYNAMIN-RELATED PROTEIN 5B (DRP5B) (also known as ARC5), a member of the dynamin family of self-assembling GTPase proteins (Gao et al., 2003; Miyagishima et al., 2003), and PLASTID DIVISION1 (PDV1) and PDV2 proteins, which contain coiled-coil domains on the cytosolic side (Miyagishima et al., 2006; Glynn et al., 2008). DRP5B is specific to plants and algae and is suggested to have evolved from a dynamin-related protein that is involved in eukaryotic cytokinesis (Miyagishima et al., 2008). PDV1 and PDV2 are specific to land plants (Miyagishima et al., 2006; Glynn et al., 2008).

Chloroplast division in land plants is initiated by stromal FtsZ ring formation at the division site (containing FtsZ1 and FtsZ2, which arose by genetic duplication after the cyanobacterial endosymbiosis) (Vitha et al., 2001; Kuroiwa et al., 2002), which is stabilized by the inner envelope spanning protein ARC6 (Vitha et al., 2003). Then, the outer envelope–spanning proteins PDV1 and PDV2, which are paralogs of each other (Miyagishima et al., 2006), are recruited to the division site through direct interaction.
between PDV2 and ARC6 (Glynn et al., 2008). In addition, a recent study showed that the recruitment of PDV1 is mediated by PARC6 (paralog of ARC6 unique to vascular plants; Glynn et al., 2009). Finally, the dynamin-related protein DRP5B is recruited by PDV1 and PDV2 (Miyagishima et al., 2006; Glynn et al., 2008), and the entire division complex is involved in the fission of the chloroplast at the division site (Figure 1A).

In algae and meristematic cells in land plants, chloroplast (or plastid) division keeps pace with cell division to ensure their passage to daughter cells during cell division. By contrast, cells of land plants regulate the chloroplast division rate in accord with cell differentiation, thereby controlling the distinct size and number of chloroplasts (Possingham and Lawrence, 1983; Boffey and Lloyd, 1988). For example, small developing chloroplasts in young emerged leaves actively divide while the division rate slows down as leaves and chloroplasts mature (Boffey and Lloyd, 1988). Although several components that drive chloroplast division have been characterized, little is known about how the division machinery is controlled so as to modulate the rate of division in land plants.

Here, we report that the level of PDV proteins, land plant–specific components of the division apparatus, determines the rate of chloroplast division in the angiosperm Arabidopsis thaliana and the moss Physcomitrella patens. The analyses also show that the PDV level is upregulated by the plant hormone cytokinin. Our results suggest that acquisition of PDV proteins by the common ancestor of land plants has linked the cell differentiation program and chloroplast division and has enabled land plant cells to change the size and number of chloroplasts based on cell differentiation.

RESULTS

Artificial Increase or Decrease of the PDV1 and PDV2 Levels Gives Rise to an Increase or Decrease in the Chloroplast Division Rate in Arabidopsis

To identify the factors that modulate the rate of chloroplast division, we searched for genes that accelerate chloroplast division. We found that the expression levels of PDV1 and PDV2 are upregulated by the plant hormone cytokinin. Our results suggest that acquisition of PDV proteins by the common ancestor of land plants has linked the cell differentiation program and chloroplast division and has enabled land plant cells to change the size and number of chloroplasts based on cell differentiation.

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division when the genes are overexpressed. To this end, we screened ~10,000 independent lines of full-length cDNA-overexpressing (FOX) system of Arabidopsis (Ichikawa et al., 2006). In each FOX line, a full-length cDNA is expressed under the cauliflower mosaic virus 3SS promoter (Ichikawa et al., 2006). As a result, we isolated six independent lines that grow normally and contain a larger number of smaller chloroplasts in the expanded leaves than the wild type. Of these, five lines contained a 3SS promoter-PDV2 transgene, and the immunoblot analysis using anti-PDV2 antibodies showed that PDV2 protein was overexpressed in the line (see Supplemental Figure 1 online; the absence of the band in the pdv2/pdv2 null mutant in Figure 1D indicate that the antibodies are specific to PDV2). To further confirm whether the phenotype is linked to the overexpression of PDV2, we prepared a PDV2 overexpresser by a newly constructed 3SS-PDV2 transgene. An immunoblot analysis showed that PDV2 protein is overexpressed in the line compared with the wild type (Figure 1D) and the plant displayed the same phenotype as the isolated FOX lines (Figures 1B to 1D). These results indicate that PDV2 overexpression accelerates chloroplast division.

