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Peroxisomes: versatile organelles with diverse roles in plants

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Summary

Peroxisomes are small ubiquitous organelles delimited by a single membrane and lacking genetic material. However, these simple-structured organelles are highly versatile in morphology, abundance and protein content in response to various developmental and environmental cues. In plants, peroxisomes are essential for growth and development and perform diverse metabolic functions, many of which are carried out coordinately by peroxisomes and other organelles physically interacting with peroxisomes. Recent studies have added greatly to our knowledge of

peroxisomes, including the diverse proteome, regulation of division and protein import, pexophagy, matrix protein degradation, solute transport, signaling, redox homeostasis and various metabolic and physiological functions. This review summarizes our current understanding of plant peroxisomes, focusing on recent discoveries. Problems and future efforts needed to better understand these organelles are also discussed. Knowledge gained will be important not only to the understanding of eukaryotic cell biology and metabolism, but also to agricultural efforts aimed at improving crop performance and defense.

Key words: peroxisomes, proteome, peroxisome protein import, division, inter-organellar interaction, pexophagy, β-oxidation, metabolism

I. Introduction

Eukaryotic cells contain various subcellular compartments (organelles) that each host a specific set of cellular activities. As one of the last discovered major organelles (De Duve & Baudhuin, 1966), peroxisomes are approximately 0.1-1 μm in diameter, delimited by a single membrane and lacking genetic materials. Despite their small size and simple structure, peroxisomes are highly dynamic morphologically and metabolically, and play essential roles in the development of animals and plants. Severe impairment of peroxisome biogenesis and function can lead to embryo lethality in plants and infant fatality in mammals (Hu *et al.*, 2012; Dasouki, 2017; Pan *et al.*, 2018a).

Plant peroxisomes contain at least 200 proteins involved in a wide range of physiological functions, including primary and secondary metabolism, development, and response to abiotic and biotic stresses (reviewed in Hu *et al.*, 2012; Reumann & Bartel, 2016; Kao *et al.*, 2018; Pan & Hu, 2018a; Pan *et al.*, 2019). Peroxisomes are physically and metabolically linked to various other organelles, such as chloroplasts,

mitochondria and oil bodies (Wanders *et al.*, 2016; Oikawa *et al.*, 2019). The recent decade witnessed a rapid expansion of our knowledge of plant peroxisomes, thanks to advances in bioinformatics, mass spectrometry-based proteomics and advanced microscopy, as well as traditional genetic and biochemical approaches. Here, we present an overview of plant peroxisomal proteome, biogenesis, quality control and remodeling, interaction with other organelles, metabolic functions and transporters, focusing more on recent progress. We also provide perspectives on future research needed to fully understand these fascinating organelles. We apologize to researchers whose work we are unable to discuss in this review due to space limitation.

II. Decoding the peroxisomal proteome

Plant peroxisomes display a high level of functional complexity, plasticity and specificity, as exemplified by the presence of numerous peroxisomal pathways unique to plants (reviewed in Pan & Hu, 2018a). Indexing the peroxisomal proteome is prerequisite to fully understanding their roles in plant physiology. Most peroxisomal matrix proteins contain one of the two types of peroxisome targeting signals: PTS1, a C-terminal tripeptide, and PTS2, a nonapeptide near the N-terminus (Reumann & Chowdhary, 2018). Studies using high throughput approaches, such as mass spectrometry (MS)-based proteomic analysis and algorithm prediction of PTS1-containing proteins, have significantly expanded our knowledge of proteins and biochemical reactions in plant peroxisomes. Many novel peroxisomal proteins, such as those involved in methylglyoxal detoxification, phylloquinone biosynthesis, pseudouridine catabolism, CoA biosynthesis, and putative regulatory proteins have been discovered by peroxisomal proteome analysis (reviewed in Pan & Hu, 2018a).

Peroxisome proteome analyses have been performed on various plant species and organs, including greening and etiolated Arabidopsis cotyledons (Fukao *et al.*, 2002, 2003), Arabidopsis green leaves (Reumann *et al.*, 2007, 2009), non-green Arabidopsis suspension cell cultures (Eubel *et al.*, 2008), etiolated Arabidopsis seedlings (Quan *et al.*, 2013), etiolated soybean cotyledons (Arai *et al.*, 2008) and spinach leaves

(Babujee *et al.*, 2010). A recent study extended the analysis to peroxisomes isolated from Arabidopsis leaves undergoing dark-induced senescence, revealing a higher number of proteins involved in the detoxification of reactive oxygen species (ROS) and new peroxisomal proteins with potential roles in fatty acid metabolism and stress response (Pan *et al.*, 2018a). These studies demonstrated that the core proteome of plant peroxisomes is conserved throughout development. Therefore, as previously proposed (Pracharoenwattana and Smith, 2008), all plant peroxisomal subtypes should simply be named peroxisomes rather than individually as leaf peroxisome, glyoxysome in seed and germinating seedling, gerontosome in senescing tissue and so on. In addition to experimental proteomics, plant-specific PTS1 prediction algorithms, including PredPlantPTS1 (Lingner *et al.*, 2011) and PPero (Wang *et al.*, 2017), successfully predicted hundreds of known plant peroxisomal proteins, as well as many novel ones.

In vivo protein targeting analysis using fluorescence microscopy is usually needed to confirm the localization of candidate proteins obtained from proteomic, bioinformatic, genetic and biochemical studies. Cautions should be taken when selecting fluorophores. In transient expression systems using Arabidopsis seedlings and tobacco leaves, certain fluorophore combinations that weakly heterodimerize can cause false positives as a result of the so-called piggy-back mechanism of peroxisomal protein import (Falter *et al.*, 2019). However, this phenomenon has not been observed in Arabidopsis protoplasts, onion epidermal cells, or stable transgenic plants. In addition to the piggy-back mechanism, false positive targeting to peroxisomes may also occur when the fluorophore tag affects protein folding or masks the true targeting signal, exposing PTS-like sequences that are otherwise inactive. When overexpressed proteins saturate the import machinery of a non-peroxisomal organelle, the excess proteins may also be redirected to peroxisomes. However, whether these speculated mechanisms indeed occur in planta, especially in stable transgenic lines, is unclear.

Proteome studies followed by in vivo targeting verification discovered that plant

peroxisomes contain at least 200 proteins that represent a larger proteome and more diverse metabolic pathways than their counterparts in animals and yeasts (reviewed in Pan & Hu, 2018a). This may be partially attributed to the existence of photosynthesis and photorespiration in plants, which require a higher anti-oxidant capacity to achieve oxidative homeostasis. Alternatively, plants may depend more on peroxisome-derived signals and metabolites to respond to environmental changes. Despite these important findings, the plant peroxisome proteome is still far from being completely understood due to several reasons. First, most proteome analyses were performed with leaf tissue or cotyledons, whereas many other tissues such as root, reproductive organs and seed, have yet been analyzed. Second, peroxisomal proteome analyses have not been performed in monocotyledon species, including major crops like rice, wheat and maize, which are not oilseeds and thus may contain functions different from those of dicots. Third, existing algorithms still cannot accurately predict proteins containing non-canonical PTS1 and those with PTS2. Fourth, identification of peroxisomal membrane proteins (PMPs) remains technically challenging for both proteomics and bioinformatics.

