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REVIEW

Plant Peroxisomes: Biogenesis and Function

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Peroxisomes are eukaryotic organelles that are highly dynamic both in morphology and metabolism. Plant peroxisomes are involved in numerous processes, including primary and secondary metabolism, development, and responses to abiotic and biotic stresses. Considerable progress has been made in the identification of factors involved in peroxisomal biogenesis, revealing mechanisms that are both shared with and diverged from non-plant systems. Furthermore, recent advances have begun to reveal an unexpectedly large plant peroxisomal proteome and have increased our understanding of metabolic pathways in peroxisomes. Coordination of the biosynthesis, import, biochemical activity, and degradation of peroxisomal proteins allows for highly dynamic responses of peroxisomal metabolism to meet the needs of a plant. Knowledge gained from plant peroxisomal research will be instrumental to fully understanding the organelle's dynamic behavior and defining peroxisomal metabolic networks, thus allowing the development of molecular strategies for rational engineering of plant metabolism, biomass production, stress tolerance, and pathogen defense.

INTRODUCTION

Peroxisomes were one of the last major cellular organelles to be discovered (De Duve and Baudhuin, 1966), and their importance in plant metabolism, particularly with respect to fatty acid β-oxidation, the glyoxylate cycle, and photorespiration, was soon realized (reviewed in Beevers, 1979; Huang et al., 1983). In recent years, it has become clear that peroxisomes are highly dynamic organelles, both morphologically and metabolically, and are involved in a wide range of plant processes, including primary carbon metabolism, secondary metabolism, development, abiotic stress response, and pathogen defense. With this understanding, the names of microbody, glyoxysome, peroxisome, and gerontosome, which were used to define some specialized peroxisome activities, are now subsumed within the general name of peroxisome (Pracharoenwattana and Smith, 2008). Here, we review recent advances in plant peroxisome research and provide perspectives on the future research needed to fully understand the dynamics and functions of these organelles.

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PEROXISOME BIOGENESIS

The Role of the Endoplasmic Reticulum in Peroxisome Biogenesis

A Historical Perspective

The biogenetic relationship between the endoplasmic reticulum (ER) and peroxisomes has been highly contentious (reviewed in Mullen and Trelease, 2006; Schlüter et al., 2006; Tabak et al., 2006). Peroxisomes were initially thought to form exclusively by vesiculation of specialized ER regions. Nascent soluble and membrane-bound protein constituents were thought to be synthesized cotranslationally on the ER before sequestration, along with membrane lipids, into an expanding vesicle that eventually buds off from a specific segment of the (smooth) ER to produce a new functional peroxisome (Figure 1A). This ER vesiculation model (Beevers, 1979) was supported by microscopy observations of peroxisomes commonly associated with the ER in plants (Huang et al., 1983) and by pulse-chase studies indicating that both peroxisomal proteins and phospholipids in the peroxisomal membrane first passed through the ER (Moore, 1982; Lord and Roberts, 1983).

However, new techniques and reevaluation of older data resulted in the ER vesiculation model losing favor to the growth and division model (Trelease, 1984; Lazarow and Fujiki, 1985). In

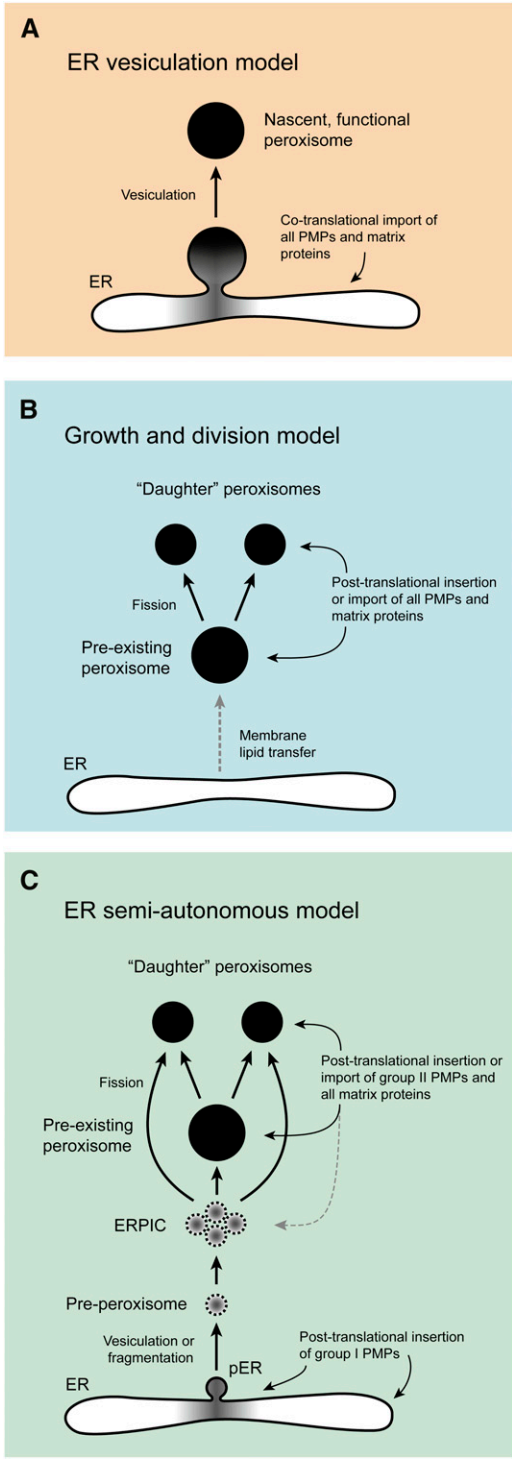

Figure 1. Models for the Biogenesis of Peroxisomes in Plants.

In the ER vesiculation model **(A)**, all of the protein constituents of the peroxisomal boundary membrane and matrix are considered to be synthesized cotranslationally on the ER and then sequestered into a specialized region of the ER, where an expanding smooth membrane vesicle eventually buds off to yield (de novo) a nascent, functional peroxisome. By contrast, in the growth and division model **(B)**, all PMPs and matrix proteins are synthesized on free polyribosomes in the cytosol and sorted posttranslationally to preexisting and new (daughter) peroxisomes, resulting in their growth. Daughter peroxisomes arise from preexisting peroxisomes by fission, and the ER somehow provides the membrane lipids necessary for peroxisome growth (e.g., via ER-peroxisome contact sites and/or lipid transfer proteins). In the ER semiautonomous model **(C)**, some PMPs (group I PMPs) are posttranslationally inserted either directly into the pER subdomain or first into the general ER and then routed to the pER. The subsequent transport of these group I PMPs (and membrane lipids) from the pER to preexisting and daughter peroxisomes involves the (de novo) formation (via vesiculation or fragmentation) of putative preperoxisomal carriers that travel to, or from, an ERPIC. All matrix proteins and group II PMPs are sorted posttranslationally from the cytosol to daughter peroxisomes and preexisting peroxisomes, and perhaps preperoxisomes at the ERPIC, the former of which arise by fission (as depicted in more detail in Figure 2).

this model, peroxisomes, like chloroplasts and mitochondria, were considered fully autonomous, increasing in size by posttranslational import of protein constituents from the cytosol and forming only from the division of preexisting organelles (Figure 1B). The ER was thought to serve only as a source of membrane lipids for the enlargement of preexisting peroxisomes (e.g., via phospholipid transfer proteins and/or ER-peroxisome contact sites).

Studies in yeasts and Chinese hamster ovary cells (Kunau, 1998) identified a set of peroxins encoded by *PEX* genes that are required for peroxisome biogenesis. The growth and division paradigm was challenged by demonstrations that mutant yeast and mammalian cells lacking certain *PEX* genes, such as *PEX3* and *PEX19*, were devoid of any obvious peroxisomal structures, yet the organelles appeared after reintroduction of the wild-type gene (South and Gould, 1999; Hettema et al., 2000). Also conflicting with the idea that peroxisomes are strictly autonomous were observations from in vivo trafficking studies of peroxisome membrane proteins (PMPs) in yeasts, mammals, and plants, which demonstrated that at least some PMPs sorted indirectly to peroxisomes by way of the ER (reviewed in Titorenko and Rachubinski, 2009).

The current working model for peroxisome biogenesis incorporates aspects of both earlier models plus latest data and considers peroxisomes to be semiautonomous, arising by two distinct pathways: de novo biogenesis from specific regions of the ER and by growth and fission of preexisting peroxisomes (Figure 1C). This ER semiautonomous model for peroxisome biogenesis includes at least one important new feature: the involvement of ER-derived preperoxisomes (i.e., vesicles or membrane fragments/lamellae) that deliver phospholipids and some PMPs to preexisting peroxisomes and/or fuse together in a controlled, step-wise fashion to form a new peroxisome (Trelease and Lingard, 2006; Titorenko and Rachubinski, 2009).

There is a growing appreciation that the processes underlying the de novo synthesis and growth and fission of peroxisomes may not be controlled completely independently (Koch and Brocard, 2011) and that these processes may vary considerably depending on the species, cell type, or physiological status of the organism. Hence, a unified model of peroxisome biogenesis may not be easy to attain. For instance, in mammals and yeast, both de novo synthesis from the ER and fission contribute to the formation of new (daughter) peroxisomes, albeit to different

extents (Nagotu et al., 2010), whereas in plants there is no direct evidence for the de novo synthesis of peroxisomes from the ER. Instead, the ER appears to serve as a platform from which selected membrane components are derived and trafficked by an unknown carrier to preexisting peroxisomes, which undergo growth and division to produce new peroxisomes.

Membrane Protein Trafficking from the ER to Peroxisomes

The understanding of the ER-to-peroxisome pathway in plants is based primarily on studies of two types of PMPs: (1) cottonseed (*Gossypium hirsutum*) and pumpkin (*Cucurbita maxima*) ascorbate peroxidase (APX), a carboxy tail-anchored integral membrane protein that plays a key role in protecting plant cells by scavenging toxic reactive oxygen species (Yamaguchi et al., 1995; Bunkelmann and Trelease, 1996); and (2) *Arabidopsis thaliana* PEX16, an integral membrane peroxin (Karnik and Trelease, 2005; Nito et al., 2007). Like most other PMPs that traffic to peroxisomes via the ER (referred to as group I PMPs), APX3 and PEX16 contain ER targeting elements that are distinct from typical signal peptide or signal anchor sequences and overlap with or are adjacent to the elements responsible for their subsequent targeting from the ER to peroxisomes (Mullen and Trelease, 2000; Karnik and Trelease, 2007). While the precise nature of these ER targeting signals is not known, APX relies on a posttranslational targeting process that involves ATP and various chaperones (Mullen et al., 1999; Shen et al., 2010). Whether PEX16 and any other plant PMPs that traffic to peroxisomes via the ER use the same or a different posttranslational pathway remains to be investigated.

Another important, but poorly characterized, aspect of the ER-peroxisome relationship in plants is the nature of the peroxisomal ER (pER) subdomain, a region of the ER at which preperoxisomes are proposed to be formed (Mullen et al., 1999; Lisenbee et al., 2003). The PMPs APX3 and *Arabidopsis* PEX10 localize to subdomains of the rough ER (Lisenbee et al., 2003; Flynn et al., 2005; Karnik and Trelease, 2005, 2007). However, whether these regions are identical and how the intra-ER sorting and segregation of APX and PEX10 (or any other PMP in the ER) is accomplished has not been elucidated. By contrast, *Arabidopsis* PEX16 localizes throughout the general ER and not to a specific subdomain, as does PEX16 in mammals (Kim et al., 2006) and certain yeasts (Titorenko and Rachubinski, 1998). *Arabidopsis* PEX16 also exists in peroxisomes under steady state conditions (Karnik and Trelease, 2005) and a *pex16* knockdown mutant possesses fewer and enlarged peroxisomes (Nito et al., 2007), suggesting that, as in mammals, plant PEX16 performs multiple roles depending on its subcellular location. For instance, PEX16 may modulate peroxisome morphology (Nito et al., 2007) via its potential role as peroxisomal membrane receptor (Matsuzaki and Fujiki, 2008). At the ER, however, PEX16 might regulate the early steps of peroxisome biogenesis, including acting as a receptor for other PMPs and orchestrating their subsequent sorting into the pER (Karnik and Trelease, 2005; Nito et al., 2007), although, to date, no experimental evidence for such a role in plants has been presented. In addition, PEX16 appears to participate in the biogenesis of other plant-specific subcellular compartments, such as protein and oil bodies, which also are derived from the ER (Lin et al., 1999).

