Peroxisome division and proliferation in plants

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Abstract
Peroxisomes are eukaryotic organelles with crucial functions in development. Plant peroxisomes participate in various metabolic processes, some of which are co-operated by peroxisomes and other organelles, such as mitochondria and chloroplasts. Defining the complete picture of how these essential organelles divide and proliferate will be instrumental in understanding how the dynamics of peroxisome abundance contribute to changes in plant physiology and development. Research in Arabidopsis thaliana has identified several evolutionarily conserved major components of the peroxisome division machinery, including five isoforms of PEROXIN11 proteins (PEX11), two dynamin-related proteins (DRP3A and DRP3B) and two FISSION1 proteins (FIS1A/BIGYIN and FIS1B). Recent studies in our laboratory have also begun to uncover plant-specific factors. DRP5B is a dual-localized protein that is involved in the division of both chloroplasts and peroxisomes, representing an invention of the plant/algal lineage in organelle division. In addition, PMD1 (peroxisomal and mitochondrial division 1) is a plant-specific protein tail anchored to the outer surface of peroxisomes and mitochondria, mediating the division and/or positioning of these organelles. Lastly, light induces peroxisome proliferation in dark-grown Arabidopsis seedlings, at least in part, through activating the PEX11b gene. The far-red light receptor phyA (phytochrome A) and the transcription factor HYH (HY5 homologue) are key components in this signalling pathway. In summary, pathways for the division and proliferation of plant peroxisomes are composed of conserved and plant-specific factors. The sharing of division proteins by peroxisomes, mitochondria and chloroplasts is also suggesting possible co-ordination in the division of these metabolically associated plant organelles.

Introduction
Peroxisomes exist in almost all eukaryotic cells. These small organelles, 0.1–1 μm in diameter, are delimited by single membranes and do not contain their own genome. However, they perform diverse and crucial metabolic functions, including fatty acid metabolism through β-oxidation and degradation of ROS (reactive oxygen species), such as H2O2 [1]. Peroxisome disorders in humans are caused by impaired biogenesis or function of peroxisomes and can lead to death during infancy or early childhood [2]. Peroxisomes are also essential to plant development, resulting in embryonic lethality when functions of core proteins required for peroxisome biogenesis are disrupted. In addition to lipid metabolism and H2O2 degradation, other pathways and functions demonstrated or indicated to be mediated by plant peroxisomes include photorespiration, the glyoxylate cycle, jasmonic acid biosynthesis, IBA (indole-3-butryric acid) metabolism, signalling and plant–pathogen interactions [3–5]. In total, 85 and 61 peroxisomal genes have been identified in humans and Saccharomyces cerevisiae respectively [1], in contrast with the over 130 genes validated to date to encode peroxisomal proteins in Arabidopsis (http://www.peroxisome.msu.edu/). This difference in peroxisomal proteome size may suggest that plant peroxisomes house more pathways and perform more complex functions compared with peroxisomes from yeasts and mammals.

Upon developmental, metabolic and environmental changes, peroxisomes are capable of varying their morphology, abundance and content to adapt to the new conditions. It is commonly believed that, besides arising de novo from subdomains of the ER (endoplasmic reticulum), peroxisomes can also multiply via division. Division and proliferation (i.e. induced division) both follow the events of peroxisome elongation/growth/tubulation, membrane constriction and fission, resulting in the duplication or multiplication of peroxisomes. A number of components of the peroxisome division/proliferation machineries have been identified from the fungal and animal kingdoms [6–9]; dissection of the molecular events underlying these processes in plants has also begun in recent years.

Three evolutionarily conserved components in the peroxisome division machinery
Using the reference plant species Arabidopsis thaliana, several proteins or protein families involved in the division/proliferation of peroxisomes have been identified...
through sequence-based searches for homologues to yeast peroxisome division proteins and GFP (green fluorescent protein)/YFP (yellow fluorescent protein)-based peroxisome morphological mutant screens [4,10]. These proteins, i.e. PEX11 (PEROXIN11), DRP3 (dynamin-related protein 3) and FIS1 (FISSION1), represent three evolutionarily conserved components of the peroxisome division and proliferation machineries across species.

