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PEROXISOME DIVISION IN ARABIDOPSIS THALIANA

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PEROXISOME DIVISION IN *ARABIDOPSIS THALIANA*

By

Xinchun Zhang

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ABSTRACT

PEROXISOME DIVISION IN *ARABIDOPSIS THALIANA*

By

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Peroxisomes are versatile, single-membrane bound organelles with diverse functions in eukaryotes. Their division is controlled by at least three types of proteins, PEROXIN11 (PEX11), FISSION1 (FIS1) and Dynamin-Related Proteins (DRPs), in yeast and humans. The five PEX11 proteins promote peroxisome elongation, which initiates peroxisome division, while DRP3A plays a role in peroxisome fission, the late step of peroxisome division in *Arabidopsis thaliana*. To further determine the molecular architecture of peroxisome division *in planta*, we used forward and reverse genetic strategies to search for more players involved in this process. Four new components of the peroxisome division machinery in *Arabidopsis* were identified: DRP3B, DRP5B, FIS1A and FIS1B.

DRP3B is a homolog of DRP3A, and both proteins are involved in mitochondrial division. DRP3B appears as puncta marking the fission sites or potential fission sites, not only on mitochondria, but on peroxisomes. Disruption of DRP3B causes defects in peroxisome fission. *drp3A drp3B* double mutants display stronger deficiencies than each *drp3* single mutant in peroxisome abundance, seedling establishment and plant growth, suggesting that DRP3A and DRP3B are functionally redundant.

DRP5B is the only known DRP serving as a component of the chloroplast division complexes. We addressed a new role for DRP5B in peroxisome division. Subcellular localization analysis shows that DRP5B not only forms a ring on chloroplast as previously reported, but also is co-localized with peroxisomes. Mutations in the *DRP5B*

gene lead to aggregated peroxisomes with membrane constriction. Furthermore, impaired peroxisome functions caused by loss of DRP5B affect seedling establishment and plant growth in *Arabidopsis*. Taken together, DRP5B mediates peroxisome division.

FIS1A and FIS1B that are dual-targeted to peroxisomes and mitochondria function in the division of both organelles. Overexpression of each *FIS1* gene increases the abundance of both mitochondria and peroxisomes, by contrast, loss of *FIS1* results in number reduction of both organelles showing incomplete fission and enlarged size. Domain truncation studies show that the C-terminal transmembrane domain is required for FIS1 targeting to peroxisomes. Moreover, *FIS1* silencing experiments demonstrate that FIS1A and FIS1B play rate-limiting and partially overlapping roles in promoting the fission of peroxisomes and mitochondria. In summary, FIS1A and FIS1B are involved in the fission of peroxisomes and mitochondria.

Lastly, bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation (Co-IP) assays demonstrate that DRP5B interacts with itself, and also with both DRP3A and DRP3B. These physical interactions suggest that the three DRPs may assemble together to exert their functions on peroxisomal membrane fission. Additionally, FIS1 and PEX11 proteins physically interact with DRP5B, DRP3A and DRP3B *in vivo* and *in vitro*, indicating that two families of transmembrane proteins, FIS1s and PEX11s, might anchor DRPs to different organelles. In conclusion, our data support the view that PEX11, DRPs and FIS1 orthologs are common conserved proteins of the peroxisomal division apparatus across eukaryotic species, and plant-specific targeting mechanisms by which DRPs are recruited to different organelles may have been evolved.

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LIST OF ABBREVIATIONS

2,4-DB	2,4-dichlorophenoxybutyric acid
IBA	Indole 3-butyric acid
35S	Cauliflower mosaic virus 35S promoter
arc	Accumulation and replication of chloroplasts
bar	BASTA resistant
BiFC	Bimolecular fluorescence complementation
Co-IP	Co-Immunoprecipitation
Col-0	*Columbia* ecotype 0
C-terminus	Carboxy terminus
ER	Endoplasmic reticulum
DRP	Dynamin-Related Protein
DsRed	*Discosoma sp.* red fluorescent protein
EMS	Ethyl methanesulfonate
FIS1	Fission1
GFP	Green fluorescent protein
GTPase	Guanosine triphosphatase
Ler	*Landsberg erecta* ecotype
N-terminus	Amino terminus
PEX	Peroxin
PTS1	Peroxisomal targeting signal 1

PTS2	Peroxisomal targeting signal 2
YFP	Yellow fluorescent protein
UBQ	Ubiquitin

Chapter 1 Introduction - Peroxisome Division

1.1 Peroxisomes are vital organelles in eukaryotic cells

Peroxisomes are pleomorphic, single-membrane-bound organelles that have diverse metabolic and biochemical functions in eukaryotes (Titorenko and Rachubinski, 2001; Wanders, 2004; Wanders and Waterham, 2006). Their functions are often specialized by species, cell types and environmental cues. Fatty acid β-oxidation and hydrogen peroxide (H_2O_2) detoxification are two widely distributed and well-conserved functions for peroxisomes. Plant glyoxysomes that are specialized peroxisomes in germinating seeds, harbor the glyoxylate cycle (Escher and Widmer, 1997; Graham, 2008). Peroxisomes in some yeast are equipped with enzymes for methanol or amine oxidation and assimilation (Veenhuis et al., 1983; Opperdoes, 1987). Additionally, mammalian peroxisomes carry the enzymes involved in ether lipid synthesis and cholesterol synthesis (Mannaerts *et al.*, 2000; Wanders, 2000; Wierzbicki, 2007).

Plant peroxisomes are related to broad ranges of cellular metabolisms in addition to glyoxylate cycle, such as photorespiration in leaves and nitrogen metabolism in roots (Graham and Eastmond, 2002; Baker et al., 2006). Peroxisomal functions are not limited in cellular metabolisms, their roles are also defined in embryogenesis, photomorphogenesis, biosynthesis of jasmonic acid and conversion of the protoauxin indole-3-butyric acid (IBA) into active auxin, indole-3-acetic acid (IAA), as well as plant pathogen resistance (Hu et al., 2002; Weber, 2002; Fan et al., 2005; Woodward and Bartel, 2005b, a). Interestingly, a novel peroxisomal function has been just reported in peroxisome-associated matrix protein degradation (Lingard et al., 2009). Together, plant peroxisomes exert their functions in a variety of plant-specific processes.

1.2 The biogenesis of peroxisomes - Protein import into peroxisomes

In contrast to their functional heterogeneity, the biogenesis of peroxisomes follows a common pathway relying on the peroxins (PEX) encoded by *PEX* genes. PEX proteins are involved in the processes by which matrix and membrane proteins are assembled into the organelle, as well as those involved in the control of peroxisome size, volume and number. Till now, 32 Pex proteins have been found in yeast, 16 in human and 15 in plants (Kiel et al., 2006; Orth et al., 2007). Different PEXs function in various aspects of peroxisome biogenesis, including 1) protein import into peroxisomes, a process including recognition of peroxisome targeting proteins through specific receptors, docking and translocation of proteins to peroxisomal matrix and receptor recycling, 2) peroxisome *de novo* biogenesis, and 3) peroxisome division (Gould and Valle, 2000; Lazarow, 2003; Vizeacoumar *et al.*, 2003; Vizeacoumar *et al.*, 2004; Thoms and Erdmann, 2005).

Peroxisomes do not contain DNA or protein translation machinery, so all their proteins are encoded by nuclear genes and imported into preoxisomal matrix post-translationally. The import of matrix proteins into peroxisomes is a unique process, which differs substantially from the import mechanisms into the ER, mitochondria or chloroplasts. A major breakthrough in the elucidation of the mechanism of protein import into peroxisomes was the identification of the first peroxisomal targeting signal (PTS1) at the C-terminus of luciferase of the firefly *Photinus pyralis* (Gould et al., 1987; Gould et al., 1989). The majority of the peroxisomal matrix proteins contain a C-terminal PTS1, and some have an N-terminal PTS2. The PTS1- or PTS2-containing matrix proteins are recognized by soluble receptors, PTS1 by PEX5 (PEX5 cargo), and PTS2 by PEX7

(PEX7 cargo) in the cytosol, which guide them to a docking site at the peroxisomal membrane (Baker and Sparkes, 2005). *Arabidopsis* PEX5 and PEX7 interact with each other, and silencing experiments of *PEX5* and *PEX7* show that PEX7 is required for PTS2-protein import, whereas reducing PEX5 affects both PTS1- and PTS2-protein import (Nito *et al.*, 2002; Baker and Sparkes, 2005). In our research, fluorescence proteins tagged with PTS1 are used to mark peroxisomes in plant cells.

The PTS1 receptor PEX5 loaded with matrix proteins can associate with some peroxisomal membrane proteins, referred to as docking and translocation machinery. Yeast Pex13, Pex14 and Pex17 are in charge of docking Pex5/Pex7-cargo at the membrane of peroxisomes (Eckert and Erdmann, 2003). In *Arabidopsis*, PEX13 and PEX14 control intracellular transport of both PTS1 and PTS2 containing proteins into three different types of peroxisomes, and silencing of *PEX14* causes defects in peroxisome morphology, seed germination and photorespiration (Hayashi et al., 2000; Nito et al., 2007). In yeast, three RING domain-containing peroxins, Pex2, Pex10 and Pex12, help the translocation of the receptor cargo complex loaded with matrix proteins into peroxisome matrix, however, the mechanism of translocation of folded proteins across the membrane and the cargo release still remain mysterious (Gould and Collins, 2002; Platta and Erdmann, 2007). Orthologs of peroxisomal RING domain-containing proteins in *Arabidopsis* are essential for plant growth and development. (Hu *et al.*, 2002; Fan *et al.*, 2005; Nito *et al.*, 2007).

After translocation, Pex8 helps release the receptors in yeast (Agne *et al.*, 2003). Further downstream, the putative ubiquitin-conjugating enzyme Pex4 and the AAA ATPases

Pex1 and Pex6 are required for receptor recycling and dislocation (Platta and Erdmann, 2007). *Arabidopsis* PEX1, PEX4 and PEX6 play similar roles to their yeast orthologs in matrix protein import (Zolman and Bartel, 2004; Nito et al., 2007). Additionally, mutation in *AtPEX6* results in significantly reduced levels of AtPEX5, which suggests the role for AtPEX6 in receptor recycling (Zolman and Bartel, 2004). A recent research in *Arabidopsis* reveals that PEX4, PEX5, PEX6, and PEX22 are involved in peroxisome-associated matrix protein degradation (PexAD) of damaged or obsolete matrix proteins (Lingard *et al.*, 2009).

1.3 The biogenesis of peroxisomes – Interactions of peroxisomes with the ER

Peroxisomes have long been viewed as semiautonomous organelles that exist outside the secretory and endocytic pathways of vesicular flow. However, it has now become clear that peroxisomes are evolutionarily derived from the Endoplasmic Reticulum (ER) (Hoepfner et al., 2005; Kragt et al., 2005; Tam et al., 2005; Matsuzaki and Fujiki, 2008).

In yeast, the peroxisomal membrane peroxins Pex3 and Pex16 have been shown to reach the peroxisome via the ER, and peroxisomes bud off from the ER in a Pex19-dependent manner (Hoepfner et al., 2005; Kragt et al., 2005; Tam et al., 2005; Matsuzaki and Fujiki, 2008). Similarly, human PEX3, PEX16 and PEX19 are essential for the formation of the peroxisomal membrane and the localization of membrane proteins (Matsuzaki and Fujiki, 2008). AtPEX16 is the only plant peroxin ortholog known to coexist at steady state within peroxisomes and ER, suggesting its ER-related roles in peroxisome biogenesis (Karnik and Trelease, 2005).

In plants, several other proteins are known to reach the peroxisome through the ER rather than by direct import from the cytosol. For instance, ascorbate peroxidase (APX) in plant cells can be detected in a distinct portion of the ER, suggesting that they are targeted from the cytosol directly to a preexisting subdomain of the ER membrane (Mullen et al., 1999; Mullen et al., 2001). Tomato bushy stunt virus (TBSV) replication protein p33 expressed on its own in plant cells is sorted initially from the cytosol to peroxisomes. And then, p33 is transported via peroxisome-derived vesicles and together with resident peroxisomal membrane proteins (PMPs), to the ER (McCartney et al., 2005). However, whether plant peroxisomes are originated from the ER is yet to be determined.

1.4 The biogenesis of peroxisomes – Peroxisome division

Many observations indicate that peroxisomes can not only arise from the ER, but also possess the ability to undergo division (Thoms and Erdmann, 2005; Lingard and Trelease, 2006; Orth et al., 2007). Peroxisome division can be divided into three overlapping steps including elongation, membrane constriction and final fission steps (Figure 1.1). Peroxin 11 (PEX11) proteins are implicated to promote the elongation step of peroxisomes, while Fission 1 (FIS1) and Dynamin-Related Proteins (DRPs) are required for the final fission step (Thoms and Erdmann, 2005).

1.4.1 PEX11 proteins are involved in the early steps of peroxisome division

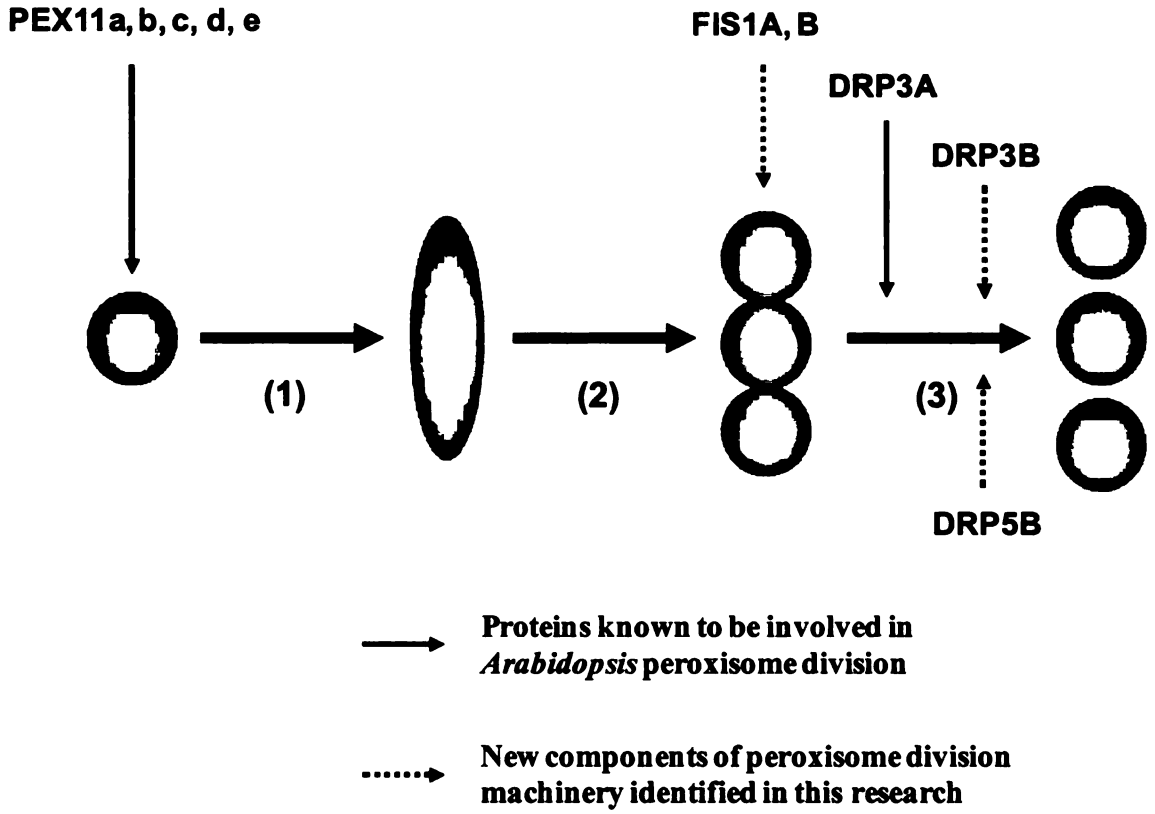


Figure 1.1 Model of peroxisome proliferation and division.

Peroxisome division is a process consisting of three partially overlapping steps, namely (1) elongation, (2) membrane constriction and (3) fission.

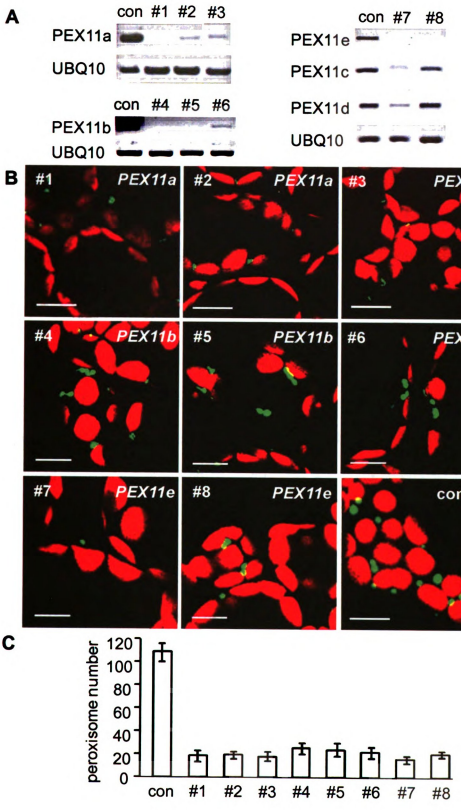
Figure 1.2 Peroxisome phenotypes conferred by reducing the expression of *PEX11* genes in *Arabidopsis*.

(A) RT-PCR analysis of *PEX11* and *UBQ10* transcripts from RNAi plants in which the expression of *PEX11a* (lines 1 to 3), *PEX11b* (lines 4 to 6), and *PEX11c* to *PEX11e* (lines 7 and 8) is reduced. Controls (con) are YFP-PTS1 plants.

(B) Confocal microscopy of cells from (B). Bars=10 μm. Green=YFP-PTS1 peroxisomal marker, red=autofluorescence of chlorophyll.

(C) Numerical analysis of peroxisomes in leaf mesophyll cells from 4-week-old T2 RNAi plants. Numbers shown were obtained from epifluorescence images captured from 150 mm 3 150 mm of a cell (n > 17). Error bars indicate SD.

Orth, T., Reumann, S., Zhang, X., Fan, J., Wenzel, D., Quan, S., and Hu, J. (2007). The PEROXIN11 protein family controls peroxisome proliferation in *Arabidopsis*. The Plant Cell 19, 333-350.


A
con #1 #2 #3
PEX11a
UBQ10
con #4 #5 #6
PEX11b
UBQ10
con #7 #8
PEX11e
PEX11c
PEX11d
UBQ10
B
#1 PEX11a
#2 PEX11a
#3 PEX11a
#4 PEX11b
#5 PEX11b
#6 PEX11b
#7 PEX11e
#8 PEX11e
control
C
peroxisome number
120
100
80
60
40
20
0
con #1 #2 #3 #4 #5 #6 #7 #8

S. cerevisiae Pex11 was the first identified factor involved in peroxisome division. Overexpression of *Pex11* promotes peroxisomal elongation, whereas deletion of Pex11 causes peroxisomes to be greatly enlarged (van Roermund et al., 2000; Thoms and Erdmann, 2005). New members of the Pex11 family were found in yeast. *Pex25* was discovered as a gene that was induced in yeast cells upon growth on oleate, and Pex27 was found by homology to Pex25. Mutations in Pex25 or Pex27 result in enlarged peroxisomes (Rottensteiner et al., 2003b; Rottensteiner et al., 2003a; Tam et al., 2003). A triple mutant (*pex11Δ pex25Δ pex27Δ*) shows severe peroxisomal protein import defects and is unable to utilize oleate for growth (Rottensteiner et al., 2003b; Rottensteiner et al., 2003a; Tam et al., 2003). These *S. cerevisiae* Pex11p family members share significant sequence similarity in their C-terminal segments, and all members homo-oligomerize, which is essential for their function (Rottensteiner et al., 2003b; Rottensteiner et al., 2003a; Tam et al., 2003). Overexpression of mammalian *PEX11α* and *PEX11β* but not *Pex11γ* induced peroxisome proliferation in different cell types. Moreover, PEX11β is more efficient in promoting peroxisome elongation than PEX11α (Thoms and Erdmann, 2005; Schrader and Fahimi, 2006).

To study functions of PEX11 proteins in plants, five PEX11 orthologs in the *Arabidopsis* proteome were characterized in our lab. Overexpression of each *PEX11* gene promotes the elongation of peroxisomes, conversely, silencing of *PEX11* gene results in a dramatic reduction in peroxisome number, as shown in Figure 1.2 (Lingard and Trelease, 2006; Orth et al., 2007). Based on changes in peroxisomal number, volume and morphology in *PEX11* overexpression and silencing lines, we conclude that the five *Arabidopsis* PEX11 proteins play partially overlapping, but distinct roles in peroxisome proliferation, and

participate in peroxisomal elongation, the early step of peroxisome proliferation. Although it is well-known that PEX11 proteins function in the early steps of peroxisome division in various eukaryotes, the molecular mechanism by which PEX11 proteins promote peroxiome proliferation is still not fully understood.

Data shown in chapter 4 showed that PEX11s interact with Dynamin-Related Proteins (DRPs) *in vivo* and *in vitro*, suggesting that PEX11 proteins might function by recruiting DRPs to peroxisomes (Chapter 4). Our data provide a clue for the molecular function of PEX11 in other organisms since no direct interactions between PEX11s and DRPs have ever been reported (Thoms and Erdmann, 2005). However, whether plant PEX11s play roles in anchoring DRPs to peroxisomes need to be further determined.

Besides, many other yeast peroxins, such as Pex23, Pex24, Pex28, Pex29, Pex30, Pex31 and Pex32, also have more or less impacts on the morphology, number and size of peroxisomes. (Eckert and Erdmann, 2003; Vizeacoumar et al., 2004; Kiel et al., 2006).

1.4.2 FIS1 and DRPs cooperate in peroxisome fission

Fis1, known to function in the mitochondrial fission, was recently found to play a role in peroxisome fission as well in yeast and mammalian cells. Fis1 has a transmembrane domain at the C-terminal tail responsible for its targeting to the membrane of peroxisomes and mitochondria (Koch et al., 2005; Kobayashi et al., 2007). Overexpression of *Fis1* promotes peroxisome division, while its silencing causes tubulation in mammalian and yeast cells (Koch et al., 2005; Kobayashi et al., 2007).

Morphological observations of peroxisomes and mitochondria in *fis1* mutants indicate Fis1 might play a role in membrane constriction in mammals (Serasinghe and Yoon, 2008). To further determine the components of peroxisome division machinery in *Arabidopsis*, we characterized the roles of two FIS1 proteins in *Arabidopsis* (Chapter 2 and 3). Consistent with the functions of their orthologs in mammalian and yeast cells, FIS1A and FIS1B are involved in the division of both peroxisomes and mitochondria (Lingard et al., 2008; Zhang and Hu, 2009).

Dynamin-related proteins (DRPs) are large GTPases involved in many processes including the division of mitochondria and chloroplasts. Progress in recent years has revealed that peroxisomes, like plant chloroplasts and mitochondria of fungal, plant and animal origin, divide using DRPs (Mano et al., 2004; Thoms and Erdmann, 2005). During organelle division, DRPs act late on the cytosolic side, after some other division machinery has constricted the membranes (Osteryoung and Nunnari, 2003; Thoms and Erdmann, 2005).

One important DRP required for peroxisomal fission in glucose-grown *S. cerevisiae* appears to be vacuolar protein sorting-associated protein 1 (Vps1) (Hoepfner et al., 2001). The *vps1* mutant exhibits only one or two giant peroxisomes that may form long tubules oriented along actin cables. However, cells grown in oleate have been reported to require the yeast DRP Dnm1, Fis1, and Mdv1 and Caf4. Furthermore, Mdv1 and Caf4 are cytosolic WD proteins that bind to Fis1 and Dnm1 in yeast (Kuravi et al., 2006; Motley and Hettema, 2007; Motley et al., 2008). In addition, the four proteins are key components of mitochondrial division in yeast (Hoppins et al., 2007). Similarly, silencing

of DLP1, a DRP in mammalian cells, leads to highly elongated peroxisomal tubules which are constricted, but cannot be completely separated (Koch et al., 2003). DLP1 is also involved in mitochondrial division (Hoppins et al., 2007).

All eukaryotic species tested contain DRPs. *Arabidopsis* DRP3A dual-functions in the division of mitochondria and peroxisomes (Mano et al., 2004; Fujimoto et al., 2009). It plays a role in peroxisome fission (Figure 1.1). Since *Arabidopsis* has 16 DRPs, some of which have multiple functions, it is possible that there are more DRPs involved in peroxisome division. In the thesis, two new DRPs were identified for the peroxisome division apparatus in *Arabidopsis* (Chapter 2 and Chapter 4). DRP3B, a homolog of DRP3A, is also involved in peroxisome division (Chapter 2). A surprise, however, is that DRP5B (also called ARC5), a component of the chloroplast division machinery, also plays a critical role in peroxisome division (Chapter 4).

In summary, the PEX11 proteins induce peroxisome elongation which initiates peroxisome division, while FIS1 and DRP proteins are involved in the fission of peroxisomes (Figure 1.1). The relationships among these proteins are still elusive in higher plants. For examples, it is yet to be addressed whether plant peroxisomal membrane proteins, FIS1 and PEX11 orthologs, also function in recruiting DRPs to peroxisomes If these proteins cooperate in organelle division also remains unknown. In this dissertation, we investigated these questions by testing the physical interactions among PEX11s, FIS1s and DRPs.

1.4.3 The link among PEX11s, FIS1s and DRPs

As DRPs lack targeting sequence, some other factors must determine their association with organelles (Thoms and Erdmann, 2005). Fis1 is a tail-anchored membrane protein with N-terminal tetratricopeptide repeats (TPR) for the binding of cytosolic fission factors. Fis1 recruits DRP1 to peroxisomes and mitochondria in mammalian cells (Yoon et al., 2003; Kobayashi et al., 2007). In yeast, Fis1 recruits Dnm1 to peroxisomes and mitochondria for fission through the soluble adaptors, Mdv1 and Caf4 that interact with both Dnm1 and Fis1 (Kuravi et al., 2006; Motley and Hettema, 2007; Motley et al., 2008). In this dissertation, the direct physical interactions between Arabidopsis FIS1s and DRPs were identified using two independent approaches, suggesting that FIS1s may recruit DRPs to peroxisomes and mitochondria in plants (Chapter 4).

PEX11 is another candidate that might help anchor DRPs to peroxisomes based on genetic evidence. Overexpression of *PEX11β* increases preoxisome proliferation, however, this effect is not present when mammalian *DLP1* is silenced in mammalian cells (Li and Gould, 2003). Although direct interaction between PEX11 β and DLP1 was not detectable, a protein complex containing PEX11β, Fis1 and DLP1 was shown to form (Kobayashi et al., 2007). These findings suggest an interconnection between PEX11s and DRPs. In *Arabidopsis*, PEX11 proteins form homo- and hetero-oligomers, and FIS1B interacts with five PEX11 proteins (Lingard et al., 2008). Our research revealed that PEX11 proteins also interact with FIS1A, more importantly, they interact with DRPs, indicating that PEX11 proteins may also play a role in DRP recruitment (Chapter 4). Our results support the current opinions about relationship among PEX11s, FIS1s and DRPs in peroxisome division.

1.5 Objectives

The major objective of this study was to determine the molecular architecture of peroxisome division and to obtain more knowledge about how components of peroxisomal division machinery contribute to plant growth and development.