Because previous studies showed that PDV2 mediates the recruitment of DRP5B to the chloroplast division site together with PDV1, a protein paralogous to PDV2 (Miyagishima et al., 2006), we also examined the effect of PDV1 overexpression by the 3SS promoter on chloroplast division. Leaf cells of the PDV1 overexpresser contained a larger number of smaller chloroplasts than the wild type (Figures 1B to 1D). Although the effect of PDV1 overexpression was less evident than that of PDV2, the difference between the wild type and the PDV1 overexpresser was still significant (t test, one-tailed, P < 0.01; Figure 1C). When both PDV1 and PDV2 were simultaneously overexpressed, the number of chloroplasts further increased and the size was reduced compared with the single gene overexpressers (Figures 1B to 1D). These results indicate that PDV1 and PDV2 independently accelerate chloroplast division when the genes are overexpressed.

It was previously reported that the pdv2/PDV2 heterozygous T-DNA insertional mutant contains a smaller number of larger chloroplasts than the wild type (Miyagishima et al., 2006). The phenotype of pdv2/PDV2 is intermediate between those of the wild-type and pdv2/pdv2 plants (Miyagishima et al., 2006) (Figures 1B to 1D). By immunoblot analysis, we confirmed that PDV2 is absent in pdv2/pdv2 and found that the PDV2 level is reduced in pdv2/PDV2 compared with the wild type (Figure 1D). Taken together, the above results indicate that the levels of PDV1 and PDV2 positively correlate with the rate of chloroplast division. By contrast, overexpression of DRP5B had no effect on chloroplast division (see Supplemental Figure 1 online). Previous studies showed that overexpression of FtsZ1, FtsZ2 (Stokes et al., 2000), or ARC6 (Vitha et al., 2003) in addition to some other proteins related to the division machinery impairs chloroplast division (Colletti et al., 2000; Itoh et al., 2001; Raynaud et al., 2004).

Figure 2. The PDV2 Level, but Not the Other Chloroplast Division Protein Levels, Decreases in Accord with the Increase of Chloroplast Size during Leaf Development.

(A) Change of chloroplast size during leaf development. Chloroplasts in a young emerging leaf (1) and expanding leaves (2 to 4) of the wild-type plant were observed by Nomarski optics. Chloroplasts are observably still dividing in the first true leaf (4, indicated by arrow). Bars = 2 mm (left) and 5 μm (right, panels 1 to 4).

(B) Immunoblot analyses showing the levels of chloroplast division proteins during leaf development. PDV2 and FtsZ2-1 are detected by anti-PDV2 and anti-FtsZ2-1 antibodies, respectively. DRP5B and ARC6 levels were analyzed by anti-GFP antibodies using wild-type plants expressing GFP-DRP5B and ARC6-GFP by their respective promoters. The same amount of protein extracted from leaves corresponding to stages 1 to 4 (indicated in [A], a sample of stage 1 including both the young emerging leaves and shoot apexes) was loaded in each lane. Three biological replicates showed the same result.

(C) Histochemical GUS staining of PDV2 promoter-GUS, FtsZ2-1 promoter-GUS, and DRP5B promoter-GUS transgenic plants. Approximately three-week-old plants grown on agar plates were stained. Magnified images of the centers of the rosettes are also shown (insets). ARC6 promoter-GUS transgenic plants were also prepared, but we could not obtain a staining signal. Three independent transgenic lines for each promoter showed the same results. Bar = 3 mm.
resulting in giant chloroplasts. The acceleration of chloroplast division has not been observed in the overexpression or disruption of genes that are reported to be involved in the division process. Taken together, it is suggested that levels of PDV1 and PDV2 determine the rate of chloroplast division.

**PDV Levels, but Not Those of Other Division Components, Decrease during Leaf Development along with a Decrease in the Chloroplast Division Rate**

Since these changes in the PDV levels were caused by the expression of transgenes or by mutations, next we examined whether the levels of PDV1 and PDV2 translated from endogenous genes actually change so as to modulate the chloroplast division rate in the wild-type plant. To this end, we examined the levels of PDV and other chloroplast division proteins during leaf development. During leaf development, the frequency of chloroplasts with a division site constriction decreases, while the size of chloroplasts increases as the leaf gets older (Possingham and Lawrence, 1983; Boffey and Lloyd, 1988; Pyke, 1999) (Figure 2A).

Immunoblot analyses showed that the PDV2 level is highest in the apical meristem and young emerging leaves and decreases during leaf development (Figure 2B). By contrast, the DRP5B level increased, but the FtsZ2-1 (the antibodies are specific to FtsZ2-1 of three FtsZ proteins of Arabidopsis; Suzuki et al., 2009), and ARC6 levels remained constant during leaf development (Figure 2B). Promoter-β-glucuronidase (GUS) fusion assays also showed that the activity of the PDV2 promoter is highest around the shoot apical meristem, in contrast with the FtsZ and DRP5B promoters (Figure 2C). These results indicate that the level of PDV2, but not that of FtsZ, ARC6, and DRP5B, decreases during leaf development.