Among the peroxisome division factors, the yeast Pex11p protein was the first to be identified [11,12]. The function of this protein, which is conserved in a wide range of species, is believed to be promoting peroxisome elongation/tubulation, an early step in peroxisome division. Two additional S. cerevisiae proteins, Pex25p and Pex27p, are partially redundant in function with Pex11p [13–15]. In mammals, PEX11α, -β and -γ are three PEX11 homologues contributing to peroxisome elongation to various degrees [1–2]. Overall, loss-of-function pex11 mutants show a reduction in the peroxisome population, whereas overexpressing the gene results in increased peroxisome elongation and proliferation. Some PEX11 genes are also transcriptionally regulated in response to environmental, metabolic and developmental cues. Taken together, PEX11s are positive regulators of peroxisome division, playing a rate-limiting role in the early stage of the process [1,6–8,10].

The studies of Arabidopsis PEX11 proteins have been carried out in cell cultures and transgenic plants [16–18]. Arabidopsis contains five isoforms of PEX11, PEX11a–e, which are further grouped into three subfamilies (PEX11a, PEX11b and PEX11c–e) based on sequence similarity. All five PEX11 homologues are peroxisomal integral membrane proteins, inducing peroxisome proliferation at various degrees and in a partially redundant fashion [16–18]. Like their counterparts in yeasts and mammals, Arabidopsis PEX11 proteins are most likely to be involved in initializing peroxisome division; PEX11c–e were able to partially rescue the phenotypes of the yeast pex11-null mutant as well [16,17]. Despite the extensive characterization of PEX11 proteins in various species, their biochemical function is still elusive.

DRPs compose the second group of conserved factors in peroxisome division. Dynamins and DRPs are large GTPases that mediate membrane fission by forming collar-like oligomeric complexes around the neck of constriction sites and acting as mechanochemical enzymes through GTP hydrolysis [9,19,20]. Unlike PEX11, DRPs involved in peroxisome division also participate in the division of mitochondria (i.e. yeast Dnm1p and mammalian Drp1/DLP1) or protein sorting to the vacuole (i.e. yeast Vps1p). Null mutants of the yeast DNM1 and VPS1 (vacuolar protein sorting 1) genes and mammalian cells in which the Drp1 (or DLP1) gene is silenced exhibit elongated peroxisomes that have already been constricted, the so-called ‘beads on a string’ phenotype [21–23]. Ectopic expression of PEX11 in cells lacking a functional Drp1 only results in tubulation of peroxisomes, but fails to process peroxisome fission. Taken together, these data suggest that these DRPs are responsible for executing the fission step [23–25].

Arabidopsis has approx. 16 DRP proteins categorized into six subfamilies (DRP1–6) based on their sequence and structure similarity [26]. A phylogenetic study of dynamin proteins across diverse species showed that DRP3 proteins (DRP3A and DRP3B) belong to the same subclade in which Dnm1p and Drp1 are located [27], suggesting functional conservation between DRP3A, DRP3B and other members of this subgroup. Indeed, DRP3A and DRP3B are involved in the division of both peroxisomes and mitochondria in Arabidopsis, as drp3 mutants show impaired division in both organelles and inhibited plant growth [28–33]. DRP3A has a dominant role over DRP3B in peroxisome division, whereas the two proteins are functionally equivalent in the division of mitochondria [30,31,33].

Since most DRPs lack putative lipid binding or transmembrane domains, they are believed to be cytosolic proteins recruited to the destination membranes via cytosolic adaptors and/or membrane-bound receptors. Multiple lines of evidence have suggested that FIS1, a protein dual-targeted to peroxisomes and mitochondria, functions as a membrane-bound receptor recruiting DRP directly or indirectly to the organelar membranes [23,24,31,34–38]. FIS1 is a C-TA (C-terminal tail-anchored) membrane protein with a TPR (tetratricopeptide repeat) domain at the N-terminus and a single segment of transmembrane domain at the C-terminal end. The TPR domain is a protein–protein interaction domain facing towards the cytosol, with a predicted role in recruiting DRPs or the DRP complex to the target membrane. Mammalian Fis1 has been shown to directly recruit Drp1 to peroxisomal or mitochondrial membranes. Suppression of FIS1 by siRNA (small interfering RNA) phenocopied the Drp1 siRNA mutant, whereas ectopic expression of the FIS1 gene caused an increase in the number of peroxisomes and mitochondria, suggesting a rate-limiting role of Fis1 in peroxisomal/mitochondrial fission [24,34].