III. Peroxisome protein import and proliferation/division

Since all peroxisomal proteins are encoded in the nucleus, they need to be imported into peroxisomes from the cytosol through a process mediated by peroxins (or PEX proteins), most of which are conserved across kingdoms (Hu *et al.*, 2012; Cross *et al.*, 2016). Recent molecular genetic studies have shed light on the function of plant PEX proteins and revealed regulatory mechanisms.

Peroxisomal membrane proteins target to the peroxisome by direct insertion into the peroxisomal membrane from the cytosol, or by trafficking via the ER. The import of peroxisomal membrane proteins in Arabidopsis involves three well conserved peroxins, PEX19 (AtPEX19A and AtPEX19B) as the chaperone for PMPs, PEX3 (AtPEX3A and AtPEX3B) as the membrane anchor for PEX19, and PEX16 that recruits PEX3 to the ER prior to the formation of pre-peroxisomes (Figure 1) (Pan &

Hu, 2018a; Burkhart *et al.*, 2019). Arabidopsis PEX16 also recruits PMPs to the ER in an PEX3/PEX19-independent manner (Hua *et al.*, 2015).

In plants, newly synthesized matrix proteins are recognized and bound by the receptors PEX5 and PEX7 in the cytosol, before the receptor-cargo complex is docked on the membrane at the docking complex formed by PEX13 and PEX14 (Figure 1). PEX5 can be recycled from the peroxisomal matrix back to the cytosol, facilitated by the cooperative efforts of the ubiquitin conjugating enzyme PEX4 and its membrane anchor PEX22, three RING-type ubiquitin ligases PEX2, PEX10 and PEX12, and two AAA ATPases PEX1 and PEX6 that are tethered to their membrane anchor PEX26/APEM9 (Figure 1) (reviewed in Reumann & Bartel, 2016; Cross *et al.*, 2016; Kao *et al.*, 2018; Pan & Hu, 2018a).

In Arabidopsis *pex6* or *pex26* mutants, the reduced level of PEX5 protein can be partially restored by inhibiting activity of the proteasome or peroxisome-associated ubiquitination machinery, indicating that PEX5 is degraded by the ubiquitin-proteasome system when its export is impaired (Gonzalez *et al.*, 2017). Consistent with the role of the RING-type ubiquitin ligases in receptor ubiquitination followed by degradation, the Arabidopsis *pex12-1* mutant has increased PEX5 and PEX7 protein levels (Kao *et al.*, 2016). Interestingly, proteasome-mediated degradation of Arabidopsis PEX5 can be increased by elevated temperature (Kao & Bartel, 2015). Moreover, the *pex1-1* allele partially rescued *pex6-1* defects without restoring PEX5 levels, but enhanced *pex26* defects, implying that the plant PEX1-PEX6 complex may have novel roles in peroxisome homeostasis and function (Gonzalez *et al.*, 2018). Furthermore, Arabidopsis PEX14 plays a positive role in drought tolerance through modulation of the expression of stress-responsive genes, ROS metabolism, and metabolic homeostasis (Shi *et al.*, 2015), which may reflect the function of the entire peroxisome as PEX14 is required for matrix protein import.

Besides the RING peroxins, two homologous Arabidopsis E3 ubiquitin ligases, SP1

(suppressor of plastid protein import locus 1) and SPL1, also modulate peroxisome protein import (Figure 1). SP1 was shown to possess E3 ubiquitin ligase activity (Pan & Hu, 2017) and promote the degradation of PEX13 and PEX14, most likely through the ubiquitin-proteasome pathway, whereas SPL1 has an opposite effect on PEX13 degradation (in tobacco transient expression system) and on peroxisomal function (Pan & Hu, 2016; Pan *et al.*, 2018b). SP1 was also shown to negatively regulate chloroplast protein import by targeting several TOC (translocon at the outer envelope membrane of chloroplasts) proteins for destabilization (Ling *et al.*, 2012). Both 35S- and native *SP1* promoter-driven SP1-YFP showed clear localization to peroxisomes, mitochondria and chloroplasts in multiple transgenic plants (Pan *et al.*, 2016; Pan & Hu, 2018b). In another study, SP1 was reported to only localize to chloroplasts when transiently expressed in protoplasts, and transgenic Arabidopsis plants expressing 35Spro:SP1-YFP or SP1pro:SP1-YFP were unable to be obtained (Ling *et al.,* 2017). These discrepancies may be partially due to the fact that SP1 may require certain length of time or mechanism to accumulate in mitochondria and peroxisomes, which could be easier to detect in certain experimental systems than in others. MUL1 is the mammalian homolog of SP1 and SPL1 that localizes to mitochondria and, via mitochondrion-derived vesicles, to peroxisomes (Braschi *et al.*, 2010). MUL1 is involved in mitochondrial fission and hyperfusion, mitophagy, maintenance of mitochondrial integrity, and mitochondrial antiviral response, as a SUMO or ubiquitin ligase (summarized in Pan & Hu, 2018b). Therefore, this protein family may have a conserved role in plants and animals in modulating protein import and/or dynamics of multiple organelles.

Peroxisomes can also proliferate by division of pre-existing peroxisomes, which consists of two stages: 1) elongation/tubulation mediated by PEX11 proteins; and 2) membrane constriction and fission mediated by dynamin-related proteins DRP3A and DRP3B, together with their probable membrane anchors FISSION 1A (FIS1A) and FIS1B (Figure 2) (Kaur & Hu, 2009). The DRP3-FIS1 complex is shared by peroxisomes and mitochondria, whereas PMD1 (Peroxisomal and mitochondrial

division 1) and DRP5B are two plant specific peroxisome fission factors that are shared with mitochondria and chloroplasts, respectively (Figure 2) (Pan & Hu, 2011; Kaur *et al.*, 2014).