The research presented in this dissertation is a progression of the studies involving *Arabidopsis* peroxisome division. Forward and reverse genetic strategies were undertaken to search for more plays in the process of peroxisome divison. Four new components, DRP3B, DRP5B and two FIS1 proteins have been identified for the division machinery of peroxisomes. They are involved in peroxisome fission. In addition, analyzing these new components of peroxisome division apparatus suggested that they play roles in plant growth and development, which provides further information about the specific functions of peroxisomes *in planta*. Bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation (Co-IP) assays demonstrate that peroxisomal membrane proteins, PEX11s and FIS1s, interact with DRPs *in vivo* and *in vitro*. These two types of membrane proteins may anchor DRPs to peroxisomes. More importantly, the direct physical interactions between PEX11s and DRPs were identified for the first time in all eukaryotic cells.

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Chapter 2 Two Small Protein Families, DYNAMIN-RELATED PROTEIN3 and FISSION1, Are Required for Peroxisome Fission in *Arabidopsis*

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Abstract

Peroxisomes are multifunctional organelles that differ in size and abundance depending on the species, cell type, developmental stage, and metabolic and environmental conditions. The PEROXIN11 protein family and the DYNAMIN-RELATED PROTEIN3A (DRP3A) protein have been shown previously to play key roles in peroxisome division in Arabidopsis. To establish a mechanistic model of peroxisome division in plants, we employed forward and reverse genetic approaches to identify more players involved in this process. In this study, we identified three new components of the Arabidopsis peroxisome division apparatus: DRP3B, a homolog of DRP3A, and FISSION1A and 1B (FIS1A and 1B), two homologs of the yeast and mammalian FIS1 proteins that mediate the fission of peroxisomes and mitochondria by tethering the DRP proteins to the membrane. DRP3B partially targets to peroxisomes and causes defects in peroxisome fission when the gene function is disrupted. The *drp3A drp3B* double mutants display stronger deficiencies than each single mutant parent in peroxisome abundance, seedling establishment, and plant growth, suggesting partial functional redundancy between DRP3A and DRP3B. In addition, FIS1A and FIS1B are each dual-targeted to peroxisomes and mitochondria; their mutants show growth inhibition and contain peroxisomes and mitochondria with incomplete fission, enlarged size, and reduced number. Our results demonstrate that both DRP3 and FIS1 protein families contribute to peroxisome fission in Arabidopsis and support the view that DRP and FIS1 orthologs are common components of the peroxisomal and mitochondrial division machineries in diverse eukaryotic species.

Introduction

Plant peroxisomes orchestrate a wide array of metabolic activities such as fatty acid β–oxidation, the glyoxylate cycle, photorespiration, jasmonate biosynthesis, H_2O_2 detoxification, and metabolism of nitrogen and indole-butyric acid (Hayashi and Nishimura, 2003; Nyathi and Baker, 2006; Olsen and Harada, 1995; Reumann and Weber, 2006; Zolman *et al.*, 2000). Peroxisomes can form de novo from the endoplasmic reticulum (ER) or arise from division/proliferation of pre-existing peroxisomes via multiple steps involving organelle elongation/enlargement, membrane constriction, and peroxisome fission (Fagarasanu *et al.*, 2007; Hoepfner *et al.*, 2005; Motley and Hettema, 2007; Titorenko and Mullen, 2006). Peroxisome division (from one to at least two peroxisomes) takes place constitutively or under induced conditions; induced division (or peroxisome proliferation) is often referred to as the increase in peroxisome abundance/volume in response to environmental and metabolic stimuli (Yan *et al.*, 2005).

Several major components of the peroxisome division machineries are conserved in eukaryotes. For example, orthologs of the peroxisomal membrane protein PEROXIN11 (PEX11) in fungi, animals, trypanosomes, and plants promote the first step of peroxisome division, namely, peroxisome elongation/tubulation (Fagarasanu *et al.*, 2007). Arabidopsis contains five PEX11 isoforms, PEX11a to –e, all of which are targeted to peroxisome membranes and able to induce peroxisome elongation and population increase with some degrees of functional specificity and redundancy (Lingard and Trelease, 2006; Nito *et al.*, 2007; Orth *et al.*, 2007). Decreasing the expression of individual *PEX11* or a subfamily of *PEX11* genes led to reduction in the total number of

peroxisomes (Orth *et al.*, 2007) or slightly enlarged peroxisomes (Nito *et al.*, 2007). Arabidopsis plants overexpressing individual *PEX11* genes displayed significant peroxisome tubulation and increase in peroxisome abundance (Orth *et al.*, 2007). The precise mode of action for PEX11 proteins remains elusive; membrane modification through phospholipid binding, metabolite transport, and recruitment of downstream proteins are some of the proposed functions (Fagarasanu *et al.*, 2007; Thoms and Erdmann, 2005).

The second class of conserved constituents of the peroxisome division apparatus consists of dynamin-related proteins (DRPs), which mediate peroxisome fission after membrane constriction occurs (Fagarasanu *et al.*, 2007). Dynamin and dynamin-related proteins are large self-assembling GTPases involved in the fission and fusion of membranes by positioning into spiral-like structures around the membranes and acting as mechanochemical enzymes or signaling GTPases (Hoppins *et al.*, 2007; Koch *et al.*, 2004; Osteryoung and Nunnari, 2003; Praefcke and McMahon, 2004). DRPs share with the conventional dynamins an N-terminal GTPase domain, the middle domain (MD), and a C-terminal GTPase effector domain (GED), but lack the pleckstrin homology domain (PH) for binding to membrane lipids and the C-terminal proline- and arginine-rich domain (PRD) that mediates interactions with SH3 motif-containing proteins (Thoms and Erdmann, 2005). *Saccharomyces cerevisiae* Vps1p and Dnm1p and the mammalian DLP1/Drp1 proteins are DRPs required for peroxisome division, besides their roles in mitochondrial division (Dnm1p and DLP1/Drp1) and Golgi (DLP1/Drp1) and vacuole (Vps1p) morphogenesis (Hoepfner *et al.*, 2001; Koch *et al.*, 2004; Koch *et al.*, 2003; Kuravi *et al.*, 2006; Li and Gould, 2003; Schrader, 2006; Wilsbach and Payne, 1993). Of

the 16-member superfamily of dynamins and DRPs in Arabidopsis (Hong *et al.*, 2003), family 3 consists of DRP3A and DRP3B, which share 77% amino acid sequence identity (Figure 1a). Both proteins are involved in mitochondrial division, whereas DRP3A also controls the division of peroxisomes (Arimura *et al.*, 2004; Arimura and Tsutsumi, 2002; Logan *et al.*, 2004; Mano *et al.*, 2004). Whether or not DRP3B functions in peroxisome fission is unclear.

The third group of proteins with a conserved function in peroxisome division, at least in yeasts and mammals, is FISSION1 (FIS1). FIS1 orthologs are integral membrane proteins dual-targeted to peroxisomes and mitochondria, acting as adaptors for the mammalian DLP1 and yeast Dnm1 proteins by recruiting these DRPs to the organelles to execute membrane fission (Kobayashi *et al.*, 2007; Koch *et al.*, 2003; Koch *et al.*, 2005; Kuravi *et al.*, 2006). Structural features shared by FIS1 orthologs include a highly conserved C-terminal transmembrane domain (TMD) and a tetratricopeptide repeat (TPR)-like binding domain that spans over two-thirds of the protein from the N-terminus and mediates protein-protein interaction (Figure 1b). Arabidopsis contains two homologs of FIS1 (FIS1A and FIS1B) that share 58% protein sequence identity (Figure 1b). The Arabidopsis mutants of *BIGYIN* (*FIS1A*) displayed a reduced number of mitochondria and an increase in mitochondrial size (Scott *et al.*, 2006). It remains to be determined whether these two Arabidopsis FIS1 proteins are involved in controlling the number and size of peroxisomes and whether FIS1B is also required for mitochondrial division.

We are interested in elucidating molecular pathways underlying the environmental and metabolic control of the abundance of plant peroxisomes, which will ultimately lead to

answers to the question of how peroxisomal dynamics correlates with plant physiology and development. Transmission electron microscopic studies demonstrated that by mostly unknown mechanisms plants increase their peroxisome numbers in response to environmental stresses such as ozone, the herbicide isoproturon, the hypolipidemic drug clofibrate, and high light (de Felipe *et al.*, 1988; Ferreira *et al.*, 1989; Oksanen *et al.*, 2003; Palma *et al.*, 1991). We recently provided evidence that light induces the proliferation of peroxisomes in Arabidopsis seedlings through the far-red light receptor phytochrome A (phyA) and the bZIP transcription factor HY5 HOMOLOG (HYH), which coordinately activate the expression of the *PEX11b* gene (Desai and Hu, 2008). To further dissect molecular pathways governing the environmental control of plant peroxisome abundance, we need first to establish a precise mechanistic model for peroxisome division in plants. All players in the division machinery need to be identified, as some of them could be targets for plant peroxisome proliferators. To this end, we screened for mutants deficient in peroxisome division and conducted reverse genetic studies to characterize plant orthologs of the yeast and mammalian proteins involved in peroxisome division. In this report we demonstrate the role of DRP3B and the two FIS1 proteins in peroxisome division in Arabidopsis. The role for the FIS1 proteins in mitochondrial division is also illustrated.

Results

DRP3A and DRP3B are partially redundant in controlling peroxisome fission

Figure 2.1 Sequence alignments of DRP3 and FIS1 proteins.

(a) Alignment of Arabidopsis DRP3A and DRP3B and the yeast *S. cerevisiae* Dnm1p protein. The arrowhead points to the mutation in *pdd1*. (b) Alignment of Arabidopsis FIS1A and FIS1B and the *S. cerevisiae* Fis1p protein. Positions of the tetratricopeptide (TPR)-like domain in the three proteins are: Fis1p, 6-129; FIS1A, 36-142; FIS1B, 92-125. The putative transmembrane domain (TMD) is underlined. Shaded sequences represent identical amino acid residues in both (a) and (b).

(a)

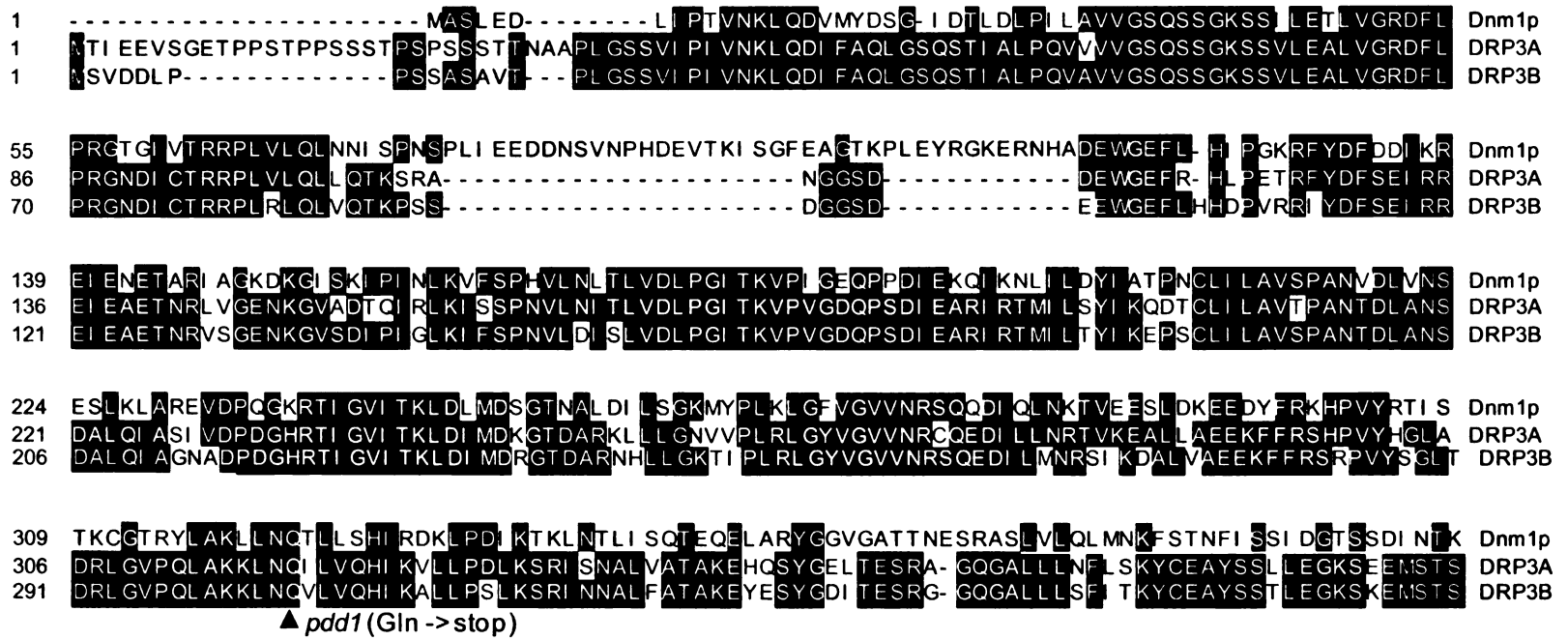


Figure 2.1 (a) continued

Figure 2.1 continued

To identify more players in the peroxisome division and proliferation pathways in Arabidopsis, we performed ethyl methane sulfonate (EMS) mutagenesis on seeds from the peroxisome marker plant YFP-PTS1, which expresses the yellow fluorescent protein with the PEROXISOME-TARGETING SIGNAL TYPE1 (Ser-Lys-Leu) sequence attached to the C-terminus (Desai and Hu, 2008; Fan *et al.*, 2005; Orth *et al.*, 2007). Screening of the M_2 population for peroxisome division/proliferation deficient mutants (*pdd*) enabled us to identify several classes of mutants showing changes in peroxisome size, shape, or number from the wild-type plants (Figure 2a). The *pdd1* mutant exhibited highly aggregated/inseparable (Figure 2b) and massively elongated (Supplemental Figure 1) peroxisomes, phenotypes reminiscent of those of the *aberrant peroxisome morphology 1* (*apm1*) mutant alleles, which contain mutations in the *DRP3A* gene (Mano *et al.*, 2004). Sequencing of the *DRP3A* gene in the *pdd1* mutant revealed a nonsense mutation at ^{319}Gln (Figure 1a and Figure 2i).

Phylogenetic analyses of DRP sequences from various species suggested that DRP3A and DRP3B are more closely related to the yeast Vps1p and Dnm1p and the human Drp1 than to members of the other *Arabidopsis* DRP families (Arimura and Tsutsumi, 2002b; Konopka, 2008). Searches of public Arabidopsis microarray databases (https://www.genevestigator.ethz.ch/; (Zimmermann *et al.*, 2004) showed that *DRP3A* and *DRP3B* are both fairly ubiquitously expressed (Supplemental Figure 2). Given the role of DRP3A, Vps1p, Dnm1p, and Drp1 in peroxisome division, it is highly likely that DRP3B is also involved in the same process in Arabidopsis. To test this hypothesis, we obtained two T-DNA insertion mutants of *DRP3B*, *drp3B-1* (*SALK_045316*) and *drp3B-2* (*SALK_112233*), as well as two additional mutant alleles of *DRP3A*, *drp3A-1*

(*SALK_008706*) and *drp3A-2* (*SALK_147485*) (Figure 2i). Semi-quantitative reverse transcriptase-PCR (RT-PCR) of RNA from the mutants gave evidence that the expression of *DRP3A* or *DRP3B* was strongly reduced in the respective mutants (insets in Figure 2c-f).

The *YFP-PTS1* peroxisomal marker gene was introduced into these mutants via *Agrobacterium*-mediated transformation (Clough and Bent, 1998); transgenic plants were analyzed for peroxisome phenotypes. Both *drp3B* mutant alleles expressing *YFP-PTS1* contained many peroxisome aggregates that were constricted but failed in fission (Figure 2e-f). These phenotypes were comparable to some extent with those observed in *pdd1*, *drp3A-1*, and *drp3A-2* (Figure 2b-d). However, the long and extended tails associated with peroxisomes that were frequently seen in the *drp3A* alleles (Figure 2b-d) and named by Scott et al. (2007) as "peroxules", were not observed in the *drp3B* mutants (Figure 2e-f). Most of the individual peroxisomes in the *drp3A* and *drp3B* mutants also appeared larger than those in the wild type (Figure 2b-f). Thus, DRP3B, like its homolog DRP3A, is required for peroxisome division. However, DRP3B's role may be weaker than that of DRP3A.

Single mutants of *DRP3A* and *DRP3*B each showed deficiency in peroxisome division, suggesting that the functions of DRP3A and DRP3B may not be completely overlapping. To further address this question, we generated *drp3A drp3B* double mutants. For convenience in genotyping, the two T-DNA insertion lines of *DRP3A* were used to make

Figure 2.2 Peroxisome phenotypes of the *drp3* mutants

(a) Genomic structures of *DRP3A* and *DRP3B*. Boxes represent exons, with the coding region in black. Positions of the mutant alleles are also indicated. (b) Confocal micrographs of leaf mesophyll cells from wild-type and *drp3* mutant plants expressing the *YFP-PTS1* peroxisomal marker gene and grown for 4 weeks. Green signals show YFP-PTS1-labelled peroxisomes; red signals indicate chloroplasts. Scale bars = 10 μm. (c) RT-PCR analyses of RNA from corresponding mutants. In each inset, the left lane is wild type and the right lane is the mutant; the top panel represents transcripts of the individual *DRP3* gene and bottom panel is the *UBIQUITIN10* transcript. (d) Quantification of total YFP fluorescence and peroxisome number per 2500 μm^2 of the mesophyll cells in *drp3* mutants ($n>8$, $p<0.05$).

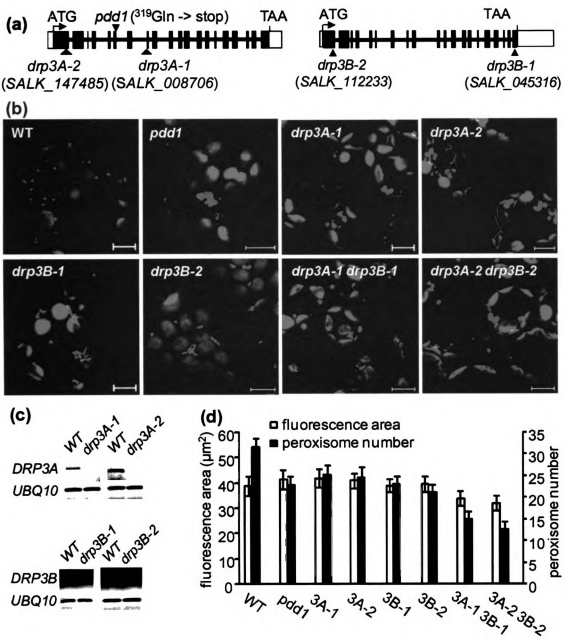
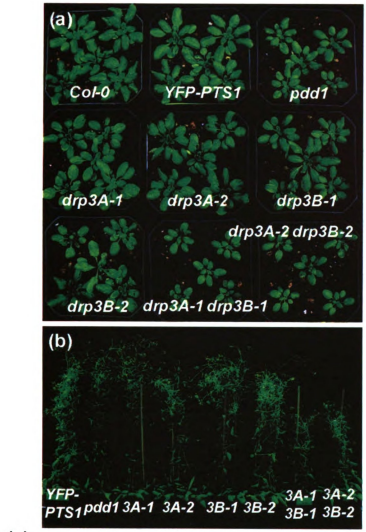

(a)
ATG
pdd1 (319Gln -> stop)
TAA
ATG
TAA
drp3A-2
(SALK_147485)
drp3A-1
(SALK_008706)
drp3B-2
(SALK_112233)
drp3B-1
(SALK_045316)
(b)
WT
pdd1
drp3A-1
drp3A-2
drp3B-1
drp3B-2
drp3A-1 drp3B-1
drp3A-2 drp3B-2
(c)
WT
drp3A-1
WT
drp3A-2
DRP3A
UBQ10
WT
drp3B-1
WT
drp3B-2
DRP3B
UBQ10
(d)
fluorescence area
peroxisome number
fluorescence area (μm2)
peroxisome number
0
10
20
30
40
50
60
0
5
10
15
20
25
30
35
WT
pdd1
3A-1
3A-2
3B-1
3B-2
3A-1 3B-1
3A-2 3B-2

Figure 2.3 Growth and germination phenotypes of the *drp3* mutants

(a-b) Wild type and *drp3* mutants grown for three (a) and seven weeks (b). (c) Sucrose-dependence assay of *drp3* mutants. Hypocotyl lengths of dark-grown seedlings grown for 5 days on MS plates with or without 1% sucrose were measured. Error bars indicate standard deviations ($n > 50$; $p<0.05$).


(a)
Col-0
YFP-PTS1
pdd1
drp3A-1
drp3A-2
drp3B-1
drp3A-2 drp3B-2
drp3B-2
drp3A-1 drp3B-1
(b)
YFP-PTS1
pdd1
3A-1
3A-2
3B-1
3B-2
3A-1 3B-1
3A-2 3B-2
(c)
W/O Sucrose
W Sucrose
hypocotyl length (mm)
20
16
12
8
4
0
YFP-PTS1
pex14
pdd1
drp3A-1
drp3A-2
drp3B-1
drp3B-2
drp3A-1 drp3B-1
drp3A-2 drp3B-2

crosses. Two double mutants were obtained: *drp3A-1 drp3B-1* and *drp3A-2 drp3B-2*. Similar to the single mutants, the double mutants (F_2) also contained many clumped peroxisomes that were each slightly larger than those of the wild type (Figure 2g-h). However, these morphological defects of peroxisomes in the double mutants were not stronger than those of the single mutants. To determine whether the total number of peroxisomes was changed in these mutants, we used ImageJ software to quantify peroxisome abundance. We measured the planar area of YFP fluorescence and the number of peroxisomes in a given field in leaf mesophyll cells, using information extrapolated from at least 8 confocal images from each genotype. Whereas the total area of fluorescence per 2500 μm^2 remained virtually unchanged from wild type to *drp3* single mutants, it was decreased in the two *drp3A drp3B* double mutants (Figure 2j). Compared with the wild type, the number of peroxisomes per 2500 μm^2 was noticeably decreased in the single mutants; the double mutants contained the lowest number of peroxisomes (Figure 2j). Of the two double mutants, *drp3A-1 drp3B-1* has a slightly weaker phenotype, likely due to the fact that there was still a small amount of *DRP3A* mRNA detected in *drp3A-1* (Figure 2c).

At the seedling stage, *pdd1*, *drp3A-1,* and *drp3A-2* grew more slowly than the wild-type *YFP-PTS1* control plants, whereas *pdd1* exhibited the strongest growth inhibition (Figure 3a). It seems that despite our inability to detect *DRP3A* transcripts in *drp3A-2*, it is not a null mutant. The *drp3B* mutant alleles showed comparable phenotypes with those of *drp3A*. The two double mutants displayed stronger defects in plant size than the single mutants (Figure 3a) and were slightly pale-green at the seedling stage (data not shown).

Adult plants of the wild type and single mutants were largely undistinguishable in appearance, whereas double mutants remained reduced in plant size (Figure 3b). The pale-green phenotype reflects defects in photorespiration, a pathway in which peroxisomes and mitochondria play essential roles. Reduced division in both peroxisomes and mitochondria in the *drp3* mutants obviously reduced plant growth.

To determine whether disruption of the *DRP3* genes led to impaired seedling establishment, we measured hypocotyl lengths of dark-grown seedlings germinated in the presence or absence of sucrose. The *pex14* null mutant, which is defective in a peroxisome biogenesis factor involved in peroxisomal matrix protein import and has a sugar-dependent phenotype (Fan *et al.*, 2005; Orth *et al.*, 2007), was used as a control (Figure 3c). On sucrose-free medium, hypocotyl elongation was more inhibited in the *drp3A drp3B* seedlings than in the single mutants and the wild type; this deficiency was largely rescued by exogenous sucrose (Figure 3c). It is likely that the *drp3* mutants were defective in lipid metabolism as a result of reduced division of peroxisomes and mitochondria, two key venues of this physiological process. Hence, insufficient energy in the form of carbohydrates was available for the seedlings to become established. Collectively, results from the sugar-dependence assays and the peroxisome and plant growth phenotype analyses of the *drp3* mutants provide evidence that the DRP3A and DRP3B mediate peroxisome division in a partially redundant manner.

Subcellular localization of DRP3B

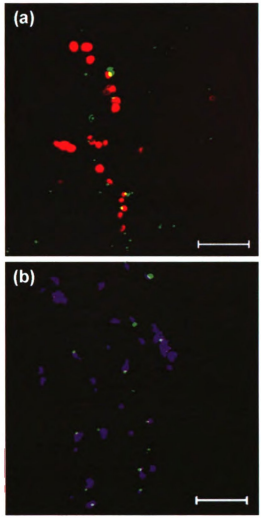


Figure 2.4 Subcellular localization of DRP3B

Confocal images were taken from leaf epidermal cells of 4-week-old plants co-expressing *CFP-PTS1* and *YFP-DRP3B*. Bars = 10 μm.

(a) Association of YFP-DRP3B (green signals) with some CFP-PTS1-labelled peroxisomes (red signals). (b) Association of YFP-DRP3B (green signals) with some MitoTracker-stained mitochondria (magenta signals).

Given that DRP3B clearly plays a role in peroxisome division, we next sought to determine whether this protein indeed sorted to peroxisomes. Full-length cDNA encoding *DRP3B* (At2g14120) was cloned to the C-terminus of YFP in a plant expression vector driven by the *CaMV35S* promoter. This construct was co-expressed with *cyan fluorescent protein* (*CFP*)-*PTS1* in Arabidopsis. Transgenic plants expressing both transgenes exhibited many YFP signals that were tightly associated with small circular structures labeled with CFP-PTS1 (Figure 4a). Likewise, many YFP-DRP3B proteins were also associated with mitochondria, which were stained by MitoTracker (Figure 4b).

Instead of distributing throughout these organelles, the YFP-DRP3B protein targeted to spots on peroxisomes and mitochondria or showed juxtaposition to these compartments (Figure 4). A similar localization pattern was previously shown for DRP3A and DRP3B on mitochondria (Arimura *et al.*, 2004; Arimura and Tsutsumi, 2002) and for DRP3A on peroxisomes (Mano *et al.*, 2004); locations of these spots were suggested to be tips and possible sites for membrane constriction (Arimura *et al.*, 2004; Arimura and Tsutsumi, 2002). Taken together, our data demonstrate that the DRP3B protein is partially localized to peroxisomes in addition to targeting to mitochondria.

The two *Arabidopsis* FIS1 proteins are localized to both peroxisomes and mitochondria

In yeast and mammals, DRP proteins are tethered to the membrane of peroxisomes and mitochondria by the small membrane-anchored protein FIS1 before they participate in division by pinching off small organelles from tubules that are already constricted

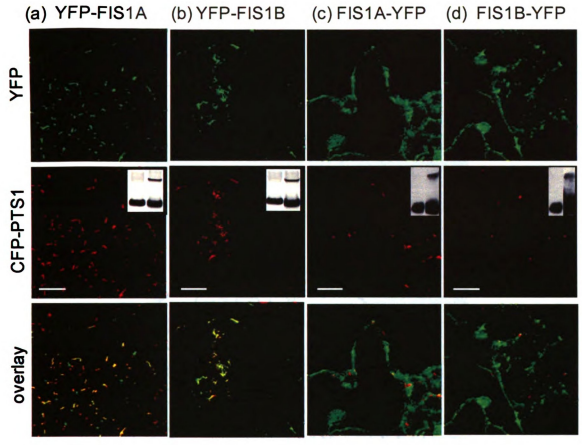


Figure 2.5 Peroxisome targeting of the FIS1 proteins.