Arabidopsis PEX10, which is reported to sort either indirectly to peroxisomes via the ER in suspension cells (Flynn et al., 2005) or directly to peroxisomes from the cytosol in leaves (Sparkes et al., 2005), also appears to perform multiple functions, including the biogenesis of ER-derived protein and oil bodies (Schumann et al., 2003), the maintenance of ER morphology, the formation of cuticular wax (Kamigaki et al., 2009), peroxisome and chloroplast connections (Schumann et al., 2007), and, as discussed further below, the import of matrix proteins (Nito et al., 2007; Prestele et al., 2010). The relative distribution of PEX10 in the ER and peroxisomes might exemplify how plant peroxisome biogenesis varies depending on the species and/or cell type. Likewise, *Arabidopsis* PEX3 is reported to target directly to peroxisomes from the cytosol (Hunt and Trelease, 2004), whereas its homologs in yeast and mammals sort to peroxisomes via the ER (Hoepfner et al., 2005; Toro et al., 2009) and participate in PMP import and the formation of preperoxisomal membrane carriers (e.g., vesicles) (van der Zand et al., 2010). Whether plant PEX3 functions independently of the ER is still an open question, particularly if the protein sorts rapidly through the ER as it does in other organisms (Hoepfner et al., 2005; Agrawal et al., 2011), making transient intermediates difficult to detect.

No solid data exist on the preperoxisomal membrane carriers that would originate from the pER and ultimately sort to preexisting or nascent (daughter) peroxisomes in plants, but factors necessary for forming preperoxisomes are beginning to be identified in other organisms, such as Sec20p, Sec39p, and Dsl1p (Perry et al., 2009) as well as Sec16B (Yonekawa et al., 2011). In plants, small preperoxisomal membrane vesicles may bud from the ER and perhaps, prior to their fusion with preexisting peroxisomes, coalesce in a so-called ER-peroxisome intermediate compartment (ERPIC) (Mullen and Trelease, 2006; Trelease and Lingard, 2006), consistent with the proposed ER-to-peroxisome vesicular transport pathways in certain yeasts and mammalian cells (reviewed in Titorenko and Rachubinski, 2009). Alternatively or in addition, plant preperoxisomes may exist as large pleomorphic structures of clustered peroxisomal tubules, reminiscent of the lamellar extension that detaches en block from the ER in mouse dendritic cells (Geuze et al., 2003). Independent of their structural features, one important functional attribute of the preperoxisomal membrane vesicles in plants (and in other organisms) is that they are competent in importing matrix proteins (Mullen et al., 1999) and group II PMPs that bypass the ER (i.e., PMPs that sort directly to peroxisomes from the cytosol, such as the *Arabidopsis* 22-kD PMP [PMP22]) (Murphy et al., 2003).

Another intriguing possibility is that plant peroxisomes might remain physically attached to the ER, analogous to recent models for oil body–ER connectivity (Chapman et al., 2012). Some support for this premise comes from live-cell imaging of peroxisome tubular extensions (peroxules) in *Arabidopsis* (Sinclair et al., 2009). The growth and retraction of peroxules appears to occur along tracks defined by ER tubules (and perhaps driven by cytoskeleton interactions) and at speeds (i.e., seconds) that argue against the idea that these morphological changes in peroxules simply result from the acquisition of (new) membranes from the ER via preperoxisomal carriers (Mathur, 2009). However, because

no ultrastructural studies have revealed any direct connections between ER and peroxisome membranes in any organism, peroxisome-ER connectivity has to be considered carefully. For instance, the reported dynamic behavior of peroxules in plants could be due to peroxisome-ER contact sites, akin to that proposed in yeast (Raychaudhuri and Prinz, 2008).

Peroxisome Multiplication by Growth and Division

In addition to de novo formation from the ER, peroxisomes also multiply through division, which occurs constitutively (i.e., in association with the cell cycle) or inducibly (i.e., peroxisome proliferation). Peroxisome division begins with organelle elongation/tubulation and ends in fission, resulting in the formation of two or more peroxisomes (reviewed in Koch and Brocard, 2011; Schrader et al., 2011). *Arabidopsis* proteins that operate in peroxisome division have been identified through sequence similarity-based searches using yeast proteins, forward genetic screens, and in silico analysis followed by cell biological validations (reviewed in Kaur and Hu, 2009; Aung et al., 2010). As discussed below, plant peroxisome division machineries consist of evolutionarily conserved and plant-specific factors. Moreover, several plant peroxisomal division proteins are shared with mitochondria and chloroplasts, a strategy that might enable plants to coordinate divisions of these metabolically-linked organelles.

Peroxisome Elongation/Tubulation: PEROXIN11 Proteins Serve as Key Factors

Saccharomyces cerevisiae Pex11p was the first peroxisome division protein identified. Ectopic expression of Sc-*PEX11* leads to the elongation/tubulation and/or increased numbers of peroxisomes, whereas the yeast *pex11* null mutants contain one or two giant peroxisomes per cell (Erdmann and Blobel, 1995; Marshall et al., 1995). PEROXIN11 (PEX11) homologs have been identified as multigene families in various lineages (Hu, 2009; Schrader et al., 2011). *Arabidopsis* has five PEX11 homologs categorized into three subfamilies based on sequence (i.e., PEX11a, PEX11b, and PEX11c to e) (Figure 2A). These five isoforms are integral PMPs capable of inducing peroxisome elongation and/or number increase (Figure 2B) (Lingard and Trelease, 2006; Nito et al., 2007; Orth et al., 2007; Lingard et al., 2008). Heterologous expression of plant or mammalian PEX11 homologs complements the yeast mutant phenotype to various degrees, demonstrating the conserved role of PEX11 across kingdoms (Orth et al., 2007; Koch et al., 2010).

A recent study in *Penicillium chrysogenum* showed a role for Pex11p (and possibly other PEX11 homologs) in membrane remodeling. The conserved N-terminal amphipathic helix of Pc-Pex11p binds to liposomes that have membrane lipid content resembling that of the peroxisome membrane and induces liposome tubulation and membrane curvature, possibly through insertion into the cytosolic leaflet of the phospholipid bilayer (Koch and Brocard, 2011; Opaliński et al., 2011). Despite sequence and structural similarities, individual PEX11 family members may have distinct functions (Koch and Brocard, 2011; Huber et al., 2012). The differential roles played by *Arabidopsis* PEX11 proteins is indicated by the findings that (1) PEX11a has a distinct membrane topology from the other isoforms (Lingard and Trelease, 2006), and (2) only members of the PEX11c-e subfamily complement the yeast *pex11* mutants (Orth et al., 2007; Koch et al., 2010).

Being a highly abundant component of the peroxisome membrane and rate-limiting factor in early peroxisome division, PEX11 is regulated at both transcriptional and posttranslational levels in yeast and mammals (Gurvitz and Rottensteiner, 2006; Michalik et al., 2006; Knoblach and Rachubinski, 2010). In *Arabidopsis* synchronized cell cultures, the expression of *PEX11* and genes encoding other key division proteins is regulated by the cell cycle, which correlates with peroxisome duplication (Lingard et al., 2008). A phytochrome A–mediated light signaling pathway induces *PEX11b* expression during dark-to-light transitions, in which the bZIP transcription factor HY5 HOMOLOG binds to the *PEX11b* promoter (Figure 1A) (Desai and Hu, 2008). Salt stress, abscisic acid, and jasmonic acid (JA) also regulate the expression of *Arabidopsis* and/or rice (*Oryza sativa*) *PEX11* genes (Nayidu et al., 2008; Mitsuya et al., 2010). Whether plant PEX11 proteins are subjected to posttranslational modifications is unclear.

Role of Dynamin-Related Proteins DRP3 and DRP5B and FISSION1 in Fission

Following elongation/tubulation, peroxisome division proceeds with membrane constriction and fission, a process mediated by a protein complex consisting of the integral membrane-anchored protein FISSION1 (FIS1), a dynamin-related protein (DRP), and some lineage-specific cytosolic adaptor proteins (Benard and Karbowski, 2009).

Dynamins and DRPs are mechano-chemical enzymes or signaling GTPases that form oligomeric rings around membranes, enforcing membrane fission or fusion through GTP hydrolysis (Praefcke and McMahon, 2004. At least three of the 16 *Arabidopsis* DRPs are involved in peroxisome fission. The closely related DRP3A and DRP3B proteins are dual localized and shared by peroxisomal and mitochondrial divisions, with DRP3A playing a major role in peroxisome fission (Mano et al., 2004; Fujimoto et al., 2009; Zhang and Hu, 2009; Kaur and Hu, 2009 and references therein) (Figure 2). Interestingly, DRP5B (ARC5), a DRP distantly related to DRP3, targets to chloroplasts and peroxisomes and facilitates the division of both organelles (Gao et al., 2003; Zhang and Hu, 2010) (Figure 2). Besides having enlarged, dumbbell-shaped chloroplasts, *drp5B* mutants also contain aggregated peroxisomes that are impaired in fission (Figure 2B) and are partially compromised in peroxisomal functions (Zhang and Hu, 2010). Whereas DRP3A and DRP3B are members of an ancient family of DRPs involved in peroxisome and mitochondrial division, DRP5B evolved more recently in the plant/algal lineage (Miyagishima et al., 2008) to mediate chloroplast and peroxisome division.

Most eukaryotic DRPs lack a lipid binding or transmembrane domain (TMD) and are only recruited to the division sites by interacting directly or indirectly with a membrane-bound receptor (Praefcke and McMahon, 2004). A yeast DRP, Dnm1p, is recruited to peroxisomes and mitochondria by Fis1p, which is tethered to the organelles by its C terminus and extends its N-terminal tetratricopeptide repeat domain into the cytosol (Motley and Hettema, 2007). Both *Arabidopsis* FIS1 homologs, FIS1A (BIGYIN) and FIS1B, are dual targeted to peroxisomes and mitochondria and play rate-limiting roles in initiating organelle fission (Scott et al., 2006; Lingard et al., 2008; Zhang

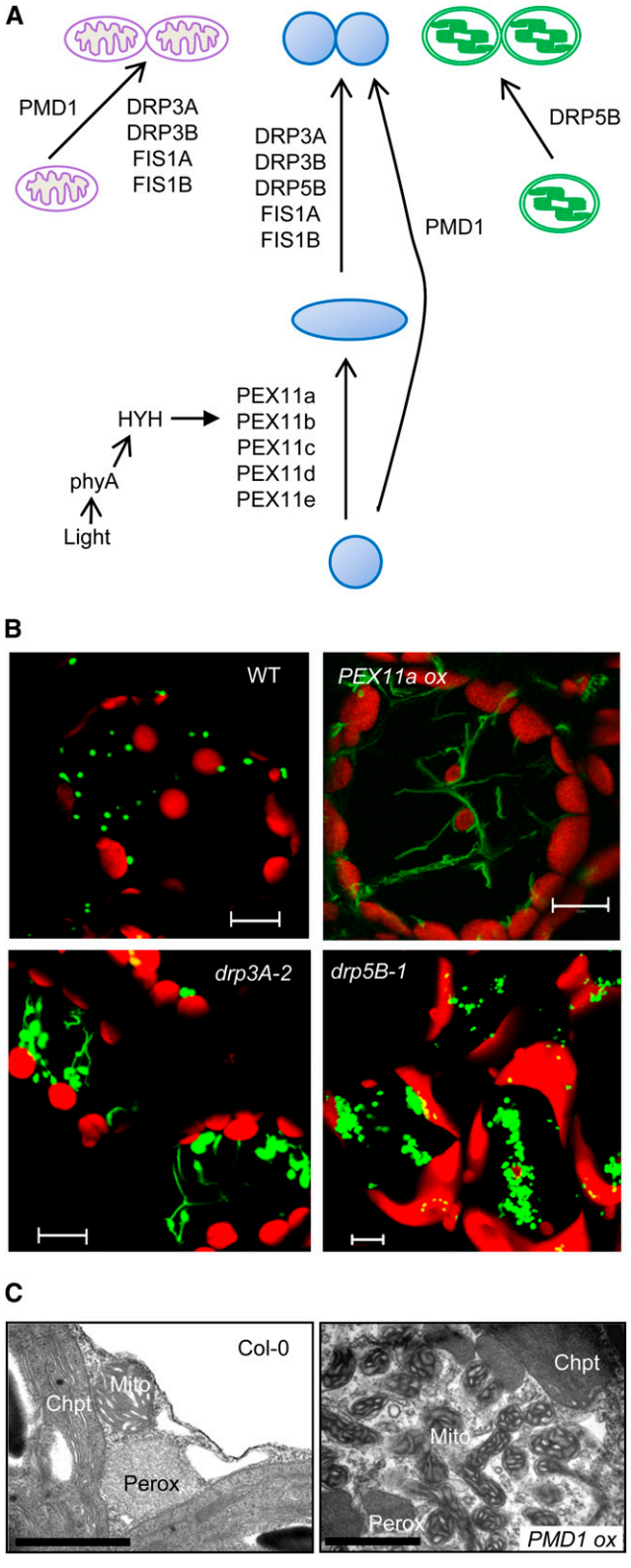

Figure 2. Proteins That Mediate Peroxisome Division in *Arabidopsis*.