Regulatory mechanisms by which peroxisomes proliferate in response to environmental cues are beginning to be uncovered. The transcription of Arabidopsis *PEX11b* is induced by the HY5 HOMOLOG (HYH) transcription factor in response to light stimulation through phytochrome A (phyA), and repressed by another nuclear protein, Forkhead-Associated Domain Protein 3 (FHA3) (Figure 2) (Desai & Hu, 2008; Hu & Desai, 2008; Desai *et al.*, 2017). Arabidopsis *PEX11e* is up-regulated by salt stress, and both *PEX11e* overexpression and salt stress can increase peroxisome number, but *PEX11e* overexpression does not improve salt tolerance (Mitsuya *et al.*, 2010). In contrast, rice seedlings overexpressing *PEX11* (Os03g0302000) have increased salt tolerance compared to wild-type and *OsPEX11*-RNAi seedlings (Cui *et al.*, 2016b). Hence, PEX11 may have acquired distinct functions in stress response in different plant lineages during evolution. PMD1 binds to actin and functions genetically downstream of MAP kinase 17 (MPK17), a putative regulator of salt-induced peroxisome proliferation (Figure 2) (Frick & Strader, 2017).

In summary, core proteins in peroxisome protein import, such as the major PEX proteins, and proteins in peroxisome division, such as PEX11, FIS1 and DRP3, are significantly conserved in eukaryotes. However, some lineage-specific factors also exist, and the regulatory mechanisms of peroxisome protein import and division in plants seem to be mostly unique.

IV. Peroxules and peroxisome interaction with other organelles

Oxidative stress promotes the formation of peroxules, which are extensions of the peroxisome membrane (Sinclair *et al.*, 2009). In Arabidopsis, peroxule formation can be induced by cadmium or arsenic treatment in a PEX11a- and ROS-dependent manner, followed by peroxisome proliferation, suggesting the important role of

PEX11a and peroxules in plant stress response (Rodríguez-Serrano *et al.*, 2016).

Accumulating evidence has demonstrated convincingly that peroxules are key to the interaction of peroxisomes with organelles known to have close metabolic and physical ties with peroxisomes, including ER, oil bodies, mitochondria and chloroplasts (Sinclair *et al.*, 2009; Thazar-Poulot *et al.*, 2015; Gao *et al.*, 2016; Jaipargas *et al.*, 2016). It is possible that during plant response to physiological signals, peroxules serve as a platform to connect peroxisomes and other organelles to facilitate the exchange of metabolites and proteins. Such signals most likely include reactive oxygen species (ROS), as shown in the case of peroxisomal interaction with ER and mitochondria (Sinclair *et al.*, 2009; Jaipargas *et al.*, 2016).

The interaction between peroxisomes and other organelles is controlled by physiological and environmental signals. For example, peroxisome-oil body interaction is increased in Arabidopsis seedlings deficient in fatty acid degradation, while exogenously applied sucrose can reduce this interaction, indicating that this interaction facilitates lipid catabolism and is negatively regulated by cellular sugar levels (Cui *et al.*, 2016a). In photosynthetic tissues, the interaction between peroxisomes, mitochondria and chloroplasts is expected to be beneficial to the flow of intermediates in photorespiration, a carbon-recycling pathway that accompanies photosynthesis (see VI. 4). Consistent with this view, the area and strength of the interactions were found to be increased by light (Oikawa *et al.*, 2015; Jaipargas *et al.*, 2016). Direct physical tethering between peroxisomes and chloroplasts has also been verified in tobacco and Arabidopsis, using advanced optic technologies such as femtosecond laser and optical tweezer (Oikawa *et al.*, 2015; Gao *et al.*, 2016).

In non-plant organisms, several molecular mechanisms are involved in the formation of contact sites between peroxisomes and other organelles. In mammals, acyl-CoA binding domain containing 4 (ACBD4), ACBD5, vesicle-associated membrane protein-associated proteins A (VAPA) and VAPB mediate ER-peroxisome interaction,

and synaptotagmin 7 (SYT7) and phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] are involved in lysosome-peroxisome interaction (reviewed in Castro *et al.*, 2018). In yeast cells, Pex3 and inheritance of peroxisomes 1 (Inp1) participate in ER-peroxisome interaction, and Pex34, fuzzy onions homolog 1 (Fzo1), Pex11 and mitochondrial distribution and morphology 34 (Mdm34) participate in mitochondrion-peroxisome interaction (reviewed in Castro *et al.*, 2018). Although physical contacts between peroxisomes and other organelles have been repeatedly documented in microscopic studies (Oikawa *et al.*, 2019), proteins that mediate these interactions have not been conclusively determined in plants. In mammalian and yeast cells, vesicle carriers can mediate transportation of proteins and possibly other kinds of cargo from other organelles to peroxisomes (Neuspiel *et al.*, 2008; Lam *et al.*, 2011). This type of transportation has not been directly demonstrated for plant peroxisomes.

V. Peroxisomal quality control and proteome remodeling

The development and maintenance of peroxisomes require protein maturation, degradation and recycling as well as autophagic degradation of the whole organelle (Baker & Paudyal, 2014). Arabidopsis peroxisomes contain several proteases and peptidases, such as LON2 (Lon protease 2), DEG15 (Degradation of periplasmic protein 15), SCPL20 (Serine carboxypeptidase-like protein 20), RDL1 (Response to drought21A-like 1) and PXM16 (Peroxisomal M16 metalloprotease) (Figure 3) (reviewed in van Wijk, 2015; Pan & Hu, 2018a). LON2 facilitates sustained matrix protein import in mature peroxisomes and the degradation of matrix proteins during peroxisome remodeling (Lingard and Bartel, 2009; Farmer *et al.*, 2013; Goto-Yamada *et al.*, 2014) (Figure 3). Analysis of watermelon DEG15 suggested that DEG15 can be a processing peptidase as a dimer to cleave PTS2 from PTS2-containing proteins, or a general protease in its monomeric form (Helm *et al.*, 2007) (Figure 3). SCPL20 is involved in β-oxidation and plant pathogen response (Floerl *et al.*, 2012; Quan *et al.*, 2013), and RDL1 plays a role in β–oxidation, seed viability and stress response (Quan *et al.*, 2013). The exact role of RDL1, SCLP20 and PXM16 remains to be elucidated.

Plant peroxisomal proteome varies to some extent in different developmental stages and tissue types. During oil seed germination, the glyoxylate cycle enzymes are replaced by photorespiration enzymes (Paudyal *et al.*, 2017). This remodeling of the plant peroxisomal proteome most likely involves the simultaneous action of several processes, such as LON2-mediated degradation of the glyoxylate cycle enzymes and pexophagy-mediated degradation of the obsolete peroxisomes (Figure 3). Dysfunction of both LON2 and pexophagy results in the stabilization of the glyoxylate cycle enzymes, which cannot be accomplished by removing only one of these two factors (Farmer *et al.*, 2013; Goto-Yamada *et al.*, 2014). Besides its protease activity, LON2 also has chaperone activity that suppresses pexophagy and peroxisome remodeling (Goto-Yamada *et al.*, 2014). Remodeling of the peroxisomal proteome is also attributed to light- and sugar-dependent transcriptional changes, and possibly other proteases, the ubiquitination system and proteins involved in peroxisome biogenesis (Goto-Yamada *et al.*, 2015).