Confocal images were taken from leaf epidermal cells of 4-week old plants expressing *CFP-PTS1* combined with *YFP-FIS1* or *FIS1-YFP*, as indicated on top. Each inset is an immunoblot analysis of proteins extracted from wild-type plants expressing *CFP-PTS1* only (left lane) and plants co-expressing *CFP-PTS1* and the indicated *FIS1* construct (right lane). The α–GFP antiserum detected CFP-PTS1 (bottom band) and the FIS1-YFP (or YFP-FIS1) fusion proteins (top band). Bars = 10 μm.

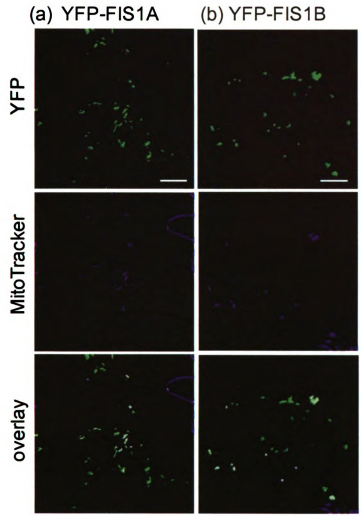


Figure 2.6 Co-localization of YFP-FIS1 fusion proteins with mitochondria. All images were captured from epidermal cells of 6-week-old leaves from the *YFP-FIS1* transgenic plants stained by Mito-Tracker. Scale bars = 10 μm.

(Kobayashi *et al.*, 2007; Koch *et al.*, 2003; Koch *et al.*, 2005; Kuravi *et al.*, 2006). Data collected from online microarray databases (https://www.genevestigator.ethz.ch/) (Zimmermann *et al.*, 2004) revealed that both *FIS1A* and *FIS1B* from Arabidopsis are constitutively expressed. The expression level of *FIS1A* is higher than that of *FIS1B* in most tissues, whereas *FIS1B* shows very high expression in pollen (Supplemental Figure 2). FIS1A (BIGYIN) was previously shown to control the size and number of mitochondria (Scott *et al.*, 2006). However, whether FIS1B plays a role in mitochondrial division and whether these two FIS1 proteins are targeted to mitochondria have not been shown clearly. Here, we characterized Arabidopsis FIS1A and FIS1B to determine whether they play a role in the division both peroxisomes and mitochondria.

Subcellular localization of these two proteins was tested. We transformed *35S* promoter-driven constructs containing *YFP-FIS1* or *FIS1-YFP* into Arabidopsis plants that were already expressing the peroxisomal marker protein CFP-PTS1. Transgenic plants expressing YFP-FIS1A or YFP-FIS1B fusions displayed partial co-localization of the YFP signals with CFP-PTS1 (Figure 5a-b). Unlike DRP3B, which concentrated at spots on the peroxisome (Figure 4), the FIS1 proteins were evenly distributed along peroxisomes (Figure 5a-b). In contrast, when fused to the N-terminus of YFP, FIS1A and FIS1B were mostly diffused in the cytosol and nucleus (Figure 5c-d), indicating that the C-terminus of FIS1, which contains the transmembrane domain, is important for targeting FIS1 to the peroxisome. These data suggest that Arabidopsis FIS1A and FIS1B are partially targeted to peroxisomes and that the C-terminus of the proteins is required for the targeting. We also stained leaf epidermal cells from transgenic plants expressing *YFP-FIS1A* or *YFP-FIS1B* with the MitoTracker dye. Confocal microscopy showed that

some of the YFP-FIS1A and YFP-FIS1B fusion proteins clearly co-localized with MitoTracker (Figure 6a-b), thus validating the partial mitochondrial localization of these two proteins.

FIS1A and FIS1B are involved in the fission of peroxisomes and mitochondria

We obtained loss-of-function mutants to further examine the role of FIS1 proteins in the division of peroxisomes and mitochondria. A *fis1A* mutant (SALK_086794) has a T-DNA insertion in the last exon (Figure 7a) and is the same allele (*bigyin1-2*) used by Scott *et al* (2006) for mitochondrial phenotype analysis. Using RT-PCR analysis, we were unable to detect *FIS1A* transcripts in this mutant (Figure 7b), which was later crossed into the *YFP-PTS1* background. Since T-DNA insertion lines for *FIS1B* were not available, we utilized RNAi to reduce the expression of this gene. The full-length cDNA of *FIS1b* (513 bp) was cloned into the pFGC5941 dsRNAi vector as inverted repeats; the *35S*-driven construct was later transformed into plants expressing *YFP-PTS1*. We generated a total of 59 T_1 transformants that contained both sense and antisense repeats of *FIS1B*, seven of which were randomly picked for RT-PCR analysis. Two RNAi lines, R15 and R47, which showed silencing of *FIS1B* but wild-type levels of *FIS1A* mRNA, (Figure 7c), were selected for further analysis in T_3. The *fis1A* T-DNA insertion mutant and the *FIS1B* RNAi lines both displayed growth inhibition compared with the wild-type plant (Figure 7d).

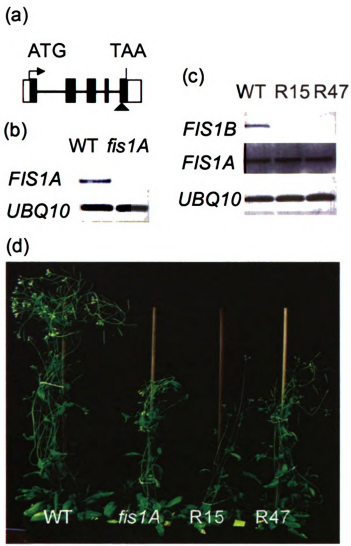
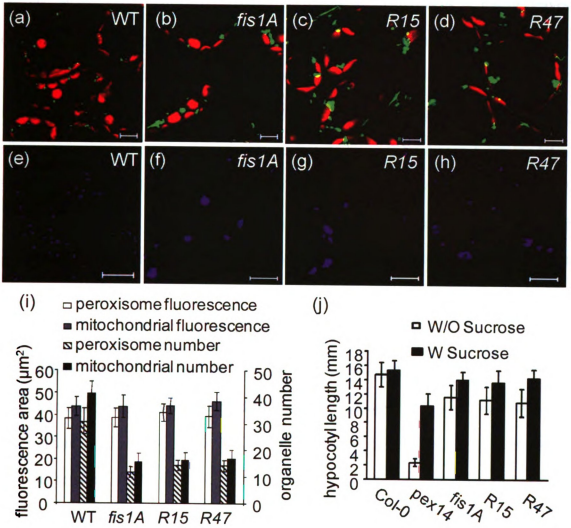


Figure 2.7 Growth phenotype of the *fis1* mutants.

(a) Schematic of the *FIS1A* gene. Boxes indicate exons; coding region is in black. The arrowhead indicates the position of the T-DNA insertion in the *fis1A* mutant. (b-c) RT-PCR analysis of *FIS1A*, *FIS1B*, and *UBIQUITIN10* transcripts in wild-type, *fis1A*, and the two *FIS1B* RNAi lines. (d) Growth comparison of 6-week-old *fis1* mutants and the wild-type plants. Two plants were grown in each pot.

Figure 2.8 Peroxisomal and mitochondrial phenotypes of the *fis1* mutants.

(a-h) Confocal micrographs of leaf mesophyll (a-d) or leaf epidermal (e-h) cells from 6-week-old wild-type and *fis1* mutant plants, all of which contained the *YFP-PTS1* peroxisomal marker gene. Green signals, YFP-PTS1-tagged peroxisomes; red signals, autofluorescent chloroplasts; magenta signals, MitoTracker-stained mitochondria. Bars = 10 μm. (i) Quantification of total peroxisome (YFP) and mitochondrial (MitoTracker) fluorescence and the number of these two types of organelles within 2500 μm^2 of leaf cells in wild type and *fis1* mutants ($n>8$, $p<0.05$). (j) Sucrose-dependence assays of the *fis1* mutants. Hypocotyl lengths of 5-d-old etiolated seedlings grown on MS plates with or without 1% sucrose were measured ($n > 50$; $p<0.05$). Error bars are standard deviations in (i-j).


(a) WT
(b) fis1A
(c) R15
(d) R47
(e) WT
(f) fis1A
(g) R15
(h) R47
(i)
peroxisome fluorescence
mitochondrial fluorescence
peroxisome number
mitochondrial number
fluorescence area (μm²)
organelle number
WT fis1A R15 R47
(j)
W/O Sucrose
W Sucrose
hypocotyl length (mm)
Col-0 pex14 fis1A R15 R47

Confocal microscopic analysis of peroxisomes in the mesophyll cells of rosette leaves demonstrated that these mutants contained many enlarged peroxisomes, some of which were clustered together and failed in fission, in contrast to the mostly spherical and separated peroxisomes in the wild-type plants (Figure 8a-d). Quantification of YFP fluorescence area and peroxisome abundance (per 2500 μm^2) from over 8 images from each genotype revealed that, whereas the total volume of peroxisomes (measured by YFP fluorescence area in the given field) remained largely constant, the number of peroxisomes was significantly reduced in the *fis1* mutants (Figure 8i). The *fis1A* mutant (*bigyin-2*, SALK_086794 used in this study) was shown previously to contain enlarged mitochondria as well as a reduced mitochondrial number per cell (Scott *et al.*, 2006). When stained with MitoTracker, the two *FIS1B* RNAi lines (R15 and R47) also showed many mitochondria that were enlarged in size and decreased in number, similar to the *fis1A* mutant (Figure 8e-h). MitoTracker fluorescence area per 2500 μm^2 remained constant between wild type and the single *fis1* mutants, yet the total number of mitochondria strongly decreased in the mutant lines (Figure 8i). Sugar-dependence assays were also performed on the *fis1* mutants. Both *fis1A* and the two *FIS1B* RNAi lines showed partial growth inhibition on sucrose-free medium (Figure 8j), indicating weak deficiencies in lipid metabolism during germination. Taken together, our results illustrate that FIS1A and FIS1B are targeted to both peroxisomes and mitochondria and are required for the division of both types of organelles in Arabidopsis.

Discussion

In Arabidopsis, the PEX11 protein family and the DRP3A protein have been shown to be involved in peroxisome division (Lingard and Trelease, 2006; Mano *et al.*, 2004; Nito *et al.*, 2007; Orth *et al.*, 2007). In this study, we identified three additional components of the Arabidopsis peroxisome division apparatus, DRP3B, FIS1A, and FIS1B. Whereas PEX11 proteins are primarily responsible for the elongation/tubulation of peroxisomes, DRP3A/3B and FIS1A/1B proteins mediate the fission of peroxisomes.

We provided genetic evidence that DRP3A and DRP3B play partially redundant roles in peroxisome division, seedling establishment, and plant growth. First, some of the YFP-DRP3B proteins were found to be associated with spots on peroxisomes, similar to what was discovered for DRP3A (Mano *et al.*, 2004). Second, single and double mutants of *DRP3A* and *DRP3B* were impaired in peroxisome division, whereas the *dry3A drp3B* double mutants showed stronger phenotypes than either single mutant parent in peroxisome number, sugar dependence, and plant stature and pigmentation. The degree of dwarfness in the *DRP3A* null allele *pdd1* shown in this study seemed to be weaker than *apm1-6*, the strongest mutant allele of *DRP3A* identified from a previous study, which contained a ^{71}Gly->Asp substitution (Mano *et al.*, 2004). This phenotypic difference implies that the truncated DRP3A protein encoded by *pdd1* may still be partially functional, whereas mutation of the N-terminal GTPase domain in *apm1-6* may have completely abolished the function of this protein. Alternatively, the mutant protein encoded by the *apm1-6* allele may have a dominant negative effect by disrupting the function of both endogenous DRP3 proteins and possibly other DRPs that play a role in the division of peroxisomes and mitochondria. To this end, it would be necessary to obtain a mutant in which both DRP3 proteins are completely non-functional, in order to

determine the full capacity of this subfamily of DRPs in peroxisome division and plant development. Furthermore, given that the *drp3A* and *drp3B* single mutants each displayed apparent morphological deficiencies in peroxisomes, each gene should maintain some unique functions in peroxisome division. The slightly different peroxisomal phenotypes of the *drp3A* and *drp3B* mutants shown in this study may provide support for this prediction.

Dynamins and dynamin-related proteins are engaged in endocytosis, cell division and expansion, intracellular vesicle trafficking, and division of organelles such as plastids, mitochondria, peroxisomes, and Golgi vesicles (Osteryoung and Nunnari, 2003; Praefcke and McMahon, 2004). The complete functional spectra of many of the Arabidopsis DRPs have not been characterized. Members from different DRP subfamilies can be involved in the same function. For example, in addition to DRP3A and DRP3B (Arimura *et al.*, 2004; Arimura and Tsutsumi, 2002b; Logan *et al.*, 2004; Mano *et al.*, 2004), two members of family 1 were also shown to participate in mitochondrial division. Mutants of *DRP1C* (*ADL1C*) and *DRP1E* (*ADL1E*) exhibited abnormal mitochondrial elongation; the two proteins also partially co-localized with a mitochondrial marker (Jin *et al.*, 2003). Thus, it is likely that other Arabidopsis DRP subfamilies are also involved in peroxisome division. In addition, the same DRP may participate in the fission of multiple types of membranes. Such examples include the yeast Vps1p protein (Vacuolar Protein Sorting protein *1*) that plays a role in the division of peroxisomes and biogenesis of vacuoles, and the mammalian DLP1 protein that participates in the fission of peroxisomes, mitochondria, and Golgi bodies (Hoepfner *et al.*, 2001; Koch *et al.*, 2003; Li and Gould, 2003). Therefore, despite the finding that DRP3A is involved in the division of only

peroxisomes and mitochondria (Mano *et al.*, 2004), we cannot completely exclude the possibility that DRP3B also targets to other subcellular compartments and contributes to the division or morphogenesis of other organelles. Peroxisomes and mitochondria both move fast. Thus, we were unable to clearly address the question of whether or not YFP-DRP3B targets to spherical structures other than peroxisomes and mitochondria, by visualizing YFP-DRP3B, peroxisomes, and mitochondria simultaneously in a single image (data not shown).

We also show in this study that the two Arabidopsis FIS1 homologs, FIS1A and FIS1B, are targeted to both peroxisomes and mitochondria and play significant roles in the division of these two organelles. FIS1 is one of the very few proteins known to target to the membrane of both peroxisomes and mitochondria. The C-terminus seems critical for FIS1A and FIS1B to target to peroxisomes (Figure 5) in Arabidopsis, consistent with the finding that the C-terminal region of hFIS1 (including the transmembrane domain) is both necessary and sufficient for targeting to both peroxisomes and mitochondria in human cells (Koch *et al.*, 2005). An open question remains as to how targeting signals are specified within the C-terminus of the FIS1 protein and which organelle-specific proteins mediate these targeting events. Among the three essential components of the mitochondrial division machinery in yeast, namely, Dnm1p, Fis1p, and Mdv1p (or its homolog Cav4p), Mdv1p/Cav4p (molecular adaptor) appears to be species-specific and does not have apparent structural orthologs in higher eukaryotes (Hoppins *et al.*, 2007). It is possible that some unidentified proteins exclusively localized to peroxisomes mediate the specific targeting of FIS1 to peroxisomes in Arabidopsis and in other eukaryotes as well.

Our study shows that despite having deficiencies in peroxisome fission, peroxisomal volume (indicated by fluorescent areas) in the *drp3* and *fis1* single mutants remains largely unchanged from that of the wild type. This compensation of the reduced number of peroxisomes by enlarged individual peroxisomes may be a mechanism utilized by the cell to maintain enough volume of the organelles in order to carry out their normal function. However, when both members of the gene family are dysfunctional, as in the case for the *drp3* double mutants, this balance was lost. We expect to see a similar trend in the *fis1* double mutants, which will be generated in the lab. Besides partial redundancy in function, we also expect to see unique function for each FIS1. For example, it is possible that FIS1A and FIS1B each have its specific DRP target. Whereas ectopic expression of Arabidopsis *DRP3* genes (this study and Mano *et al.*, 2004) or the human *DLP1* gene (Li and Gould, 2003) did not cause any apparent peroxisome phenotypes, overexpressing YFP-FIS1 fusion proteins seems to lead to some degree of increased proliferation and clustering of peroxisomes (Figure 5a-b vs. 5c-d). Overproducing Myc-hFIS1 in mammalian cells led to more numerous peroxisomes and segmented mitochondria, suggesting that FIS1 is the limiting factor for peroxisomal and mitochondrial fission (Koch *et al.*, 2005). To address this question in Arabidopsis, we will need to express untagged FIS1 or FIS fused with small tags to avoid possible dominant negative effects caused by attaching the 27-kDa YFP protein to the wild-type FIS1.

A very recent study using Arabidopsis suspension cell cultures failed to show co-localization of myc-FIS1A or myc-FIS1B with peroxisomes that were immunolabelled with α-catalase antibodies; however, an increase in the number of peroxisomes was

observed in cells expressing myc-FIS1B (Lingard *et al.*, 2008). This study also demonstrated that FIS1B has a role in cell-cycle associated peroxisome duplication and targeted to peroxisomes only after it was co-expressed with a PEX11 protein, whereas FIS1A does not seem to be involved in peroxisome duplication (Lingard *et al.*, 2008). In contrast, our study clearly demonstrates that, on their own, both YFP-FIS1A and YFP-FIS1B are able to localize to peroxisomes and mitochondria and that both proteins are involved in peroxisome fission in Arabidopsis plants. GFP and myc-fusions of the mammalian FIS1 protein (hFis1) also target to both mitochondria and peroxisomes when expressed by themselves (Koch *et al.*, 2005). It is possible that the role of FIS1A and FIS1B in cell-cycle associated peroxisome division in cell cultures differs from their role in peroxisomal division in intact Arabidopsis plants.

Despite their distinct evolutionary origins (endosymbiotic vs. ER-derived) and different membrane structures (double membrane vs. single membrane), mitochondria and peroxisomes share some of the same DRPs and their anchor proteins in the division machinery across plant, fungal, and animal kingdoms (this study; (Schrader, 2006; Schrader and Yoon, 2007). However, given that peroxisomes and mitochondria are functionally linked in many metabolic activities, such as lipid metabolism and photorespiration — two of the major functions involving plant peroxisomes, it is not surprising that the divisions of these two organelles are coordinated at some levels. The peroxisome phenotypes of *fis1a* and the *FIS1b* RNAi mutants are similar to those of the *drp3A* and *drp3B* mutants, consistent with the notion that FIS1 and DRP3 proteins work closely in the same pathway. In mammalian cells hFIS1 and DLP1 physically interact *in vivo* and *in vitro* (Yoon *et al.*, 2003). However, our co-immunoprecipitation (co-IP)

assays using HA-FIS1 and YFP-DRP3 proteins failed to show the co-existence of DRP3A/3B and FIS1A/1B in the same protein complex (data not shown). Using bimolecular fluorescence complementation (BiFC), Lingard *et al.* (2008) did not detect interaction between DRP3A and FIS1A/FIS1B in Arabidopsis cultured cells, either. Thus, it is possible that the interaction between FIS1 and DRP in Arabidopsis is rather transient; alternatively, other proteins may bring DRP3 to FIS1 at the organelle membrane, which would represent a unique feature for plant peroxisomal/mitochondrial fission.

PEX11, DRP, and FIS1 represent conserved members of the peroxisome division machineries. Recently, ternary complexes containing mammalian DLP1, FIS1, and PEX11β were identified through chemical linking methods, which began to link the machineries controlling peroxisome elongation and fission together, suggesting that these three groups of proteins coordinate their functions in peroxisome multiplication (Kobayashi et al. 2007). Lately, a novel tail-anchored membrane protein, Mff, was identified from mammalian cells; this protein promotes the fission of both mitochondria and peroxisomes independently from the FIS1 protein and does not have an obvious homolog in yeast (Gandre-Babbe and van der Bliek, 2008). It is unclear whether a plant homolog of Mff exists. In addition, a number of yeast peroxisomal membrane proteins, such as Pex28p, Pex29p, Pex30p, Pex31p, and Pex32p, which are known to be specifically involved in controlling peroxisome size and abundance by means of largely unknown mechanisms (Thoms and Erdmann, 2005), do not seem to have cognate orthologs in plants. Proteins that mediate peroxisomal membrane constrictions are largely

unidentified in any given species. As such, further genetic and biochemical studies need to be conducted to reveal plant- and peroxisome-specific players in peroxisome division.

Materials and Methods

Plant growth

All plants were in the Columbia-0 (*Col-0*) background and were germinated under 16-h light (60 μE m^{-2} sec^{-1})/8-h dark conditions on 0.6% (w/v) agar plates with ½ Murashige and Skoog basal salt mixture (½MS) supplemented with 1% (w/v) sucrose. After two weeks, plants were transferred to soil and grown under a photosynthetic photon flux density of 70–80 μmol m^{-2} sec^{-1} at 21°C with a 14-h light/10-h dark period. Wild-type plants expressing the *CFP-PTS1* or *YFP-PTS1* transgene (Desai and Hu, 2008; Fan *et al.*, 2005; Orth *et al.*, 2007) were used to visualize peroxisomes in plants.

Generation of constructs and transgenic plants

DNA fragments used for cloning in this study were amplified by PCR using the High-Phusion DNA polymerase according to manufacture's instructions (New England Biolabs Inc.). A standard gateway cloning system (Invitrogen) was used to make the constructs. The Gateway-compatible PCR products of *DRP3B*, *FIS1A*, and *FIS1B* were cloned into binary vectors containing the *attR1-Cm^r-ccdB-attR2* integration region using One-Tube Format Protocol. Constructs and primers used for gateway cloning are listed in Supplemental Figure 3. The resulting constructs were transformed into *A. tumefaciens*

(C58C1) via electroporation. Agrobacteria containing the constructs were later transformed into *CFP-PTS1* or *YFP-PTS1* plants using the floral-dip method (Clough and Bent, 1998). Stable primary transformants were selected on ½ MS medium containing kanamycin (50 μg/ml; for DRP3B-YFP and FIS1-YFP) / glufosinate ammonium (10 μg/mL; Crescent Chemical, Augsburg, Germany, for YFP-FIS1) and gentamycin (60 μg/mL; for CFP-PTS1) and then transferred to soil for further characterization.

Characterization of the T-DNA insertion mutants and generation of *FIS1B* RNAi plants

drp3A-1 (*SALK_008706*), *drp3A-2* (*SALK_147485*), *drp3B-1* (*SALK_045316*), *drp3B-2* (*SALK_112233*) and *fis1A* (*SALK_086794*) seeds were obtained from the ABRC (Ohio State University). Homozygous mutants were identified by PCR analysis of genomic DNA using gene-specific forward (LP) and T-DNA left border primers (LBb1, 5'-GCGTGGACCGCTTGCTGCAACT-3') and gene-specific reverse primer (RP). PCR products were further sequenced to confirm the insertion of the T-DNA in the gene. The primers for genotyping are shown in Supplemental Figure 3. *YFP-PTS1* was expressed in the *drp3a-1*, *drp3a-2*, *drp3b-1*, *drp3b-2* and *fis1a* mutants to visualize peroxisomes. The double mutants *drp3A-1 drp3B-1* and *drp3A-2 drp3B-2* were identified through genotyping from an F_2 generation from crosses between the single mutants.

Gene-specific primers (listed in Supplemental Figure 3) were used to amplify a 513-bp full-length cDNA fragment of *FIS1B*. The amplified fragment was cloned in pFGC5941 in sense and antisense orientations as described (Orth *et al.*, 2007). The *FIS1B* RNAi construct was transformed into *YFP-PTS1* plants and T_1 plants were screened on ½ MS

agar plates containing 50 μg / mL kanamycin and 10 μg / mL glufosinate ammonium. To make sure that both sense and antisense repeats of *FIS1B* are present, we genotyped T_1 primary transformants using primers upstream (forward) and downstream (reverse) of the insertion sites (Supplemental Figure 3).

Sugar-dependence assay

Seeds of wild type and mutants were plated on ½ MS agar growth medium with or without 1% sucrose, following 4 days of cold treatment. All seeds were allowed to germinate and grow in the dark for 5 days. Five-day-old etiolated seedlings were scanned using an EPSON scanner. Hypocotyl length was then measured using ImageJ (http://rsb.info.nih.gov/ij/). For hypocotyl length measurements, $n>50$, $p<0.05$.

Reverse transcription (RT)-PCR analysis of SALK lines and RNAi lines

Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. First-strand cDNA synthesis was performed using the Invitrogen Reverse Transcriptase, Superscript II (Invitrogen), in a 20-μl standard reaction containing oligo dT primers. PCR amplification was carried out using primers specific for *DRP3A*, *DRP3B*, *FIS1A*, *FIS1B* and *UBQ10* genes (Supplemental Figure 3). PCR (Promega) parameters were: 95°C 2 min, followed by 26 cycles of 95°C 30 s, 54°C 30 s, 72°C 1min, and a final elongation step at 72°C for 10 min. Amplified DNA was run on 0.8% agarose gel.

Immunoblot analysis

Total protein was extracted from leaf discs of 4-week-old plants. Homogenized leaf tissue was kept in 1×SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, boiled for 5 min, and centrifuged for 2 min. The supernatant was run on SDS-PAGE gels and transferred to Immobilon-P membrane for blotting (Millipore Corp., Bedford, MA). Primary antibody used to detect YFP and CFP proteins was a rabbit polyclonal GFP antibody (Santa Cruz Biotechnology, Inc.). The secondary antibody used was goat anti-rabbit IgG (LI-COR Biosciences).

Confocal laser scanning microscopy and image analysis

For in vivo detection of CFP and YFP, Arabidopsis tissue was mounted in water and viewed using a confocal laser scanning microscope (Zeiss Meta 510) to obtain confocal images of fluorescence proteins. To analyze subcellular localizations of FIS1A and FIS1B in mitochondria, leaves were treated with 500 nM MitoTracker Red CMXRos (Mitochondrion-Selective Probes, Invitrogen) according to (Arimura and Tsutsumi, 2002a). We used 458-nm, 514-nm, 543-nm, and 633-nm lasers for excitation of CFP, YFP, MitoTracker, and chlorophyll, respectively. For emission, we used 465-510 nm band pass (CFP), 520-555 band pass (YFP), 560-614 band pass (Mitotracker), and 650 nm long pass (chlorophyll) filters. All images were obtained from single optical sections of 0.14 μm in depth.

We used ImageJ (http://rsb.info.nih.gov/ij/) to measure fluorescence area and count organelle numbers in 50 μm x 50 μm confocal images. Color confocal images from single channels (YFP or MitoTracker) were converted to grayscale in 8-bits. The scale for measurement was based on scale bars on the confocal images. We used manual settings of the Threshold function to designate black pixels (peroxisomes or mitochondria) as objects to be measured or counted, and then the Analyze Particles function for fluorescence area measurements and organelle counting. Organelles that were clumped together without clear boundaries in between, which likely indicates that there were incomplete fissions, were treated as a single organelle. The counting of the organelles was also validated manually. Standard deviations and statistical significance for the data were calculated using the Excel program (Microsoft). For all organelle counting and fluorescence measurements, $n>8$, $p<0.05$.