(A) A molecular model of peroxisome division in *Arabidopsis*. Peroxisome elongation is promoted by the PEX11 proteins, among which *PEX11b* can be transcriptionally activated by light through a phyA-mediated signal transduction pathway. The fission machineries of peroxisomes and mitochondria share at least five components: DRP3A, DRP3B, FIS1A, FIS1B, and PMD1. DRP5B is a common fission factor for peroxisomes and chloroplasts. PMD1 appears to function independently from PEX11 and the FIS1-DRP3 complex by an unknown mechanism. For mitochondrial and chloroplast division, only factors shared with peroxisomes are depicted.

(B) Confocal micrographs of leaf mesophyll cells showing peroxisome phenotypes in plants ectopically expressing *PEX11a* and loss-of-function mutants of *DRP3A* and *DRP5B*. WT, the wild type. Bars = 10 μm. (*Images reprinted from Orth et al. [2007]*, *Figure 5; Zhang and Hu [2009]*, *Figure 2; Zhang and Hu [2010]*, *Figure 1*.)

(C) Transmission electron micrographs of leaf mesophyll cells showing the organelle phenotype of plants overexpressing *PMD1*. Bars = 1 μm. (*Images reprinted from Aung and Hu [2011]*, *Figure 4*.)

and Hu, 2008, 2009) (Figure 2A). Whether At-FIS1 is required for targeting DRP3A/3B to the organelles has not been verified. Given that DRP5B has a Pleckstrin Homology domain, which presumably binds to lipids (Praefcke and McMahon, 2004), it may not need a receptor for peroxisome association. Physical interactions between FIS1 and PEX11 have been detected in mammals and plants (Kobayashi et al., 2007; Lingard et al., 2008; Zhang and Hu, 2010), indicating a possible, direct functional link between the peroxisome elongation and fission machineries.

Possible kingdom-specific factors also exist in the FIS1-DRP complex. Yeast Mdv1p and Caf4p are two homologous and partially redundant proteins, each possessing a WD40 repeat and a coiled-coil domain and acting as cytosolic adaptors for DRP recruitment (Tieu et al., 2002; Griffin et al., 2005; Motley et al., 2008). Functional orthologs of Mdv1p and Caf4p have not been identified from mammals or plants.

Peroxisome Division Factors that Act Independently from PEX11, FIS1, and/or DRPs

Mff (for Mitochondrial fission factor) is a mammalian-specific coiled-coil protein, which is tethered to mitochondrial and peroxisome membranes and recruits Drp1 to the organelles in a Fis1-independent manner (Gandre-Babbe and van der Bliek, 2008; Otera et al., 2010). In the yeast *Yarrowia lipolytica*, peroxisome division can be triggered when the β-oxidation enzyme acyl-CoA oxidase binds to the PMP Pex16p, which subsequently induces lipid biosynthesis in the membrane and the formation of a division complex containing the DRP Vps1p (Guo et al., 2003, 2007). Some *Arabidopsis* mutants defective in β-oxidation or NAD^+ transport contain larger but fewer peroxisomes (Graham et al., 2002; Baker et al., 2006; Mano et al., 2011), suggesting that accumulation of acyl-CoA or other molecules within the peroxisome may regulate division.

Arabidopsis PEROXISOMAL and MITOCHONDRIAL DIVISION FACTOR1 (PMD1) is a plant-specific organelle division factor that acts independently from PEX11 and the FIS1-DRP3 complex (Aung and Hu, 2011) (Figure 2A). PMD1 is dual targeted to the membranes of peroxisomes and mitochondria. Loss-of-function *pmd1* mutants contain enlarged peroxisomes and elongated mitochondria, and ectopic expression of the gene leads to increased numbers of the organelles, which are often aggregated (Figure

2C). Surprisingly, PMD1 fails to show physical or genetic interaction with any of the known organelle division proteins, indicating that it is not an Mff counterpart. Furthermore, the PMD1 homolog, PMD2, which can form complexes with PMD1, is localized only to mitochondria and exclusively involved in mitochondrial morphogenesis (Aung and Hu, 2011). The mechanism by which PMD1 and PMD2 impact peroxisome and mitochondrial division and morphogenesis remains to be elucidated.

Protein Import

Identification of Genes Required for Matrix Protein Import

With the exception of some PMPs that traffic to peroxisomes via the ER (see above), nascent peroxisomal proteins are imported from the cytosol. The plant peroxins that recognize and transport peroxisomal proteins (Figure 3) have been identified by a combination of forward and reverse genetic approaches. Forward genetic strategies have taken advantage of the role of peroxisomes in bioactivation of auxin precursors. Indole-3-butyric acid (IBA) and 2,4-dichlorophenoxybutryic acid (2,4-DB) undergo β-oxidation to form indole-3-acetic acid (IAA) and 2,4-D, respectively, resulting in the inhibition of root and hypocotyl elongation. Therefore, IBA- or 2,4-DB–resistant mutants that display an elongated phenotype but remain sensitive to the product (IAA or 2,4-D) are readily identified (Hayashi et al., 1998; Zolman et al., 2000; Strader et al., 2011). These screens have identified mutants in both β-oxidation and *PEX* genes. As mutants defective in β-oxidation are often dependent upon exogenous Suc for establishment, screens for sucrose-dependent (*sdp*) mutants identified additional genes (Eastmond, 2006, 2007). Mislocalization of peroxisome-targeted fluorescent fusion proteins has been used to isolate mutants defective in peroxisome protein import (Mano et al., 2006; Goto et al., 2011). Finally, putative peroxins have been identified in silico and characterized through reverse genetic approaches (Baker et al., 2010 and references therein).

The Matrix Protein Import Pathway

The majority of matrix proteins are synthesized with one of two import signals: PTS1 (for peroxisomal targeting signal type 1), a C-terminal tripeptide, or PTS2, an N-terminal nonapeptide. PTS1 sequences typically conform to the consensus of [small]-[basic]-[aliphatic], as exemplified by the sequence SKL. PTS2 sequences have the consensus R[L/I/Q] X_5 HL (Lanyon-Hogg et al., 2010 and references therein). Details on permissible PTS1 signals and their in silico prediction are described later.

Following translation, PTS1 proteins interact with their receptor PEX5 in the cytosol (Figure 3). PEX5 is highly conserved and contains two functional domains: an N-terminal peroxisomal docking domain and a C-terminal domain formed from two sets of three tetratricopeptide repeats, which provide a binding pocket for PTS1 (Lanyon-Hogg et al., 2010). Homology modeling of *Arabidopsis* PEX5 on a human PEX5-PTS1 protein structure suggests that all the important interactions are conserved (Lanyon-Hogg et al., 2010). These structural studies indicate that the mechanism of PTS1 recognition by PEX5 is likely to be conserved; however, targeting studies show some species-specific differences that are likely to reflect subtle differences in the geometry of the PTS1 binding pocket that remain to be fully understood.

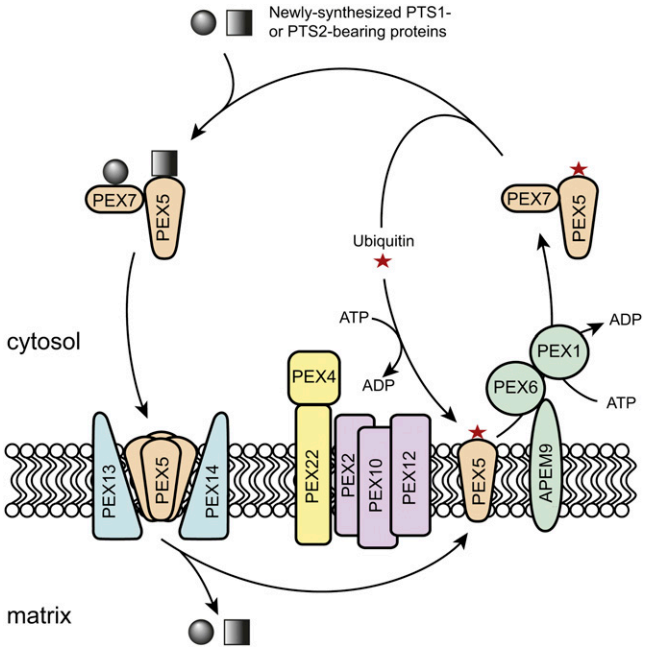

Figure 3. Schematic Diagram of Matrix Protein Import into Peroxisomes.

Cytosolic PEX5 and PEX7 recognize their cargo proteins (square and round shapes) via binding of specific targeting sequences, PTS1 and PTS2, respectively. Cargo-loaded PEX5 associates with the membrane via interactions with PEX13 and PEX14 and probably also via interactions with the lipid phase. PEX7 cannot dock to the membrane on its own and depends on physical interaction with PEX5 for docking. PTS1- and PTS2-bound cargo is released to the matrix, and the receptors are recycled back into the cytosol via a mechanism that probably requires ATP-dependent ubiquitination of PEX5 (represented by a star) by PEX4 and the RING complex comprised of PEX2, PEX10, and PEX12. The ubiquitinated PEX5 is then removed from the membrane via the action of the AAA ATPases PEX6 and PEX1, which are tethered by APEM9. The route that PEX7 takes through the pathway, in particular whether it accompanies PEX5 throughout the import cycle, is unknown.

PTS2 proteins interact with their receptor PEX7 prior to peroxisome entry (Figure 3), but the molecular details of this interaction are unclear. Unlike PEX5, PEX7 cannot mediate interaction with the peroxisome membrane alone but requires accessory proteins. *Arabidopsis* PEX5 acts as the coreceptor for PEX7 (Nito et al., 2002). Downregulation of PEX5 by RNA interference (RNAi) compromises both PTS1 and PTS2 import (Hayashi et al., 2005), and mutation of a conserved Ser in the *pex5-1* mutant reduces PTS2 import, while PTS1 import remains functional (Woodward and Bartel, 2005b). The *Arabidopsis pex5-10* mutant, which contains a large N-terminal deletion, has both PTS1 and PTS2 import defects, but the PTS2 import defect can be rescued by expression of a construct comprising the N-terminal domain of PEX5 (Khan and Zolman, 2010), confirming that the PEX5 N-terminal domain is required for PEX7 interaction.

In mammalian PEX5, multiple WX_3F/Y motifs within the N terminus bind to the N-terminal domain of the PMP PEX14 (Neufeld et al., 2009). *Arabidopsis* PEX14 is an integral PMP important for PTS1 and PTS2 import (Hayashi et al., 2000). The topology of PEX14 is somewhat controversial (Oliveira et al., 2002); therefore, it is unclear whether the critical interaction between PEX5 and PEX14 takes place on the cytosolic side of the membrane, within the membrane, or even within the matrix. The latter possibility would suggest that PEX14 is not the initial docking point for PEX5. In this context, it is interesting that yeast and human PEX5 can spontaneously insert into lipid membranes in vitro (Kerssen et al., 2006) and that residual protein import can occur without PEX14 in *Hansenula polymorpha* (Salomons et al., 2000) and *Arabidopsis* (Monroe-Augustus et al., 2011).

PEX5/7 docking at the peroxisome membrane also involves PEX13 (Figure 3). At-PEX13 was identified from the *aberrant peroxisome morphology* (*apm*) collection as a mutant showing partial mislocalization of a green fluorescent protein (GFP)-PTS1 peroxisome marker to the cytosol (Mano et al., 2006). A null *pex13* allele was subsequently identified as *abstinence by mutual consent* with defective male-female gametophyte recognition (Boisson-Dernier et al., 2008). PEX7 also binds to the N terminus of PEX13 (Mano et al., 2006). There is still uncertainty about the order, stoichiometry, and affinity of binding interactions among PEX5, PEX7, their cargoes, PEX14, and PEX13; however, the general consensus is that import is driven by thermodynamically favorable binding interactions (for more detailed discussion of this point, see Lanyon-Hogg et al., 2010). The mechanism of protein translocation is also uncertain, but yeast PEX5 and PEX14 appear to form a transient pore that can open to a diameter of up to 9 nm (Meinecke et al., 2010).