Pexophagy is involved in peroxisomal quality control, as blocking pexophagy causes accumulation of aggregates of damaged catalase, clustered peroxisomes and an increased number of peroxisomes in both stress and non-stress conditions (Kim *et al.*, 2013; Shibata *et al.*, 2013; Yoshimoto *et al.*, 2014; Calero Muñoz *et al.*, 2019). Excessive ROS accumulation and catalase deficiency are linked to peroxisome damage and pexophagy (Hackenberg *et al.*, 2013; Tyutereva *et al.*, 2018; Luo & Zhuang, 2018). The plant pexophagy receptor for ATG8, a ubiquitin-like protein connecting the condemned organelle to the autophagic machinery, is still elusive. However, several ATG8-interacting proteins have been identified. During cadmium-induced pexophagy, ATG8 co-localizes with catalase and NBR1 (Neighbor of BRCA1 gene 1) in the electron dense peroxisomal core, suggesting that catalase and NBR1 are involved in pexophagy and NBR1 may function as a pexophagy receptor (Calero‐Muñoz *et al.*, 2019). A bioinformatics approach, named hfAIM (high fidelity ATG8 interacting motif), identified 9 peroxisomal PEX proteins in

Arabidopsis that contain putative hfAIM, among which PEX6 and PEX10 were further verified by BiFC (Bimolecular fluorescence complementation) (Xie *et al.*, 2016). An independent yeast two-hybrid screen also identified PEX10 as an ATG8-interacting protein, suggesting PEX10 to be a promising candidate for a receptor in pexophagy (Marshall *et al.*, 2019) (Figure 3). A recent study showed that autophagy mediates glucose-promoted peroxisomal degradation in roots, and that ATG8 physically interacts with a peptide that contains the Walker B motif of PXA1 (Peroxisomal ABC transporter 1)/CTS (Comatose)/PED3 (Peroxisome defective 3) (Huang *et al.*, 2019) (Figure 3), making PXA1 another possible receptor for pexophagy. Whether full-length PXA1 interacts with ATG8 in planta has not been shown. More rigorous studies are needed to determine directly whether any of these ATG8-interacting proteins is a pexophagy receptor in plants.

VI. Peroxisomal metabolism

Plant peroxisomes have diverse functions, housing metabolic pathways such as fatty acid degradation, the glyoxylate cycle, phytohormone biosynthesis, photorespiration and ROS catabolism. Fatty acid β-oxidation, which occurs exclusively in peroxisomes in plants as opposed to in mitochondria and peroxisomes in animals, participates in fatty acid catabolism and the biosynthesis of several major phytohormones, including jasmonic acid (JA), indole-3-acetic acid (IAA) and salicylic acid (SA). Substrates of β-oxidation, such as fatty acyl-CoA (FA-CoA), 12-oxo-phytodienoic acid (OPDA), indole-3-butyric acid (IBA) and cinnamic acid (CA), are imported by the ATP-dependent transporter PXA1. Recent studies linked more metabolic pathways to peroxisomes, adding to the complexity of plant peroxisomal metabolism (reviewed in Reumann & Bartel, 2016; Kao *et al.*, 2018; Pan & Hu, 2018a).

1. Fatty acid breakdown

After import into the peroxisome, fatty acids are esterified with CoA and catabolized into acetyl-CoA via β-oxidation. Each β-oxidation cycle is a four-step cascade catalyzed by three enzymes: acyl-CoA oxidase (ACX), multifunctional protein (MFP)

that catalyzes both a hydration and an oxidation step, and 3-ketoacyl-CoA thiolase (KAT), producing an acetyl-CoA and a FA-CoA that is shortened by two carbons and subjected to the next round of β-oxidation (Figure 4). Reducing FA import into the peroxisome in Arabidopsis does not affect peroxisomal size, whereas impairing the FA β-oxidation pathway enlarges peroxisomes, possibly due to the accumulation of β-oxidation intermediates inside peroxisomes (Graham *et al.*, 2002; Rinaldi *et al.*, 2016). Arabidopsis mutants of FA degrading enzymes typically show reduced seed oil mobilization that results in impaired seedling establishment, which can be ameliorated by exogenously applied sucrose (reviewed in Hu *et al.,* 2012). Some of these mutants exhibit defects in pollen fertility, embryo development and germination, which may not be solely explained by deficiency in FA degradation (reviewed in Pan *et al.,* 2019).

Although the core β-oxidation pathway is sufficient to metabolize straight-chain saturated fatty acids, the metabolism of unsaturated fatty acids requires auxiliary enzymes (reviewed in Graham, 2008). One of these auxiliary enzymes in Arabidopsis is *ECH2*, which encodes an enoyl-CoA hydratase that, when mutated, leads to accumulation of 3-hydroxyoctenoate (C8:1-OH) and 3-hydroxyoctanoate (C8:0-OH), putative hydrolysis products of the catabolism of α-linolenic acid and linoleic acid, and poor seedling development due to toxic effects of the accumulated intermediates (Li *et al.*, 2019a).

FA degradation is an important part of plant primary metabolism, which, when blocked, can affect other carbon metabolic processes and lipid homeostasis. Disrupting FA β-oxidation in Arabidopsis affects membrane lipid homeostasis in leaves (Fan *et al.*, 2014). Deficiencies in FA turnover in Arabidopsis starch biosynthetic mutants results in strong growth defects, increased levels of membrane lipids, triacylglycerol and soluble sugars, and altered fatty acid flux between the chloroplast- and ER-localized lipid biosynthetic activities, indicating a role for FA

breakdown in the crosstalk between starch and lipid metabolic pathways (Yu *et al.*, 2018). Disruption of Arabidopsis *PEX16* causes decreased levels of oil, increased levels of starch and accumulation of various soluble metabolites during seed development, suggesting the role of peroxisomes in FA biosynthesis and in crosstalk between lipid and starch metabolism (Lin *et al.*, 2004, 2006). FA β-oxidation also affects nuclear epigenetic modifications, as Arabidopsis *acx4* mutants have reduced nuclear histone acetylation and increased DNA methylation, and *mfp2* and *kat2* mutants show DNA hyper-methylation (Wang *et al.*, 2019). Hence, FA β-oxidation seems to have a broader impact on plant cellular processes than previously known.