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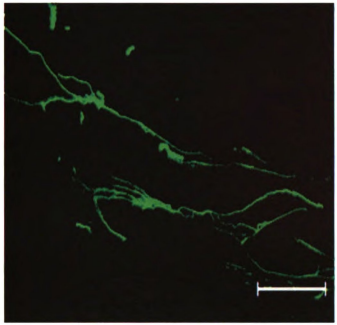

Supplemental Figure 2.9 Elongated peroxisomes in the *pdd1* mutant root cell. Green signals are YFP-PTS1-labelled peroxisomes. Scale bar = 20 μm.

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Chapter 3 FISSION1A and FISSION1B proteins mediate the fission of peroxisomes and mitochondria in Arabidopsis

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Abstract

Peroxisomes and mitochondria are metabolically diverse organelles that act in concert in a number of pathways in eukaryotes, including photorespiration and lipid mobilization in plants. The division machineries of these two types of organelles also share several components such as dynamin-related proteins (DRPs) and their organelle anchor, the FISSION1 (FIS1) protein. In Arabidopsis, members of the DRP3 and FIS1 small protein families, namely, DRP3A, DRP3B, FIS1A, and FIS1B, are each dual-targeted to peroxisomes and mitochondria and are required for the division of both organelles; DRP3A and DRP3B play partially overlapping roles. To further determine the contribution of FIS1A and FIS1B to the division of peroxisomes and mitochondria, we analyzed plants overexpressing *FIS1A* and *FIS1B* and mutants in which the functions of both proteins are disrupted. Domains in FIS1A and FIS1B required for peroxisomal targeting were also dissected. Our results demonstrate that FIS1A and FIS1B play rate-limiting and partially redundant roles in promoting the fission of peroxisomes and mitochondria. Furthermore, although the C-terminal half of the FIS1 proteins is both necessary and sufficient for targeting these proteins to peroxisomes, the role of the extreme C-terminal end adjacent to the transmembrane domain may differ among diverse species in peroxisomal targeting.

Introduction

Peroxisomes are ER-derived and single-membrane eukaryotic organelles that mediate a variety of oxidative metabolic pathways (Beevers, 1979; Titorenko and Mullen, 2006; Van den Bosch et al., 1992). Plant peroxisomes play essential roles in many developmental and physiological processes such as embryogenesis, oilseed germination, photorespiration, jasmonate biosynthesis, and metabolism of nitrogen and indole-butyric acid (Hayashi and Nishimura, 2003; Nyathi and Baker, 2006; Olsen and Harada, 1995; Reumann and Weber, 2006; Zolman et al., 2000). Peroxisomes are also called "organelles at the crossroads", because during metabolism they often act in concert with other subcellular compartments within close physical proximity. For example, peroxisomes (glyoxysomes) in germinating oilseed seedlings interact with oil bodies and mitochondria and act coordinately with these two organelles during lipid mobilization; fatty acid β-oxidation and the glyoxylate cycle are crucial steps in the process, both taken place inside peroxisomes. In addition, leaf peroxisomes are also physically and functionally associated with chloroplasts and mitochondria during photorespiration through the glycolate recycling pathway, (Beevers, 1979).

Peroxisomes are highly dynamic, capable of changing their complement, shape, and abundance in response to developmental and metabolic stimuli (Purdue and Lazarow, 2001). In plants, the abundance of peroxisomes can vary in response to environmental signals (de Felipe et al., 1988; Ferreira et al., 1989; Oksanen et al., 2003; Palma et al., 1991). Recently, a phytochrome A-dependent signaling pathway was shown to mediate the light-induced proliferation of peroxisomes in Arabidopsis seedlings (Desai and Hu,

2008). Plant peroxisomes, like their counterparts in animals and fungi, can multiply by division through several partially overlapping steps, namely, organelle elongation, membrane constriction, and fission (Fagarasanu et al., 2007; Yan et al., 2005). To dissect signaling pathways underlying the control of peroxisome abundance under various environmental influences in plants, we need to first identify constituents of the machinery that controls the division and multiplication of these organelles.

In Arabidopsis, the first step of peroxisome division, i.e., peroxisome elongation, is promoted by a five-member family of peroxisomal membrane proteins called PEROXIN11 (PEX11). Each AtPEX11 isoform, PEX11a to PEX11e, is able to induce peroxisome elongation and number increase (Lingard and Trelease, 2006; Nito *et al.*, 2007; Orth *et al.*, 2007). Despite our lack of knowledge of their precise biochemical function, PEX11 orthologs in diverse species play largely conserved roles (Fagarasanu et al., 2007; Thoms and Erdmann, 2005). In support of this, Arabidopsis PEX11c and PEX11e partially completed the mutant phenotype of the *pex11* null mutant in *Saccharomyces cerevisiae* (Orth *et al.*, 2007).

A later step in peroxisome division, namely, membrane fission, is governed by at least two types of dual-targeted proteins: dynamin-related proteins (DRPs) and FISSION1 (FIS1), which function coordinately. A subset of DRPs in yeast and animals are involved in the fission of peroxisomes and mitochondria (Hoepfner et al., 2001; Koch et al., 2004; Koch et al., 2003; Kuravi et al., 2006; Li and Gould, 2003; Schrader, 2006; Wilsbach and Payne, 1993) by serving as mechanochemical enzymes and/or signaling GTPases (Hoppins et al., 2007; Koch et al., 2004; Osteryoung and Nunnari, 2003; Praefcke and

McMahon, 2004). Mammalian and yeast FIS1 proteins are C-terminal tail-anchored membrane proteins of peroxisomes and mitochondria, which use their cytoplasmically exposed N-terminal region containing the tetratricopeptide repeat (TPR) domain to interact with the DRPs (James et al., 2003; Kobayashi et al., 2007; Koch et al., 2003; Koch et al., 2005; Kuravi et al., 2006; Mozdy et al., 2000; Stojanovski et al., 2004; Yoon et al., 2003). In Arabidopsis, members of the DRP3 family, DRP3A and DRP3B, regulate peroxisomal fission in a partially redundant manner (Mano et al., 2004; Zhang and Hu, accepted with revision); they are also involved in mitochondrial fission (Arimura et al., 2004; Arimura and Tsutsumi, 2002a; Logan et al., 2004; Mano et al., 2004). The AtFIS1 family also constitutes two isoforms, FIS1A and FIS1B, which facilitate the division of both peroxisomes and mitochondria (Zhang and Hu, accepted with revision; Scott et al., 2006). FIS1B was recently shown to be involved in cell cycle-associated replication of peroxisomes in Arabidopsis cell cultures, whereas FIS1A did not seem to play a role in this process (Lingard et al., 2008).

Yeast and mammals each have a single FIS1 protein (James et al., 2003; Kobayashi et al., 2007; Koch et al., 2003; Koch et al., 2005; Kuravi et al., 2006; Mozdy et al., 2000; Stojanovski et al., 2004; Yoon et al., 2003), whereas Arabidopsis contains two FIS1 variants. Our previous study showed that both FIS1A and FIS1B are dual-targeted to peroxisomes and mitochondria. In addition, T-DNA insertion mutant of *FIS1A* and RNAi lines of *FIS1B* both showed growth inhibition and contained peroxisomes and mitochondria with incomplete fission, enlarged size, and number decrease (Zhang and Hu, accepted with revision). To further determine whether FIS1A and FIS1B each play specific roles in the fission of peroxisomes and mitochondria, we analyzed Arabidopsis

plants ectopically expressing *FIS1A* or *FIS1B* and mutants in which the functions of both FIS1A and FIS1B are disrupted. We also dissected FIS1A and FIS1B to determine domains crucial for peroxisomal targeting, in order to compare targeting mechanisms utilized by FIS1 orthologs in plants and mammals.

Results

Ectopic expression of FIS1A and FIS1B leads to an increase in peroxisomal and mitochondrial abundance

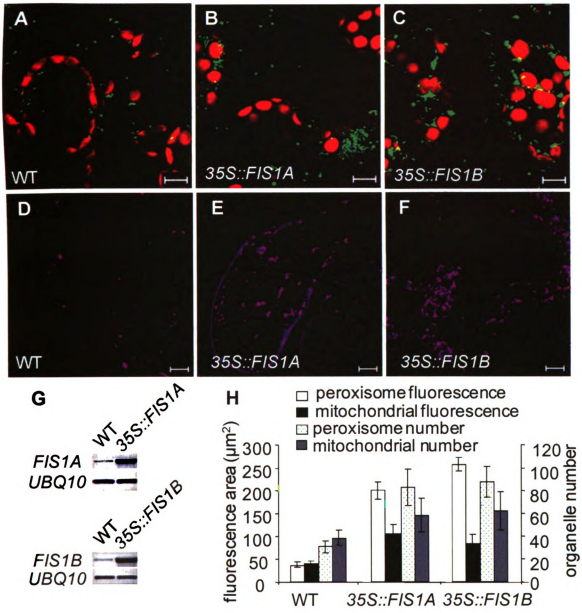
In our previous study of FIS1 localization, overexpression of YFP-FIS1A or YFP-FIS1B appeared to cause an increase in the number of peroxisomes and mitochondria, as well as aggregation of these organelles (Zhang and Hu, accepted with revision). These results suggest a role for FIS1A and FIS1B as limiting factors in peroxisomal and mitochondrial division. However, we could not exclude the possibility that these phenotypes were rendered by a dominant negative effect of FIS1 proteins tagged with YFP, as YFP proteins on the surface of the organelles may interact with each other and interfere with the proper function of FIS1. To unequivocally determine the contribution of FIS1A and FIS1B to peroxisomal and mitochondrial fission and to see whether or not these two proteins have distinct functions in promoting organelle division, we generated plants expressing untagged FIS1A or FIS1B under the control of the 35S promoter (*35S::FIS1*). To visualize morphological changes of peroxisomes, we used plants containing the peroxisomal marker CFP-PTS1 (PEROXISOME TARGETING SIGNAL TYPE 1; a tripeptide consisting of Ser-Lys-Leu), which were generated and characterized in the lab

Figure 3.1 Overexpression of *FIS1A* and *FIS1B* increases the fission of peroxisomes and mitochondria.

(A-F) Confocal laser scanning microscopic images of leaf mesophyll cells **(A-C)** and leaf epidermal cells **(D-F)** from 4-week-old Arabidopsis plants expressing CFP-PTS1. In **(A-C)**, green signals indicate CFP-PTS1-labelled peroxisomes; red signals indicate chloroplasts. In **(D-F)**, fluorescent signals represent MitoTracker-stained mitochondria. Scale bars = 10 μm.

(G) RT-PCR analysis of RNA extracted from the respective *FIS1*-overexpressing plants.

(H) Quantification of total fluorescence (CFP or MitoTracker) and organelle (peroxisome or mitochondrial) number per 2500 μm^2 of the cells ($n=10$, $p<0.05$).


A
B
C
WT
35S::FIS1A
35S::FIS1B
D
E
F
WT
35S::FIS1A
35S::FIS1B
G
WT
35S::FIS1A
FIS1A
UBQ10
WT
35S::FIS1B
FIS1B
UBQ10
H
peroxisome fluorescence
mitochondrial fluorescence
peroxisome number
mitochondrial number
fluorescence area (μm²)
organelle number
300
250
200
150
100
50
0
120
100
80
60
40
20
0
WT
35S::FIS1A
35S::FIS1B

in previous studies (Fan et al., 2005; Orth et al., 2007), as the background for transformation.

We obtained 43 transgenic plants containing the *35S::FIS1A* transgene and 47 plants expressing the *35S::FIS1B* transgene. After RT-PCR and confocal laser scanning microscopic (CLSM) analyses of a subset of the transgenic plants, we selected two representative lines from the T_3 generation for detailed imaging analysis. Both *FIS1A*- and *FIS1B*-overexpressing plants displayed markedly increased peroxisomal abundance; peroxisomal aggregation was more evident in the *FIS1B*-overexpressing plants (Figure 1, a-c). To quantify this increase, we used ImageJ software to measure the area of CFP fluorescence and the number of peroxisomes. 2500 μm^2/cell from 10 confocal microscopic images obtained from each genotype was used for the measurements. The area of CFP fluorescence in plants overexpressing *FIS1A* or *FIS1B* increased to approximately 5-6 times from that of the wild-type CFP-PTS1 plant (Figure 1g). Likewise, the number of peroxisomes also increased to about 3 fold in the *FIS1*-overexpressing plants compared with the wild type (Figure 1g).

We used the mitochondrial dye MitoTracker to stain leaf cells of the transgenic plants and subsequently confocal microscopy to examine changes in mitochondria. A significant increase in mitochondrial abundance was also shown in the *FIS1*-overexpressing plants (Figure 1, d-f). Quantification analysis showed a 1.5- to 2-fold increase in the area of MitoTracker fluorescence and the number of mitochondria in the transgenic plants compared with the wild type, although these increases were not as dramatic as those seen in peroxisomes (Figure 1g).

Despite having a strong induction of peroxisomal and mitochondrial volume (measured by fluorescence area), plants ectopically expressing *FIS1A* or *FIS1B* did not exhibit obvious differences in appearance from the wild type under normal growth conditions, nor did they have distinct germination or growth rate while germinating on agar plates supplemented with or without sucrose (data not shown). Thus, although elevated levels of *FIS1A* or *FIS1B* gave rise to significant increases in the abundance of peroxisomes and mitochondria, they did not cause obvious physiological changes to the plants.

FIS1A and FIS1B are partially redundant in promoting organelle fission

Single *fis1* mutants, whose transcripts of *FIS1A* or *FIS1B* were undetectable by RT-PCR, showed similar phenotypes, that is, they were slightly smaller than the wild-type plants and contained peroxisomes and mitochondria that were enlarged in size, reduced in number, and clustered together (Zhang and Hu, accepted with revision). These findings indicate that the two homologous proteins FIS1A and FIS1B are not completely redundant in function and may each carry some unique roles in organelle fission. To test this hypothesis, a *fis1A fis1B* double mutant was needed. A T-DNA insertion mutant of *FIS1A* (SALK_086794) was characterized in previous studies and found to contain undetectable levels of the *FIS1A* mRNA (Scott et al., 2006; Zhang and Hu, accepted with revision), whereas a *fis1B* knockout mutant was unavailable. To this end, we created *fis1A fis1B* double mutants by using RNAi to silence *FIS1B* in the *fis1A* mutant background. The *FIS1B* RNAi construct was generated in our previous study and was

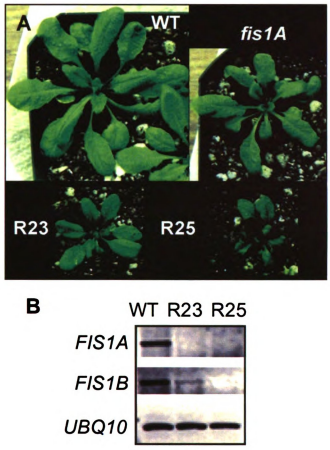


Figure 3.2 Plant phenotype of *fis1* mutants.

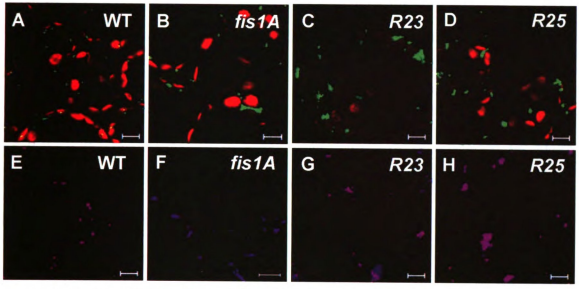
(A) Plants grown for 3 weeks. R23 and R25 are *fis1A* plants in which the *FIS1B* gene is also silenced.

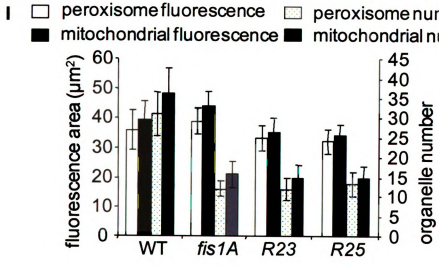
(B) RT-PCR analysis of RNA from R23 and R25.

Figure 3.3 Peroxisomal and mitochondrial phenotypes of the *fis1* mutants.

(A-H) Confocal micrographs of leaf mesophyll cells **(A-D)** and leaf epidermal cells **(E-H)** from 6-week-old wild-type and *fis1* mutant plants. All plants express the *YFP-PTS1* peroxisomal marker gene. R23 and R25 are *fis1A* plants in which the expression of *FIS1B* is also reduced. In **(A-D)**, green signals indicate YFP-PTS1; red signals are chloroplasts. In **(E-H)**, fluorescent signals represent mitochondria stained by MitoTracker. Bars = 10 μm.

(I) Quantification of total YFP or MitoTracker fluorescence area and the number of peroxisomes or mitochondria within 2500 μm^2 of the cells ($n>8$, $p<0.05$).


A WT
B fis1A
C R23
D R25
E WT
F fis1A
G R23
H R25


I
peroxisome fluorescence
peroxisome number
mitochondrial fluorescence
mitochondrial number
fluorescence area (μm²)
organelle number
0
10
20
30
40
50
60
0
5
10
15
20
25
30
35
40
45
WT
fis1A
R23
R25

proved to be effective in reducing *FIS1B* expression; *fis1A* mutant expressing the YFP-PTS1 peroxisomal marker was also generated in the same study (Zhang and Hu, accepted with revision). 14 transgenic *fis1A* plants containing the full-length *FIS1B* RNAi transgene and showing various levels of *FIS1B* expression were obtained; two lines (R23 and R25) with strong reduction in *FIS1B* gene expression were selected for future analysis. R23 and R25 exhibited stronger growth inhibition than the *fis1A* single mutant (Figure 2a). RT-PCR analysis confirmed that the *FIS1B* gene was significantly silenced in these two lines (Figure 2b).

We previously showed that although the number of peroxisomes in the *fis1A* and *fis1B* single mutants was decreased, the total volume of these organelles, as measured by fluorescence area, remained largely constant from the wild type to the mutants (Zhang and Hu, accepted with revision). These data suggest that plants seem to be able to compensate for the mild division deficiencies by increasing the size of individual peroxisomes. Confocal microscopic analysis of YFP-PTS1 and MitoTracker signals in the double mutants revealed no major differences in peroxisomal and mitochondrial appearance and number between the double mutants and the *fis1A* single mutant parent (Figure 3). All mutants contained clumped and enlarged peroxisomes and mitochondria (Figure 3, a-h), similar to the phenotype shown in the *fis1A* mutant (Zhang and Hu, accepted with revision; Figure 3). However, quantification data revealed that, although the number of these organelles was largely unchanged, the total volume of peroxisomes and mitochondria was slightly lower in the double mutants than in the *fis1A* single mutant (Figure 3i). Hence, the plant growth and organelle phenotypes collectively led us to

conclude that FIS1A and FIS1B have overlapping and unique functions in controlling the division of mitochondria and peroxisomes.

Targeting of FIS1 proteins to peroxisomes

The C-terminal tail (aa 92-152) of hFIS1 is both necessary and sufficient for targeting this protein to peroxisomes and mitochondria in human cells (Koch et al., 2005). Furthermore, the transmembrane domain (TMD) along with a short basic segment at the C-terminal end is essential for mitochondrial and peroxisomal targeting of hFIS1 (Koch et al., 2005; Stojanovski et al., 2004). In our previous study, we demonstrated in Arabidopsis that FIS1A and FIS1B, when tagged with YFP at N-terminus (YFP-FIS1), were dual-targeted to peroxisomes and mitochondria, whereas FIS1 proteins with YFP fused to the C-terminus (FIS1-YFP) were mainly diffused in the cytosol and the nucleus, supporting the notion that the C-terminal end of the FIS1 proteins is important for proper organelle targeting in plants as well (Zhang and Hu, accepted with revision). In light of these findings, questions arose as to what specific signals in the same FIS1 protein are being recognized by the different targeting machineries of peroxisomes and mitochondria, and whether FIS1 orthologs in diverse species utilize similar targeting mechanisms. As a first step toward answering these questions, we determined regions in the AtFIS1 proteins sufficient for peroxisomal targeting and tested whether the short basic segment downstream from TMD is also essential for peroxisomal targeting in plants. Here we chose to focus on the peroxisomal aspect of targeting due to our primary interests in this organelle.

Figure 3.4 Sequence alignment of FIS1 proteins and immunoblot analysis of truncated FIS1 proteins expressed in tobacco leaves.

(A) Alignment of Arabidopsis FIS1A and FIS1B and the human FIS1 proteins. The putative transmembrane domain (TMD) is underlined. Domains used in some of the truncation constructs are: FIS1A^{NT}, aa 1-85; FIS1A^{CT}, aa 86-170; FIS1B^{NT}, aa 1-101; FIS1B^{CT}, aa 102-167; FIS1A^{TMD+CE}, aa 139-170; FIS1B^{TMD+CE}, aa 141-167. The boxed region indicates TMD+CE (extreme C-terminal end).

(B) Immunoblot analysis of proteins extracted from tobacco leaves co-expressing YFP-fusions of truncated FIS1 proteins and the peroxisomal marker protein CFP-PTS1. Proteins were detected by the α-GFP antibody. Samples are: lane 1, protein marker; lane 2, tissue expressing CFP-PTS1 only; lanes 3-10, leaves co-expressing CFP-PTS1 and YFP-FIS1A^{NT}, YFP-FIS1A^{CT}, YFP-FIS1B^{NT}, YFP-FIS1B^{CT} , YFP-FIS1A^{TMD+CE}, YFP-FIS1B^{TMD+CE}, YFP-FIS1A$^{\Delta 167-170}$, and YFP-FIS1B$^{\Delta 166-167}$, respectively. Asterisks on the left of the protein bands point to the corresponding YFP-FIS1 fusions. The arrow indicates position of the CFP-PTS1 protein band.

A

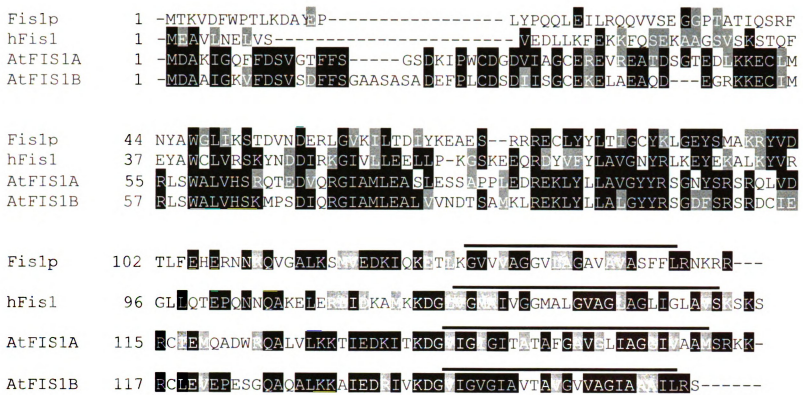

Fis1p 1 -MTKVDFWPTLKDAYEP---------- LYPQQLEILRQQVVSEGGPTATIQSRF
hFis1 1 -MEAVLNELVS---------- VEDLLKFEKKFQSEKAAGSVSKSTQF
AtFIS1A 1 -MDAKIGQFFDSVGTFFS----- GSDKIPWCDGDVIAGCEREVREAIDSGFEDLKKECIM
AtFIS1B 1 -MDAAIGKVFDSVSDFFSGAASASADEFPLCDSDIISGCEKELAEAQD---EGRKKECIM
Fis1p 44 NYAWCLIKSTDVNDERLGVKILTDIYKEAES--RRRECLYYLTIGCYKLGEYSMAKRYVD
hFis1 37 EYAWCLVRSKYNDDIRMGIVLLEELLP-KGSKEEQRDYVFYLAVGNYRLKEYEKALKYVR
AtFIS1A 55 RLSWALVHSRQTEDVQRGIAMLEASLESSAPPLEDREKLYLLAVGYYRSGNYSRSRQLVD
AtFIS1B 57 RLSWALVHSKMPSDIQRGIAMLEALWVNDTSAVKLREKLYLLALGYYRSGDFSRSRDCIE
Fis1p 102 TLFEHERNNKQVGALKSWEDKIQKSTLKGVVVAGGVLAGAVLWASFFLRNKRR---
hFis1 96 GLLQTEPQNNQAKELEKNIEKAVKKDGDWGKAIVGGMALGVAGAGIGLAMSKSKS
AtFIS1A 115 RCLEAQADWKQALVLKKTIEDKITKDGGWIGGITATAPGAVGLIAGSIVAAMSRKK-
AtFIS1B 117 RCLEVEPESGQAQALKKAIEDKIVKDGGWIGVGIAVTAAGVVAGIAVILRS------

Figure 3.4 continued

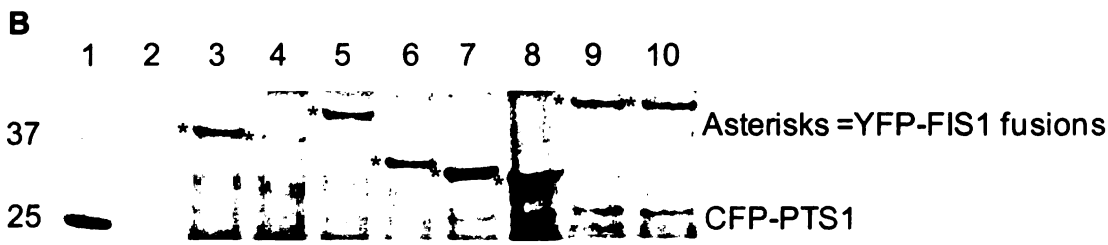


Figure 3.5 C-terminus of FIS1A and FIS1B is sufficient for peroxisomal targeting.

Confocal images were taken from leaf epidermal cells of 4-week-old tobacco plants co-infiltrated with Agrobacteria containing CFP-PTS1 and YFP-FIS1 truncations. Bars = 10 μm.

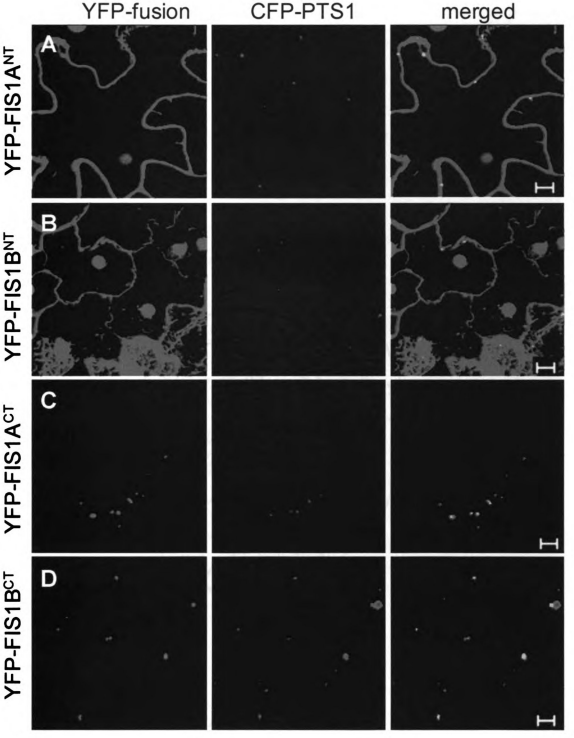
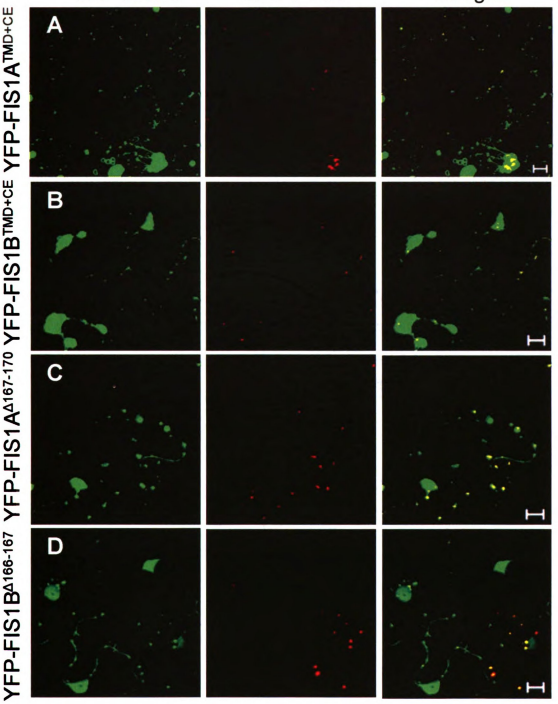

YFP-fusion
CFP-PTS1
merged
A
YFP-FIS1A^{NT}
B
YFP-FIS1B^{NT}
C
YFP-FIS1A^{CT}
D
YFP-FIS1B^{CT}

Figure 3.6 Analysis of the role for TMD and the C-terminal end of FIS1A and FIS1B in peroxisomal targeting.