Not surprisingly, FIS1 orthologues in Arabidopsis (FIS1A/BIYG1 and FIS1B) have been identified as peroxisomal and mitochondrial division factors, whereby loss-of-function mutants contain reduced peroxisomal and mitochondrial abundance, and plants overexpressing each gene showcase a significant increase in peroxisomal/mitochondrial number [31,38,39]. Although FIS1s have been characterized as one of the positive regulators in organelle division, their ability to directly or indirectly recruit DRPs has yet to be demonstrated in Arabidopsis. The fact that peroxisomes and mitochondria share common division factors prompted the speculation that these proteins might be key links in the possible co-ordinated division of these two organelles, which are associated through metabolic pathways, such as lipid mobilization during oilseed germination, glycolate recycling during plant photorespiration and β-oxidation in animals [10,25].

In yeasts, the membrane-bound receptor Fis1p functions together with two WD40-repeat cytosolic adaptors, Mdv1p and its parologue, Caf4p, forming a receptor complex [40–43]. Owing to the lack of apparent functional or structural homologues of Mdv1p and Caf4p in other species,
those two proteins are currently defined as yeast-specific factors in peroxisomal and mitochondrial division.

**DRP5B is a plant/algal-specific DRP in the division of both peroxisomes and chloroplasts**

The DRPs subfamily of *Arabidopsis* DRPs consists of DRP5A and DRP5B (ARC5) [26]. Phylogenetic analysis of dynamin and dynamin-like proteins demonstrated that DRP5B and its orthologues, which only exist in plants and chloroplast-containing algae, arose from a group of DRPs (including DRP5A) involved in cytokinesis [27]. We recently found that DRP5B (ARC5), which was originally identified as a chloroplast division protein [44], plays an additional role in peroxisome division [44a]. In addition to enlarged and dumb-bell-shaped chloroplasts showing impaired division, highly clustered peroxisomes unable to complete fission and/or enlarged peroxisomes are also evidenced in the *drp5B* mutants. GFP–DRP5B, driven by the 35S constitutive promoter or the native *DRP5B* promoter, co-localized with the peroxisomal marker CFP–PTS1 (cyan fluorescent protein–peroxisome targeting signal type 1), besides being targeted to a discontinuous ring at the chloroplast division site. Mutants of key components of the chloroplast division apparatus, such as *arc3* and *arc6*, show normal peroxisomal morphology and abundance and undisrupted peroxisomal localization of GFP–DRP5B. This observation supports the view that the peroxisome division defect in the *drp5B* mutant is not a secondary effect from impaired chloroplast division. Furthermore, major peroxisome-related functions, such as photorespiration and fatty acid β-oxidation, are compromised in the *drp5B*-null mutants. Thus, unlike DRP3A and DRP3B, whose functions in peroxisomal and mitochondrial division are conserved across diverse species, DRP5B is a plant/algal-specific dynamin that exerts its function in the division of chloroplasts and peroxisomes.

Using BiFC (bimolecular fluorescence complementation) and co-IP (co-immunoprecipitation) assays with proteins transiently expressed in tobacco leaves, we have demonstrated further that DRP5B is capable of homodimerization and heterodimerization with DRP3A and DRP3B [44a], consistent with the view that dimer formation is necessary for the GTPase activity of dynamins [45]. In addition, DRP5B also forms complexes with FIS1A (but not FIS1B) and at least four of the five PEX11 isoforms, further strengthening the notion that proteins involved in the early (elongation) and late (fission) steps of peroxisome proliferation may act cooperatively. Establishment of functional dependence between these proteins, for instance, whether PEX11 and/or FIS1 functions are required for the recruitment of DRP5B to peroxisomes, will be needed to paint a clearer picture of this putative co-operation.

It is unclear whether the role for DRP5B in peroxisome division is replaceable by those of DRP3A and DRP3B. The contribution of DRP5B may be distinct, because sequences and domain structures between DRP5B and DRP3 are quite different and the pattern for DRP5B localization on peroxisomes seems to differ from those previously reported for DRP3A and DRP3B [30,31,33]. Further genetic analysis (e.g. making *drp3A* *drp3B* *drp5B* triple mutants) and biochemical characterization of DRP5B and DRP3 should shed light on this matter.