To compare the levels of the chloroplast division proteins at the chloroplast division site, green fluorescent protein (GFP) fusion proteins were expressed by their respective promoters and observed by fluorescence microscopy. Consistent with the results of the immunoblot and GUS analyses, strong fluorescence signals of GFP-PDV1 and GFP-PDV2 at the chloroplast division site were clearly observed in young, emerging leaves, but the signals were hardly detected in older, expanding leaves (Figure 3). By contrast, the FtsZ-GFP and ARC6-GFP signals at the division site were observed in both young and older leaves (Figure 3). Strong GFP-DRP5B signals at the division site were observed in older leaves but hardly at all in young emerging leaves (Figure 3). Despite the absence of signals strong enough to allow the observation of GFP-PDV1 and GFP-PDV2 in expanding leaves, or GFP-DRP5B in young emerging leaves (Figure 3), we detected the signals of these GFP fusion proteins at the chloroplast division site throughout leaf development when the proteins were overexpressed (Figure 3, bottom panels). Therefore, the barely detectable GFP signals by fluorescence microscopy (Figure 3) and faint but still detectable signals by immunoblot analyses (Figure 2B) are probably indicative of low levels of the proteins in the division apparatus, instead of the absence of these proteins.

The above results indicate that the PDV levels in the chloroplast division apparatus, but not FtsZ, ARC6, or DRP5B,
decrease during leaf development, suggesting that the stoichiometric relationship between the components of the division apparatus changes during chloroplast development. Given the results of overexpression and the reduction of PDV levels (Figure 1), it is suggested that the decrease of PDV levels during leaf development in turn decreases the rate of chloroplast division, thereby increasing the size of chloroplasts. Supporting this conclusion, the size and number of chloroplasts in young emerging leaves are similar in the wild type and the PDV1 and PDV2 overexpressers (Figure 4), but constant overexpression depresses the chloroplast increase in size in the older, expanding leaves (Figure 4).

Overexpression of Cytokinin-Responsive Transcription Factor CRF2 or Exogenous Cytokinin Treatment Increases PDV2 Level, but Not Those of Other Division Proteins, in Parallel with an Increase in the Chloroplast Division Rate

Further supporting the relationship between cell differentiation and change in the chloroplast division rate, another FOX line, in which chloroplast division is accelerated, turned out to be an overexpresser of CRF2 (Rashotte et al., 2006) (see Supplemental Figure 2 online). Transgenic plants overexpressing CRF2 as the result of a newly constructed 35S-CRF2 transgene displayed a similar phenotype (Figures 5A and 5C), indicating that the phenotype is indeed caused by CRF2 overexpression. Immunoblot analyses showed that the PDV2 level, but not the level of FtsZ, DRP5B, or ARC6, was increased in the CRF2 overexpresser (Figure 5D), suggesting that the increase of the PDV2 level is the cause of the acceleration of chloroplast division in the CRF2 overexpresser.

CRF2 is a putative transcription factor that, together with other paralogous CRF proteins, mediates transcriptional responses to the plant hormone cytokinin (Rashotte et al., 2006). In land plants, cytokinin regulates numerous growth and developmental processes, including cell division, shoot initiation, and apical...

![Figure 4. Effect of PDV1 and PDV2 Overexpression on the Young Emerging Leaves and Expanding Leaves.](image-url)

Chloroplasts of the wild type and PDV1 and PDV2 overexpressers in emerging and expanding leaves corresponding to stages 1 and 4 in Figure 2 A. Bar = 5 μm.

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![Figure 5. CRF2 Overexpression or Cytokinin Treatment Increases the PDV2 Level and Increases the Number and Decreases the Size of Chloroplasts.](image-url)

(A) Chloroplasts of wild-type and transgenic plants overexpressing CRF2 (35S-CRF2) in single leaf mesophyll cells. Tips of the first true leaves were cut from ~3-week-old plants grown on agar plates. Bar = 20 μm.

(B) Effect of cytokinin treatment on the size and number of chloroplasts. Chloroplasts in single mesophyll cells of the cotyledon are shown. Wild-type seeds were germinated and grown for 10 d on agar plates with (+BA) or without (−BA) 5 μM BA. Bar = 20 μm.

(C) RT-PCR analyses showing the CRF2 transcript was increased in 35S-CRF2 transgenic plants. Total RNA extracted from rosettes was used for RT-PCR. UBQ1 was used as the internal control. The number of PCR cycles was 28 for CRF2 and 26 for UBQ1. The signals were estimated by ethidium bromide staining. Two biological replicates showed the same result.

(D) Immunoblot analyses comparing the levels of the chloroplast division proteins between the wild type and CRF2 overexpresser. The same amount of total protein extracted from ~3-week-old russets was loaded in each lane. Rubisco large subunit stained by Ponceau S is shown as the loading control. Three biological replicates showed the same result.