After import into the matrix, cargo is unloaded and the receptors are recycled. Again, there is a paucity of mechanistic data and cargo unloading remains an obscure process. In yeast, Pex5p reexport requires the three RING finger peroxins Pex2p, Pex10p, and Pex12p, the ubiquitin-conjugating enzyme Pex4p and its membrane anchor Pex22p, and the two AAA ATPases Pex1p and Pex6p, which are tethered to the membrane by Pex15p. The prevailing model (Figure 3) invokes Pex5p monoubiquitination by Pex4p (E2) and Pex12p (E3) and ATP-dependent dislocation of ubiquitinated Pex5p from the membrane via Pex1p and Pex6p (Grou et al., 2009). Although there is no direct evidence for PEX5 ubiquitination in plants, the machinery is conserved. The finding that the very mild *pex13-1* mutant exacerbates the phenotypes of mutants in the early part of the pathway but ameliorates the phenotypes of mutants in the recycling limb of the pathway points to a need to balance receptor import and export (Ratzel et al., 2011).

Knockout mutants of *Arabidopsis PEX2*, *PEX10*, and *PEX12* are embryo lethal (Hu et al., 2002; Schumann et al., 2003; Sparkes et al., 2003; Fan et al., 2005), and RNAi lines all show PTS1 and PTS2 import defects and Suc dependence following germination (Nito et al., 2007). In addition to these typical *pex* defects, some of the RING finger peroxin mutants display additional phenotypes, for example, an RNAi line with strong *PEX10* suppression also has variegated leaves, fused floral organs, aberrant ER morphology, and a defect in cuticular wax synthesis (Kamigaki et al., 2009). A transgenic *Arabidopsis* line expressing a *PEX10* with a mutated RING finger also shows defects in photorespiration and interaction between chloroplasts and peroxisomes (Schumann et al., 2007). A gain-of-function mutant of *PEX2* (*TED3*) suppresses the photomorphogenetic defects of *det1-1* (Hu et al., 2002). If indeed the RING finger peroxins are E3 ligases, they could potentially target proteins other than the import receptors.

The *pex4* RNAi mutant has a PTS1 protein import defect (Nito et al., 2007), and partial loss-of-function mutations in *PEX4* and *PEX22* confer mild defects that are enhanced in the double mutant (Zolman et al., 2005), supporting the notion that PEX4 and PEX22 function in the same pathway. Indeed, *Arabidopsis* PEX22 and PEX4 interact and together can complement the *S. cerevisiae pex4* or *pex22* mutants (Zolman et al., 2005).

PEX1 and *PEX6* RNAi lines have a PTS1 protein import defect (Nito et al., 2007), and a missense allele of *pex6* was isolated as an IBA-resistant mutant (Zolman and Bartel, 2004). *pex6* plants are small, pale, and have reduced seed set. At the cellular level, peroxisomes are enlarged and PEX5 levels are reduced. Recently, the membrane anchor for PEX1 and PEX6 has been identified from the collection of *apm* mutants. APEM9 is an integral PMP that binds PEX6 and recruits the PEX1-PEX6 complex to the peroxisome membrane (Goto et al., 2011).

Degradation of the PTS1 Receptor PEX5

As discussed above, PEX5 monoubiquitination is required for PEX5 recycling in yeast and mammals, and the conservation of the responsible ubiquitin-conjugating enzyme (PEX4), ubiquitin protein ligases (PEX2, PEX10, and PEX12), and AAA ATPases (PEX1 and PEX6) in plants suggests that the PEX5 recycling mechanism also occurs in plants (Figure 3). Intriguingly, these receptor-recycling peroxins resemble proteins needed during ER-associated protein degradation (ERAD), the process of ubiquitination, retrotranslocation, and proteasomal degradation of misfolded ER proteins (Schlüter et al., 2006). Further supporting an ERAD analogy are the observations that yeast and mammalian PEX5 are polyubiquitinated and degraded by the proteasome when not efficiently recycled (Platta et al., 2004) in a process termed RADAR (for receptor accumulation and degradation in the absence of recycling) (Léon et al., 2006). Although plant PEX5 ubiquitination has not been directly demonstrated, the Cys residue that is ubiquitinated in other eukaryotes (Carvalho et al., 2007; Williams et al., 2007) is conserved in *Arabidopsis* PEX5. In addition, the *Arabidopsis pex6-1* missense allele has reduced PEX5 levels, and overexpressing *PEX5* partially restores peroxisome function in *pex6-1* (Zolman and Bartel, 2004), suggesting that a RADAR mechanism also operates in plants. Reducing PEX4 function (Zolman et al., 2005) in the *pex6-1* background restores PEX5 levels while exacerbating *pex6-1* physiological and molecular defects (Ratzel et al., 2011), suggesting that PEX4 is needed for both the ubiquitination that promotes PEX5 recycling and the ubiquitination that triggers RADAR. The apparent conservation of RADAR processes suggests that this degradation prevents a deleterious buildup of PEX5 in the peroxisomal membrane.

In addition to low PEX5 levels observed in *pex6-1* mutants (Zolman et al., 2005; Ratzel et al., 2011), PEX5 levels are reduced in light-grown *pex7* mutants (Ramón and Bartel, 2010),

suggesting that the dependence of PEX7 on PEX5 for cargo delivery in plants (Hayashi et al., 2005; Woodward and Bartel, 2005a) is mirrored by a dependence of PEX5 on PEX7 for stability. Whether the apparent PEX5 instability in *pex7* mutants reflects inefficient recycling leading to RADAR or instability in the cytosol remains to be determined.

Peroxisomal Proteases and Matrix Protein Degradation

Two peroxisomal proteases are implicated in peroxisome biogenesis. Originally purified from watermelon (*Citrullis vulgaris*) cotyledons, DEG15 is a trypsin-like Ser protease that cleaves PTS2 proteins to remove the N-terminal region both in vitro and in vivo (Helm et al., 2007; Schuhmann et al., 2008). Beyond a slight resistance to the inhibitory effects of IBA (Lingard and Bartel, 2009) and 2,4-DB (Schuhmann et al., 2008), the *Arabidopsis deg15* null mutant does not display growth or germination defects that would ascribe a physiological benefit to removing the PTS2 sequence following peroxisome entry. Indeed, yeasts lack a peroxisomal DEG15 ortholog and do not remove PTS2 sequences upon import (Helm et al., 2007). The evolutionary advantage that has conserved the PTS2 removal process in plants and mammals remains to be identified.

LON proteases are members of the AAA ATPase family originally discovered in bacteria, where they degrade both aberrant and regulatory proteins (reviewed in Van Melderen and Aertsen, 2009). In plants, LON isoforms are found in chloroplasts, mitochondria, and peroxisomes (Ostersetzer et al., 2007); LON2 is the peroxisomal LON isoform. In *Arabidopsis lon2* mutants, matrix proteins correctly localize in 4-d-old cotyledon cells but mislocalize to the cytosol in older seedlings; similarly, a PTS2-GFP reporter sorts to peroxisomes in *lon2* root tip cells but is largely cytosolic in mature root cells (Lingard and Bartel, 2009). The delayed onset of matrix protein sorting defects in *lon2* mutants suggests that LON2 facilitates continued matrix protein import in mature peroxisomes and is a previously unrecognized peroxin. It will be interesting to discover the LON2 substrate(s) that hinders matrix protein import if not efficiently degraded. The increasing severity of *lon2* import defects with age contrasts with several other *pex* mutants; for example, the severe matrix protein import defects of young *pex14* seedlings lessen as seedlings mature (Hayashi et al., 2000; Monroe-Augustus et al., 2011), and *pex5-10* mutants recover normal pigmentation upon maturation (Khan and Zolman, 2010).

Although we are beginning to understand how proteins are delivered to the peroxisome matrix, little is known about how excess plant peroxisomes or peroxisomal proteins are degraded. A specialized form of autophagy, pexophagy, is important in removing excess peroxisomes in yeast and mammals (reviewed in Manjithaya et al., 2010), but pexophagy has not been reported in plants. Peroxisomal sequestration likely protects the cytosol from hydrogen peroxide (H_2O_2) produced by various peroxisomal oxidases. Although peroxisomes house catalase and other enzymes that decompose this H_2O_2, the protective capacity of the peroxisome can be exceeded (Eastmond, 2007). Moreover, certain matrix proteins, such as the glyoxylate cycle enzymes isocitrate lyase (ICL) and malate synthase (MLS; see below), are susceptible to oxidative damage both in vitro and in vivo (Yanik and Donaldson, 2005; Eastmond, 2007; Anand et al., 2009), which may necessitate a degradation pathway that responds to oxidative damage. In addition, obsolete proteins are removed during developmental peroxisomal remodeling. For example, ICL and MLS are degraded when seedlings transition from fatty acid β-oxidation to photosynthesis (Nishimura et al., 1996). This degradation is accelerated in a catalase mutant (Lingard et al., 2009), suggesting that oxidative damage by H_2O_2 promotes peroxisome-associated protein degradation. Furthermore, ICL and MLS must enter peroxisomes to be efficiently degraded (Lingard et al., 2009), suggesting that degradation is triggered following import or that the responsible protease is peroxisomal. However, insertion alleles disrupted in any of the five predicted peroxisomal proteases (DEG15/At1g28320, LON2/At5g47040, PXM16/At2g41790, At2g18080, and At2g35615) display normal ICL and MLS degradation (Lingard and Bartel, 2009), indicating that if ICL and MLS degradation is accomplished by a peroxisomal protease, it acts redundantly or remains to be identified. Interestingly, one of the receptor-recycling peroxins, PEX4, facilitates ICL and MLS degradation (Lingard et al., 2009), consistent with the alternative possibility that damaged and obsolete proteins actively exit peroxisomes for cytosolic proteasomal degradation, perhaps using the same ERAD-resembling machinery that is used to recycle (or destroy) PEX5. It will be interesting to learn whether PEX5, which is essential for the entry of peroxisomal matrix proteins, also assists in the exit of these proteins when they are damaged or obsolete.

PEROXISOMAL FUNCTIONS

Plant peroxisomes mediate a multitude of processes crucial to development. Peroxisomes are the sole site of fatty acid β-oxidation in plant cells and are involved in generating two phytohormones: IAA and JA. They play an important role in photorespiration in conjunction with mitochondria and chloroplasts. In addition to these processes, plant peroxisomes also participate or are implicated in a plethora of other metabolic and signaling pathways, such as the glyoxylate cycle, detoxification, generation of signaling molecules, biosynthesis of salicylic acid, and the metabolism of urate, polyamines, sulfite, and branched-chain amino acids (reviewed in Kaur et al., 2009). Recent studies have also revealed roles for peroxisomes in plant immune response (Lipka et al., 2005; Coca and San Segundo, 2010; Rojas et al., 2012) and the biosynthesis of biotin (Tanabe et al., 2011), *S*-allantoin (Lamberto et al., 2010), phylloquinone (Widhalm et al., 2012), and isoprenoids (Sapir-Mir et al., 2008; Tholl and Lee, 2011).

Peroxisomal β-Oxidation

Fatty Acid β-Oxidation

Fatty acid oxidation is an essential process in the mobilization of seed oil reserves, which are laid down during seed development predominantly as triacylglycerol (TAG) and mobilized to support postgerminative growth prior to the seedling developing photosynthetic competence (Graham, 2008). Oil body–associated TAG lipases SUGAR DEPENDENT1 (SDP1) (Eastmond, 2006) and SUGAR DEPENDENT1 LIKE release free fatty acids and together account for 95% of TAG lipase activity

(Kelly et al., 2011). Fatty acids (and other substrates of β-oxidation) are transported into peroxisomes by the peroxisomal ATP binding cassette (ABC) transporter protein CTS/PXA1/PED3 (see details below). Mutants deficient in fatty acid degradation lack the energy or metabolites necessary for seedling establishment into a photosynthetic plant and thus produce short hypocotyls when grown in the dark, a phenotype that can be rescued by Suc. A severe β-oxidation block results in strongly reduced germination (Baker et al., 2006). In addition to roles in early seedling development, fatty acid β-oxidation also has important roles in remobilization of reserves during senescence and in survival in extended periods of darkness (Dong et al., 2009; Kunz et al., 2009; Slocombe et al., 2009).