The finding that DRP5B has dual roles in the division of chloroplasts and peroxisomes provides another line of evidence for shared organelle division factors in plants. Chloroplasts and peroxisomes act in concert in metabolic processes such as photorespiration and jasmonic acid biosynthesis. Whether the use of shared fission components represents a mechanism to enable co-ordinated divisions between these two organelles awaits further investigation.

**The coiled-coil membrane protein PMD1 is a putative peroxisomal and mitochondrial division factor in *Arabidopsis***

In addition to the conserved core proteins in peroxisome division, i.e. *PEX11*, DRP and FIS1 proteins, two additional families of yeast proteins, *Pex28p*/*Pex29p* and *Pex30p*/*Pex31p*/*Pex32p*, have been implicated in mediating peroxisome separation and regulating size/number of peroxisomes [7]. It has not been determined whether these PEX proteins and the Mdv1p/Caf4p WD40 linker proteins have functional orthologues in plants. Despite lacking Mdv1p/Caf4p-like proteins, animals utilize at least one of their own tail-anchored factors, Mff (mitochondrial fission factor), in the division of both mitochondria and peroxisomes [46]. Extensive genetic screens in *Arabidopsis* have not yet led to the identification of major players in peroxisome division, besides DRP3A. Several recent in-depth proteomic analyses of *Arabidopsis* peroxisomes [47–49] have not revealed potential candidates in organelle division either. To identify novel, especially plant-specific, components of the peroxisome division machinery, we used an *in silico* approach and searched for uncharacterized *Arabidopsis* proteins containing well-known protein–protein interaction domains, as well as putative transmembrane domains (K. Aung and J. Hu, unpublished work). Since coiled-coil proteins have been shown to be involved in organelle division and positioning in several model organisms (see below), we first focused on finding the peroxisomal coiled-coil proteins.

A coiled-coil domain is defined by having heptad repeats, with each repeat containing hydrophobic residues in the first and fourth positions, and charged/polar residues in the fifth and seventh positions. Coiled–coil proteins are known to homo- (self-interaction) or hetero- (interaction with other partners) dimerize and function in regulating gene expression, maintaining the structure of Golgi stacks, and attaching functional protein complexes to centrosomes, centromeres and the nuclear envelope [50]. The role of coiled-coil proteins in organelle division has been shown in several organisms. For example, the homologous *Arabidopsis* coiled-coil proteins PDV1 and PDV2 (plastid division 1 and 2) are anchored to the outer envelope membrane of chloroplasts through the
transmembrane domain, using the N-terminal cytoplasmic coiled-coil domain to recruit DRP5B (ARC5) directly or indirectly to the division site [51,52]. The mammalian Mff is a C-TA protein with a cytosolic coiled-coil domain, serving as a metazoan-specific component in mitochondrial and peroxisome division [46]. Coiled-coil proteins are also involved in organelle positioning. One such example is CHUP1 (chloroplast unusual positioning protein 1), which is targeted to the outer envelope of chloroplasts and contains a cytosolic coiled-coil domain. CHUP1 plays a role in maintaining the association of chloroplasts with actin filaments; loss-of-function chup1 mutants fail to mount chloroplastic photo-avoidance responses [53,54].

We searched the ARABI-COIL database (http://www.coiled-coil.org/arabidopsis/) for putative membrane-bound, long coiled-coil proteins, i.e. those that contain one coiled-coil domain of at least 70 amino acids or more than one coiled-coil domain with at least 90 amino acids in total [50]. Over a dozen expressed (and uncharacterized) proteins fit our selection criteria and were subjected to in vivo subcellular localization analysis using fluorescent protein fusions. One protein was found to localize to peroxisomes and mitochondria, and was thus named PMD1 (K. Aung and J. Hu, unpublished work).