(E) Immunoblot analyses comparing the levels of the chloroplast division proteins between wild-type seedlings germinated on medium with (+BA) or without (−BA) BA. The same amount of total protein extracted from 10-d-old seedlings was loaded in each lane. PDV2, FtsZ2-1, GFP-DRP5B, and ARC6-GFP were detected as in Figure 2B. Three biological replicates showed the same result.

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meristem function (Kieber, 2002; Kakimoto, 2003). Therefore, the above results suggest that the PDV2 level is regulated by a developmental program responsive to cytokinin. To examine the relationship between PDV levels and cytokinin, wild-type seeds were germinated on medium containing cytokinin (6-benzyladenine [BA]). We then compared the size and number of chloroplasts and the PDV2 level between plants treated or untreated with cytokinin. Chloroplasts in the cotyledon cells of cytokinin-treated plants were smaller and more numerous than those grown without cytokinin (Figure 5B). The same pattern of difference was observed when detached true leaves were put on the medium with or without cytokinin. Immunoblot analyses of total plantlets showed that the PDV2 level, but not the level of FtsZ, DRP5B, or ARC6, was increased in plants germinated on the cytokinin-containing medium (Figure 5E). These results suggest that cytokinin, or a developmental program induced by cytokinin, upregulates PDV2, at least in part through an upregulation of CRF2, which leads to an increase in the chloroplast division rate.

The PDV Protein Level Determines the Rate of Chloroplast Division in the Moss

The results obtained in Arabidopsis suggest that PDV1 and PDV2 determine the rate of chloroplast division, at least in the angiosperms. Homologs of PDV1 and PDV2 are encoded in the genomes of other lineages, but are only evident in land plants, suggesting that the genes for these proteins were acquired by the ancestral plant during the transition to terrestrial habitats (Miyagishima et al., 2006; Glynn et al., 2008) (Figure 6A; see Supplemental Figure 3 online). To obtain evolutionary insights into the function of PDV proteins, we examined whether PDV proteins also rate-determine chloroplast division in mosses, mosses having branched earliest in land plant evolution (Kenrick and Crane, 1997). To this end, we used the moss P. patens since the draft genome sequence is available and earlier studies showed involvement of FtsZ in chloroplast division by gene disruption in this organism (Strepp et al., 1998).

By BLAST and PSI-BLAST (Altschul et al., 1997) searches, we identified four P. patens genes encoding proteins homologous to angiosperm PDV1 and PDV2 (Figure 6A; see Supplemental Figure 3 online). Phylogenetic analyses revealed the conservation of PDV1 and PDV2 in angiosperms (Figure 6A). By contrast, the...
PDVs Determine Chloroplast Division Rate

Recent studies have reported several components of the plastid division apparatus. Of these components, FtsZ and ARC6, which function inside chloroplasts, are descended from a cyanobacterial ancestor, and cytosolic DRP5B evolved from an eukaryotic membrane fission system (Figure 1A). These three components are well conserved in green algae and land plants (Miyagishima, 2005; Maple and Moller, 2007; Yang et al., 2008) (Figure 7). DRP5B is also conserved in red algae and stramenopiles, the chloroplast in which was established by secondary endosymbiosis of a red algae (Yoon et al., 2002; Reyes-Prieto et al., 2007; Gould et al., 2008), suggesting that DRP5B became integrated in the division apparatus prior to the branching of the green and red lineages of photosynthetic eukaryotes ~1 billion years ago (Miyagishima, 2005). Given the conservation of FtsZ, ARC6, and DRP5B, it is suggested that the division apparatus consisting of these three components was established at a relatively early point in chloroplast evolution. By contrast, PDV proteins, connecting the bacterial and eukaryotic division components, are specific to land plants, suggesting that they became integrated into the division apparatus when land plants evolved from green algae (Miyagishima et al., 2006; Glynn et al., 2008) (Figures 6A and 7), which is estimated to be ~0.4 to 0.5 billion years ago (Kenrick and Crane, 1997).

Our results show that increases or decreases of the PDV levels increase or decrease the rate of chloroplast division (Figures 1B to 1D), although changes in the levels of the other division components either impair division or have no effect. The levels of PDV translated from the nuclear genome, but not those of other division components, actually decreased during leaf development, in parallel with the decrease of the division rate (Figure 2B). Cytokin treatment or CRF2 overexpression increases the PDV levels, but not those of the other division components, in parallel with the increase in the division rate (Figure 5). These results suggest that PDV proteins determine the rate of chloroplast division based on the cell differentiation system that evolved in the ancestor of land plants. Given the evolutionary distribution of the chloroplast division proteins and the conservation of PDV function as the rate-determining factor of chloroplast division in the moss P. patens (Figure 6), it is suggested that the integration of PDV proteins into the division machinery enabled land plant cells to change chloroplast size and number in accord with the fate of cell differentiation (Figure 7).