Following peroxisomal import, straight-chain saturated fatty acyl-CoAs undergo a cycle of oxidation, hydration, oxidation, and thiolysis, leading to release of acetyl-CoA and an acyl-CoA molecule that has been shortened by two carbons (Figure 4; Graham, 2008). The first step is catalyzed by a family of acyl-CoA oxidases, ACX1-5 in *Arabidopsis*, with differing but partially overlapping chain length specificities (Graham, 2008 and references therein; Khan et al., 2012). These enzymes are flavin adenine dinucleotide linked, and the electrons are passed to molecular oxygen to produce H_2O_2. The resulting 2-*trans*-enoyl CoA is the substrate for the multifunctional protein, which contains both hydratase and dehydrogenase domains.

There are two peroxisomal multifunctional proteins in *Arabidopsis*: MFP2 (Rylott et al., 2006) and AIM1 (Richmond and Bleecker, 1999). MFP2 is the major seedling form; its mutant shows a typical β-oxidation deficiency phenotype (Rylott et al., 2006). The *mfp2* mutant is not resistant to pro-auxins, whereas the *aim1* mutant is. Consistent with this resistance, AIM1 prefers short-chain substrates (Richmond and Bleecker, 1999; Arent et al., 2010). MFP2's hydratase activity prefers longer chains (Rylott et al., 2006) but shows little activity on acyl-CoAs above 14 carbons in length (Arent et al., 2010), suggesting that there is a yet undiscovered long-chain hydratase.

The final step of core β-oxidation is the thiolytic cleavage of 3-ketoacyl CoA by thiolase to produce acetyl-CoA and a shortened acyl-CoA. Of the three peroxisomal thiolases, PED1/KAT2 is the major seedling form (Hayashi et al., 1998; Germain et al., 2001). The *ped1*/*kat2* mutant has a more severe β-oxidation deficient phenotype than the *mfp2* mutant, but interestingly both *mfp2* (Rylott et al., 2006) and *kat2* (Germain et al., 2001) have enlarged peroxisomes, suggesting that intraperoxisomal accumulation of acyl-CoAs could result in peroxisomal expansion or inhibition of division (Graham et al., 2002).

The core β-oxidation machinery metabolizes straight-chain saturated fatty acids. However, peroxisomes also metabolize unsaturated fatty acids with double bonds at both odd and even positions, which requires accessory enzymes to convert these molecules into suitable substrates (Goepfert and Poirier, 2007; Graham, 2008). For the degradation of fatty acids with double bonds at the odd position (e.g., C18:Δ9*cis* [oleic acid]), the peroxisomal $\Delta^{3,5}\Delta^{2,4}$ dienoyl CoA isomerase encoded by At-*DCI* is essential (Goepfert et al., 2005). For even double bonds, an epimerase activity that is part of the multifunctional protein or a separate enoyl-CoA hydratase (ECH) is required (Goepfert et al., 2006).

The acyl-CoA oxidase reaction produces H_2O_2, which is metabolized by catalase. However, under conditions of high H_2O_2 production, such as during TAG mobilization in early seedling growth, a membrane-bound system comprising ascorbate peroxidase and monodehydroascorbate reductase acts as a second line of defense to prevent H_2O_2 leakage into the cytosol. A mutant in monodehydroascorbate reductase (*sdp2*) has compromised β-oxidation due to excess H_2O_2 that causes oxidative inactivation of the TAG lipase SDP1 (Eastmond, 2007).

The product of β-oxidation, acetyl-CoA, can be respired by mitochondria (Kunze et al., 2006) or can enter the glyoxylate cycle, where citrate synthase (CSY), ICL, and MLS convert it to succinate and malate used for gluconeogenesis (Pracharoenwattana and Smith, 2008). *Arabidopsis* CSY2 and CSY3 convert acetyl-CoA to citrate for export to mitochondria; the double mutant is unable to germinate without Suc, and physical removal of the seed coat fails to degrade its oil bodies and is resistant to 2,4-DB (Pracharoenwattana et al., 2005). The *icl1* mutant germinates and degrades oil bodies, presumably respiring the acetyl-CoA, but has reduced survival in periods of extended darkness (Eastmond et al., 2000). *mls* mutants have mild phenotypes, suggesting MLS is partially dispensable for gluconeogenesis and lipid metabolism (Cornah et al., 2004)

The hydroxyacyl-CoA dehydrogenase activity of MFP produces NADH. Reoxidation of NADH and, therefore, continued β-oxidation depends on a malate-oxaloacetate shuttle that involves peroxisomal and cytosolic isoforms of malate dehydrogenase (MDH). Double mutants defective in the two peroxisomal MDH genes, *PMDH1* and *PMDH2*, germinate but are Suc dependent for establishment, are resistant to 2,4-DB, and mobilize TAGs slowly (Pracharoenwattana et al., 2007).

JA Production

The major functions of jasmonates, phytohormones regulating development and stress response, include wounding and pathogen responses, stamen development, and pollen release. This hormone family is comprised of several related lipid-derived compounds: JA, its precursor 12-oxo-phytodienoic acid (OPDA), and JA derivatives, including the methyl ester and the Ile conjugated forms (reviewed in Acosta and Farmer, 2010). Production of active jasmonates occurs sequentially in three locations: chloroplasts, peroxisomes, and the cytosol.

Chloroplast-localized reactions convert polyunsaturated fatty acids to OPDA, which is released via an unknown mechanism (Acosta and Farmer, 2010). Following peroxisomal import, the OPDA reductase OPR3 converts OPDA to OPC8:0 (3-oxo-2-(2′-[*Z*]-penenyl) cycopentane-1-octanoic acid). OPR3 has reductase activity in vitro (Costa et al., 2000; Schaller et al., 2000), and *opr3* was found as a male-sterile mutant rescued specifically by JA application (Stintzi and Browse, 2000).

Three rounds of peroxisomal β-oxidation convert OPC8:0→ OPC6:0→OPC4:0→JA. OPCL1 activates OPC8:0, and ACX1 and ACX5, AIM1, and PED1/KAT2 are implicated in the core β-oxidation of JA precursors. These isozyme assignments were inferred from three observations: (1) *OPCL1*, *ACX1*, and *KAT2* mRNAs strongly and rapidly accumulate in response to JA, as part of a positive feedback mechanism (Cruz Castillo et al.,

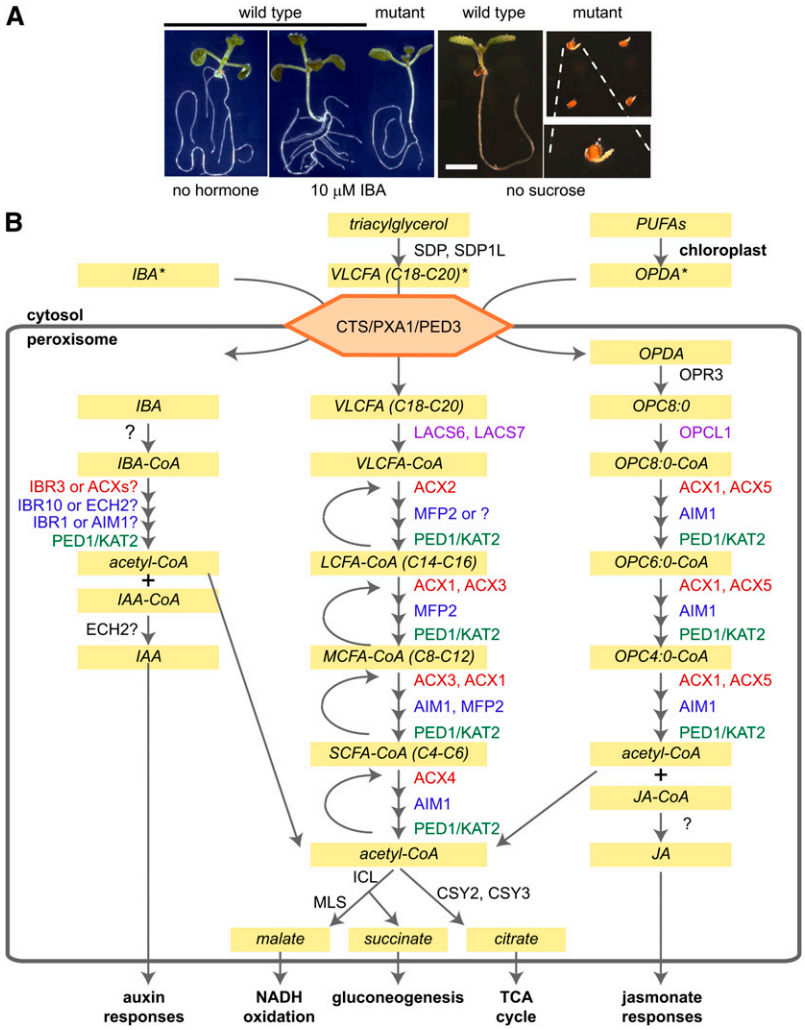

Figure 4. Proteins Acting in Peroxisomal β-Oxidation.

(A) Mutants disrupting peroxisomal function frequently have IBA response and Suc-dependent phenotypes. Left, wild-type seedlings grown with applied IBA have shorter primary roots and abundant secondary roots, whereas peroxisome-defective mutants (e.g., *pxa1* mutant shown) do not respond to IBA application because of their inability to β-oxidize IBA to IAA. Right, wild-type seedlings germinate and grow normally without an external carbon source, but peroxisome-defective mutants have disruptions in seedling establishment, ranging from failed to delayed development. Bar = 2 mm. (*Images reprinted from Zolman et al. [2001], cover photo, and Adham et al. [2005], Figure 8.*)

(B) Major metabolic pathways in peroxisomes use a core set of enzymes. Fatty acid β-oxidation (center) in developing seeds involves conversion of very-long-chain fatty acids (VLCFA) stored as TAG through long-chain fatty acid (LCFA), medium-chain fatty acid (MCFA), and short-chain fatty acid (SCFA) intermediates. Each round of β-oxidation releases two carbons as acetyl-CoA. IBA (left) and OPDA (right) are metabolized in parallel pathways that use an overlapping but distinct set of enzymes; OPDA is produced from polyunsaturated fatty acids (PUFAs) in a multistep pathway in chloroplasts. *, CTS/PXA1/PED3 may import unmodified substrates or CoA derivatives. For all pathways, substrate activation by acyl-CoA synthetases is shown in purple, the initial oxidation enzymes are in red, the hydration/oxidation intermediate steps (frequently performed by a multifunctional enzyme) are shown in blue, and the thiolysis step is shown in green; if known, specific isozymes catalyzing the reaction are indicated. Peroxisomal acetyl-CoA is a central intermediate in various branches of cellular metabolism, including (1) the conversion to succinate via the glyoxylate cycle, which enters gluconeogenic pathways to produce Glc; (2) the production of malate, necessary for the malate-oxaloacetate shuttle to reoxidize NADH produced by MFP2/AIM1; and (3) the conversion to citrate, which enters the tricarboxylic acid (TCA) cycle.

2004; Koo et al., 2006); (2) OPCL1 (Koo et al., 2006; Kienow et al., 2008) and ACX1 (Li et al., 2005) are biochemically active on JA intermediates; and (3) RNAi lines and *opcl1*, *acx1*, *aim1*, and *ped1/kat2* mutants have decreased JA biosynthesis (Cruz Castillo et al., 2004; Afitlhile et al., 2005; Pinfield-Wells et al., 2005; Koo et al., 2006; Delker et al., 2007). Moreover, disruptions of *ACX1* or *PED1/KAT2* delay systemic responses (Cruz Castillo et al., 2004), and a tomato (*Solanum lycopersicum*) *acx1* mutant has reduced defense against chewing insects (Li et al., 2005).

The modification of JA to JA-Ile, the active component in JA signaling, occurs in the cytoplasm (reviewed in Acosta and Farmer, 2010). OPDA, JA, and JA-Ile have unique roles in plant cells. The transition between organelles may regulate the ratio of jasmonates and thereby affect the types or intensity of responses.

OPDA regulates seed germination. Whereas mutants blocked in β-oxidation can be rescued for establishment by Suc supplementation, indicating an insufficient supply of carbon and energy from fatty acid metabolism, severe mutants in core β-oxidation functions cannot germinate unless the testa is manually ruptured (Russell et al., 2000; Pinfield-Wells et al., 2005; Footitt et al., 2006). Peroxisomal transport or activity mutants, including *cts/pxa1/ped3*, *ped1/kat2*, and *acx1 acx2* double mutants, accumulate OPDA and, paradoxically, JA, in seeds. However, a *pxa1 opr3* double mutant, which accumulates high OPDA but lacks JA, maintains the germination defect, indicating that peroxisomal import and metabolism of OPDA is important for germination (Dave et al., 2011). Moreover, OPDA and ABA act synergistically to increase levels of the transcription factor ABI5 (Dave et al., 2011). *ABI5* is also upregulated in the *ped3* allele, which in turn leads to higher levels of polygalacturonase-inhibiting proteins; removal of pectin using exogenous polygalacturonase can overcome the germination block in *ped3* (Kanai et al., 2010).