Shown are confocal images of 4-week-old tobacco leaf epidermal cells co-expressing CFP-PTS1 and YFP-fusions of FIS1 truncations. Bars = 10 μm.


YFP-fusion
CFP-PTS1
merged
A
YFP-FIS1A^{TMD+CE}
B
YFP-FIS1B^{TMD+CE}
C
YFP-FIS1A$^{\Delta 167-170}$
D
YFP-FIS1B$^{\Delta 166-167}$

Arabidopsis FIS1A and FIS1B proteins are 58% identical in amino acid sequence and contain predicted molecular weight of 18.7 and 17.9 kDa, respectively. They share approximately 28% sequence identity with the 17-kDa human hFIS1 protein; an alignment of the Arabidopsis and human FIS1 proteins revealed strong conservation at the C-terminus, especially in the putative TMD (Figure 4a). Basic residues are also found to flank TMD at the 3' end of the Arabidopsis FIS1 proteins: FIS1A has an arginine and two lysines (diK motif), whereas FIS1B only contains an arginine (Figure 4a). To determine sequences in the AtFIS1 proteins sufficient for peroxisomal targeting, we expressed in tobacco leaf epidermal cells a series of truncated FIS1A and FIS1B proteins, all of which were attached to the C-terminus of YFP. *35S* promoter-driven truncated *FIS1* constructs used in the analysis included: *YFP-FIS1A/B*^{NT} (N-terminal half), *YFP-FIS1A/B*^{CT} (C-terminal half), YFP-FIS1A/B^{TMD+CE} (C-terminus including TMD and the adjacent C-terminal end), YFP-FIS1A$^{\Delta 167\text{-}170}$, and YFP-FIS1B$^{\Delta 166\text{-}167}$. Two days after inoculation of the *FIS1* truncation constructs combined with *CFP-PTS1* via Agrobacterial infiltration, expression of the YFP-fusion proteins in tobacco leaves were confirmed by immunoblot analysis using α–GFP antibodies (Figure 4b).

Co-localization of the YFP-fusion proteins with the peroxisomal marker CFP-PTS1 was tested by confocal microscopy. Both YFP-FIS1A^{NT} and YFP-FIS1B^{NT} were present in the nucleus, cytosol, and possibly on the plasma membrane (Figure 5, a-b), re-affirming that signals required for peroxisomal targeting do not reside in the N-terminal regions of the FIS1 proteins. In contrast, YFP-FIS1A^{CT} and YFP-FIS1B^{CT} showed punctate fluorescent signals largely co-localized with CFP-PTS1. However, increases in peroxisome number and aggregation, phenotypes caused by overexpressing YFP-FIS1A

and YFP-FIS1B proteins (Zhang and Hu, accepted with revision), did not occur in cells overexpressing YFP-FIS1^{CT}. These results are consistent with the notion that the C-terminus of FIS1 proteins is necessary and sufficient for peroxisomal targeting but is insufficient to confer protein function in promoting peroxisomal fission, in other words, the N-terminus is required for proper protein function (Koch et al., 2005).

YFP-FIS1A^{TMD+CE} constructs were used to further delineate domains in the C-terminus of the FIS1 proteins sufficient for peroxisomal targeting. YFP-FIS1A^{TMD+CE} contained the last 32 aa of FIS1A, whereas YFP-FIS1B^{TMD+CE} contained the last 27 aa of FIS1B (Figure 4a). These two fusion proteins targeted to peroxisomes and to structures characteristic of the nucleus and plasma membrane (Figure 6, a-b). Thus, the TMD domain and its 3' flanking sequences seem to contain major signals required for peroxisomal targeting, but other sequences outside this region are also needed for efficient and accurate targeting to peroxisomes.

The short C-terminal segment adjacent to the TMD was shown to be essential to peroxisomal and mitochondrial targeting of hFIS1 (Koch et al., 2005; Yoon et al., 2003). To determine whether the same region in AtFIS1 is also required for peroxisomal targeting in plants, we deleted this short stretch of sequence from the C-terminal end of the AtFIS1 proteins: YFP-FIS1A$^{\Delta 167\text{-}170}$ was deleted for the last four amino acids (SRKK) of FIS1A and YFP-FIS1B$^{\Delta 166\text{-}167}$ was deleted for the last two amino acids (RS) of FIS1B. Both proteins were largely targeted to small and spherical structures, many of which overlapped with CFP-PTS1, although localization to structures characteristic of the nucleus and plasma membrane was also evident (Figure 6, c-d). These data suggest that

the diK motif and other basic residues at the C-terminal end of Arabidopsis FIS1 are involved but not critical in the peroxisomal targeting of these proteins. As such, the role of the C-terminal segment adjacent to TMD may differ from plants to mammals in targeting FIS1 to peroxisomes.

Discussion

Overexpressing myc-hFIS1 in human COS-7 cells led to a dramatic increase in the number of small and punctiform peroxisomes and a pronounced fragmentation of mitochondria (Koch et al., 2005; Yoon et al., 2003). Similarly, elevating levels of At*FIS1A* and *FIS1B* also significantly increased the number of peroxisomes and mitochondria in plants (Figure 1). Thus, the function of FIS1 orthologs in the division of peroxisomes and mitochondria is well conserved in diverse species. In contrast to the dramatically elongated peroxisomes displayed in plants overexpressing each of the five Arabidopsis PEX11 proteins (Orth et al., 2007), plants overexpressing *FIS1A* or *FIS1B* primarily show completely divided and sometimes clumped peroxisomes. This fits with the model that PEX11 proteins are responsible for the initial step of peroxisome division, i.e., peroxisome elongation, whereas FIS1 proteins are mediating a later step in the process, namely, peroxisome fission. Both PEX11 and FIS1 proteins are apparently limiting factors in the division process: PEX11's role is restricted to peroxisomes, whereas FIS1 proteins perform dual functions.

Plants ectopically expressing FIS1A and FISB show slightly distinct peroxisome phenotypes, that is, peroxisomes in *FIS1A*-overexpressors tend to be more completed in

fission than those in plants overexpressing *FIS1B* (Figure 1). This difference may reflect distinct roles of these two proteins in peroxisome fission. Consistent with this view is the fact that single mutants of *FIS1A* or *FIS1B* each have deficiency in peroxisomal (and mitochondrial) fission and are inhibited in growth (Zhang and Hu, accepted with revision; this study). It is likely that each Arabidopsis FIS1 isoform may interact with specific downstream effector proteins such as DRPs in mediating the division of peroxisomes and mitochondria. Difference in the function of FIS1A and FIS1B have also been shown in Arabodopsis suspension cultured cells, whereby FIS1B but not FIS1A was shown to have a role in cell cycle-associated peroxisome replication (Lingard et al., 2008).

It is surprising that our double mutants only show slightly stronger phenotypes than *fis1A* or *fis1B* single mutants (this study; Zhang and Hu, accepted with revision), given that the expression of both genes were greatly reduced in these plants. It is likely that other proteins with little sequence identity with FIS1 perform similar functions on the membrane of these two types of organelles.

Human cells with reduced levels of *hFIS1* contained elongated and segmented peroxisomes that have been constricted but not separated (Koch et al., 2005). However, Arabidopsis mutants in which the functions of FIS1A, FIS1B, or both, are disrupted are not elongated but rather enlarged (Zhang and Hu, accepted with revision; this study). Similarly, *hFIS1* RNAi cells displayed extended mitochondrial tubules, whereas these organelles in the *fis1* mutants in Arabidopsis are mostly enlarged in size (Zhang and Hu, accepted with revision; Figure 3 of this study). This difference in peroxisomal and

mitochondrial morphology in the mutants may reflect distinct mechanisms utilized by diverse species in coping with deficiencies in organelle division.

Although FIS1 orthologs in different organisms exert conserved functions in peroxisomal fission, targeting signals in these proteins seem to be less conserved. For example, signals sufficient for peroxisome targeting reside in the C-terminal half of the FIS1 protein in both mammals and plants, yet the exact regions to which these signals are restricted may differ. The last 26 amino acids of hFIS1 were successfully targeted to peroxisomes and mitochondria (Koch et al., 2005), whereas AtFIS1 proteins containing the corresponding domain plus a few extra amino acids upstream (i.e., YFP-FIS1^{TMD+CE}) not just target to peroxisomes but also localize to the nucleus and plasma membrane (Figure 6). In addition, hFIS1 protein lacking the last five amino acid at the C-terminal end (SKSKS; Figure 4a) were diffused in the cytosol and failed to localize to peroxisomes (Koch et al., 2005). In contrast, AtFIS1 proteins missing the corresponding segment at the extreme C-terminus are still largely localized to peroxisomes (Figure 6), suggesting that this small region is not essential for peroxisomal targeting in Arabidopsis. Previous studies of hFIS1 protein showed that the two lysine residues (diK motif; Figure 4a) at the extreme C-terminus are required for mitochondrial targeting, as replacing both lysine residues with alanines led to mis-targeting of the hFIS1 protein to the ER (Stojanovski et al., 2004). Given the targeting pattern of the C-terminal end-deleted Arabidopsis FIS1 proteins in our study, we predict that this C-terminal segment may not be essential for mitochondrial targeting in plants, either. More detailed dissection of the C-terminal region is required to precisely locate residues required for FIS1 targeting to peroxisomes vs. mitochondria in plants, since such information cannot be accurately derived from

studies of FIS1 orthologs in other kingdoms. In fact, targeting mechanism may even differ in different research systems of the same organism. For example, on their own, neither myc-FIS1A nor myc-FIS1B was able to target to peroxisomes labeled by α–catalase antibodies in Arabidopsis suspension cell cultures (Lingard et al., 2008).

Peroxisomes and mitochondria, two subcellular compartments with different evolutionary origins, distinct structures, and unique metabolic function, share the same DRP and FIS1 proteins in their division machines. This fact may bear some physiological significance. Given that plant peroxisomes and mitochondria act in collaboration in two of the most important physiological processes in plants, namely, lipid metabolism and photorespiration (Beevers, 1979), it is likely that these organelles also coordinate to some degrees in multiplication in order to carry out these collaborative processes to successful completion.

Taken together, our analysis of gain- and loss-of-function mutants of the Arabidopsis *FIS1A* and *FIS1B* genes and peroxisomal targeting analysis of truncated FIS1 proteins have revealed that FIS1 orthologs in diverse species contain conserved as well unique features in their targeting mechanism and in their role in mediating the fission of peroxisomes and mitochondria. In order to uncover additional and plant-specific features of organelle division, we need to perform further forward genetic and biochemical screens to identify novel components of the division machines.

Materials and Methods

Plant Growth

Seedlings (all in *Col-0* background) were germinated under 16h light (60 $\mu E\ m^{-2}\ sec^{-1}$)/8h dark cycles and 21°C on plates containing 0.6% (w/v) agar, ½ Murashige and Skoog salt mixture (½MS), and 1% (w/v) sucrose. 2w plants were transferred to soil and grown under a photosynthetic photon flux density of 70–80 $\mu mol\ m^{-2}\ sec^{-1}$ at 21°C with 14h light/10h dark cycles. The wild-type plants expressing the *YFP-PTS1* or *CFP-PTS1* transgene were generated from previous studies (Desai and Hu, 2008; Fan et al., 2005; Orth et al., 2007). The *fis1A* mutant was characterized in a previous study (Zhang and Hu, accepted with revision).

Construct generation and plant transformation

We used the proofreading High-Phusion DNA polymerase to amplify DNA fragments used for cloning, with conditions suggested by the manufacturer (New England Biolabs Inc.). Primers used to amplify The *FIS1* genes were: FIS1A forward GGGGTACC ATGGATGCTAAGATC and reverse ACGCGTCGACTCATTTCTTGCGAGAC; and FIS1B forward GGGGTACCATGGACGCGGCGATAG and reverse ACGCGTCGACTTAGCTGCGTAATATG. The PCR products were digested by *Kpn*I and *Sal*I and individually cloned into a binary vector containing the 35S promoter. The *FIS1B* RNAi construct was made in a previous study (Zhang and Hu, accepted with revision).

Standard gateway cloning system (Invitrogen) was used to make the *FIS1* truncation constructs. The Gateway-compatible PCR products of *FIS1* truncations were cloned into binary vectors containing *YFP-attR1-Cm^r-ccdB-attR2* integration region using One-Tube Format Protocol. Primers used in PCR amplifications are as follows:

YFP-FIS1A^{NT}:
Forward
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGATGCTAAGATCGG,
Reverse GGGGACCACTTTGTACAAGAAAGCTGGGTGTCAAGGGG
CACTGCTTTC.

YFP-FIS1A^{CT}:
Forward GGGGACAAGTTTGTACAAAAAAGCAGGCTTC ATG
CCATTGGAGGACCG,
Reverse
GGGGACCACTTTGTACAAGAAAGCTGGGTGTCATTTCTTGCGAGACATCGC.

YFP-FIS1B^{NT}:
Forward
GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGGACGCGGCGATAGGG,
Reverse GGGGACCACTTTGTACAAGAA AGCTGGGTG
TTATCTTGAAAAGTCACC.

YFP-FIS1B^{CT}:
Forward GGGGACAAGTTTGTACAAAAAAGCAGGCTTTC
ATGAGCCGGGATTGTAT,
Reverse
GGGGACCACTTTGTACAAGAAAGCTGGGTGTTAGCTGCGTAATATGGCTGC.

YFP-FIS1A^{TMD+CE}:
Forward
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAAGGATGGTGTTATAG GG,
Reverse
GGGGACCACTTTGTACAAGAAAGCTGGGTGGCTTGCATGCCTGCAGGTCC.

YFP-FIS1B^{TMD+CE}:
Forward
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAAAGATGGTG TGATTGGC,

Reverse
GGGGACCACTTTGTACAAGAAAGCTGGGTGGCTTGCATGCCTGCAGGTCC.

YFP-FIS1A$^{\Delta 167\text{-}170}$:
Forward
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGATGCTAAGATCGG,
Reverse GGGGACCACTTTGTACAAGAAAGCTGGGTTTTACATCGCTG
CTACGATACC.

YFP-FIS1B$^{\Delta 167\text{-}168}$:
Forward
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGACGCGGCGATAGGG
Reverse
GGGGACCACTTTGTACAAGAAAGCTGGGTTTTATAATATGGCTGCAGC
AATAC.

The resulting constructs were transformed into *A. tumefaciens* (C58C1) via electroporation. We used the floral-dip method (Clough and Bent, 1998) to transform the *35S::FIS1A/1B* constructs into wild-type plants already expressing CFP-PTS1, and to transform the *FIS1B* RNAi construct (Zhang and Hu, accepted with revision) into *fis1A* mutants already expressing YFP-PTS1 (Zhang and Hu, accepted with revision). Stable primary transformants were selected on ½ MS medium containing kanamycin (50 μg/ml; for *35S::FIS1*) combined with gentamycin (60 μg/mL; for CFP-PTS1) to select for FIS1 overexpressing plants, and kanamycin (50 μg/ml; for YFP-PTS1) plus glufosinate ammonium (10 μg/mL; Crescent Chemical, Augsburg, Germany, for *FIS1B* RNAi) to select for *fis1A* mutants containing the *FIS1B* RNAi transgene. For tobacco infiltration, Agrobacteria containing the YFP-fusion constructs were co-infiltrated with *CFP-PTS1* in leaves of four-week-old *Nicotiana tabacum* (cv. Petit Havana) plants grown at 25°C (Goodin et al., 2002). Method for identification of plants in which *FIS1B* is silenced is described previously (Zhang and Hu, accepted with revision).

Reverse transcription (RT)-PCR analysis of overexpression and RNAi lines

Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen) using protocols suggested by the manufacturer. First-strand cDNA was synthesized using the Invitrogen Reverse Transcriptase, Superscript II (Invitrogen). PCR amplification was carried out using the following gene-specific primers: FIS1A (At3g57090) forward ATGGATGCTAAGATCGGACAATTC, reverse GCGAGACATCGCTGCTACGATA CC; FIS1b (At5g12390) forward ATGGACGCGGCGATAGGGAAGGT, reverse GCTGCGTAATATGGCTGCAGCAA; *UBQ-10* (At4g05320) forward TCAATTCTCTCTACCGTGATCAAGATGCA, reverse GGTGTCAGAACTCTC CACCTCAAGAGTA. PCR conditions were: 95°C 2 min, 26 cycles of 95°C 30 s, 54°C 30 s, 72°C 1min, and a final elongation step at 72°C for 10 min. Amplified DNA was run on 0.8% agarose gel.

Immunoblot analysis

After 48 hours of Agrobacterial infiltration, we ground tabacum leaf discs in liquid nitrogen and then suspended the leaf powder in 1×SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. The samples were boiled for 5 min followed by centrifugation for 2 min. The supernatant was run on SDS-PAGE gels and transferred to Immobilon-P membrane for blotting (Millipore Corp., Bedford, MA). Primary antibody used to detect YFP and CFP proteins was a rabbit polyclonal GFP antibody (Santa Cruz Biotechnology, Inc.). The secondary antibody was goat anti-rabbit IgG (LI-COR Biosciences).

Confocal laser scanning microscopy and organelle quantification

Confocal laser scanning microscopes (Zeiss Meta 510 or Zeiss Pascal) were used to obtain images of fluorescence proteins in plant cells. To detect YFP and CFP, plant tissue was mounted in water before analysis. For detection of mitochondria, leaves were first treated with 500 nM MitoTracker Red CMXRos (Mitochondrion-Selective Probes, Invitrogen) according to a previous study (Arimura and Tsutsumi, 2002b). Lasers used for fluorophore excitation were: CFP, 458 nm; YFP, 514 nm; MitoTracker, 543 nm, and chlorophyll, 633 nm. For emission, the following filters were used: 465-510 nm band pass for CFP, 520-555 band pass for YFP, 560-614 band pass for MitoTracker, and 650 nm long pass for chlorophyll. All images were acquired from single optical sections.

ImageJ (http://rsb.info.nih.gov/ij/) was used to measure fluorescence area and organelle number in 50 μm X 50 μm of confocal images. Confocal images obtained from YFP or MitoTracker single channels were first converted to grayscale (8-bits). Scale for measurement was set based on scale bar on the confocal images. We used manual settings of the Threshold function to designate objects (organelles) to be measured or counted and the Analyze Particles function to measure fluorescence area and count the number of organelles. Organelles aggregated together without clear separation from each other would be treated as a single one. The Excel program (Microsoft) was used to calculate standard deviations and statistical significance. For all organelle counting and fluorescence measurement shown in Figure 1 and Figure 3, >8 images from each plant were analyzed, $p<0.05$.

Accession numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers: hFIS1, NP 057152; FIS1A, Q9M1J1 (At3g57090); FIS1B, Q94CK3 (At5g12390).

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Chapter 4 The Arabidopsis Chloroplast Division Protein DYNAMIN-RELATED PROTEIN5B also Mediates Peroxisome Division

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Abstract

Peroxisomes are highly dynamic organelles involved in various metabolic pathways. The division of peroxisomes is controlled by the PEROXIN11 (PEX11) proteins that initiate peroxisome elongation, and the dynamin-related proteins (DRPs) and FISSION1 (FIS1) proteins that function together to mediate peroxisome fission that is the late step of peroxisome division. In Arabidopsis, DRP3A/DRP3B and FIS1A/FIS1B are two pairs of homologous proteins known to be shared by peroxisomal and mitochondrial division. Here we report that DRP5B, a DRP distantly related to DRP3 and originally identified as a chloroplast division protein, also contributes to peroxisome division. DRP5B is dual-targeted to peroxisomes and chloroplasts. Mutations in the *DRP5B* gene lead to peroxisome division defects and compromised peroxisome functions. Using bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation (Co-IP) assays, we further demonstrate that DRP5B forms homodimers and interacts with DRP3A, DRP3B, FIS1A, and all five Arabidopsis PEX11 isoforms. Specific interactions among individual DRP, FIS1, and PEX11 proteins occur on peroxisomes and mitochondria, prompting the hypothesis that distinct targeting mechanisms may have been created in plants to recruit DRP5B, DRP3A, and DRP3B to various organelles and that proteins involved in the early and late stages of peroxisome division may act coordinately.

Introduction

Peroxisomes are ubiquitous eukaryotic organelles that participate in diverse metabolic functions. In plants, these single membrane-bound subcellular structures are involved in biochemical and physiological processes such as photorespiration, fatty acid metabolism, hydrogen peroxide degradation, synthesis of jasmonic acid, and metabolism of indole- 3-butyric acid, and are essential to embryo viability. Peroxisomes are often found to be in intimate physical contact with other subcellular compartments such as mitochondria and chloroplasts, and act in concert with these organelles in a number of metabolic pathways (reviewed in Kaur et al., 2009).

Peroxisomes are highly dynamic, changing their abundance in response to environmental, metabolic, and development cues for proper functioning under diverse conditions (Purdue and Lazarow, 2001; Yan et al., 2005). Despite continuous debates over the evolutionary origin of peroxisomes, it is commonly believed that these organelles arose from the endoplasmic reticulum (ER) during evolution and can also be formed *de novo* in the ER in cells previously deprived of peroxisomes, at least in yeast (Hoepfner et al., 2005; Gabaldon et al., 2006; Schluter et al., 2006; Titorenko and Mullen, 2006). Evidence from yeasts also demonstrate that peroxisomes multiply primarily from pre-existing peroxisomes through either constitutive or induced division; the latter is also called proliferation. Both division and proliferation involve peroxisome elongation (growth), constriction and fission, during which at least two peroxisomes are formed from a single pre-existing peroxisome (Yan et al., 2005; Fagarasanu et al., 2007). Previous studies have identified a number of key factors in peroxisome division/proliferation, among which

PERXIN11 (PEX11), dynamin-related proteins (DRPs), and FISSION1 (FIS1) represent three evolutionarily conserved families of proteins that control various stages of division and proliferation (reviewed in Kaur et al., 2009). PEX11 proteins are exclusively involved in the early step of peroxisome division, whereas DRP and FIS1 are shared by the division apparatus of peroxisomes and mitochondria, executing the fission of these organelles in diverse species (Delille et al., 2009).

PEX11 is believed to play a rate-limiting role in initiating peroxisome elongation/tubulation, the first step of peroxisome division. This conclusion is based on the fact that overexpressing *PEX11* promotes peroxisomal elongation, whereas deletion or silencing of the gene(s) causes fewer and/or larger peroxisome. Yeast species each carry a single PEX11, whereas three isoforms of PEX11 (PEX11α, -β, and -γ) exist in mammals, with PEX11β being essential for embryo viability (Yan et al., 2005; Fagarasanu et al., 2007; Kaur and Hu, 2009). Arabidopsis contains five PEX11 isoforms categorized into three subfamilies, PEX11a, PEX11b, and PEX11c to –e, all of which are integral membrane proteins of the peroxisome performing functions similar to those of their yeast and animal orthologs (Lingard and Trelease, 2006; Orth et al., 2007). Members of the Arabidopsis PEX11 family are partially redundant in function and display distinct expression patterns; among them PEX11b plays a specific role in mediating the phytochrome A-dependent light induction of peroxisome proliferation in seedlings (Orth et al., 2007; Desai and Hu, 2008). The PEX11 protein (Pex11p) in *Saccharomyces cerevisiae* is able to form homooligomers, resulting in the inhibition of its function (Marshall et al., 1996). Mammalian PEX11β self interacts in two-hybrid and co-immunoprecipitation (co-IP) assays (Kobayashi et al., 2007), and results from

bimolecular fluorescence complementation (BiFC) assays in Arabidopsis cultured cells support the ability for all five Arabidopsis PEX11 isoforms to homo- and heterodimerize (Lingard et al., 2008). The biological consequences of PEX11 dimerization in plants and animals and the molecular mechanism for the function of PEX11 proteins in any given species remain elusive.

Dynamins and DRPs are large GTPases involved in biological processes such as endocytosis, intracellular vesicle trafficking, cytokinesis, and organelle division, by self-assembling into ring-like structures around membranes and mediating their fusion and fission (Osteryoung and Nunnari, 2003; Koch et al., 2004; Praefcke and McMahon, 2004; Hoppins et al., 2007). Mutations in the mammalian *Drp1* (*DLP1*) and the yeast *Dnm1* or *Vps1* genes lead to fewer and enlarged/elongated peroxisomes that have already undergone membrane constriction, indicating the function of DRPs in the final fission of these organelles. Drp1 and Dnm1p are also involved in mitochondrial division, whereas Vps1p has an additional role in vacuole morphogenesis (Yan et al., 2005; Fagarasanu et al., 2007). Arabidopsis has 16 DRPs, which are divided into six families based on protein structure and sequence similarity (Hong et al., 2003). DRP3 family includes DRP3A and DRP3B, two proteins sharing 77% amino acid sequence identity and dual localized to peroxisomes and mitochondria. Peroxisomal and mitochondrial division deficiencies are observed in *drp3A* and *drp3B* mutants, with the former displaying stronger peroxisome and plant growth phenotypes than the latter (Mano et al., 2004; Fujimoto et al., 2009; Zhang and Hu, 2009). Consistent with the notion that dimer formation is central to the GTPase activity of DRPs (Praefcke and McMahon, 2004), DRP3A and DRP3B homo- and heterodimerize in yeast two-hybrid assays (Fujimoto et al., 2009). Although the

drp3A drp3B double mutants display defects in organelle division and plant growth, the plants are not severely impaired, implying that other members of the Arabidopsis DRP superfamily may be at work in peroxisomal fission (Zhang and Hu, 2009).