2. The glyoxylate cycle and acetate-malate shunt

The glyoxylate cycle is a major peroxisomal function in seeds and post-germinative seedlings, where the peroxisomal enzymes citrate synthase (CSY), isocitrate lyase (ICL) and malate synthase (MLS) convert acetyl-CoA derived from FA β-oxidation into 4-carbon metabolites that can be consumed by gluconeogenesis and mitochondrial respiration (reviewed in Graham, 2008) (Figure 4). Thus, mutants in this pathway show typical phenotypes of mutants disrupted in FA degradation, e.g., impaired seedling establishment after germination that can be ameliorated by exogenous sucrose. Since peroxisomal MDHs do not seem to be involved in the glyoxylate cycle (Pracharoenwattana *et al.*, 2007), a cytosolic MDH was speculated to oxidize malate from the glyoxylate cycle (Graham, 2008). The expression of maize *CSY* is induced during seed germination and by light in leaves, and both of these changes involve methylation of the *CSY* promoter, suggesting epigenetic control of the glyoxylate cycle (Eprintsev *et al.*, 2018).

Arabidopsis acetate non-utilizing 1 (ACN1) is a peroxisomal short-chain acyl-CoA synthetase that produces acetyl-CoA from free acetate, thus also contributing to the pool of acetyl-CoA to be fed into the glyoxylate cycle (Figure 4). ACN1 only consumes a small fraction of the total cellular acetate, yet it affects primary metabolism and prevents carbon leakage from peroxisomes during lipid mobilization

in seedlings (Allen *et al.*, 2011). ACN1 was recently shown to be the starting point of the acetate-malate shunt, a process that converts acetate to malate in peroxisomes and very interestingly, modulates guard cell turgor and drought tolerance (Dong *et al.*, 2018).

3. Biosynthesis of phytohormones

The β-oxidation pathway also contributes to the production of several key phytohormones, including IAA, JA and SA (Figure 5). IBA is an endogenous auxin precursor that is converted into the active auxin IAA in peroxisomes. Peroxisome-originated auxin plays important regulatory roles in the development of lateral root, cotyledon, root hair and apical hook in seedlings (reviewed in Kao *et al.*, 2018).

Benzoic acid (BA) is a precursor of the defense hormone SA. Dissection of BA β-oxidative in *Petunia hybrida* and Arabidopsis led to the model that cinnamic acid (CA) is imported into the peroxisome by PXA1 (Arabidopsis) (Bussell *et al.*, 2014), followed by the enzymatic cascade catalyzed by cinnamoyl-CoA ligase (CNL), bifunctional CA-CoA hydratase/dehydrogenase (CHD), 3-ketoacyl thiolase 1 (KAT1) and thioesterase 1 (TE1) (Petunia) (Moerkercke *et al.*, 2009; Colquhoun *et al.*, 2012; Qualley *et al.*, 2012; Klempien *et al.*, 2012; Adebesin *et al.*, 2018) (Figure 5). Phenotypes of the rice *aim1* (*abnormal inflorescence meristem 1*) mutant in redox gene expression and root development can be rescued by SA treatment, suggesting that rice AIM1, whose homolog in Arabidopsis (AtAIM1) is a multifunctional protein (MFP) in β-oxidation, participates in SA biosynthesis to regulate ROS levels for proper function of the root meristem (Xu *et al.*, 2017).

JA is important for plant defense and reproduction (Wasternack & Strnad, 2018). In the octadecanoid pathway for JA biosynthesis, the chloroplast-synthesized JA precursors 12-oxo-phytodienoic acid (OPDA) and dnOPDA are imported into peroxisomes, reduced by OPDA reductase (OPR), and activated to CoA esters that go

through the β-oxidation cycles to produce JA (Figure 5) (reviewed in Pan *et al.*, 2018a; Wasternack & Strnad, 2018). A recent study in Arabidopsis revealed a peroxisomal OPR-independent pathway for JA biosynthesis, where OPDA skips reduction by the peroxisomal OPR and is directly converted to 4,5-didehydro-JA via β-oxidation in peroxisomes before being reduced to JA by an OPR in the cytosol (Chini *et al.*, 2018).

Peroxisomal OPRs are conserved in Arabidopsis, tomato, rice, maize and wheat, but show species-specific mutant phenotypes. The loss-of-function mutant of the rice peroxisomal OPR, OG1 (or OsOPR7), is fertile but impaired in carbohydrate transport into lodicules during anthesis (Li *et al.*, 2018b). By contrast, Arabidopsis *opr3* mutant is male sterile (Sanders *et al.*, 2000), and the maize *opr7 opr8* double mutant is defective in sex determination and defense (Yan *et al.*, 2012). Moreover, maize Silkless 1 (SK1), a peroxisomal UDP-glycosyltransferase that suppresses JA accumulation, is also involved in sex determination (Hayward *et al.*, 2016).

The first enzyme in β-oxidation, Acyl-CoA oxidase (ACX), is encoded by a multigene family. ACX isoforms important for wound-induced JA biosynthesis have been identified in Arabidopsis, tomato and tea plants (Cruz Castillo *et al.*, 2004; Xin *et al.*, 2019). Other proteins involved in peroxisome biogenesis or β-oxidation, such as Arabidopsis ACX5, MFP2, KAT2 and PEX6, are also important for JA biosynthesis and as a result, plant defense and reproduction (Wasternack & Strnad, 2018).

4. Photorespiration

When seedlings begin photosynthesis, photorespiration becomes the most prominent function of peroxisomes. Photorespiration salvages and converts 2-phosphoglycolate (2-PG), a toxic product of the oxygenase activity of the photosynthetic enzyme Rubisco, to 3-phosphoglycerate, which re-enters the Calvin-Benson cycle. Photorespiration spans multiple subcellular compartments, with peroxisomes at the center of this pathway. The peroxisome-localized photorespiratory enzymes include

glycolate oxidase (GOX), glutamate:glyoxylate aminotransferase (GGT), serine:glyoxylate aminotransferase (SGAT) and hydroxypyruvate reductase 1 (HPR1), while the NADH-producing enzyme peroxisomal malate dehydrogenase (pMDH) and the H_2O_2-degrading enzyme catalase (CAT) are indirectly involved. Disruption of the peroxisomal photorespiratory enzymes negatively affects growth, which can be compensated to various degrees by elevated CO_2, under which conditions Rubisco's oxygenase activity is suppressed (reviewed Timm *et al.*, 2016).

A high-throughput photometric screen of an Arabidopsis peroxisomal mutant library found photorespiratory mutants to exhibit activated cyclic electron flow (CEF) around photosystem I and accumulate higher levels of H_2O_2 under high light conditions. The authors speculated that impaired photorespiration disturbs the balance of ATP and NADPH and causes the accumulation of H_2O_2, which activates CEF to produce ATP to compensate for the imbalance of ATP and NADPH (Li *et al.*, 2018a). Using dynamic light conditions that are more mimicking the natural environment, this study was able to reveal photosynthetic deficiencies in some peroxisomal mutants, such as *gox1* (glycolate oxidase 1) and *pxn1* (peroxisomal NAD^+ transporter), which otherwise show no apparent phenotypes in constant laboratory light conditions, suggesting the importance of these peroxisomal proteins in photosynthetic performance under high/dynamic lights. A follow-up study showed that in *hpr1* mutants, 2-PG accumulation inhibits the activity of triose phosphate isomerase (TPI), an enzyme in the Calvin-Benson cycle, causing a Glc-6P-phosphate shunt and higher rates of CEF (Li *et al.*, 2019).