The JA biosynthetic pathway was proposed in the 1980s (Vick and Zimmerman, 1983). Although great strides have been made identifying the peroxisomal components, several questions remain. An unknown thioesterase presumably is required to cleave the jasmonoyl-CoA to release JA. The transporter facilitating JA export also remains unknown. In addition, there is a high degree of redundancy in JA transport and biosynthesis, and residual JA still accumulates in single mutants. For instance, *opr3* accumulates JA in certain conditions (Chehab et al., 2011), and *opcl1* accumulates JA to ~60% of wild-type levels, allowing many expression targets to still be induced (Koo et al., 2006). Similarly, only in the *acx1 acx5* double mutant is fertility and wound-induced JA biosynthesis lost (Schilmiller et al., 2007). In addition, different tissues may regulate JA synthesis differently. For instance, Dave et al. (2011) reported high JA levels in *cts-2* seeds, but studies on the same allele showed almost no JA in leaves (Theodoulou et al., 2005). Similarly, *acx1 acx5* makes no JA in wounded leaves but produces JA in flowers and following fungal infections (Schilmiller et al., 2007). Further studies, including analysis of additional mutant combinations, could define the full complement of proteins involved in JA biosynthesis, but mutant analysis will require examination in multiple conditions for a complete understanding.

Peroxisomal Conversion of IBA to IAA

IAA is the principal form of auxin, a phytohormone regulating many aspects of development by influencing cell division and elongation. IBA is structurally similar to IAA but has a butyl instead of acetyl side chain; IBA is known for efficacy in root induction and is applied to cuttings or seedlings to ensure strong root development (reviewed in Woodward and Bartel, 2005b). Feeding studies have shown that IAA can be converted to IBA; IBA formation is hypothesized to relieve high IAA levels. IBA is also converted back to IAA, increasing free (active) IAA to match plant needs. Conversion of IBA to IAA removes the two extra side-chain carbons in a β-oxidation–like pathway (Fawcett et al., 1960). Because of the structural differences, IBA can be considered a protoauxin, which is transported (reviewed in Strader and Bartel, 2011) or stored (reviewed in Simon and Petrášek, 2011) without auxin activity.

Our understanding of IBA activity is based on forward genetic screens, which revealed IBA metabolism to be a peroxisomal process. The predicted pathway for IBA metabolism parallels fatty acid β-oxidation: IBA is imported into peroxisomes, activated by CoA, and converted to IAA-CoA via the core β-oxidation steps (Figure 4). Mutants defective in *AIM1* and *PED1/KAT2* show pleiotropic phenotypes, including fatty acid and JA defects (described above) and resistance to 2,4-DB (Hayashi et al., 1998; Richmond and Bleecker, 1999; Hayashi et al., 2002) and IBA (Zolman et al., 2000, 2001), indicating IBA-to-IAA conversion is disrupted. Therefore, AIM1 could catalyze the middle two steps of IBA metabolism, similar to fatty acid metabolism. PED1/KAT2 could act as a thiolase to release two side-chain carbons, producing IAA-CoA and acetyl-CoA (Hayashi et al., 1998; Zolman et al., 2000).

Alternatively, *ibr1*, *ibr3*, *ibr10*, and *ech2* only show IBA response phenotypes, suggesting that the corresponding enzymes may act specifically on IBA intermediates. IBR3 encodes an acyl-CoA dehydrogenase/oxidase, which could convert IBA-CoA to the α,β-unsaturated thioester (Zolman et al., 2007). Two enoyl-CoA hydratases are implicated in IBA responsiveness: IBR10 (Zolman et al., 2008) and ECH2 (Strader et al., 2011). Although ECH2 and IBR10 have similar domain structures, complementation experiments indicate that they are not redundant (Strader et al., 2011). In addition to hydratase activity, ECH2 also has a hot dog domain common in thioesterases and therefore may be acting at the last step to convert IAA-CoA to IAA (Strader et al., 2011). Finally, IBR1, also identified as SDRa (Wiszniewski et al., 2009), encodes a short-chain dehydrogenase/reductase (Zolman et al., 2008), which may catalyze the fourth step of IBA β-oxidation. AIM1-IBR1 redundancy at the dehydrogenase/reductase step could explain why the *ibr1* defects are less severe than those of other mutants (Strader et al., 2011).

Strader et al. (2010) demonstrated reduced IAA production from labeled IBA in *pex6*, *pxa1*, and the *ibr1 ibr3 ibr10* triple mutant, confirming roles for peroxisomes and these enzymes in IAA production. However, the precise enzymatic assignments require biochemical confirmation; in particular, IBR10 and ECH2 placement and potential redundancy between AIM1 and IBR1 will require activity assays for resolution.

ACX activity on IBA-CoA also remains questionable. *acx* mutant analysis revealed that all five ACX enzymes promote IBA responsiveness (Adham et al., 2005) and *acx1 acx2* double mutants have decreased IBA-to-IAA conversion (Strader et al., 2010). IBR3 and multiple ACX enzymes may catalyze this reaction in an overlapping manner or based on expression. However, ACX enzymes show substrate chain length specificities (see above) that seemingly contradict the idea that all five act directly on IBA. Alternatively, IBR3 may act directly on IBA substrates while ACX activity affects IBA oxidation indirectly,

perhaps based on limiting peroxisomal CoA pools (Adham et al., 2005). Furthermore, two steps remain unresolved. The *aae18* synthetase mutant is 2,4-DB resistant but responds normally to IBA (Wiszniewski et al., 2009); whether a different protein activates IBA (perhaps redundantly) remains to be determined. IAA export to the cytosol has not been defined either.

Finally, we do not know how the conversion of IBA to IAA is regulated or triggered, although one hypothesis is that low IAA levels stimulate IBA metabolism. IBA response mutants have reduced lateral root systems, smaller root meristems, defective cotyledon expansion, shorter root hairs, and reduced hypocotyl and stamen elongation (reviewed in Strader and Bartel, 2011), demonstrating the importance of this conversion in multiple aspects of plant growth and development.

Photorespiration

The Classical Pathway

The most prominent role of peroxisomes in photosynthetic tissues is their participation in photorespiration. The oxidative C_2 cycle is a salvage pathway for phosphoglycolate produced by the oxygenase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) to the Calvin cycle intermediate phosphoglycerate. This pathway is one of the most sophisticated examples of subcellular compartmentalization and spatial and temporal coordination, as it combines enzymatic reactions in, and intermediate and cofactor exchange between, chloroplasts, peroxisomes, mitochondria and, as recently shown, the cytosol (Timm et al., 2008). Peroxisome-localized photorespiratory enzymes include glycolate oxidase (GOX), catalase, two aminotransferases, hydroxypyruvate reductase (HPR), and MDH, placing leaf peroxisomes at a central position in photorespiration (Figure 5).

Downstream of Rubisco, the photorespiratory reactions continue in the chloroplast stroma with phosphoglycolate phosphatase, which dephosphorylates 2-phosphoglycolate (Schwarte and Bauwe, 2007). Glycolate diffuses into the matrix of peroxisomes, where it is oxidized to glyoxylate by GOX concomitant with H_2O_2 production. Glyoxylate is transaminated by two peroxisomal aminotransferases, Ser-glyoxylate and Glu-glyoxylate aminotransferase, which ideally cooperate at a 1:1 stoichiometry (Liepman and Olsen, 2001, 2003; Igarashi et al., 2003, 2006). Mitochondrial Gly decarboxylase decomposes Gly to CO_2, NH_3, and NADH and transfers a C_1 unit to 5,10-methylene tetrahydrofolate. Ser hydroxymethyl transferase attaches this methylene unit to the second Gly molecule to produce Ser. Ser diffuses back to leaf peroxisomes for transamination by Ser-glyoxylate to yield hydroxypyruvate, which is reduced by HPR and NADH provided by peroxisomal MDH to form glycerate. Finally, stromal glycerate kinase (GLYK) produces the Calvin cycle intermediate 3-phosphoglycerate (Figure 25) (Reumann and Weber, 2006; Maurino and Peterhansel, 2010).

Molecular Identification of All Key Photorespiration Enzymes

Photorespiration is an essential process in land plants, as evident from the conditionally lethal phenotype of mutants deficient in the participating enzymes or transporters. However, the photorespiratory pathway of C_3 plants is inefficient in terms of energy, carbon, and nitrogen usage (see below). To fill in the knowledge gaps about photorespiratory enzymes and increase plant biomass production, photorespiration research has been revitalized recently, with major activities led by groups such as the German research consortium PROMICS (www.promics.uni-rostock.de). Major fundamental and applied biotechnological knowledge has been gained in the past few years, as described by several recent reviews (Foyer et al., 2009; Bauwe, 2010; Maurino and Peterhansel, 2010).

Molecular identification of the core photorespiration enzymes has been completed only recently. Using a candidate gene approach, the gene encoding phosphoglycolate phosphatase was revealed based on the characteristic photorespiratory phenotype of the knockout mutant (i.e., nonviability in normal air but normal growth under elevated CO_2 concentrations) (Schwarte and Bauwe, 2007). Contrary to the other core photorespiratory enzymes, deletion of peroxisomal HPR1 does not lead to ambient air sensitivity but does increase the stoichiometry of photorespiratory CO_2 release (Cousins et al., 2011). Identification of a second HPR (HPR2) suggests the existence of an efficient NADPH-dependent cytosolic bypass (Timm et al., 2008). A recent study identified a third, chloroplast-localized HPR with high specificity for glyoxylate; the triple mutant of the three *HPR* genes shows increased growth retardation, decreased photochemical efficiency, and reduced oxygen-dependent gas exchange compared with the *hpr1 hpr2* double mutant (Timm et al., 2011).

The gene encoding the last missing enzyme of the C_3 plant photorespiratory cycle, GLYK, was identified from *Arabidopsis*; its knockout mutant is unviable in normal air but able to grow under elevated CO_2 (Boldt et al., 2005). Contrary to that in C_3 plants, maize (*Zea mays*) GLYK is redox regulated by an additional, C-terminal autoinhibitory domain, which forms a disulfide bridge at night, inhibiting enzyme activity and rendering the oxidized enzyme inactive (Bartsch et al., 2008).

Photorespiration as a Prime Target for Crop Improvement

Despite being a valuable salvage pathway, the photorespiratory C_2 cycle remains inefficient because it renders (1) suboptimal conversion of fixed carbon in the form of phosphoglycolate into phosphoglycerate (maximum of three of four C atoms [i.e., 75%]), (2) loss of fixed N, and (3) loss of energy during glycolate oxidation by the production of H_2O_2 rather than NAD(P)H. Hence, the photorespiratory pathway, at least theoretically, bears a high optimization potential in C_3 plants, making it a prime target for crop improvement for increased yield and biomass production.