The PDV2 level was increased by cytokinin treatment in both Arabidopsis (Figure 5E) and P. patens (Figure 6C). CRF2 overexpression increased the PDV2 level; this suggests that induction of PDV2 is in a pathway downstream of CRF. Cytokinin accelerated leaf chloroplast division in Arabidopsis (Figure 5), whereas the size and number of chloroplasts in the moss protonema were unchanged by cytokinin treatment (Figure 6). Instead, cytokinin induced the formation of buds containing...
numerous smaller chloroplasts (Figure 6). Although the reason for the difference between Arabidopsis and P. patens is unclear at present, our results indicate that the PDV levels are regulated by a cell differentiation system responsive to cytokinin. The highest expression of PDV proteins around the shoot apical meristem and young emerging leaves in Arabidopsis (Figure 2) is consistent with a previous observation that content of cytokinin is highest in meristems (Jacqmard et al., 2002). The induction of PDV expression by cytokinin might be related to the observation that cytokinin addition or expression of the ipt gene, whose product catalyzes the rate-limiting step in cytokinin synthesis, complements a P. patens mutant defective in chloroplast division (Reski et al., 1991; Reutter et al., 1998).

In P. patens, a previous study showed that slightly enhanced levels of FtsZ by expression of transgenes seem to accelerate chloroplast division, although strong overexpression of FtsZ impaired the division as observed in Arabidopsis (Kiessling et al., 2000). However, it is still not known whether FtsZ proteins translated from endogenous genes are actually upregulated to increase the rate of chloroplast division in some points of the P. patens life cycle. Since all the four Pp PDV2 genes, but not the all the five Pp FtsZ genes, are upregulated by cytokinin treatment (Figure 6; see Supplemental Figure 4 online), increase of FtsZ is not likely to be involved in the acceleration of chloroplast division during bud formation.

In vascular plants, all plastids, including chloroplasts, are derived from small, non-green proplastids in meristematic cells (Pyke, 1999; Lopez-Juez and Pyke, 2005). The decrease of PDV levels during leaf development (Figure 2) and the highest promoter activity of PDV2 around the shoot apical meristem (Figure 2C) appear to correlate with the development of leaf chloroplasts from meristematic proplastids in Arabidopsis. However, the overexpression of PDV proteins increased the division rate of photosynthetic chloroplasts (Figure 1). In addition, our results suggest that PDV proteins also rate-determine chloroplast division in the mosses (Figure 6), which contain chloroplasts throughout the life cycle, as do algae (Boffey and Lloyd, 1988). Therefore, PDV proteins were probably acquired to modulate photosynthetic chloroplast division prior to the evolutionary emergence of the differentiation system based on the proplastids. It is also suggested that the modulatin of chloroplast division rate by PDV proteins might be a critical step for the evolution of the plastid differentiation system in vascular plants.

The change in the cellular levels of PDV proteins gives rise to two possibilities. One is that the ratio of chloroplasts in which the PDV proteins are integrated changes. The other is that the amount of PDV proteins in the division complex, that is, the stoichiometrical relationship of PDV proteins to other division components changes. Almost every chloroplast in expanding leaves exhibited FtsZ and ARC6 localization at the division site, and the fluorescent signals of these proteins at the division site appeared to be constant during leaf development (Figure 3). By contrast, the fluorescent signals of GFP-PDV1 and GFP-PDV2 were hardly detected in any chloroplast in expanding leaves in which the chloroplasts were still dividing (Figure 3). These results suggest that the state of the chloroplast division complex changes as a result of the change in the PDV levels in each division complex. Chloroplasts in the pdv1 and pdv2 mutants reportedly frequently display constriction at the division site (Miyagishima et al., 2006; Glynn et al., 2008) (Figure 1; see Supplemental Figure 1 online), suggesting that constriction is delayed in the mutants and that the levels of PDV proteins likely determine the rate of division site constriction. To understand how the division machinery is affected by the change in the PDV levels, further structural and biochemical studies are required. In this regard, the phenotypes of the pdv1 and pdv2 mutants indicated that PDV1 and PDV2 are required for cytosolic DRP5B localization after stromal FtsZ and inner envelope ARC6 assembly at the division site (Miyagishima et al., 2006; Glynn et al., 2008). However, the exact biochemical relationship between PDV proteins and DRP5B is not yet clear (i.e., whether there is direct interaction between PDV proteins and DRP5B or a requirement of other intermediate components), identification and characterization of cytosolic proteins that directly interact with PDV proteins will provide further insights into that regulation of the chloroplast division rate by PDV proteins.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana (Columbia-0) was used as the wild type. Arabidopsis seeds were surface-sterilized, sown on Murashige and Skoog (MS) plates, and stratified at 4°C for 48 h in the dark before germination. Plants were grown in controlled-environment chambers with 16 h of light (100 μmol/m²s) and 8 h of dark at 20°C. For cytokinin treatment, sterilized seeds were sown on MS plates supplemented with 5 μM BA and grown for 10 d after germination. The pdv1 and pdv2 mutants used in this study are pdv1-1 (has a premature stop codon close to the start codon) and pdv2-1 (T-DNA is inserted into the first intron), respectively (Miyagishima et al., 2006). Physcomitrella patens Bruch and Schimp subsp patens Tan was grown in controlled-environment chambers with 16 h of light (100 μmol/m²s) and 8 h of dark at 20°C on the minimal medium (BCD medium) supplemented with 0.5% glucose, 1 mM CaCl₂, and 5 mM diammonium (+)-tartrate agar plate as described (Nishiyama et al., 2000). For cytokinin treatment, protonema grown on BCD medium were transferred and then grown on BCD medium containing 5 μM BA for 4 d.