How photorespiratory glycolate enters the peroxisome is still unknown. In peroxisomes, glycolate is oxidized to glyoxylate by glycolate oxidase (GOX), a flavin mononucleotide (FMN)-containing enzyme belonging to a multi-member family of (L)-2-hydroxy acid oxidases ((L)-2-HAOX) in various species, including Arabidopsis, rice and maize (reviewed in Dellero *et al.*, 2016). Arabidopsis has five GOX proteins, with GOX1, GOX2 and GOX3 showing narrow substrate specificities against

glycolate and l-lactate, and HAOX1 and HAOX2 displaying broader substrate specificities (Esser *et al.*, 2014). Mutant and gene expression analyses in Arabidopsis showed that GOX1 and GOX2 function in photorespiration, whereas GOX3 is more involved in metabolizing l-lactate to sustain low levels of l-lactate in roots (Engqvist *et al.*, 2015), and *HAOX1* and *HAOX2* are highly expressed in seeds (reviewed in Dellero *et al.*, 2016). .

Interestingly, GOX and GOX homologs in tobacco, Arabidopsis and rice are also involved in pathogen-plant interaction, which is partially due to their H_2O_2-producing capabilities (reviewed in Dellero *et al.*, 2016). The γb protein of Barley Stripe Mosaic Virus (BSMV) can hijack GOX via physical interaction and reduce peroxisome ROS generation to facilitate infection (Yang *et al.*, 2018). The P8 protein of rice dwarf phytoreovirus (RDV) also interacts with rice GOX (Zhou *et al.*, 2007). These data suggest that GOX may be a preferred target for viral pathogens and hence potentially useful for genetic engineering to improve plant virus resistance.

5. ROS & RNS metabolism

Peroxisomes contain ROS and reactive nitrogen species (RNS) metabolism that generates hydrogen peroxide (H_2O_2) and nitrite oxide (NO) respectively (Corpas *et al.*, 2019b). H_2O_2 can be generated by many peroxisomal metabolic processes, including photorespiration, β-oxidation, superoxide dismutation, sulfite oxidation, polyamine catabolism and others. Peroxisomes are also armed with a set of potent H_2O_2 scavengers, including catalases (CAT) in the matrix and ascorbate peroxidase (APX) on the membrane. In normal conditions, peroxisomal ROS level is adequately controlled. However, stresses like heavy metal cadmium (Cd) and xenobiotic 2,4-D, and specific developmental stages like leaf senescence, can disrupt peroxisomal ROS homeostasis (Del Río & López-Huertas, 2016). Peroxisomal morphology can change in response to oxidative stress, and H_2O_2 generated from peroxisomal photorespiration can affect the expression of nuclear genes in responses to pathogen and light changes (reviewed in Sandalio & Romero-Puertas, 2015). Interestingly,

563 peroxisome-derived H_2O_2 seems to show spatial signaling specificity, as it causes
564 transcriptional responses that are different from those induced by chloroplast-derived
565 H_2O_2 (Sewelam *et al.*, 2014).
566
567 Superoxide radical and singlet oxygen can also occur in peroxisomes. The presence of
568 superoxide dismutase (SOD) activity was confirmed biochemically in various species
569 (reviewed in Corpas *et al.*, 2017), and a Cu–Zn superoxide dismutase (CSD3) was
570 identified in multiple Arabidopsis peroxisome proteomic studies (reviewed in Pan &
571 Hu, 2018a). Peroxisomes also generate RNS under stress conditions such as excess
572 Cd, and probably reactive sulfur species (RSS) as well (Corpas & Barroso, 2014;
573 Corpas *et al.*, 2019a). Proteome analysis revealed numerous peroxisomal proteins to
574 be S-nitrosylated or nitrated, implying a role of RNS in peroxisomal function
575 (Sandalio & Romero-Puertas, 2015).
576
577 As the major H_2O_2 scavenger in the peroxisome, CAT plays a role in autophagy and
578 programmed cell death (PCD) via modulating ROS levels (Hackenberg *et al.*, 2013;
579 Zhou *et al.*, 2014). Arabidopsis CAT deficient lines lack pathogen-induced
580 autophagic PCD but contains normal basal and starvation-induced autophagy,
581 suggesting that CAT plays a specific role in activating pathogen-induced autophagy
582 and autophagic PCD (Hackenberg *et al.*, 2013; Tyutereva *et al.*, 2018). Arabidopsis
583 *cat2* mutant displays impaired growth and disturbed redox state, as well as leaf
584 necrotic lesions under long days, which can be rescued by elevated CO_2 or reduced
585 peroxisomal H_2O_2 generation, suggesting that CAT2 is the main catalase to degrade
586 photorespiration-derived H_2O_2 in peroxisomes (Mhamdi *et al.*, 2010; Kerchev *et al.*,
587 2016; Waszczak *et al.*, 2016). Analysis of Arabidopsis double mutants between *CAT2*
588 and genes that encode transcription factors, cell death regulators or proteins involved
589 in hormone functions discovered critical roles for some of these proteins in executing
590 cell death in *cat2*, indicating the important roles of stress hormones and other defense
591 regulators in peroxisomal H_2O_2-mediated cell death (Kaurilind *et al.*, 2015).
592 Simultaneous mutations of all three catalases in Arabidopsis resulted in severe redox

disturbance, growth defects and transcriptional changes, suggesting a role of peroxisomal H_2O_2 in retrograde signaling (Su *et al.*, 2018).

CAT appears to be a key target for various regulatory mechanisms. In Arabidopsis, *CAT1* and *CAT2* gene expression can be regulated by physiological signals and transcription factors, such as by ABI5 in seed germination and GBF1 (G-box binding factor 1) in leaf senescence and pathogen defense (Smykowski *et al.*, 2010; Bi *et al.*, 2017; Giri *et al.*, 2017). The expression of rice *OsCATA*, *OsCATB*, and *OsCATC* is induced by different abiotic stresses through cis-elements in their promoters (Vighi *et al.*, 2016). Interactions between Arabidopsis CATs and the zinc finger protein Lesion simulating disease 1 (LSD1), RING-finger protein No catalase activity 1 (NCA1), small heat shock protein Hsp17.6CII and Calcium-dependent protein kinase 8 (CPK8), are involved in plant resistance to various abiotic stresses (Li *et al.*, 2013, 2015, 2017; Zou *et al.*, 2015). Mutants of *NCA1* display strongly reduced activities of all three Arabidopsis CATs and absence of autophagy-dependent cell death (Hackenberg *et al.*, 2013). Among these CAT-interacting proteins, only Hsp17.6CII localizes to peroxisomes (Li *et al.*, 2017), suggesting that CATs may also be present at other subcellular locations in plant cells.