A bacterial glycolate oxidation pathway was introduced into *Arabidopsis* chloroplasts for alternative conversion of glycolate into glycerate, thereby shifting CO_2 release from the mitochondrion to the chloroplast to increase CO_2 concentration in the vicinity of Rubisco and reduce its oxygenase activity. Indeed, the transgenic lines showed enhanced growth (Kebeish et al., 2007). To conserve the glycolate carbon in malate, transgenic *Arabidopsis* plants overexpressing chloroplast-targeted GOX and MLS were generated. The transgenic lines developed

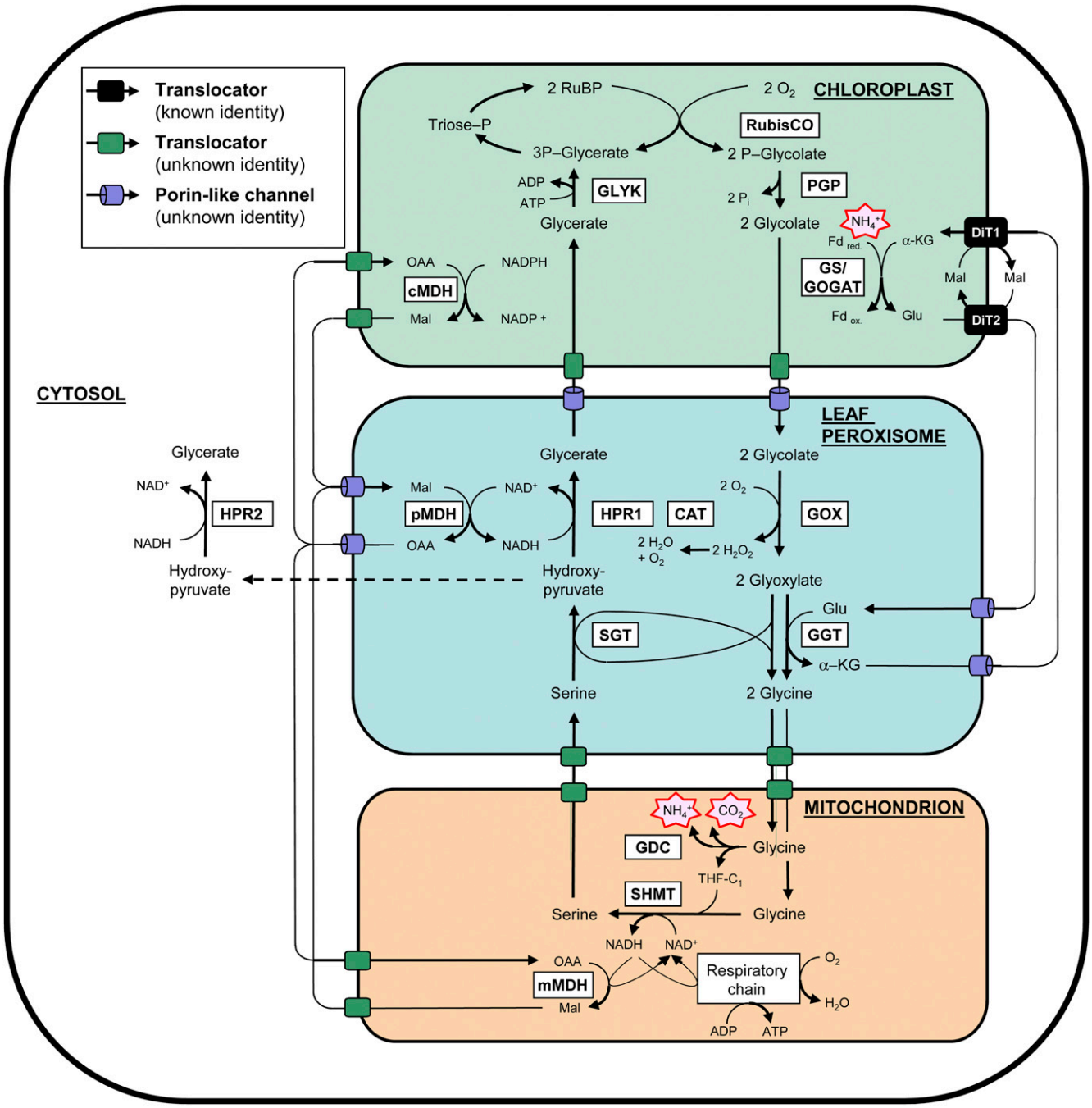

Figure 5. The Central Role of Leaf Peroxisomes in Photorespiration.

Photorespiration is compartmentalized among chloroplasts, leaf peroxisomes, mitochondria, and the cytosol. Eleven enzymes are directly involved: Rubisco, phosphoglycolate phosphatases (PGP), GOX, catalase (CAT), Glu:glyoxylate aminotransferase (GGT), Ser:glyoxylate aminotransferase (SGT), Gly decarboxylase (GDC), Ser hydroxymethyl transferase (SHMT), HPR, peroxisomal MDH (pMDH), and GLYK. Four enzymes (i.e., Glu synthase [GS], Glu:oxoglutarate aminotransferase [GOGAT], and mitochondrial/chloroplast malate dehydrogenase [mMDH/cMDH]) are indirectly involved. For the transport of photorespiratory intermediates, different translocators and a porin-like channel have been characterized biochemically (translocators, green; porin-like channel, blue) or cloned (translocator, black). Photorespiratory metabolites are abbreviated as follows: RuBP, ribulose-bisphosphate; 3-PGA, 3-phosphoglycerate; and THF, tetrahydrofolate. (*Adapted and reprinted from Reumann and Weber [2006]*, *Figure 1*.)

oxidative stress lesions under photorespiratory conditions, most likely due to enhanced H_2O_2 production in chloroplasts, but showed enhanced growth under nonphotorespiratory conditions (Fahnenstich et al., 2008; Maurino and Flügge, 2009). To bypass the peroxisomal aminotransferases and Gly-dependent ammonia production, transgenic tobacco (*Nicotiana tabacum*) plants overexpressing bacterial glyoxylate carboligase and hydroxypyruvate isomerase were generated. However, only the first enzyme was highly expressed in the transgenic plants, which exhibited stress symptoms when exposed to air, suggesting that some glyoxylate was directed into a deleterious short circuit of the photorespiratory nitrogen cycle (de F.C. Carvalho et al., 2011). These first attempts to optimize photorespiration are promising. However, because the photorespiratory pathway is more tightly integrated into the whole plant primary and secondary metabolism than previously hypothesized, these manipulations also uncover technical challenges and unexpected negative side effects and reveal the need for further studies.

Although high CO_2 levels reduce photorespiration, they often lead to a decline in the plant's nitrogen status. Indeed, atmospheric CO_2 enrichment reduced the efficiency of nitrogen use (Rachmilevitch et al., 2004). This inhibition of nitrate assimilation into organic nitrogen compounds may be largely responsible for CO_2 acclimation (i.e., the decrease in photosynthesis and growth of plants conducting C_3 carbon fixation after long exposures to CO_2 enrichment) (Bloom et al., 2010). Hence, ammonium and nitrate availability will become increasingly important in determining plant productivity as CO_2 levels rise.

PEROXISOMAL TRANSPORTERS FOR METABOLITES AND COFACTORS

Several peroxisomal metabolic pathways require an interplay with other cellular compartments, including plastids, mitochondria, and the cytosol. Consequently, a considerable number of substrates, intermediates, end products, and cofactors must be exchanged between peroxisomes and other cell compartments. Their membrane passage is mediated by transport proteins (Linka and Esser, 2012).

An ABC Transporter Importing the Substrates for β-Oxidation

Fatty acids and other β-oxidation substrates are imported by the peroxisomal ABC transporter protein CTS/PXA1/PED3 (Zolman et al., 2001; Footitt et al., 2002; Hayashi et al., 2002); similar transporters also exist in fungi and mammals (Theodoulou et al., 2006). CTS/PXA1/PED3 was independently isolated from several forward genetic screens (hence, its multiple names), underlining its pleotropic role in growth and development (reviewed in Theodoulou et al., 2006). This transporter plays a crucial role in (1) storage oil mobilization in seedlings and probably pollen (Zolman et al., 2001; Footitt et al., 2002, 2007; Hayashi et al., 2002), (2) turnover of membrane lipids, especially under carbon and energy starvation (Kunz et al., 2009; Slocombe et al., 2009), (3) JA biosynthesis (Theodoulou et al., 2005), (4) auxin biosynthesis (Zolman et al., 2001; Hayashi et al., 2002; Strader et al., 2010), (5) seed coat rupture during seed germination (Kanai et al., 2010), and (6) efficient fertilization in female reproductive tissue (Footitt et al., 2007).

CTS/PXA1/PED3 is a full ABC transporter that comprises two nucleotide binding domains (NBDs) providing the driving force for transport and two TMDs involved in substrate recognition and translocation. The transport cycle requires intramolecular communication between NBDs and TMDs, and modeling of CTS/PXA1/PED3 suggests that an interaction between NBD1 and TMD2 is critical for protein function. Mutation analysis shows distinct roles of the two NBDs in vivo (Dietrich et al., 2009).

A point of debate is whether CTS/PXA1/PED3 transports free fatty acid or CoA esterified substrates. Free fatty acids are activated to acyl-CoAs by acyl-CoA synthetases present in multiple compartments and transporter mutants accumulate long-chain acyl-CoAs (Footitt et al., 2002). The two peroxisomal long-chain acyl-CoA synthetases, LACS6 and LACS7, are essential for fatty acid mobilization and seedling development (Fulda et al., 2004). The *S. cerevisiae* equivalent transporter Pxa1p/Pxa2p transports acyl-CoAs (Verleur et al., 1997). The *Arabidopsis* CTS/PXA1/PED3 protein can complement the yeast *pxa1 pxa2* double mutant and support the metabolism of a wide range of fatty acid substrates that differ in chain length and degree of unsaturation (Nyathi et al., 2010). Furthermore, the ATPase activity of CTS/PXA1/PED3 is stimulated by acyl-CoAs but not appreciably by free fatty acids, which also supports the notion of acyl-CoAs as substrates (Nyathi et al., 2010). As proposed by Fulda et al. (2004), one possible explanation of this discrepancy is that acyl-CoAs are the substrate, but the CoA is removed during transport and acyl-CoA is resynthesized in the peroxisome by LACS6 and/or LACS7. Resolution of this issue will require in vitro transport studies using reconstituted CTS/PXA1/PED3 protein; however, this technically challenging task has not yet been achieved.

An ATP Transporter Supplying Peroxisomes with ATP

Arabidopsis PNC1 and PNC2 are members of the mitochondrial carrier family (Palmieri et al., 2011) and function as peroxisomal adenine nucleotide carriers by importing cytosolic ATP into peroxisomes to drive energy-consuming reactions, such as the activation of β-oxidation substrates. Repression of both *PNC* genes by RNAi severely impairs β-oxidation during seed storage oil mobilization (Arai et al., 2008a; Linka et al., 2008), indicating that the PNC-mediated transport pathway is the primary source for peroxisomal ATP and that another major ATP-generating system, such as substrate-level phosphorylation, may not exist in peroxisomes.

Recombinant PNC proteins function as antiporters that exchange ATP for ADP or AMP (Linka et al., 2008). In β-oxidation, PNCs import ATP in exchange for AMP released by acyl-CoA synthetases in the matrix. The influx of ATP against ADP is required, for instance, to support the activities of kinases, which have been detected by recent proteomic analysis (Reumann et al., 2007, 2009). One future task will be to elucidate other roles of the PNC proteins in supplying ATP-dependent reactions

beyond β-oxidation. Moreover, it is unknown how peroxisomes compensate their net transfer of negative charges ($ATP^{(4-)}$/$AMP^{(2-)}$ or $ATP^{(4-)}$/$ADP^{(3-)}$) across the membrane and how the nucleotide pool in plant peroxisomes is loaded in the first place.

PXN Serves as a Peroxisomal NAD^+ Transporter

The peroxisomal NAD^+ transporter PXN is an abundant protein of the peroxisomal membrane identified as PMP38 by independent proteomic approaches (Fukao et al., 2001; Reumann et al., 2007, 2009; Eubel et al., 2008) and from a screen for mutants with abnormal peroxisome morphology (Mano et al., 2011). This protein exhibits high sequence similarity to the PNCs; however, recombinant *Arabidopsis* PXN transports NAD^+ in vitro in exchange for NADH, AMP, or ADP (Bernhardt et al., 2012). Considering that NAD^+ is synthesized de novo in the cytosol (Noctor et al., 2006; Hashida et al., 2009) and that the free cytosolic NAD^+ concentration is estimated to be 0.6 mM (Igamberdiev and Gardeström, 2003), the physiological function of PXN presumably is to mediate an $NAD^+_{(in)}$/$AMP_{(out)}$ antiport, like the plastidic and mitochondrial NAD^+ transporters (Palmieri et al., 2009). A net NAD^+ influx can be achieved either by an unknown adenylate uniporter reimporting cytosolic AMP or a peroxisomal reaction generating AMP to refill the peroxisomal AMP pool. Thus, PXN might provide the cofactor NAD^+ to numerous peroxisomal redox enzymes.

Surprisingly, *Arabidopsis pxn* loss-of-function mutants do not show severe growth defects but exhibit a subtle metabolic phenotype; fatty acid degradation is slowed down in the mutant seedlings (Bernhardt et al., 2012). It is possible that an alternative NAD^+ import system exists in the peroxisomal membrane. Alternatively, plant peroxisomes may already contain sufficient NAD^+ when preperoxisomal vesicles bud from the ER, or NAD^+ may be taken up with NAD^+-dependent enzymes from the cytosol via protein import.

Diffusion of Carboxylic Acids Facilitated by a Peroxisomal Pore-Forming Channel

Based on enzyme latency analyses and electrophysiological experiments using membranes isolated from plant, mammalian, and yeast peroxisomes, peroxisomal pore-forming channels (porins) have been postulated for the passive diffusion of a broad spectrum of small solutes (Labarca et al., 1986; Lemmens et al., 1989; Reumann et al., 1995, 1997, 1998; Antonenkov et al., 2005, 2009; Grunau et al., 2009). The peroxisomal porin-like channel in spinach (*Spinacia oleracea*) leaves and germinating castor beans (*Ricinus communis*) is anion selective and facilitates the diffusion of small carboxylic acids, such as intermediates in photorespiration (e.g., glycolate, malate, Glu, and glycerate), β-oxidation, and the glyoxylate cycle (succinate and Asp) (Reumann et al., 1995, 1996, 1997, 1998). The current challenge is to assign genes that encode this observed channel activity.