Yeast and mammalian species each have a single FIS1 protein, which is anchored to the membrane of peroxisomes and the outer membrane of mitochondria by the C terminus, recruiting cytosolic DRPs to the organelle membranes through interactions via the N-terminal tetratricopeptide repeat (TPR) domain (Koch et al., 2003; Koch et al., 2005; Kuravi et al., 2006; Kobayashi et al., 2007; Serasinghe and Yoon, 2008). Arabidopsis has two FIS1 homologs, FIS1A and FIS1B, which are 58% identical at protein level and both dual-targeted to peroxisomes and mitochondria. The *fis1* loss-of-function mutants contain fewer and enlarged peroxisomes and mitochondria, whereas ectopic expression of *FIS1A or FIS1B* results in increased numbers of these organelles, reinforcing the rate-liming role for FIS1 in organelle fission (Zhang and Hu, 2008; Zhang and Hu, 2009). The C terminus of Arabidopsis FIS1A or FIS1B is necessary and sufficient for peroxisomal targeting in tobacco leaves, in a manner similar to their mammalian ortholog (Zhang and Hu, 2008). The mammalian FIS1 self-interacts on the outer membrane of mitochondria, necessitating its function in mitochondrial fission (Serasinghe and Yoon, 2008). Whether FIS1A and FIS1B also form oligomers and are responsible for recruiting DRPs to peroxisomes in plants have yet to be demonstrated.

Chemical cross-linking and co-immunoprecipitation studies in Chinese hamster ovary cells (CHO) reported the formation of a ternary heterocomplex consisting of PEX11β, Drp1 (DLP1), and FIS1 on the peroxisomal membrane, suggesting that the functions of

these proteins may be coordinated. The same study also found FIS1 to interact with PEX11β through the C-terminal region of PEX11β (Kobayashi et al., 2007). Likewise, BiFC experiments in Arabidopsis cultured cells found all five PEX11 proteins to interact with FIS1B (Lingard et al., 2008). However, attempts to show physical interaction between DLP1 and PEX11β in mammals and between PEX11s and DRP3A in Arabidopsis cell cultures have not been successful (Li and Gould, 2003; Kobayashi et al., 2007; Lingard et al., 2008). In addition, overexpression of PEX11β can no longer induce peroxisome proliferation in mammalian cells in which the expression of DLP1 was silenced through RNAi (Li and Gould, 2003). These data together suggest that besides FIS1, PEX11 may also work with DRPs for proper function/targeting of DRPs on/to peroxisomes. Whether plant PEX11, DRP, and FIS1 proteins also form a complex on peroxisomal membranes and coordinately regulate peroxisome division is unknown.

To get a complete mechanistic view of how peroxisomes divide in plants and to correlate dynamics of the abundance of these organelles with plant physiology, we searched for additional players in peroxisome division. We also began to investigate how the three classes of proteins, i.e., PEX11, DRP, and FIS1, interplay at the peroxisome in plants. Given the further expansion of PEX11, DRP, and FIS1 families in Arabidopsis compared with yeasts and mammals, it is especially important to determine whether specific interactions occur between individual isoforms of these families and on specific organelles. Here we report that Arabidopsis DRP5B, a protein previously shown to be required for plastid division, plays an additional role in the division of peroxisomes and contributes to proper peroxisomal functions. We also comprehensively analyzed the

interaction between members of the DRP, FIS1, and PEX11 protein families in Arabidopsis and provide a more detailed model of peroxisome division in plants.

Results

DRP5B (ARC5) is dual-targeted and controls the division of both peroxisomes and chloroplasts

To search for new proteins in peroxisome division, we first focused on other members of the Arabidopsis DRP superfamily. DRP5B, also called ARC5, is the only Arabidopsis DRP besides DRP3A and DRP3B known to play a direct role in organelle division. DRP5B forms a discontinuous ring at the division site on chloroplasts, executing the fission of these organelles (Gao et al., 2003). Interestingly, GFP-DRP5B was also reported to exist as "cytosolic patches" (Glynn et al., 2008), leading us to speculate that this protein may target to other organelles such as peroxisomes and exerts its function in the division of multiple types of organelles.

To investigate whether DRP5B plays a role in peroxisome division, we expressed the peroxisomal marker protein YFP-PTS1, a fusion of the yellow fluorescent protein and a C-terminal Peroxisome Targeting Signal type 1 tripeptide (PTS1, ser-lys-leu), in the *drp5B* mutants. The two *drp5B* null alleles used are *drp5B -1* (in *Ler*), which creates a stop codon in the middle of DRP5B, and *drp5B -2* (SAIL 71D_11, in *Col-0*), which has a T-DNA insertion in the 8th intron (Gao et al., 2003; Miyagishima et al., 2006). We also analyzed two *drp5A* mutant alleles, *drp5A-1* (*SALK_065118*) and *drp5A-2*

(*SALK_062383*) that have a T-DNA inserted in the 4th intron and the 7th exon, respectively (Miyagishima et al., 2008). DRP5A is the other member of the DRP5 family and shares similar domain structure with DRP5B, but it was recently shown to be involved in cytokinesis instead of chloroplast division (Miyagishima et al., 2008).

Confocal laser scanning microscopic (CLSM) image analysis of mesophyll cells from T_3 *drp5B* mutants expressing YFP-PTS1 confirmed the previously described phenotypes, i.e., enlarged chloroplasts that do not divide (Gao et al., 2003); additionally, it also revealed highly aggregated peroxisomes that are each slightly enlarged compared with those in the wild type plants (Figure 1A). Similar peroxisomal phenotypes were observed in roots and etiolated seedlings of the *drp5B* mutants (Suppl. Figure 1A-1F). To quantify the abundance and volume of peroxisomes, we used ImageJ software to measure peroxisome area and number. In *drp5B* mutants, peroxisome fluorescence area, which represents the total volume of peroxisomes, is increased, whereas peroxisome number, indicated by the number of YFP-labeled organelles (or organelle clusters without clear boundaries), is reduced (Figure 1B). These results together point to defects in peroxisome fission in the *drp5B* mutants. In contrast, no abnormalities in peroxisome morphology was observed in either of the *drp5A* mutants (Fig.1A; Suppl. Figure 1G), thus excluding the involvement of DRP5A in peroxisome division.

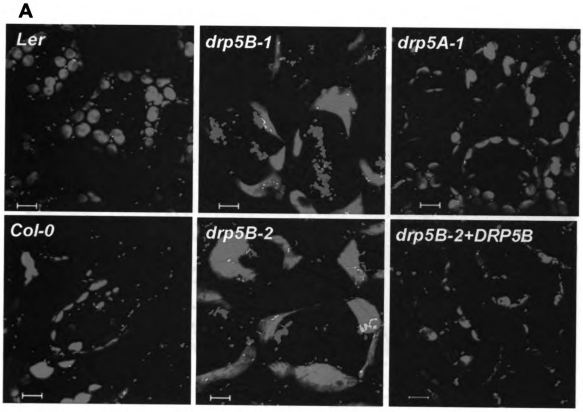
Figure 4.1 DRP5B (ARC5) is involved in the division of both peroxisomes and chloroplasts.

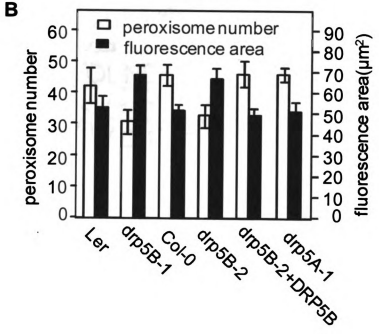
(A) Peroxisomal and chloroplast morphologies in wild-type and *drp5* mutants. Confocal images were taken from leaf mesophyll cells from four-week-old plants expressing the YFP-PTS1 peroxisome marker protein. Green signals come from YFP, and red signals are emitted from the chlorophyll. Scale bars = 10 μm.

(B) Quantification of peroxisome number and total YFP fluorescence area per 2500 μm^2 of mesophyll cells from plants shown in (A). n=8, $p<0.05$, error bars represent standard deviations.

(C) Dual targeting of GFP-DRP5B in *Col-0* plants co-expressing the *35S:DsRed2-PTS1* and *35S:GFP-DRP5B* (or *PDRP5B:GFP-DRP5B*) transgenes. Images were taken from mesophyll cells of four-week-old transgenic plants. Magenta signals represent DsRed2-PTS1. Scale bars = 10 μm.

(D) Immunoblot analysis detecting the GFP-DRP5B and DsRed2-PTS1 proteins from plants in (C), using polyclonal α-GFP and α-DsRed antibodies, respectively.


A
Ler
drp5B-1
drp5A-1
Col-0
drp5B-2
drp5B-2+DRP5B


B
peroxisome number
fluorescence area
peroxisome number
fluorescence area(μm²)
0
10
20
30
40
50
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0
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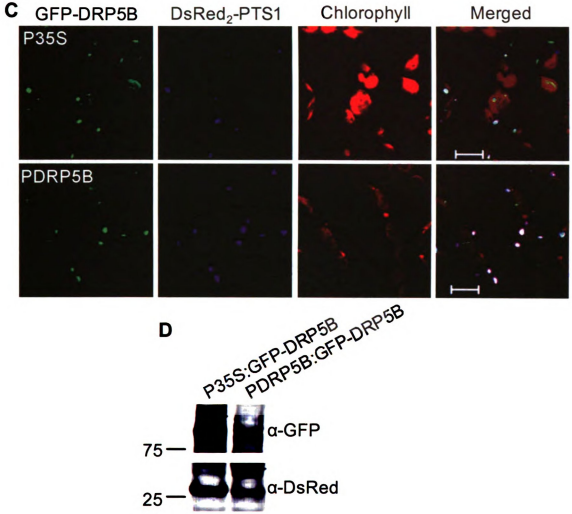
20
30
40
50
60
70
80
90
Ler
drp5B-1
Col-0
drp5B-2
drp5B-2+DRP5B
drp5A-1

Figure 4.1 continued



To further confirm that the peroxisome phenotypes in *drp5B* mutants were caused by the mutations in *DRP5B*, we expressed *DsRed2-PTS1* in *drp5B-2* mutant expressing *GFP-DRP5B* fusion gene (driven by the *DRP5B* native promoter) (Miyagishima et al., 2008). In addition to rescuing the chloroplast division deficiency, *DRP5B* also largely complemented the peroxisome phenotype in *drp5B-2*, as seen by the re-appearance of numerous spherical peroxisomes similar to those of the wild type (Figure 1A-1B). We conclude that DRP5B is not only involved in chloroplast division, but clearly plays a role in the division of peroxisomes as well.

Given that DRP5B had not been demonstrated previously to exert a function in peroxisome division, we re-checked the subcellular localization of this protein by co-expressing the peroxisome marker *DsRed2-PTS1* and *GFP-DRP5B* fusion gene in wild-type Arabidopsis. The *GFP-DRP5B* gene was under the control of the *CaMV35S* promoter (*P35S:GFP-DRP5B*) or the *DRP5B* native promoter (*PDRP5B:GFP-DRP5B*). Plants containing both *DsRed2-PTS1* and the *GFP-DRP5B* transgene were examined using confocal microscopy. GFP fluorescent signals were detected not only as a discontinuous ring structure at the chloroplast division sites, but also on peroxisomes tagged by DsRed2-PTS1, suggesting that DRP5B targets to both chloroplasts and peroxisomes (Figure 1C). The punctate structures labeled by GFP-DRP5B were never found to co-localize with mitochondrial markers (data not shown). Immunoblot analysis showed that the GFP-DRP5B and DsRed2-PTS1 proteins are indeed expressed in *P35S:GFP-DRP5B* and *PDRP5B:GFP-DRP5B* lines, with higher GFP-DRP5B expression detected in the former (Figure 1D). Despite the fact that GFP-DRP5B is functional in complementing the *drp5B* mutant phenotypes (this study and Gao et al.,

2003), no apparent differences in peroxisome appearance or abundance were found between *P35S:GFP-DRP5B* and *PDRP5B:GFP-DRP5B* lines (Figure 1C). This result is in line with previous findings that overexpressing *DRP3A* or *DRP3B* does not affect peroxisome size and number (Mano et al., 2004; Zhang and Hu, 2009), suggesting that DRP proteins by themselves are insufficient to induce organelle division.

The close functional association between plastids and peroxisomes prompted us to check peroxisome morphology in two chloroplast division mutants, *arc3* and *arc6* (Vitha et al., 2003; Glynn et al., 2008), to test out the scenario that the peroxisomal division defect in *drp5B* is caused indirectly by the abnormal division of chloroplasts. Chloroplast division is orchestrated by multiple molecular machineries composed of a number of proteins, among which ARC3 is localized in the stroma and required for the correct positioning of the division rings, and ARC6 spans the inner envelope and is responsible for recruiting DRP5B to the chloroplast surface through the outer-envelope proteins PDV1 and PDV2 (Yang et al., 2008; Okazaki et al., 2009). To visualize peroxisomes, YFP-PTS1 was introduced to *arc3* and DsRed2-PTS1 was transformed into *arc6*. Despite having dramatically enlarged chloroplasts that fail to divide, *arc3* and *arc6* do not have obvious changes in peroxisome morphology and number (Suppl. Figure 2A). Given ARC6's role in recruiting DRP5B to chloroplasts (Vitha et al., 2003; Glynn et al., 2008), we also assessed the subcellular targeting of GFP-DRP5B in the *arc6* mutant. DsRed2-PTS1 and GFP-DRP5B were co-expressed in *arc6* and progenies containing both transgenes were examined by confocal microscopy. In *arc6*, although GFP-DRP5B is not targeted to ring structures on chloroplasts, its peroxisomal localization is unaffected (Suppl. Figure 2B). These results largely rule out the possibility that peroxisome division deficiency in *drp5B*

mutants is merely a side effect of chloroplast morphology and number changes. Furthermore, DRP5B is likely the only protein shared by chloroplast and peroxisome division.

DRP5B contributes to peroxisome functions

To elucidate the impact of DRP5B on plant growth and development, especially on peroxisome-related processes, we examined its role in photorespiration, a major function of leaf peroxisomes. Photorespiration is coordinated by chloroplasts, peroxisomes, and mitochondria. It uptakes O_2 and releases CO_2 in the light, salvaging and recycling phosphoglycolate back to the chloroplast. Since this pathway is not required under high CO_2 conditions, photorespiration mutants display much stronger growth phenotypes in normal air (Kaur et al., 2009). The *pex14* mutant, which contains a T-DNA insertion in the peroxisome biogenesis factor PEROXIN14 (PEX14), serves as a positive control in this study (Figure 2) and in many of our previous studies (Fan et al., 2005; Orth et al., 2007; Zhang and Hu, 2009). After growing in ambient air for 3-4 weeks, *drp5B* mutants start to show retarded growth compared with wild-type plants, and this phenotype can be rescued by growing the mutants in elevated (3000 ppm) CO_2. In contrast, wild-type plants, *drp5B* mutants expressing GFP-DRP5B, and even other *arc* mutants such as *arc3* have similar plant sizes irrespective of the CO_2 level in the growth environment (Figure 2A; Suppl. Figure 3). These data demonstrate that DRP5B is involved in photorespiration, possibly owing to its function in the division of both peroxisomes and

Figure 4.2 The role of DRP5B in plant growth.

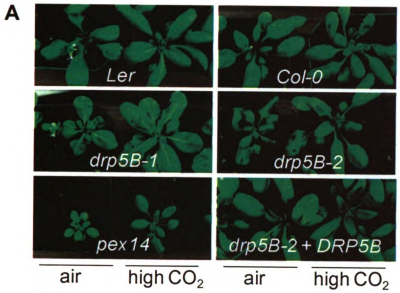
(A) Comparison of four-week-old plants grown in the air and under 3000 ppm CO_2.

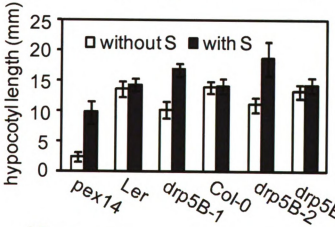
(B) Sucrose dependence assay. Hypocotyls of seedlings grown for five days in the dark on 1/2 MS media with or without the supplement of 1% sucrose (w/v) were measured ($n=60$, $p<0.05$).

(C) Effect of 2,4-DB on primary root elongation. Plants were grown for five days in the light on 1/2 LS media with or without 0.8 μM 2,4-DB ($n=60$, $p<0.05$).

(D) Effect of IBA on primary root elongation. Plants were grown for five days in the light on 1/2 LS media supplemented by IBA in the indicated concentrations ($n=60$, $p<0.05$).

(E) and (F) Expression levels of the *DRP5B* gene in different tissues (E) and at various developmental stages (F). The y axis depicts expression values assigned by GENEVESTIGATOR (https://www.genevestigator.ethz.ch/).


A
Ler
Col-0
drp5B-1
drp5B-2
pex14
drp5B-2 + DRP5B
air
high CO2
air
high CO2


B
hypocotyl length (mm)
25
20
15
10
5
0
without S
with S
pex14
Ler
drp5B-1
Col-0
drp5B-2
drp5B-2+DRP5B

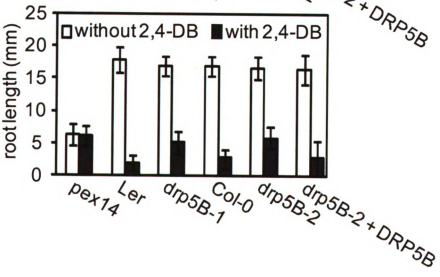
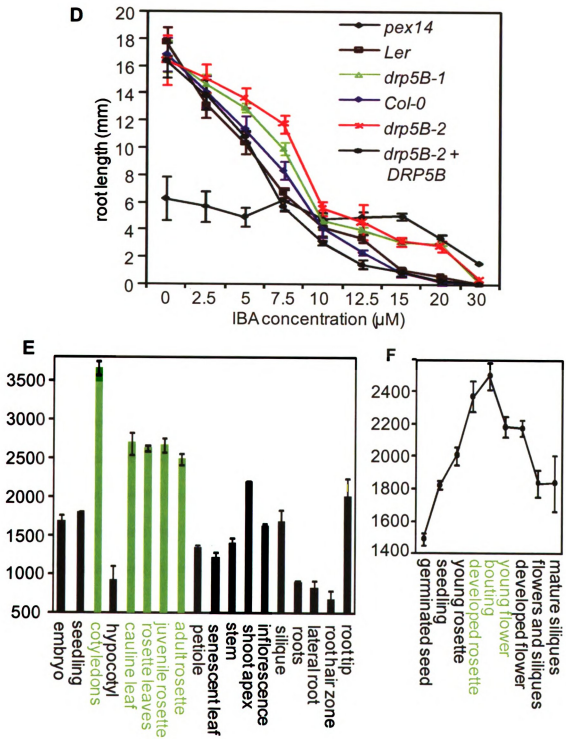

C
root length (mm)
25
20
15
10
5
0
without 2,4-DB
with 2,4-DB
pex14
Ler
drp5B-1
Col-0
drp5B-2
drp5B-2+DRP5B

Figure 4.2 continued



chloroplasts, two major participants of the glycolate recycling pathway in photorespiration. We later determined the effect that DRP5B imparts on fatty acid β–oxidation, a primary peroxisome function required for the conversion of triacylglycerol (TAG) to sucrose to fuel postgerminative seedling establishment (Kaur et al., 2009). First, *drp5B* mutant seeds were germinated in media with or without sucrose. In the absence of sucrose, hypocotyls of dark-grown *drp5B-1* and *drp5B-2* are shorter than those of wild-type and the complemented *drp5B-2* plants; this phenotype is largely rescued by application of sucrose to the media (Figure 2B). This data indicates a role of DRP5B in storage oil mobilization during hypocotyl elongation in germinating seedlings. Second, we directly examined the role of DRP5B in β–oxidation by measuring the response of the *drp5B* mutants to IBA (indole 3-butyric acid) and the synthetic auxin 2,4-DB (2,4-dichlorophenoxybutyric acid). IBA and 2,4-DB are proto-auxins that can be metabolized to the bioactive auxins IAA or 2,4-D through peroxisomal β–oxidation. Mutants impaired in β–oxidation would show resistance to the inhibitory effect of these compounds on primary root elongation (Hayashi et al., 1998; Zolman et al., 2000). Partial resistance to both 2,4-DB and IBA were observed in *drp5B* mutant seedlings, compared with the wild-type and the rescued *drp5B-2* mutant plants (Figure 2C-2D), suggesting that DRP5B is involved in peroxisomal β–oxidation during seedling establishment.

Overall, the *drp5B* mutants show relatively weaker phenotypes in fatty acid β–oxidation than in photorespiration, indicating that DRP5B may play a stronger role in green leaves than in seedlings. To determine whether this difference correlates with the expression levels of the *DRP5B* gene in development, we used the Genevestigator tool with data collected from Arabidopsis microarray databases (https://www.genevestigator.ethz.ch/) to

investigate the expression profiles of *DRP5B*. *DRP5B* is ubiquitously expressed in all tissues and throughout development, with high expression levels in green tissues such as cotyledons and cauline and rosette leaves (Figure 2E). In addition, *DRP5B*'s expression starts out at a relatively low level during seed germination, increases significantly as the plants develop leaves, reaches its peak during bolting, and declines after plants enter the reproductive phase (Figure 2F). These results support the prominent role for DRP5B in green tissues, major sites for photorespiration.

BiFC assays reveal interactions between DRP, FIS1, and PEX11 proteins

Having established a role for DRP5B in peroxisome division, we began to address the question whether this protein is able to interact with itself and with DRP3A and DRP3B, as DRP3A and DRP3B had been shown to homo- and heterodimerize in yeast two-hybrid systems (Fujimoto et al., 2009). In addition, given the reported interaction between FIS1, DRP and PEX11 in mammalian cells (see Introduction), we also wanted to test whether members of the DRP, FIS1, and PEX11 families in Arabidopsis form complexes. Interaction between proteins involved in early and late stages of peroxisome division may indicate that these distinct machineries are coordinated in function.

Figure 4.3 Interactions involving DRPs and FIS1 as detected by BiFC

Confocal images were taken from *N. tobacum* leaf epidermal cells expressing the YN- and YC-fusions along with the indicated organelle marker. YFP fluorescence (green) is an indication of BiFC, red signals come from CFP-PTS1, and magenta signals represent mitochondria stained by Mito-Tracker or chloroplasts that emit autofluorescence. The merged images show colocalization of YFP and the indicated organelles. Scale bars, 10 μm.

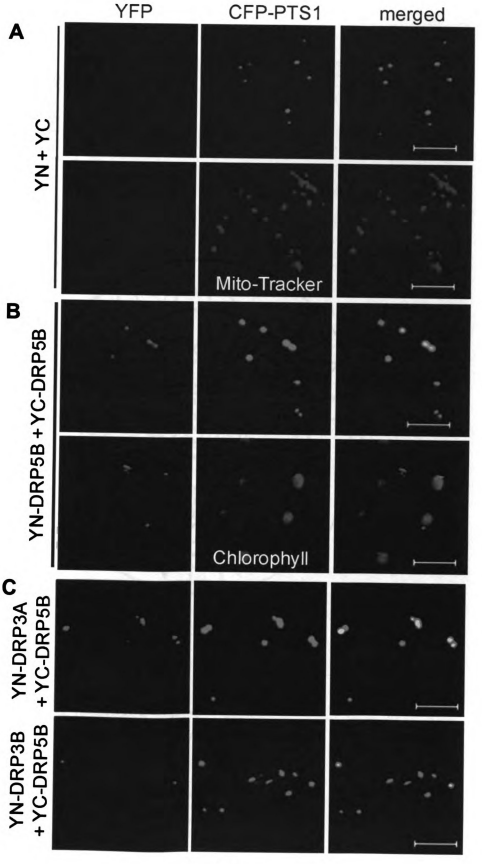

YFP
CFP-PTS1
merged
A
YN + YC
Mito-Tracker
B
YN-DRP5B + YC-DRP5B
Chlorophyll
C
YN-DRP3A + YC-DRP5B
YN-DRP3B + YC-DRP5B

Figure 4.3 continued

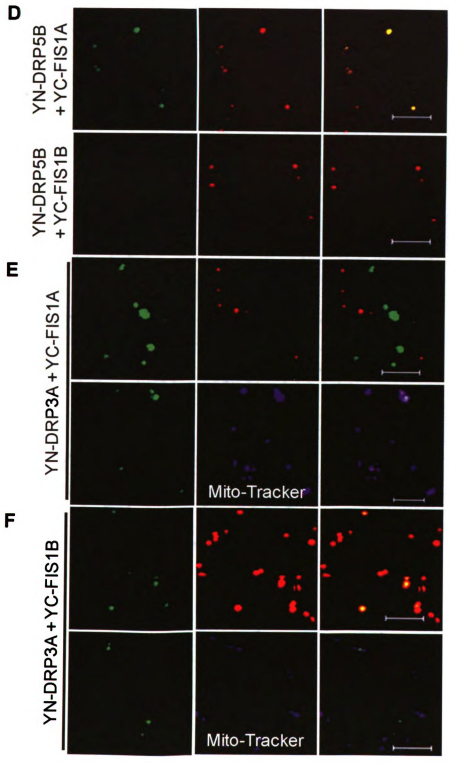
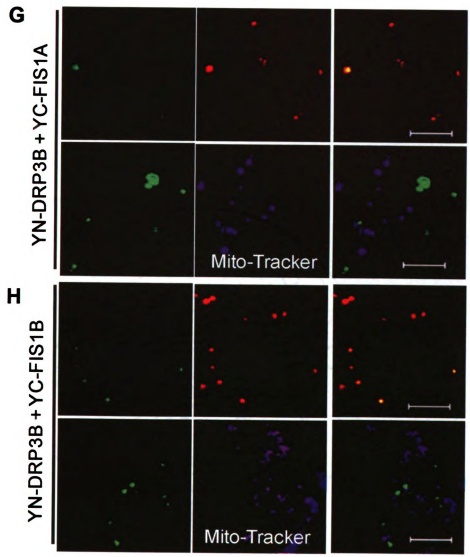


Figure 4.3 continued



To test for protein-protein interaction, we first employed bimolecular fluorescence complementation (BiFC), because this *in vivo* assay not only determines whether proteins interact or reside in close proximity, but also detects locations for such interactions. To this end, N- and C-terminal fragments of YFP (YN and YC) were respectively fused to the N terminus of each of *DRP5B, DRP3A, DRP3B*, *FIS1A*, *FIS1B*, and *PEX11a* to *-e* genes to generate YN-Gene and YC-Gene fusion constructs. For subsequent evaluation of protein expression, an HA tag was added to the N terminus of YN and a 6XHis tag was fused to the N terminus of YC. All fusion genes were driven by the *35S* promoter. Each YN- and YC-fusion pair, along with the peroxisomal maker CFP-PTS1, was transiently co-expressed in *Nicotiana tabacum* leaves using *Agrobacterium tumefaciens*–mediated transformation. Epidermal cells of the inoculated tissues were analyzed by confocal microscopy after 48 hours. To ensure that the proteins are expressed, proteins extracted from the inoculated tissues were also subjected to immunoblot analysis, using α–HA, α-His, and α–GFP antibodies to detect the YN-, YC-, and CFP-PTS1 fusion proteins, respectively (Suppl. Figure 4).

Figure 4.4 Interaction between DRPs and PEX11 proteins detected by BiFC. Images were taken from *N. tobacum* leaf epidermal cells expressing the indicated YN and YC protein pairs and the CFP-PTS1 peroxisomal marker. Scale bars, 10 μm.