Analyses of Arabidopsis FOX Lines

Seeds of ~10,000 independent FOX lines (Ichikawa et al., 2006) (Columbia-0 background) were separated to 200 pools, in which ~50 independent lines were mixed in each pool. One hundred seeds from each pool were germinated and grown for 3 weeks on MS plates. Tips from expanding leaves were put on a glass slide without fixation, covered with a cover slip, and were observed with Nomarski optics. As a result, leaf cells of 13 plants contained a larger number of smaller chloroplasts than the wild type. Among them, seven lines also showed dwarf phenotypes, while growth and shape of plantlets were normal in six lines. The inserted cDNA of the six lines was amplified by T-DNA–specific primers FOX.F and FOX.R and sequenced by primer FOX-seq.

Constructing Overexpression and Fusion Constructs and Generating Transgenic Arabidopsis

For overexpression of PDV1, PDV2, and CRF2, fragments containing respective open reading frames (orf) flanked by ~0.1 kb 5‘ upstream sequences were amplified by primers: PDV1-ox.F and PDV1-ox.R for PDV1, PDV2-ox.F and PDV2-ox.R for PDV2, and CRF2-ox.F and CRF2-ox.R
R for CRF2. The amplified PDV2 and CRF2 fragments were digested with XbaI and Smal, and the PDV1 fragment was digested with NheI and Smal (the recognition sequences of these enzymes are underlined in the sequences of respective primers). The digested products were inserted between XbaI and blunting SacI sites (downstream of cauliflower mosaic virus 35S promoter) of pBI121 vector (confering resistance to kanamycin; Clontech).

For overexpression of GFP-DRP5B, DRP5B promoter of DRP5B promoter-GFP-DRP5B construct (Miyagishima et al., 2006) was replaced by 35S promoter amplified from pBI121 vector. The generated constructs were transferred into the wild type.

For expression of GFP-PDV2 by the PDV2 promoter, two unique restriction sites were added between the PDV2 promoter and the start codon by overlap-extension PCR. We amplified a 1.2-kb 5′ upstream sequence of PDV2, including the start codon, by primers GFP-PDV2-F1 and GFP-PDV2-R1. A PDV2 orf flanked by a 60-bp 3′ downstream sequence was amplified by primers GFP-PDV2-F2 and GFP-PDV2-R2. These two amplified fragments were mixed and fused by PCR using primers GFP-PDV2-F1 and GFP-PDV2-R2. The fused fragment was cloned into pGEM-T Easy (Promega). An orf of GFP was amplified by primers 5′-GFP-BamHI.F and GFP-KpnI.R, digested with BamHI and KpnI, and cloned between a 5′ flanking region and an orf of PDV2. The resulting PDV2 promoter-GFP-PDV2 fusion was excised with NotI and then transferred into pMLBART (conferring resistance to glufosinate ammonium; Vitha et al., 2001). The construct was transformed into the wild type.

For expression of GFP-PDV1 by the 35S promoter, GFP-PDV1 fusion (Miyagishima et al., 2006) was cut out with BamHI and EcoRI and inserted into BamHI and EcoRI sites of pBI121. For expression of GFP-PDV2 by the 35S promoter, GFP-PDV2 was amplified using the PDV2 promoter-GFP-PDV2 fusion described above as template by primers PDV2-ox-F and PDV2-ox-R. The amplified fragment was digested with XbaI and Smal and inserted into XbaI and blunting SacI sites of pBI121. The generated construct was transformed into the wild type.

For expression of ARC6-GFP by the ARC6 promoter, ARC6 orf was cloned into pGEM-T Easy (Promega). An orf of GFP was amplified by primers 5′-GFP-BamHI.F and GFP-KpnI.R, digested with BamHI and KpnI, and cloned between a 5′ flanking region and an orf of PDV2. The resulting ARC6 promoter-ARC6-GFP fusion was excised with NotI and then transferred into pMLBART. The generated construct was transformed into the wild type.