Two different transporter protein classes might be considered as prime candidates for the plant peroxisomal porin channel: (1) the voltage-dependent anion-selective channel (VDAC) family, and (2) the PMP22 family. VDACs are large nonspecific diffusion pores with sieve properties in the outer mitochondrial membrane that are involved in metabolite transport (Colombini, 2004). Unexpectedly, proteomic approaches revealed VDAC homologs in cucumber (*Cucumis sativus*) and soybean (Glycine max) peroxisomes, and their localization was confirmed by immunogold labeling and fluorescence microscopy using GFP fusion proteins (Corpas et al., 2000; Arai et al., 2008b). The mouse PMP22 homolog forms a channel for small organic acids when heterologously expressed in insect cells (Rokka et al., 2009). *Arabidopsis* PMP22 is present in peroxisomal membranes (Tugal et al., 1999; Murphy et al., 2003), yet its biochemical function remains unknown. Electrophysiological experiments with the respective recombinant proteins may elucidate whether peroxisomal VDAC homologs and/or *Arabidopsis* PMP22 exhibit channel activities and mediate the transfer of metabolites across the peroxisomal membrane.

UNRAVELING THE COMPLETE ARRAY OF PLANT PEROXISOME FUNCTIONS

Without comprehensive knowledge of all metabolic reactions of plant peroxisomes, biochemical pathway manipulations have a high probability of failure due to overlapping roles of individual enzymes and shared segments of pathways. The role of β-oxidation in the production of IAA and JA is a case in point. In addition to genetic screens described earlier, proteomics is another powerful tool to catalog new functions for peroxisomes and help to provide a more rational basis for the future redesign of peroxisome metabolism.

Experimental Proteomics

The proteome of plant peroxisomes varies between plant tissues, developmental stages, and environmental conditions. To define the complete proteome, researchers have focused on soluble matrix proteins from *Arabidopsis*, soybean, and spinach (Fukao et al., 2002, 2003; Reumann et al., 2007, 2009; Arai et al., 2008a, 2008b; Eubel et al., 2008; Babujee et al., 2010). More than 100 putatively novel peroxisomal proteins, including many low-abundance and regulatory proteins, were identified. Because plant peroxisomes are difficult to separate from mitochondria and plastids, validation of peroxisome targeting using methods such as transient expression of the candidate proteins tagged by a GFP variant generally is required. Many new *Arabidopsis* proteins have been established in the past few years by the peroxisome community, with major contributions from the *Arabidopsis* Peroxisome 2010 project (www.peroxisome.msu.edu; reviewed in Kaur et al., 2009; Kaur and Hu, 2011; Reumann, 2011).

Protein identification by experimental proteomics is only the first step toward characterizing protein functions and the functional diversity of plant peroxisomes. Computational tools, including protein annotations deduced from sequence homology to known proteins, identification of conserved domains and motifs, microarray-based expression data analysis, and phylogenetic analysis, give valuable hints to the physiological function of the novel proteins. The physiological functions of a number of enzymes and metabolic pathways indicated by proteomic data

have been verified. Examples include the oxidative pentose phosphate pathway (Meyer et al., 2011), betaine aldehyde dehydrogenase (Missihoun et al., 2011), SDRa (Wiszniewski et al., 2009), and the bifunctional transthyretin-like protein involved in purine catabolism and *S*-allantoin biosynthesis (Lamberto et al., 2010), which have significantly broadened our knowledge of peroxisome metabolism.

Despite this success, experimental peroxisomal proteome studies are limited to major plant tissues and organs and by technological sensitivity and peroxisome purity. Additionally, only a few plant species are suitable for peroxisome isolation. The success of future experimental proteome research of plant peroxisomes relies on sensitive quantitative mass spectrometry technology to efficiently subtract contaminants from peroxisome fractions, isotope tagging methodologies, such as the LOPIT method (Dunkley et al., 2004), and efficient enrichment strategies to affinity purify peroxisomes or peroxisome vesicles by tagging of selected membrane proteins (Reumann, 2011).

The Prediction of Matrix Proteins from Genome Sequences

The prediction of plant peroxisomal matrix proteins from genome sequences combined with in vivo targeting validations is an alternative, large-scale approach that complements experimental proteome research (Reumann, 2011). Prediction methods, such as PeroxiP and the PTS1 predictor, and databases, such as PeroxisomeDB, were developed to predict and assemble PTS1 proteins from primarily metazoan genomic sequences (see references in Lingner et al., 2011). However, high-accuracy prediction tools have long been lacking for plants. Because ~80% of matrix proteins enter plant peroxisomes by the PTS1 import pathway (Reumann, 2004), prediction algorithms for PTS1 proteins are expected to significantly contribute to defining the plant peroxisomal proteome.

PTS1 proteins carry either a canonical (major) or noncanonical PTS1 tripeptide. Proteins with major PTS1s, such as SKL> and ARL> (> indicates the C-terminal end of the protein), often can be predicted to be peroxisomal based solely on the PTS1 tripeptide (Reumann, 2004) because major PTS1s are generally sufficient for peroxisome targeting, provided that the PTS1 tripeptide is surface exposed and not overruled by targeting signals for other compartments. Simple tripeptide-based predictions of *Arabidopsis* PTS1 proteins thus are relatively straightforward, and candidate proteins have been assembled in the database AraPerox (www3.uis.no/araperoxv1; Reumann, 2004; Reumann et al., 2004). The challenge is the prediction of proteins with noncanonical PTS1 tripeptides, such as ASL>, SLM>, and SRY>, because (1) their PTS1 tripeptide identities are insufficiently known and are more diverse than previously thought, and (2) noncanonical PTS1 tripeptides generally are weak and require auxiliary targeting-enhancing patterns located immediately upstream for function. Such enhancer patterns have been poorly defined for plants. Hence, among many proteins with the same noncanonical PTS1 tripeptide, only a few are indeed peroxisome targeted, and correct computational predictions are difficult. For instance, prediction tools developed for metazoa generally fail to correctly predict plant peroxisomal proteins with noncanonical PTS1 tripeptides (Lingner et al., 2011). The accuracy of prediction algorithms relies on the size, quality, and diversity of the underlying data set of example sequences that is used for model training and limited preexisting prediction algorithms (Emanuelsson et al., 2003; Bodén and Hawkins, 2005; Hawkins et al., 2007).

To develop prediction models specifically for plants, 60 known *Arabidopsis* PTS1 proteins, including low-abundance proteins with noncanonical PTS1s identified by proteome analyses, were used to generate a data set of more than 2500 homologous plant sequences, primarily from EST databases. Two prediction methods were developed, both of which showed high accuracy on example sequences. Due to the omission of a PTS1 tripeptide filter, the models were able to correctly infer novel PTS1 tripeptides and even include novel residues. In combination with in vivo subcellular targeting analyses, 23 newly predicted PTS1 tripeptides were established for plants and several previously unknown *Arabidopsis* PTS1 proteins identified. This prediction method (i.e., the position weight matrices model) predicts 389 *Arabidopsis* gene models to encode peroxisomal PTS1 protein variants; ~70% of them are not known to be peroxisomal. Some confirmed peroxisomal PTS1 proteins are located in a gray zone below the prediction threshold, indicating that the number of *Arabidopsis* peroxisomal proteins might exceed 400 to 500 (Lingner et al., 2011).

Despite good accuracy, prediction algorithms can be improved by increasing the representation of noncanonical PTS1 protein sequences in the underlying data set. By iterative experimental validation of newly predicted *Arabidopsis* proteins, identification of homologous ESTs, data set expansion with the addition of positive sequences, and improvement of the discriminative machine learning methods, the prediction accuracy can be further increased. Finally, we need to develop prediction algorithms for plant PTS2 proteins, which are more challenging due to the smaller number of example sequences and the variable positions of the PTS2 nonapeptide in the N-terminal domain.

PERSPECTIVES

Recent years have witnessed tremendous progress in understanding the complexity of plant peroxisomes in their dynamic biogenesis and function. However, many questions about peroxisomes remain unanswered, and new strategies and technologies are needed to address these issues.

A major challenge is to elucidate whether de novo synthesis of peroxisomes actually occurs at the ER in plants and, if so, how this process compares to those in other kingdoms. Notably, these types of questions may begin to be addressed by studies of viruses that specifically exploit peroxisomes during their infection cycle (reviewed in Mullen and Gidda, 2009; Lazarow, 2011). Certain plant RNA tombusviruses, for instance, appear to engage a pER-destined retrograde vesicle sorting pathway (McCartney et al., 2005). While the functional significance of this pathway and its existence in noninfected plant cells have not been determined, speculation that it represents an additional level of connectivity between peroxisomes and the ER is intriguing. Likewise, the sharing of division factors for peroxisomes, mitochondria, and chloroplasts suggests that these functionally connected organelles may also coordinate

the remodeling of their abundance as another mechanism for interorganellar communication. Further characterization of dual-localized proteins may shed light on these processes. Additional pathways that control peroxisome abundance, such as those regulated by the dual-targeted PMD1 or by the accumulation of acyl-CoAs and other molecules, need to be investigated further.

Many null mutants of peroxins appear to be gametophytic or embryo lethal. Alternative approaches, such as chemical genetics, can be valuable in the dissection of essential processes (Hicks and Raikhel, 2009). A group of benzimidazole compounds have been identified to differentially disrupt PTS1 protein import at nanomolar concentrations, whereas PTS2 import inhibition is only seen after long incubation at micromolar concentrations (Brown et al., 2011). Protein import in general is poorly understood at the mechanistic level, and kinetic and quantitative data on protein interactions within the import pathway would help in building and testing models.

Additional studies of the enzymes acting in peroxisomal processes are required to understand the complexity of the pathways. β-Oxidation pathways for straight-chain fatty acids and other diverse substrates have been defined, although specific isozyme assignments remain in progress, and questions of regulation must be addressed. JA and IAA are generated in peroxisomal reactions, but knowledge on key biosynthetic enzymes and how these hormones are exported to the cytosol is missing. In addition to pathways discussed in this review, recently uncovered pathways, including terpene (isoprenoid) and biotin biosynthesis pathways, must be further explored to identify the extent of peroxisomal involvement. For example, the mevalonic acid pathway that generates isoprenoid precursors of terpenes was long placed in the cytosol/ER, but recent work has localized at least four biosynthetic enzymes to peroxisomes (Reumann et al., 2007; Sapir-Mir et al., 2008; Simkin et al., 2011; Thabet et al., 2011). The relative contributions of this pathway to the terpene pool and the regulation of intermediate transport between the cytosol, peroxisomes, and the ER can now be investigated. The complex subcellular distribution of many of these pathways leads to further questions. For example, when and why did specific pathway steps shift to peroxisomes? How are peroxisomal pools of hormones, coenzymes, and cofactors regulating metabolic activities within peroxisomes or the cell as a whole? A complete description of the pathways and knowledge of all the enzymes will facilitate our understanding of the roles of peroxisomes within a cell.

To uncover the full array of peroxisomal functions and the dynamics of the peroxisomal proteome, technologies need to be improved to identify low-abundance and membrane proteins and peroxisomal proteins present in specific tissue or cell types and under certain environmental conditions. Although validations are required, the prediction that the number of plant peroxisomal proteins may exceed 400 suggests there are many additional roles of peroxisomes yet to be realized. The necessity for plants to cope with numerous abiotic and biotic stresses appears to have been a major driving force in the evolution of adaptation mechanisms in peroxisomes. Plant peroxisomes thereby emerge as a new model even for fungi and mammals in understanding and exploring stress adaptation functions.

In this postgenomic era, systems approaches using transcriptomics, genomics, proteomics, metabolomics, and computational biology will assist us in establishing a complete map of peroxisomal pathways and their regulatory networks. In addition to efforts aimed at engineering plants for improved biomass production by manipulating photorespiration and lipid metabolism, it is also time to translate peroxisomal research from reference plants to agronomically important crops. The extensive conservation of the peroxisomal proteome of *Arabidopsis* and the predicted rice peroxisomal proteome (Kaur and Hu, 2011) suggests that knowledge gained from model plants can aid in the study of peroxisomes in other prominent cereal crops.

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