YFP
CFP-PTS1
Merged
YN + YC
YN-DRP5B + YC-PEX11a
YN-DRP5B + YC-PEX11b
YN-DRP5B + YC-PEX11c
YN-DRP5B + YC-PEX11d
YN-DRP5B + YC-PEX11e

Figure 4.4 continued

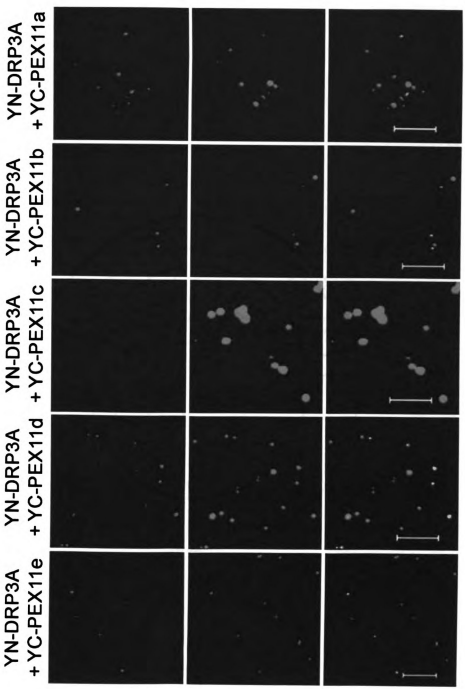
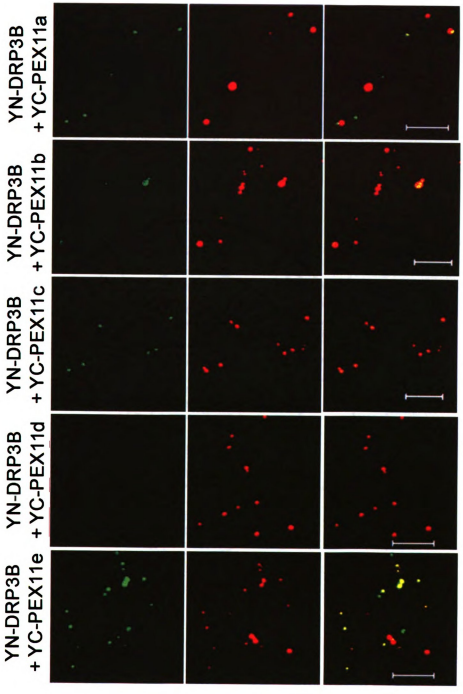


Figure 4.4 continued



As shown in Figure 3A, leaf tissues infiltrated with the empty vectors YC and YN showed no YFP signals, whereas YN-DRP5B and YC-DRP5B, when combined, conferred YFP fluorescence on CFP-PTS1-tagged peroxisomes as wells as chloroplasts that are marked by chlorophyll autofluorescence (Figure 3B). In addition, DRP5B interacts with DRP3A and DRP3B on peroxisomes (Figure 3C). These data together suggest that, similar to DRP3A and DRP3B, DRP5B is also able to interact with itself. In addition, DRP5B heterodimerizes with DRP3A and DRP3B.

In mammals, the DRP protein Drp1 is recruited to the peroxisome and mitochondrion by the membrane-anchored protein FIS1 (Koch et al., 2003; Koch et al., 2005; Kuravi et al., 2006; Kobayashi et al., 2007; Serasinghe and Yoon, 2008). Hence, it would be crucial to determine whether this is the case in plants for DRP and FIS1 homologs. If so, it would also be interesting to see whether there is any specific interaction between individual DRP and FIS1 isoforms on peroxisomes and mitochondria, given that more DRPs and FIS1 are involved in Arabidopsis compared with animals. To this end, we closely examined the interaction between the FIS1A/1B and DRP5B/3A/3B proteins. When DRP3A and DRP3B are involved, we also stained the tissue with Mito-Tracker to visualize mitochondria, besides using CFP-PTS1 for peroxisome labeling. Interestingly, DRP5B, DRP3A, and DRP3B show distinct patterns of interaction with respect to the FIS1 partner they are associated and the location of the interactions (Figure 3D-3H; Suppl. Figure 5A). DRP5B interacts with FIS1A, but not FIS1B, on peroxisomes (Figure 3D; Suppl. Fig. 5A). As for DRP3A, its interaction with FIS1A was detected on Mito-Tracker-stained mitochondria but not on CFP-PTS1-labeled peroxisomes (Figure 3E); however its interaction with FIS1B was only seen on peroxisomes (Figure 3F). DRP3B,

on the other hand, interacts with both FIS1A and FIS1B exclusively on peroxisomes (Figure 3G); no interaction was detected on mitochondria with either FIS1A or FIS1B (Figure 3H). Lastly, we investigated interactions among FIS1 proteins, as homodimerization was found to occur for the mammalian FIS1 ortholog (Serasinghe and Yoon, 2008). FIS1A and FIS1B each form homodimers on peroxisomes and mitochondria, but they do not seem to heterodimerize on either type of organelles (Suppl. Figure 5B-5C).

Lingard et al (2008) used BiFC to show in Arabidopsis cell cultures that all five Arabidopsis PEX11 proteins form homo- and heterodimers, and that they each interact with FIS1B but not FIS1A or DRP3A. In our BiFC system, we were able to reproduce the positive interaction results by Lingard et al (2008), and in addition to show association between FIS1A and PEX11a, PEX11b, PEX11d, and PEX11e (Suppl. Figure 6). When testing interaction between DRP and PEX11s, we detected peroxisome-localized association between DRP5B and each of the five PEX11 isoforms, between DRP3A and PEX11a, PEX11b, PEX11d, and PEX11e, and between DPR3B and PEX11a, PEX11b, PEX11c, and PEX11e (Figure 4). These data collectively suggest that members of the PEX11 family interact with DRP5B, DRP3A, DRP3B, and FIS1A/1B *in vivo* in Arabidopsis.

Co-Immunoprecipitation assays suggest the formation of complex by DRP, FIS1, and PEX11 proteins

To support the data obtained by BiFC, we used co-immunoprecipitation as an independent approach to test for protein-protein interaction. We constructed 35S-driven gene fusions, in which HA and FLAG tags were cloned respectively to the N terminus of the inquest proteins, and introduced the construct pairs into *N. benthamiana* leaves via Agrobacterium infiltration. Proteins from the infiltrated leaves expressing each of the HA- and FLAG-tagged protein pairs were first subjected to immunoblot analysis to ensure that the fusion proteins are expressed (Figure 5A). Subsequently, total protein extracts were incubated with agarose beads conjugated with α-HA, and proteins pulled down by α-HA were subjected to immunodetection using α-FLAG and α-HA antibodies. Detection of the two HA- and FLAG-fusion proteins in the same co-immunoprecipitation would suggest that the two proteins are in the same complex. Because of the large number of protein pairs involved, we only tested interactions in which the three DRP proteins are involved.

The Co-IP results largely corroborate with those obtained by BiFC; an additional interaction between PEX11d and DRP3B was also detected (Figure 5B). However, in contrast to the positive BiFC data between PEX11a and DRP5B/DRP3A/DRP3B and between PEX11c and DRP3B (Figure 4), PEX11a was not co-immunoprecipitated with any of the three DRP proteins and PEX11c was not pulled down by DRP3B (Figure 5B), suggesting that follow-up studies are needed to authenticate the protein-protein interactions involving PEX11a and between DRP3B and PEX11c. In summary, the co-IP results reinforce the notion that members of the DRP, FIS1, and PEX11 families interact (summarized in Table I) and potentially form complexes *in vivo*.

Figure 4.5 Co-IP assays to test the interactions involving DRP, FIS1, and PEX11 proteins.

(A) Immunoblot analysis of proteins extracted from tobacco leaves expressing the indicated HA- and FLAG-fusion proteins.

(B) Immunoblot analysis of proteins bound to anti-HA beads.

Sizes of the molecular markers (in kD) are indicated to the left of the gels. Different gels are separated by lines.

A
B
Input
Co-IP
α-FLAG
α-HA
75
25
HA-DRP5B
HA-DRP5B + FLAG-DRP5B
HA-DRP5B + FLAG-DRP3A
HA-DRP5B + FLAG-DRP3B
HA-FIS1A
HA-FIS1A + FLAG-DRP5B
HA-FIS1A + FLAG-DRP3A
HA-FIS1A + FLAG-DRP3B
HA-FIS1B
HA-FIS1B + FLAG-DRP5B
HA-FIS1B + FLAG-DRP3A
HA-FIS1B + FLAG-DRP3B
HA-PEX11a
HA-PEX11a + FLAG-DRP5B
HA-PEX11a + FLAG-DRP3A
HA-PEX11a + FLAG-DRP3B
HA-PEX11b
HA-PEX11b + FLAG-DRP5B
HA-PEX11b + FLAG-DRP3A
HA-PEX11b + FLAG-DRP3B
HA-PEX11c
HA-PEX11c + FLAG-DRP5B
HA-PEX11c + FLAG-DRP3A
HA-PEX11c + FLAG-DRP3B
HA-PEX11d
HA-PEX11d + FLAG-DRP5B
HA-PEX11d + FLAG-DRP3A
HA-PEX11d + FLAG-DRP3B
HA-PEX11e
HA-PEX11e + FLAG-DRP5B
HA-PEX11e + FLAG-DRP3A
HA-PEX11e + FLAG-DRP3B

Discussion

DRP5B plays a dual role in organelle division

DRP5B was originally identified for its function in chloroplast division (Gao et al., 2003). Here we provide several lines of evidence to demonstrate that this protein has an additional role in the division of peroxisomes and is involved in maintaining proper peroxisomal activities. First, GFP-DRP5B targets not only to chloroplast division rings but also to spherical structures labeled by the CFP-PTS1 peroxisomal marker protein. Second, besides the previously reported phenotypes such as enlarged and dumbbell-shaped chloroplasts, *drp5B* mutants also exhibit highly aggregated and/or enlarged peroxisomes that fail to divide completely. Third, peroxisomal functions such as photorespiration and fatty acid β–oxidation are compromised in the *drp5B* mutants. Lastly, deficiencies in peroxisomal morphology and function in the *drp5B* mutants can be rescued by expression of the wild-type DRP5B protein. In summary, DRP5B has joined DRP3A and DRP3B as plant DRPs involved in peroxisome division. The list of DRPs associated with peroxisome division may not be complete, as there are over a dozen Arabidopsis DRPs, most of which have not been characterized with respect to their *relevance* to peroxisomes (Hong et al., 2003).

The discovery that DRP5B also participates in peroxisome fission is somewhat unexpected. DRP3A and DRP3B are highly identical in sequence and both contain the GTPase, middle, and GTPase effector domains (Hong et al., 2003). As a result, these two proteins are interchangeable in mitochondrial division and partially redundant in the

division of peroxisomes (Fujimoto et al., 2009; Zhang and Hu, 2009). However, DRP5B shares little sequence similarity with DRP3s and contains an additional pleckstrin homology (PH) domain, which is believed to be capable of binding to membrane phospholipids (Hong et al., 2003). DRP5B and DRP3A/3B also differ in their peroxisome localization patterns. When fused to YFP or GFP, DRP3A and DRP3B were shown to be in juxtaposition to peroxisomes (Mano et al., 2004; Fujimoto et al., 2009; Zhang and Hu, 2009), whereas P35S:GFP-DRP5B or PDRP5B:GFP-DRP5B is evenly distributed along peroxisomes (Figure 1C). Finally, the "head and tail" peroxisome phenotype, which is frequently observed in *drp3A* mutants, was not shown in *drp5B* mutants. These data collectively point toward the possibility that the role for DRP5B in peroxisome division is to some extent distinct from that of DRP3A and DRP3B. To test this hypothesis, it will be crucial to determine in the future whether DRP5B can substitute the function of DRP3 in peroxisome division.

In addition to DRP3A, DRP3B, and DRP5B, another example of a single DRP participating in diverse functions comes from the Arabidopsis DRP1 family. The DRP1 family is generally believed to be involved in cytokinesis and cell expansion (Konopka and Bednarek, 2008), however, DRP1C and DRP1E were also reported to act in mitochondrial morphogenesis (Jin et al., 2003). In non-plant systems, the yeast Vps1p and Dnm1p and the mammalian DLP1 (Drp1) proteins are DRPs involved in the division/vesiculation of more than one type of organelle (Wilsbach and Payne, 1993; Hoepfner et al., 2001; Koch et al., 2003; Li and Gould, 2003; Koch et al., 2004; Kuravi et al., 2006; Schrader, 2006). These results together suggest that a given DRP, which normally lacks intrinsic organelle targeting signals, can be recruited to different types of

subcellular structures to facilitate with membrane fission. However, recruitment of DRPs seems to have some specificities. For example, DRP3A, DRP3B, and DRP5B do not participate in the fission of membrane structures other than peroxisomes, mitochondria, and chloroplasts, whereas DRP5A, despite being structurally similar to DRP5B, functions in cytokinesis instead of chloroplast division (Miyagishima et al., 2008; Fujimoto et al., 2009; Zhang and Hu, 2009).

Photorespiration, fatty acid metabolism, and jasmonic acid biosynthesis are among some of the metabolic pathways coordinated by peroxisomes and other organelles, including chloroplasts and mitochondria (Kaur et al., 2009). The efficiency of these metabolic processes is dependent on the intimate physical association and functional cooperation between the organelles involved. In Arabidopsis, DRP3 and its putative anchor, FIS1, are shared by the division machineries of peroxisomes and mitochondria, and DRP5B is shared by peroxisomes and chloroplasts. The use of shared fission components could be a mechanism to render coordinated division among the metabolically linked subcellular compartments. It will be interesting to determine whether such coordinated division truly takes places in plants, and if so, whether there are biological significances for this phenomenon.

Distinct interactions between members of the DRP, FIS1, and PEX11 families on different organelles

Table 4.1 Summary of interactions among members of the *Arabidopsis* DRP, FIS1, and PEX11 protein families

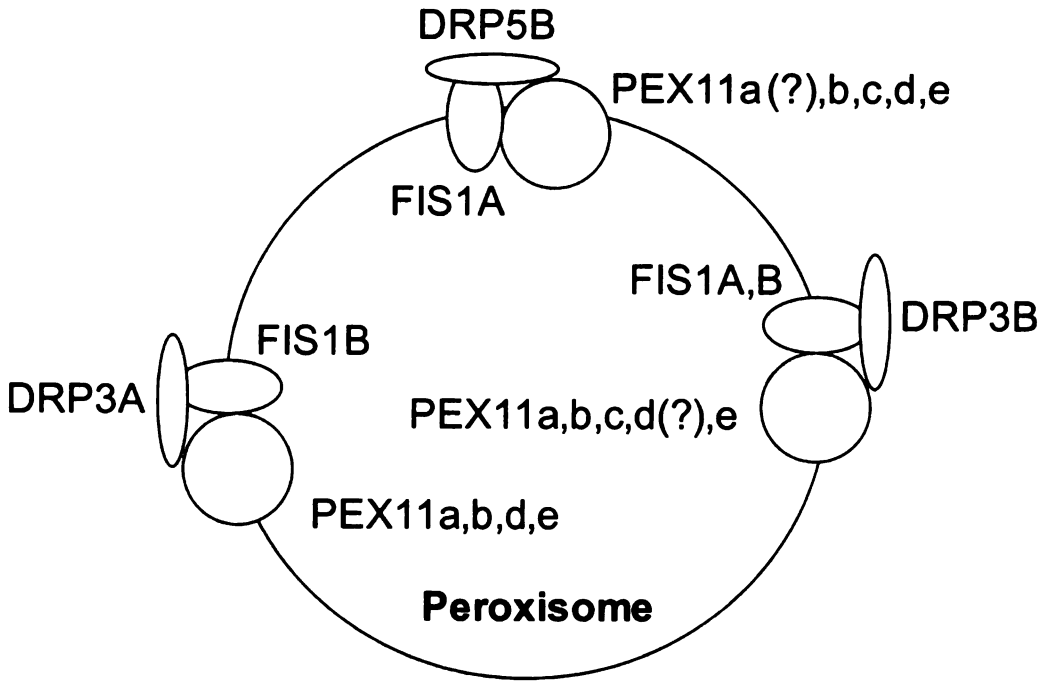
	DRP5B	DRP3A	DRP3B	FIS1A	FIS1B	PEX11a	PEX11b	PEX11c	PEX11d	PEX11e
DRP5B	+√	+√	+√	+√	-x	+x	+√	+√	+√	+√
DRP3A	+	+ *	+ *	+√	+√	+x	+√	-x	+√	+√
DRP3B	+	+ *	+ *	+√	+√	+x	+√	+x	-√	+√
FIS1A	+	+	+	+	-	+	+	-	+	+
FIS1B	-	+	+	-	+	+ *	+ *	+ *	+ *	+ *
PEX11a	+	+	-	+	+	+ *	+ *	+ *	+ *	+ *
PEX11b	+	+	+	+	+	+ *	+ *	+ *	+ *	+ *
PEX11c	+	-	+	-	+	+ *	+ *	+ *	+ *	+ *
PEX11d	+	+	-	+	+	+ *	+ *	+ *	+ *	+ *
PEX11e	+	+	+	+	+	+ *	+ *	+ *	+ *	+ *

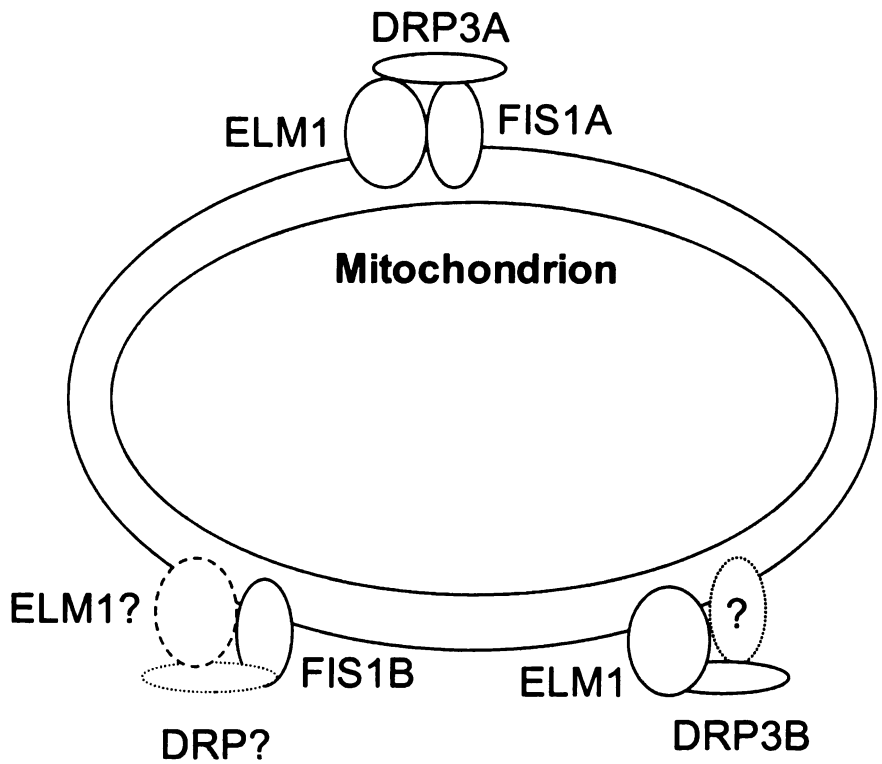
* indicate known interactions, which also serve as positive controls in this study. + or - denote interactions or lack of interactions detected by BiFC in this study. √ or x indicate interactions or lack of interactions detected by co-IP in this study.

In this study, we have provided a more detailed map of how DRP5B, DRP3A, and DRP3B may be recruited to peroxisomes. Multiple factors, such as protein expression levels, rates of protein folding, and protein stability, may contribute to variations in the detection of protein-protein interaction by BiFC, leading to false positive/negative results (Lalonde et al., 2008). To this end, we also used co-IP as an independent method to test for protein-protein interaction. Results from these two approaches confirmed the interaction among members of the DRP, FIS1, and PEX11 families *in planta*. Our data are consistent with the current knowledge about the interplay between these proteins in mammalian cells, where the DRP proteins homo-oligomerize, FIS1 helps to recruit DRP proteins, and DRP, FIS1, and PEX11 may form a ternary complex (Thoms and Erdmann, 2005; Yan et al., 2005; Delille et al., 2009). More importantly, we have been able to show specific interaction patterns for the plant DRP, FIS1, and PEX11 isoforms (depicted in Figure 6). For example, DRP5B interacts with FIS1A, but not FIS1B, on peroxisomes (this study), whereas its recruitment to chloroplasts is obviously dependent on a group of chloroplast envelope proteins, i.e., ARC6, PDV1 and PDV2 (Gao et al., 2003; Miyagishima et al., 2006; Glynn et al., 2008). DRP3A interacts with FIS1A on mitochondria, yet its interaction with FIS1B only occurs on the peroxisome. Furthermore, DRP3B is associated with both FIS1A and FIS1B exclusively on peroxisomes. Finally, although BiFC and co-IP showed discrepant results regarding the interaction between PEX11a/PEX11d and DRPs, both methods demonstrated the interaction between majority of the Arabidopsis PEX11 proteins with DRP5B, DRP3A, and DRP3B, and the protein. Given that no interaction between FIS1B and DRP3A/DRP3B is detected on lack of interaction between PEX11c and DRP3A (Figure 4 & 5). The diversification of the

Figure 4.6 A hypothetical model for the targeting of DRP5B, DRP3A, and DRP3B to peroxisome and mitochondria in *Arabidopsis*.

DRP5B is recruited to peroxisomes by FIS1A. The targeting of DRP3A to the peroxisome requires FIS1B, yet its localization to mitochondria seems to be dependent on FIS1A and ELM1. DRP3B may be recruited to peroxisomes by either FIS1A or FIS1B, whereas its targeting to mitochondria may rely on the function of ELM1 and an unknown mitochondria, FIS1B (together with ELM1) may be responsible for recruiting other DRPs on mitochondria. On the peroxisome, DRP5B, DRP3A and DRP3B each interact with at least four of the five PEX11 proteins; the significance of this interaction is yet to be determined. PEX11 proteins followed by a "?" indicate that the interaction between this PEX11 and the corresponding DRP protein was supported by BiFC or co-IP but not both methods.


DRP5B
PEX11a(?),b,c,d,e
FIS1A
FIS1A,B
DRP3B
FIS1B
DRP3A
PEX11a,b,c,d(?),e
PEX11a,b,d,e
Peroxisome


DRP3A
ELM1
FIS1A
Mitochondrion
ELM1?
FIS1B
DRP?
?
ELM1
DRP3B

DRP, FIS1, and PEX11 families in plants may have led to the specific recruitment of the DRP proteins by distinct anchor proteins on the various types of organelles (Figure 6). The lack of detectable interaction between FIS1B and any of the three DRP proteins and between DRP3B and FIS1A/FIS1B on mitochondria is intriguing. These data suggest that FIS1B may be responsible for recruiting DRPs other than DRP5B/3A/3B to mitochondria and that other mitochondrial membrane proteins may act as anchors for DRP3B (Figure 6). One candidate for such mitochondrial anchors is ELM1 (Elongated Mitochondria1), a plant-specific protein that exclusively targets to the outer membrane of mitochondria, interacts with both DRP3A and DRP3B, and is required for the mitochondrial targeting of (at least) DRP3A (Arimura et al., 2008). It is also possible that FIS1 and ELM1 are part of the same mitochondrial membrane complex responsible for recruiting DRP proteins (Figure 6). In this case, *in vivo* interaction between FIS1 and ELM1 need to be shown. In addition, yeast mitochondrial and peroxisomal divisions both require Mdv1p (or Caf4p), a cytosolic linker that interacts with both DRP and FIS1 proteins on these organelles (reviewed in Delille et al., 2009). Although the orthologs for Mdv1p/Caf4p were not identified in mammals, we cannot rule out the possibility that they exist in plants and carry out similar functions.

Transgenic Arabidopsis plants, cultured Arabidopsis cells, or tobacco epidermal cells overexpressing individual FIS1 or PEX11 isoforms exhibit marked increases in peroxisome numbers or dramatic elongation of the organelles (Lingard and Trelease, 2006; Orth et al., 2007; Zhang and Hu, 2008; Zhang and Hu, 2009). However, in our BiFC assays, YFP complementation mostly takes places on peroxisomes that are not elongated and in cells where dramatic increases in peroxisome abundance are not

observed. These results indicate that interaction between DRP, FIS1, and PEX11 proteins may take place before peroxisome division is initiated. The biological consequences of the interaction between PEX11 and FIS1/DRP, two groups of proteins responsible for different steps of peroxisome division, also remain to be determined.

Materianls and Methods

Plant materials, growth conditions, and transformation

Arabidopsis plants were germinated under 16-h light (60 $\mu E\ m^{-2}\ sec^{-1}$)/8-h dark conditions on 0.6% (w/v) agar plates with ½ MS supplemented with 1% (w/v) sucrose. After two weeks, seedlings were transplanted onto soil and grown under a photosynthetic photon flux density of 70–80 $\mu mol\ m^{-2}\ sec^{-1}$ at 21°C with a 14-h light/10-h dark period.

CFP-PTS1, YFP-PTS1 (Fan et al., 2005; Orth et al., 2007; Zhang and Hu, 2008; 2009) and DsRed2-PTS1 were used as markers to visualize peroxisomes. To determine subcellular localization of DRP5B, Arabidopsis plants expressing the *GFP-DRP5B* transgene (driven by *CaMV35S* or the *DRP5B* native promoter) were transformed with *DsRed2-PTS1*. To visualize peroxisomes in various mutant background, YFP-PTS1 or DsRed2-PTS1 was expressed in *drp5B-1*, *drp5B-2* (*SAIL 71D_11*), *drp5A-1* (*SALK_065118*), *drp5A-2* (*SALK_062383*), *arc3* and *arc6*. The *Agrobacterium tumefaciens* strain *C58C1* was used for all plant transformations, and selection of transgenic plants was performed as described previously (Zhang and Hu, 2009).

Confocal laser scanning microscopy and image analysis

For co-localization and mutant analyses, rosette leaves of four-week old Arabidopsis plants were analyzed by using a confocal laser scanning microscope (Zeiss Meta 510, http://www.zeiss.com/) to capture images of fluorescent proteins. Confocal microscopic observation was performed as previously described (Zhang and Hu, 2009). We used 458-nm, 488-nm, 514-nm, 543-nm, and 633-nm lasers for excitation of CFP, GFP, YFP, DsRed and chlorophyll, respectively. For emission, we used 465-510 nm band pass (CFP), 505-530 band pass (GFP), 520-555 band pass (YFP), DsRed2 (560-615) and 650 nm long pass (chlorophyll) filters. All images were obtained from optical sections of 6 μm in depth. ImageJ (http://rsb.info.nih.gov/ij/) was used to measure the fluorescence area and count peroxisome numbers in confocal images (50 μm X 50 μm) and statistical analysis was done as described previously (Zhang and Hu, 2009).

Sugar-dependence and 2,4-DB/IBA response assays

For sugar dependence analysis, seeds were placed on ½ MS agar plates supplemented with or without 1% (w/v) sucrose, stratified at 4°C for two days in the dark, and exposed to 24 hours of light to induce germination before being placed in dark conditions. After 5 days of seedling growth in the dark, hypocotyl length was measured using ImageJ. To study the response to 2,4-DB and IBA, 2,4-DB (0.8 μM) or IBA (final concentration 0, 2.5, 5, 7.5, 10, 12.5, 15, 20, 30 μM) was added to ½ LS agar media supplemented with 0.5% sucrose. After 2 days of stratification, seeds were kept under low-intensity light for 5 days. Hypocotyls (for sugar dependence assay) and roots (for IBA and 2,4-DB

response) were scanned using an EPSON scanner and measured using ImageJ (http://rsb.info.nih.gov/ij/). For all statistic analyses, n=60, $p<0.05$.