For expression of ARC6-GFP by the ARC6 promoter, ARC6 orf was cloned between the start codon, amplified by primers ARC6-GFP.F and ARC6-GFP.R and cloned into pGEM-T Easy (Promega). An orf of GFP was amplified by primers 5′-GFP-BamHI.F and GFP-KpnI.R, digested with BamHI and KpnI, and cloned between a 5′ flanking region and an orf of PDV2. The resulting ARC6 promoter-ARC6-GFP fusion was excised with NotI and then transferred into pMLBART. The generated construct was transformed into the wild type.

For expression of FtsZ2-1 promoter, FtsZ2-1-GUS promote, FtsZ2-1-GUS-F and FtsZ2-1-GUS.R for FtsZ2-1, FtsZ2-1-GUS.F and FtsZ2-1-GUS.R for FtsZ2-1, and FtsZ2-1-GUS.F and FtsZ2-1-GUS.R for FtsZ2-1 were cloned into pGEM-T Easy (Promega). An orf of GFP was amplified by primers 5′-GFP-BamHI.F and GFP-KpnI.R, digested with BamHI and KpnI, and cloned between a 5′ flanking region and an orf of PDV2. The resulting FtsZ2-1 promoter-FtsZ2-1-GFP fusion was excised with NotI and then transferred into pMLBART. The generated construct was transformed into the wild type.

For expression of FtsZ2-1 promoter, FtsZ2-1-GUS promote, FtsZ2-1-GUS-F and FtsZ2-1-GUS.R for FtsZ2-1, FtsZ2-1-GUS.F and FtsZ2-1-GUS.R for FtsZ2-1, and FtsZ2-1-GUS.F and FtsZ2-1-GUS.R for FtsZ2-1 were cloned into pGEM-T Easy (Promega). An orf of GFP was amplified by primers 5′-GFP-BamHI.F and GFP-KpnI.R, digested with BamHI and KpnI, and cloned between a 5′ flanking region and an orf of PDV2. The resulting FtsZ2-1 promoter-FtsZ2-1-GFP fusion was excised with NotI and then transferred into pMLBART. The generated construct was transformed into the wild type.

For expression of CRF2 promoter, CRF2 orf was amplified between the start codon and the stop codon by overlap-extension PCR. A CRF2 orf flanked by a 60-bp 3′ downstream sequence was amplified by primers CRF2-PDV2-F2 and CRF2-PDV2-R2. These two amplified fragments were mixed and fused by PCR using primers CRF2-PDV2-F1 and CRF2-PDV2-R2. The fused fragment was cloned into pGEM-T Easy (Promega). An orf of GFP was amplified by primers 5′-GFP-BamHI.F and GFP-KpnI.R, digested with BamHI and KpnI, and cloned between a 5′ flanking region and an orf of PDV2. The resulting CRF2 promoter-CRF2-GFP fusion was excised with NotI and then transferred into pMLBART. The generated construct was transformed into the wild type.

For expression of CRF2 promoter, CRF2 orf was amplified between the start codon and the stop codon by overlap-extension PCR. A CRF2 orf flanked by a 60-bp 3′ downstream sequence was amplified by primers CRF2-PDV2-F2 and CRF2-PDV2-R2. These two amplified fragments were mixed and fused by PCR using primers CRF2-PDV2-F1 and CRF2-PDV2-R2. The fused fragment was cloned into pGEM-T Easy (Promega). An orf of GFP was amplified by primers 5′-GFP-BamHI.F and GFP-KpnI.R, digested with BamHI and KpnI, and cloned between a 5′ flanking region and an orf of PDV2. The resulting CRF2 promoter-CRF2-GFP fusion was excised with NotI and then transferred into pMLBART. The generated construct was transformed into the wild type.

For expression of CRF2 promoter, CRF2 orf was amplified between the start codon and the stop codon by overlap-extension PCR. A CRF2 orf flanked by a 60-bp 3′ downstream sequence was amplified by primers CRF2-PDV2-F2 and CRF2-PDV2-R2. These two amplified fragments were mixed and fused by PCR using primers CRF2-PDV2-F1 and CRF2-PDV2-R2. The fused fragment was cloned into pGEM-T Easy (Promega). An orf of GFP was amplified by primers 5′-GFP-BamHI.F and GFP-KpnI.R, digested with BamHI and KpnI, and cloned between a 5′ flanking region and an orf of PDV2. The resulting CRF2 promoter-CRF2-GFP fusion was excised with NotI and then transferred into pMLBART. The generated construct was transformed into the wild type.