PMD1 is a plant-specific protein with four putative coiled-coil domains at the N-terminus and a putative transmembrane domain at the C-terminal end. Using transgenic Arabidopsis plants, we showed that YFP–PMD1, driven by the native PMD1 promoter, localizes to punctate spots labelled by the peroxisomal marker CFP–PTS1 or the mitochondrial marker ScCOX (S. cerevisiae cytochrome oxidase)–CFP [55]. The peroxisomal and mitochondrial targeting of PMD1 was also confirmed using a biochemical approach, in which we detected YFP–PMD1 in the purified peroxisomal and mitochondrial fractions from transgenic plants expressing the YFP–PMD1 fusion. Furthermore, PMD1 was only detected in the pellet fraction after purified leaf peroxisomes or mitochondria were treated with NaCl and strong alkaline solutions (Na₂CO₃), suggesting it to be an integral membrane protein of the peroxisome and mitochondrion. Finally, immunoblot analysis after thermolysin treatment showed that PMD1 has an extruding N-terminus, which faces toward the cytosol. We conclude that PMD1 is a C-TA protein localized to the outer surface of peroxisomes and mitochondria in Arabidopsis.

A null mutant of PMD1 contains enlarged peroxisomes and elongated mitochondria. On the other hand, plants overexpressing the gene have highly aggregated peroxisomes and mitochondria and display slowed growth compared with the wild-type plants. Collectively, these findings suggest that PMD1 has a functional role in division and/or positioning of peroxisomes and mitochondria. Similar to most of the coiled-coil proteins, PMD1 also self-interacts, as demonstrated by yeast two-hybrid, BiFC and co-IP analyses. Furthermore, dissection of the domains determined that the second coiled-coil domain is required for PMD1 to homodimerize and to induce organelle aggregation.

Physical and genetic interaction studies between PMD1, PEX11, FIS1 and DRPs will uncover the relationship between PMD1 and the core division factors and determine whether PMD1 functions in co-ordination with the PEX11-DRP-FIS1 proteins in division or in an independent pathway. Meanwhile, we also entertain the idea that PMD1 might be functional as a molecular tether to bring peroxisomes and mitochondria into close proximity. This hypothesis is based on the facts that (i) PMD1 is dual-targeted to both organelles as a C-TA protein, (ii) PMD1 homodimerizes and (iii) overexpression of PMD1 leads to highly clustered peroxisomes and mitochondria. Although its exact mode of action is yet to be determined, PMD1 has proven to be a plant-specific factor with a key role in regulating the morphology, abundance and/or possibly positioning of both peroxisomes and mitochondria.

**Light regulation of peroxisome proliferation**

Plant peroxisomes perceive a variety of signals and respond by changing their morphology, abundance and protein complement [4]. Light activates the switch from heterotrophic to autotrophic growth in germinating seedlings, where proteins required for photosynthesis and photorespiration are synthesized and imported into the organelles. Upon light treatment, Arabidopsis plants expressing the peroxisome marker protein YFP–PTS1 also exhibit a concomitant increase in peroxisome abundance in cotyledon cells, following a multi-step process resembling that of peroxisome division [56].

We partially dissected the signalling pathway by which light induces peroxisome proliferation in Arabidopsis [56]. We showed that among the five PEX11 genes, PEX11b is the only one whose expression is strongly induced by light, and that plants in which PEX11b expression is largely reduced by RNAi (RNA interference) responds only subtly to light, pointing towards a pivotal role for the PEX11b isoform in light-induced peroxisome proliferation. After examining a number of light signalling mutants in Arabidopsis, we were able to identify the far-red light receptor phyA (phytochrome A) and a bZIP transcription factor, HYH (HY5 homologue), as key regulators of PEX11b expression. The light induction of PEX11b is significantly reduced and peroxisome abundance is strongly decreased in the phyA and byb mutants, and these phenotypes were largely rescued by overexpressing PEX11b. Lastly, the nuclear transcriptional factor HYH can bind to the LRE (light-responsive element)-enriched promoter of PEX11b in gel-shift assays.

Our data collectively suggest that the increase in peroxisome proliferation during dark-to-light transition in Arabidopsis seedlings is, at least in part, a result of PEX11b gene activation. Furthermore, a phyA-dependent pathway, in which HYH is a major component, is largely responsible for this transcriptional activation [56]. The identification of components in the light signalling pathway that induces
peroxisome proliferation in Arabidopsis is a starting point for dissecting signalling pathways that regulate changes in peroxisome abundance in plants. Besides members of the PEX11 gene family, other factors, such as FIS1, DRP and PMD1, may also act as molecular targets for environmental and metabolic cues at gene or protein levels.

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