Peroxisomal membrane-associated APX proteins have a higher affinity to H_2O_2 than CATs and proposed function of preventing H_2O_2 from leaking out of the peroxisome (Kaur *et al.*, 2009). Arabidopsis APX3 and APX5 are both localized to peroxisomes (Pan *et al.*, 2018a). Peroxisomal APX level can be induced by stress, and overexpression of peroxisomal APXs confers stronger resistance to abiotic stresses in various plant species (reviewed in Anjum *et al.*, 2016). Knockdown of a peroxisomal APX in rice *cat* ameliorates the oxidative stress caused by photorespiration-derived H_2O_2, suggesting that APX and CAT are not redundantly involved in photorespiration and removal of APX may trigger a mechanism to compensate for the lack of CAT (Sousa *et al.*, 2015). The functional relation between these two major H_2O_2 scavengers in the peroxisome remains a key question for future investigation.

623

624 **6. NADPH regeneration**

625 NADPH is a critical cofactor required by several reductive biosynthetic and
626 detoxification pathways in peroxisomes. There are several sources of NADPH in the
627 peroxisome, including peroxisomal NADP-dependent isocitrate de-hydrogenase
628 (pICDH) and the oxidative pentose phosphate pathway (OPPP), which in *Arabidopsis*
629 is catalyzed sequentially by glucose-6-phosphate dehydrogenase 1 (G6PD1),
630 6-phosphogluconolactonase 3 (PGL3) and 6-phosphogluconate dehydrogenase 2
631 (PGD2) (Pan & Hu, 2018a). Arabidopsis pICDH, a NADPH-generating
632 dehydrogenase, is required for stomatal movement, and the stomatal opening defects
633 of *picdh* can be reversed by scavengers of H_2O_2 or NO, suggesting that
634 pICDH-derived NADPH may be involved in the homeostasis/signaling of
635 peroxisome-derived ROS and RNS in stomata (Leterrier *et al.*, 2016). Defects in
636 PGD2 impair the guided growth of pollen tube and male-female gametophytic
637 interaction in Arabidopsis, suggesting a role of the OPPP-generated NADPH in
638 fertilization (Hölscher *et al.*, 2016). Other peroxisomal enzymes, such as NADH
639 kinase 3 (NADK3) and possibly betaine aldehyde dehydrogenase (BADH), are also
640 postulated to produce NADPH (reviewed in Pan & Hu, 2018a).

641

642 **7. Biosynthesis of phylloquinone, biotin, CoA and ubiquinone**

643 Plant peroxisomes have an amazing metabolic diversity and intricate metabolic
644 connections with other organelles. The biosynthesis of several crucial cofactors, such
645 as phylloquinone (or vitamin K1), biotin (or vitamin B7), coenzyme A (CoA) and
646 ubiquinone (coenzyme Q), is also achieved by peroxisomes in concert with other
647 organelles (Figure 6).

648

649 Phylloquinone is an important cofactor for photosystem I as wells as a key vitamin for
650 humans. The biosynthesis of phylloquinone initiates in the plastid, goes through
651 intermediate steps in the peroxisome and finalizes in the plastid (Figure 6). Within
652 this pathway, 1,4-dihydroxy-2-naphthoyl-CoA (DHNA-CoA) thioesterases (DHNAT)

and naphthoate synthase (NS) localize in peroxisomes, while *o*-succinylbenzoyl-CoA (OSB-CoA) ligase (named AAE14 in Arabidopsis) is dual localized to chloroplasts and peroxisomes (reviewed in Basset *et al.*, 2017). The peroxisomal transporter of phylloquinone biosynthetic intermediates has not been identified, and PXA1 has been excluded (Basset *et al.*, 2017).

Biotin is a cofactor required in numerous carboxylation and decarboxylation reactions. In plants, the first committed step of biotin synthesis (catalyzed by Biotin F) is peroxisomal, whereas the later steps are mitochondrial (Figure 6). Pimeloyl-CoA, the precursor for biotin synthesis, is also speculated to be generated in peroxisomes (Figure 6) (Tanabe *et al.*, 2011). Dephospho-CoA kinase (CoAE), the enzyme for the last step of CoA synthesis, was found in the peroxisomal proteome in Arabidopsis leaves (Reumann *et al.*, 2009).

Peroxisomes are also involved in the biosynthesis of the benzenoid ring of ubiquinone, a prenylated benzoquinone acting as a vital respiratory cofactor in mitochondria. A member of the 4-coumarate-CoA ligase-like (4CL) family of the acyl-activating enzyme (AAE) super family, 4-Coumarate:CoA ligase 5 (4CL5), is involved in the phenylalanine-related biosynthetic pathway of ubiquinone in Arabidopsis by activating the propyl side chain of *p*-coumaric acid for subsequent β-oxidative reactions, and PXA1 is the likely transporter of *p*-coumaric acid (Figure 6) (Block *et al.*, 2014).

8. The mevalonic acid (MVA) pathway

Peroxisomes contain several enzymes involved in the mevalonic acid (MVA) pathway, one of the two major routes in generating precursors for the biosynthesis of isoprenoids (the other being the plastidic methylerythritol phosphate (MEP) pathway) (McGarvey & Croteau, 1995). A splicing isoform of Arabidopsis acetoacetyl-CoA thiolase 1 (AACT1.3), the enzyme catalyzing the first step of this pathway, is located in the peroxisome, whereas other AACT1 isoforms and AACT2 are cytosolic (Carrie

683 *et al.*, 2007) (Figure 7). The next three steps are catalyzed sequentially by the ER
684 associated hydroxymethylglutaryl-CoA synthase (HMGS) and two cytosolic enzymes,
685 hydroxymethylglutaryl-CoA reductase (HMGR) and mevalonate kinase (MVK),
686 generating 5-phosphomevalonate (MVP) (Simkin *et al.*, 2011) (Figure 7). The last
687 three steps are also peroxisomal, where MVP is converted to dimethylallyl
688 diphosphate (DMAPP) by the action of 5-phosphomevalonate kinase (PMK),
689 mevalonate 5-diphosphate decarboxylase (MVD) and isopentenyl diphosphate
690 isomerase (IDI), respectively (Simkin *et al.*, 2011; Pulido *et al.*, 2012) (Figure 7).
691 Male sterility of the Arabidopsis *ipi1 ipi2* double mutant can be rescued by
692 application of squalene, a sterol precursor, suggesting a role of the MVA pathway in
693 plant reproduction (Okada *et al.*, 2008).
694