GFP fluorescence was visualized without fixation in young and expanding leaves from ~3-week-old plants.

Antibodies and Immunoblotting

The polyclonal rabbit antibodies against Arabidopsis PDV2 were raised against recombinant PDV2. The full-length PDV2 coding region was amplified from Arabidopsis cDNA by primers His-PDV2-F and His-PDV2-R and was cloned into pET100 expression vector (Invitrogen). Six-His fusion proteins were expressed in Escherichia coli (Rosetta 2 DE3; Novagen), purified, and injected to rabbits to stimulate antibody production. The antisera were purified by N-hydroxysuccinimide–activated column (HTrap NHS-activated HP; GE Healthcare) conjugated with the recombinant PDV2 according to the manufacturer’s protocol.

Approximately three-week-old plants grown on MS plates were frozen in liquid nitrogen, ground with pestles, and homogenized in extraction buffer (50 mM Tris, pH 7.5, 2 mM MgCl2, 5 mM EDTA, and a protease inhibitor mixture [P2714; Sigma]). The homogenate was filtered through Miracloth (Calbiochem). Protein concentration of the homogenate was determined and then subjected to immunoblotting. To detect proteins during leaf development, leaves were separately collected in order of size and extracted. Samples containing 50 μg of proteins were subjected to immunoblot analyses.

Immunoblotting assays were performed as previously described (Nakanishi et al., 2009). Anti-PDV2 antibodies, anti-FtsZ2-1 antibodies (Nakanishi et al., 2009), and anti-GFP mouse monoclonal antibody (JL-8; Invitrogen) were diluted 1:20,000, 1:10,000, and 1:1000, respectively. The primary antibody was detected by horseradish peroxidase–conjugated goat anti-mouse or anti-rabbit antibody diluted at 1:20,000. The signal was detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and the VersaDoc 5000 imaging system (Bio-Rad). We confirmed that the signal was not saturated by comparison of the images from longer and shorter exposure times.

Analyses of Gene Expression by RT-PCR and GUS Staining

Total RNA of Arabidopsis was extracted from ~3-week-old plants using an RNeasy mini kit (Qiagen). Total RNA of P. patens was extracted from

GUS expression analyses were performed as described (Jefferson et al., 1987) with some modifications. Arabidopsis grown on MS plates were soaked in the GUS assay solution [0.5 mg/ml 5-bromo-4-chloro-3-indolylglucuronide, 0.5 mM K3Fe(CN)6, 0.5 mM K4Fe(CN)6, 0.1% (v/v) Triton X-100, and 100 mM Pi-buffered saline] and incubated at 37°C overnight for PDV2-GUS and for 3 h for the others. Then the samples were washed by 70% ethanol and soaked in ethanol and acetic acid mixture (6:1, by volumes) to stop the reaction and remove chlorophylls.

Phylogenetic Analyses

Deduced amino acid sequences of PDV1 and PDV2 homologs encoded by the 13 genes (GI numbers are indicated in Figure 6A) were collected by BLAST searches. The sequences were aligned by Clustal X 2.0 (Larkin et al., 2007) and manually refined, and 144 amino acid residues were used for the phylogenetic analyses. Maximum likelihood trees were constructed using RaxML 7.0.4 (Stamatakis, 2006) with 100 replicates using the WAG matrix of amino acid replacements assuming a proportion of invariant sites and four γ-distributed rates (WAG+I+gamma model). Bayesian inference was performed with the program MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003) using the WAG+I+gamma model. For the MrBayes consensus trees, 1,000,000 generations were completed with trees collected every 100 generations.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Phenotypes of the PDV2 Overexpresser Isolated from Full-Length cDNA Overexpression (FOX) Lines, the DRP5B Overexpresser, pdv1/pDV1, pdv1/pdv1, and pdv1/pdv1 pdv2/pdv2.

Supplemental Figure 2. Phenotypes of the CRF2 Overexpresser Isolated from FOX Lines.

Supplemental Figure 3. Amino Acid Sequence Alignment of the PDV Family of Proteins.

Supplemental Figure 4. Effect of Cytokinin Treatment on Expression of Pp FtsZ and Pp PDV2 Genes.

Supplemental Table 1. Primers Used in This Study.

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Author Contributions

K.O. and Y.K. contributed equally to experiments of PDV and cytokinin and wrote the draft of the manuscript. H.N., T.I., M.M., and S.M. designed the screening of Arabidopsis FOX lines, and H.N. and K.S. screened the mutants and identified inserted cDNA by supervision of T.I. and M.M. S.M. supervised the whole project with help from T.M., M.M., and T.I. S.M. and T.M. prepared the final version of the manuscript.

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The PLASTID DIVISION1 and 2 Components of the Chloroplast Division Machinery Determine the Rate of Chloroplast Division in Land Plant Cell Differentiation

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