Immunoblot analysis

Total protein was extracted from leaf discs of 4-week-old Arabidopsis plants or tobacco leaves. Homogenized leaf tissue was kept in 1×SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, boiled for 5 min, and centrifuged for 5 min. The supernatant was run on SDS-PAGE gels and transferred to Immobilon-P membrane for blotting (Millipore Corp., Bedford, MA). Primary antibodies used to detect proteins include: a rabbit polyclonal GFP antibody for CFP and GFP (Santa Cruz Biotechnology, Inc.), a mouse monoclonal His antibody for the 6XHis tag (Millipore Antibodies, www.millipore.com), a rabbit monoclonal HA antibody for HA tag (Cell Signaling Technology, Inc., www.cellsignal.com), and a rabbit monoclonal FLAG antibody for FLAG tag (Cell Signaling). The secondary antibody used was goat anti-rabbit IgG (α-GFP, α-HA, α-FLAG) or goat anti-mouse IgG (α-His) from LI-COR Biosciences (http://www.licor.com).

BiFC assays

The full-length coding sequence of DRP5B, DRP3A, DRP3B, PEX11a, PEX11b, PEX11c, PEX11d, PEX11e, FIS1A or FIS1B was cloned into binary vectors to generate YN-protein (N-terminal fragment of YFP fused to the N terminus of each protein) and YC-protein (C-terminal fragment of YFP fused to the N terminus of each protein), as

described previously (Bracha-Drori et al., 2004). A HA tag was added to the N terminus of YN-protein, and a 6XHis tag was added to the N terminus of YC-protein. The HA epitope sequence used in this study is YPYDVPDYA; the 6XHis sequence is KKKKKK. Mixtures of Agrobacteria (strain *C58C1*) containing each protein pair along with the peroxisomal marker CFP-PTS1 were co-infiltrated into leaves of four-week-old *Nicotiana tabacum* (*cv. Petit Havana*) plants grown at 25°C (Goodin et al., 2002), resulting in co-expression of these proteins in the same infiltrated area. Imaging analysis of epidermal cells in the infiltrated area was performed by confocal laser scanning microscopy as described above. Immunoblot analysis was also conducted on the infiltrated tissue to confirm co-expression of the proteins.

Co-Immunoprecipitation

The full-length coding sequence of the tested proteins was cloned into binary vectors to generate HA-protein (HA fused to the N terminus of each protein) and FLAG-protein (FLAG fused to the N terminus of each protein). The FLAG epitope sequence used is DYKDDDDK, and the HA epitope is YPYDVPDYA. Agrobacteria containing each HA and FLAG protein pair were co-infiltrated in leaves of four-week-old *Nicotiana tabacum* (*cv. Petit Havana*) plants grown at 25°C (Goodin et al., 2002). After 48 hours, leaf discs were collected and homogenized in lysis buffer (Nomura et al., 2006). The samples were then centrifuged at 20,000×*g* for 15 min at 4°C to remove insoluble debris. The supernatant was incubated with anti-HA agarose beads (Sigma-Aldrich) overnight at 4°C, and the mixture was centrifuged at 500×*g* for 1 min to collect agarose beads, which were then washed three times with lysis buffer and resuspended in 1×SDS-PAGE sample

buffer for immunoblot analysis. Proteins were separated on SDS-PAGE gels and transferred to Immobilon-P membrane, followed by immunodetection by α-FLAG and α-HA antibodies.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: PEX11a (At1g47750), PEX11b (At3g47430), PEX11c (At1g01820), PEX11d (At2g45740), PEX11e (At3g61070), FIS1A (At3g57090), FIS1B (At5g12390), DRP3A (At4g33650), DRP3B (At2g14120), and DRP5B (At3g19720).

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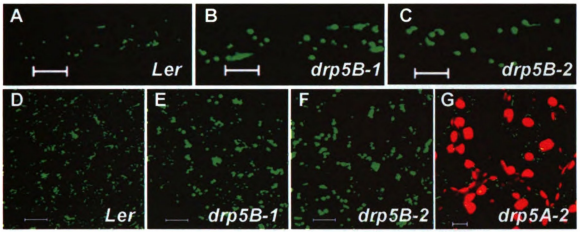
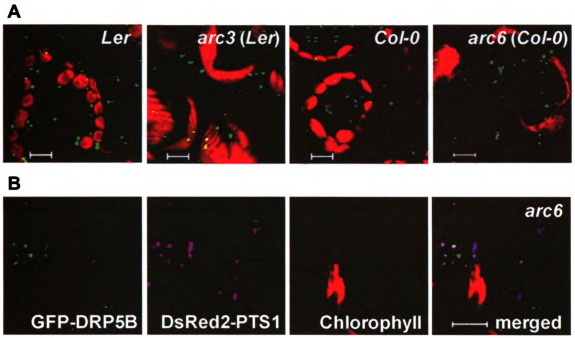


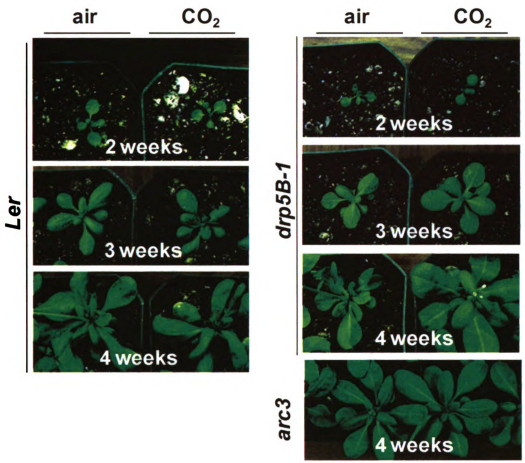
Supplemental Figure 4.7 Additional images showing peroxisomal phenotypes in *drp5B* and *drp5A* mutants.

Confocal micrographs were taken from root (A-C) and mesophyll (G) cells of four-week-old plants or from cotyledon cells of 3d dark-grown seedlings (D-F).

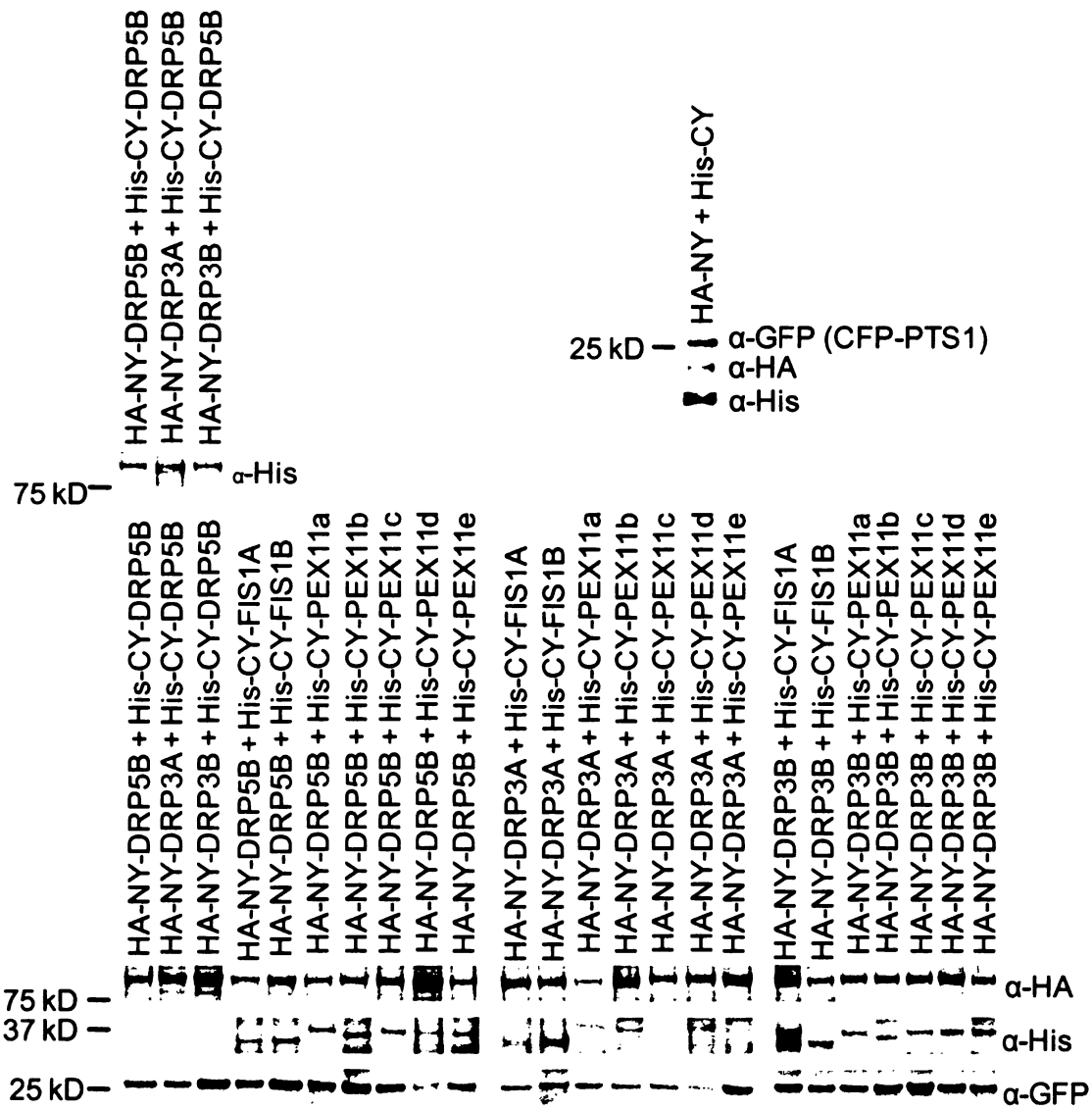


Supplemental Figure 4.8 Confocal images from Arabidopsis leaf mesophyll cells of wild-type and mutant plants

Mutants expressing YFP-PTS1(A) or the indicated fluorescent proteins (B). Scale bars = 10 μm.



Supplemental Figure 4.9 Growth phenotypes of plants in ambient air or elevated CO2.



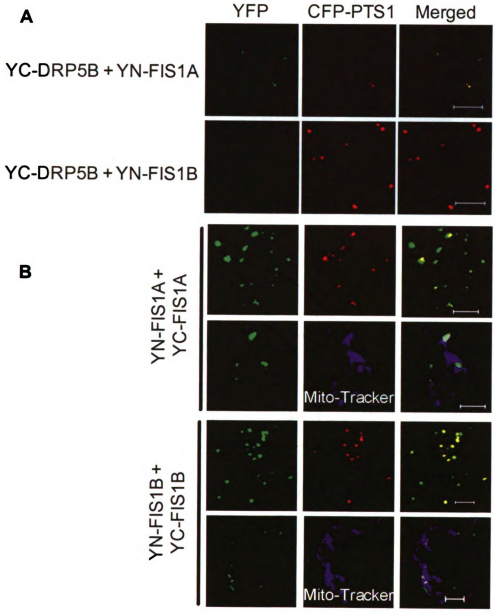
Supplemental Figure 4.10 Immunoblot analysis of proteins extracted from tissues used for BiFC assays.

Proteins extracted from the tissues inoculated with the indicated constructs were detected by the respective antibodies.

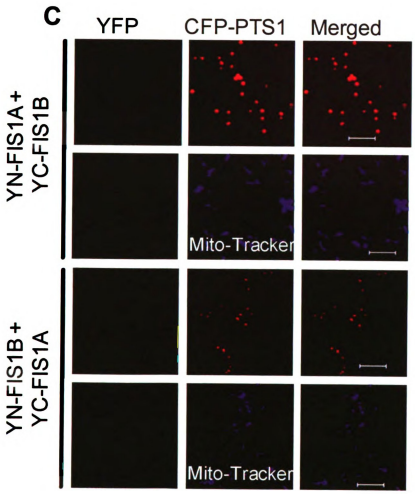
Supplemental Figure 4.11 BiFC assays testing the protein-protein interaction

The protein-protein interaction involving DRP5B and FIS1 proteins (A), and homo- (B) and heterodimerization (C) of FIS1 proteins.

Confocal images were obtained from tobacco leaves co-expressing the indicated gene fusions. Scale bars, 10 μm.


A
YFP
CFP-PTS1
Merged
YC-DRP5B + YN-FIS1A
YC-DRP5B + YN-FIS1B
B
YN-FIS1A + YC-FIS1A
Mito-Tracker
YN-FIS1B + YC-FIS1B
Mito-Tracker

Supplemental Figure 4.11 continued



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Chapter 5 Conclusions and Future Directions

5.1 Conclusions

5.1.1 DRP3A and DRP3B are functionally redundant in peroxisome fission and plant growth in Arabidopsis (Chapter 2)

DRPs have evolved in eukaryotic cells to function in diverse processes such as cytokinesis, endocytosis, membrane trafficking, organelle division, plant cell plate formation, and resistance to viral infection (Osteryoung and Nunnari, 2003; Praefcke and McMahon, 2004; Thoms and Erdmann, 2005; Miyagishima et al., 2008). Sometimes, more than one function is assigned to the same DRP. For instance, DLP1 is a mammalian DRP controlling the division of mitochondria and peroxisomes; VPS1, a DRP in yeast, is required for peroxisome morphology and also for protein trafficking to the vacuole (Kuravi et al., 2006). DRPs mediating organelle division are believed to act in membrane fission after organelle membrane is constricted.

The involvement of DRP3A in peroxisome division was observed using a forward genetic strategy in *Arabidopsis*. *drp3A* (*amp*) mutants show an aberrant mitochondrial morphology. Interestingly, peroxisomes in those mutants are elongated and enlarged with a reduction in number (Mano et al., 2004). Our lab also isolated a *drp3A* null mutant exhibiting highly aggregated/inseparable and massively elongated peroxisomes (Zhang and Hu, 2009). DRP3B is a homolog of DRP3A. Despite the high similarity in sequence between DRP3A and DRP3B, deletion of *DRP3B* results in aggregated peroxisomes with slightly reduced peroxisome number, a subtle peroxisome phenotype compared to peroxisomes in *drp3A* mutants. These observations suggest that DRP3B may plays a

minor role in peroxisome division relative to DRP3A (Zhang and Hu, 2009). Aggregated preoxisomes in both *drp3A* and *drp3B* mutants show membrane constriction, indicating that DRP3 proteins are involved in peroxisome fission after other factors already committed membrane constriction. In addition, *drp3A drp3B* double mutants display stronger deficiencies than each *drp3* single mutant in peroxisome abundance, seedling establishment and plant growth. Therefore, DRP3A and DRP3B are partially functionally redundant in peroxisome division and plant growth (Zhang and Hu, 2009). Another report about DRP3 family supports the view that DRP3B plays a weaker role in peroxisome division, and that the two DRP3 proteins are functionally redundant in plant growth, as well as in mitochondrial division (Fujimoto et al., 2009). The authors further claim that DRP3A and DRP3B may play distinct roles in peroxisome division in Arabidopsis.

5.1.2 DRP5B, a component of chloroplast division machinery, also plays a role in peroxisome division in Arabidopsis (Chapter 4)

DRP5B (ARC5) is an *Arabidopsis* DRP involved in chloroplast division (Gao et al., 2003). Our study has identified a new function for DRP5B in peroxisome division. Mutations in *DRP5B* gene result in aggregated peroxisomes showing membrane constriction. Silencing of each *DRP* gene leads to peroxisome enlargement with constricted membranes. However, peroxisomes in *drp5B* mutants are different from those in *drp3* mutants. Knowing that DRPs generally function on constricted organelles before fission in mammalian and yeast systems, and that the disruption of DRP3A, DRP3B and DRP5B in *Arabidopsis* leads to peroxisome enlargement with membrane constriction , we conclude that at least three *Arabidopsis* DRPs are involved in the fission of

peroxisomes. It has been shown that mitochondria and peroxisomes share components of their division machinery, such as FIS1 and DRP3 proteins, DRP5B is the only identified factor shared by the division machineries of chloroplasts and peroxisomes.

Subcellular localization analysis of transgenic lines expressing GFP-DRP5B driven by *35S* or *DRP5B* native promoter shows that DRP5B not only forms a ring on the cytosolic surface of chloroplasts as previously reported, but also localize to peroxisome in *Arabidopsis.* DRP3A and DRP3B appear as puncta attached on both peroxisomes and mitochondria, and these puncta mark the potential sites of organelle fission. By contrast, DRP5B is completely co-localized with peroxisomes. The functions of these three DRPs in organelle division have not yet been clear. Many questions remain unanswered, such as, whether they are functionally redundant, and if they play roles in peroxisome division coordinately.

5.1.3 FIS1A and FIS1B control the fission of peroxisomes and mitochondria in Arabidopsis (Chapters 2 and 3)

Sharing the key components of their division machineries appears to be a ubiquitous and general principle of peroxisomes and mitochondria in many eukaryotes. Fis1 protein is a C-terminal tail anchored membrane protein involved in the fission of peroxisomes and mitochondria in mammals and yeast (Yoon et al., 2003; Thoms and Erdmann, 2005; Kobayashi et al., 2007; Serasinghe and Yoon, 2008). Members of the FIS1 family, FIS1A and FIS1B, have been shown to be involved in peroxisomal and mitochondrial division in *Arabidopsis thaliana* (Lingard et al., 2008; Zhang and Hu, 2009). Ectopic expression of

each *FIS1* gene induces an increase in the numbers of peroxisomes and mitochondria, whereas silencing of *FIS1* gene results in reduced numbers of both organelles (Zhang and Hu, 2008; Zhang and Hu, 2009). Domain truncation analysis indicates that the C-terminal domain of FIS1 is required for its targeting to peroxisomes. Furthermore, analysis of plants in which both *FIS1A* and *FIS1B* are silenced shows that FIS1A and FIS1B play partially redundant roles in promoting the fission of peroxisomes and mitochondria (Zhang and Hu, 2008).

5.1.4 BiFC and Co-IP reveal the interactions among PEX11s, FIS1 and DRPs (Chapter 4)

Mammalian PEX11β forms homodimers, and interacts with Fis1 which anchors DLP1 to mitochondria and peroxisomes. Furthermore, Fis1, Pex11β, and DLP1 form ternary complexes and function in a concerted manner (Kobayashi et al., 2007). This provides clues for the peroxisome research in plants. BiFC analysis using protoplasts indicates that five *Arabidopsis* PEX11 proteins form homodimers and heterodimers, and interact with FIS1B (Lingard et al., 2008). Although PEX11s, FIS1s and DRPs are three types of conserved proteins involved in peroxisome division in various eukaryotic cells, whether these proteins act coordinately *in planta* remain largely unknown. Our research of protein-protein interactions among PEX11, FIS1 and DRP proteins shows that these proteins might function in protein complexes.

We performed a comprehensive protein interaction analysis among DRPs, PEX11s and FIS1s by using BiFC, which not only tells if two proteins interact, but also shows where

interaction happens. Another approach, Co-IP, was employed to confirm protein-protein interaction conferred by BIFC assay.

Consistent with previous results from yeast two-hybrid analysis, our BiFC and Co-IP assays show that DRP3A and DRP3B form homo- and hetero-dimers (Fujimoto et al., 2009). Our results also demonstrate that DRP5B forms homodimer, and interacts with DRP3A and DRP3B on peroxisomes, suggesting that these DRP proteins may hetero-oligomerize and further assemble together to exert their roles in the fission of peroxisomes.

In our study, BiFC and Co-IP assays show that DRP5B does not interact with FIS1B, but only with FIS1A on peroxisomes. DRP3A interacts with FIS1A exclusively on mitochondria, while it interacts with FIS1B only on peroxisomes. In addition, DRP3B interacts with both FIS1A and FIS1B on peroxisomes. Taken together, FIS1 proteins interact with DRPs *in vivo* and *in vitro*. The physical interactions between FIS1s and DRPs provide evidence that FIS1 proteins may act as an anchor for DRPs to peroxisomes and mitochondria *in planta*.

Genetic evidence shows that PEX11 and DRP interact in different organisms. A double deletion of Pex28 and Pex29, which play roles in peroxisome abundance, can be complemented by the overexpression of *Vps1* or *Pex25*, indicating a genetic interplay between the PEX11 family and DRP family in yeast (Vizeacoumar et al., 2003; Thoms and Erdmann, 2005). Interestingly, when mammalian DLP1 was reduced by RNAi, overexpression of PEX11β could no longer induce peroxisome proliferation. This

provides the genetic evidence that PEX11 proteins and DRPs act co-coordinately in peroxisome division in mammals. However, evidence of physical interaction is absent (Li and Gould, 2003; Kobayashi et al., 2007). Therefore, the physical interactions between PEX11s and DRPs have been speculated to be indirect or transient.

In our research, we were able to detect interactions between DRPs and PEX11s using transient expression in tobacco leaves. DRP5B interacts with each of the five PEX11 proteins. DRP3A and DRP3B also interact with PEX11 proteins *in vivo* and *in vitro*. However, some BiFC and Co-IP results are discrepant from each other. For example, Co-IP assay could not detect any interaction between PEX11a and DRPs, while BiFC assay shows that PEX11a interacts with DRP3A, DRP3B and DRP5B. Despite the discrepancy, our data support the view that DRPs and PEX11s may act in protein complexes. Another interesting phenomenon we observed is that PEX11 proteins interact with DRPs only on small peroxisomes before they elongate, suggesting that these two types of proteins may interact with each other at certain time and the interaction might be transient.

In summary, DRP3A, DRP3B and DRP5B are three proteins involved in peroxisome fisssion, and they physically interact with each other, suggesting that they may assemble and function together in *Arabidopsis*. FIS1 and PEX11 proteins are two types of peroxisomal membrane proteins that may serve as adaptors for DRP recruitment to different organelles. Besides, two FIS1 proteins interact with members of PEX11 protein family. These findings indicate that PEX11, FIS1 and DRP proteins may form ternary complexes and play roles in organelle division, especially peroxisome fission, in a concerted manner in plants. However, it is still unclear whether PEX11 and FIS1 proteins

recruit DRPs to peroxisomes in *Arabidopsis*. And, questions remain as to how these three types of proteins cooperate in peroxisome division. Elucidation of the genetic relationship among these three types of proteins will provide answers to the question that if they function coordinately in plant peroxisome division. Plant-specific targeting mechanisms by which DRPs are recruited to different organelles may have been created.

5.2 Future directions

5.2.1 Cooperation of PEX11s, FIS1s and DRPs in plant peroxisome divison?

PEX11 or FIS1 silencing results in a decrease in peroxisome numbers. Loss of the DRPs, DRP3A, DRP3B or DRP5B has an effect on peroxisome number reduction in *Arabidopsis*. Conversely, *PEX11* overexpression can induce peroxisome proliferation, ectopic expression of *FIS1* increases peroxisome abundance, whereas *DRP* overexpression does not have an impact on peroxisome number or morphology (Mano et al., 2004; Thoms and Erdmann, 2005; Fujimoto et al., 2009; Zhang and Hu, 2009). These data are consistent with the current model of peroxisome division in which PEX11 family members act earlier by causing membrane elongation, and then FIS1 serves as an anchor for DRPs that function in organelle membrane scission. Furthermore, physical interactions among these proteins promote speculation that plant PEX11s, FIS1 and DPRs may form tertiary complexes and cooperate in peroxisome division in plants.

ELM1 (Elongated Mitochondria), a plant-specific protein that exclusively targets to the outer membrane of mitochondria, interacts with both DRP3A and DRP3B, and is

required for the mitochondrial targeting of DRP3A (Arimura et al., 2008). Since both FIS1 and ELM1 interact with DRP3A and DRP3B, it is possible that they act as a protein complex on mitochondrial membrane to recruit DRP proteins. However, physical interactions between FIS1 and ELM1 proteins are yet to be identified (Arimura et al., 2008). In addition, Mdv1p/Caf4p is a cytosolic adaptor interacting with both Dnm1 and Fis1 proteins in yeast mitochondrial and peroxisome division (Delille et al., 2009). It is an interesting question whether other FIS1-interacting linker proteins that are functionally similar to Mdv1 and Caf4, exist in higher plants.

Future work will determine if and how these various proteins coordinate in peroxisome division. To further investigate the roles of DRPs in plants, we are trying to generate *drp3A drp3B drp5B* triple mutants and to express *DRP5B* in the *drp3A drp3B* double mutants. Future results will answer if they are functionally redundant in peroxisome division and plant growth. Analyzing the subcellular localization of DRPs in *pex11* or *fis1* knockout mutants will tell whether PEX11s and FIS1s are involved in DRP recruitment to organelles. In addition, ectopic expression of *PEX11* or *FIS1* in *drp* mutants will further provide clues for the question of how these proteins cooperate in plant peroxisome division. These studies will provide new insights into the molecular mechanism underlying organelle division. A recent report showed that the targeting of Fis1 to peroxisomes depends on PEX19, a factor involved in peroxisomal membrane protein import in mammalian cells (Delille and Schrader, 2008). Whether the targeting of FIS1 also relies on PEX19, or other peroxisomal membrane proteins, remains unknown in plants. It raises the question of how PEX11 and FIS1 proteins are targeted to organelles.

5.2.2 Interrelationship of plant organelle division

Growing new evidence in mammals, yeast and plants suggests that peroxsiomes and mitochondria exhibit a greater co-dependent relationship than previously assume (Hoepfner et al., 2001; Li and Gould, 2003; Thoms and Erdmann, 2005; Schrader and Yoon, 2007; Fujimoto et al., 2009; Zhang and Hu, 2009). Peroxisomes and mitochondria are metabolically linked in the β-oxidation of fatty acids in all eukaryotic cells. They also share some of the fission factors, FIS1 and DRP proteins. In addition, a new outer-membrane mitochondria-anchored protein ligase (MAPL) was found to be associated with peroxisomes. MAPL-containing vesicles fuse with a subset of peroxisomes, which marks a novel vesicular transport pathway from mitochondria to peroxisomes in mammalian cells (Neuspiel et al., 2008). Therefore, peroxisomes and mitochondria are interconnected not only metabolically, but also physically in their division and mitochondrion-to-peroxisome vesicle transport. It is of interest to explore if there is a vesicle transport pathway between mitochondria and peroxisomes in plants and what is the fate of the putative vesicle remnant.

Peroxisomes and mitochondria share less common aspects with chloroplasts, except that photorespiration is accomplished in the three compartments in plant cells. Our DRP5B work represents the first report of a crosstalk between the division of chloroplasts and peroxisomes. We have identified an unexpected DRP, DRP5B, as a direct link between the two organelles concerning their divisions that had been known to operate in separate manners. There is so far no evidence for shared components in the biogenesis of mitochondria and chloroplasts. Many aspects of peroxisome biology are still mysterious.

What determines the specific assembly of the shared division components on peroxisomes, mitochondria or chloroplasts, and how is their division coordinated? What are the biological contributions of this coordination?

5.2.3 Peroxisome biogenesis at large

Peroxisome biogenesis in a cell can be considered to be regulated by a number of incompletely understood processes, including peroxisome proliferation by division, peroxisome *de novo* biogenesis, peroxisome inheritance, peroxisome degradation by pexophagy, as well as peroxisome abundance responding to environmental cues (Thoms and Erdmann, 2005). The research in this dissertation has contributed to establishing a mechanistic model of peroxisome division and proliferation in plants. However, our knowledge about the other aspects of peroxisome biogenesis is rather limited. Addressing these questions will bring new insights into our understanding of peroxisome biogenesis and their specific roles in plant growth and development. Peroxisomal research *in planta* will provide important clues for the studies of peroxisomes in other eukaryotes.

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