695 **9. Catabolism of polyamines, urate, pseudouridine, sulfite and methylglyoxyal**
696 The metabolic diversity of plant peroxisomes is also exemplified by the degradation
697 of many bioactive or toxic metabolites, such as polyamines (PA), urate, pseudouridine,
698 sulfite and methylglyoxyal.
699

700 PAs, including the diamine putrescine (Put), triamine spermidine (Spd) and tetramine
701 spermine (Spm), are regulatory molecules in plant development and stress response
702 (Alcázar *et al.*, 2006). PAs can be oxidatively deaminated in peroxisomes by
703 flavin-containing polyamine oxidases (PAOs) and copper-containing amine oxidases
704 (CuAOs) (Figure 8) (Kusano *et al.*, 2015). PAO and CuAO are both encoded by
705 multi-gene families that each contain peroxisomal members. In Arabidopsis, PAO2,
706 PAO3 and PAO4 catalyze the back-conversion of spermine to putrescine, and CuAO2
707 and CuAO3 catalyze the terminal oxidation of putrescine to 4-aminobutanal (ABAL)
708 (reviewed in Kusano *et al.*, 2015; Pan & Hu, 2018a) (Figure 8). The final step of PA
709 catabolism is catalyzed by BADH, which is also named aldehyde dehydrogenase
710 10A9 (ALDH10A9), converting ABAL to 4-aminobutyrate (GABA) (Zarei *et al.*,
711 2016) (Figure 8). CuAO3 is involved in ABA-induced ROS generation and stomatal
712 closure (Qu *et al.*, 2014), and auxin signaling and IBA-dependent lateral root

development via H_2O_2 production (Qu *et al.*, 2017).

Plants fully catabolize purine nucleotides to remobilize nitrogen resources. The enzymatic route of purine ring catabolism spans across several subcellular compartments. The three peroxisomal steps are catalyzed sequentially by urate oxidase (UOX or uricase) and the dual-functional allantoin synthase (ALNS) (Werner & Witte, 2011) (Figure 8). Accumulation of uric acid in Arabidopsis *uox* mutant compromises peroxisome maintenance and seedling establishment, establishing a link between uric acid toxicity and peroxisomal fatty acid catabolism (Hauck *et al.*, 2014).

Pseudouridine is structurally similar to uridine and represents the most abundant non-classical nucleoside in RNA (Charette & Gray, 2000). Arabidopsis peroxisomes contain PfkB and IndA, both of which presumably catalyze pseudouridine degradation (Pan & Hu, 2018a). Sulfite is a toxic by-product of sulfur assimilation that can be oxidized to sulfate by the peroxisomal sulfite oxidase (SO) (Nowak *et al.*, 2004) (Figure 8). Methylglyoxal, a toxic by-product of glycolysis, can be removed by the sequential action of glyoxalase I (GLX1) and GLX2, among which GLX1 was found to be peroxisomal in Arabidopsis (Quan *et al.*, 2010) but GLX2 has not been identified in peroxisomes.

10. Amino acid metabolism

Plant peroxisomes contain proteins involved in amino acid biosynthesis and degradation. Peroxisomes isolated from mungbean hypocotyls exhibit the capability to degrade various branched-chain amino acids (BCAAs), including valine, leucine and isoleucine (Gerbling & Gerhardt, 1989). Arabidopsis peroxisomal 3-hydroxyisobutyryl (HIBYL)-CoA hydrolase 1 (CHY1), and probably its homologs CHY1H1 and CHY1H2 as well, can catabolize valine and possibly other BCAAs (Zolman *et al.*, 2001; Lingner *et al.*, 2011). Although BCAA synthesis is known to occur in chloroplasts, ALS-interacting protein 1 (AIP1) and AIP3 are dual localized to peroxisomes and chloroplasts and interact with acetolactate synthase (ALS), the first

743 enzyme in BCAA synthesis, indicating a possible role of peroxisomes in BCAA
744 synthesis besides degradation (Dezfulian *et al.*, 2017).
745
746 Based on known activity of its bacterial and mammalian homologs, Arabidopsis
747 sarcosine oxidase (SOX) was speculated to oxidize secondary or tertiary amino acids
748 (Goyer *et al.*, 2004). Tomato OAS9, an O-acetylserine(thiol)lyase (OASTL)-like
749 protein, was hypothesized to play a role in cysteine biosynthesis and leaf senescence
750 (Liu *et al.*, 2018). Arabidopsis aspartate aminotransferase isoform 3 (ASP3) and
751 cobalamin-independent met synthase 1 (ATMS1) are also potential players in amino
752 acid metabolism (reviewed in Pan & Hu, 2018a).
753
754 **VII. Peroxisomal solute transporters**
755 A number of metabolic pathways span across peroxisomes and other subcellular
756 compartments; therefore, metabolites and cofactors must move across the peroxisomal
757 membrane, and at least some of these movements should rely on membrane
758 transporters. To date, several peroxisomal membrane proteins have been identified to
759 specifically transport β-oxidation substrates - i.e. fatty acids, OPDA, CA and IBA,
760 and large cofactors - i.e. ATP, NAD^+ and CoA (Charton *et al.*, 2019).
761
762 The Arabidopsis full-size ABC transporter PXA1/CTS/PED3 can cleave acyl-CoA
763 and import free FAs into the peroxisomal matrix. PXA1 also interacts with long-chain
764 acyl-CoA synthetases LACS6 and LACS7, which reactivate free FA to acyl-CoA to
765 feed into β-oxidation (De Marcos Lousa *et al.*, 2013). In addition, PXA1 transports
766 precursors for the biosynthesis of IAA, JA, BA and the benzenoid moiety of
767 ubiquinone (Figures 5 & 6) (Block *et al.*, 2014; Li *et al.*, 2016). PXA1 is also
768 important in acetate metabolism, as a loss-of-function *PXA1* mutant named *acn2*
769 (*acetate non-utilizing 2*) is compromised in metabolizing acetate in seedlings (Hooks
770 *et al.*, 2007). PXA1 interacts with CGI-58, a regulator of lipid metabolism and
771 signaling (Park *et al.*, 2013). Consistent with its broad function, *PXA1* knockout
772 mutants have defects in lateral root development, seed dormancy and germination,

773 fertilization and leaf necrosis (Li *et al.*, 2016).

774

775 Besides transporters, Acyl-CoA-binding proteins (ACBPs) can also mediate lipid
776 transfer across membranes (Xiao & Chye, 2011). Arabidopsis ACBPs have not been
777 found in the peroxisome, but rice ACBP6 is peroxisomal and its overexpression
778 partially recovered the β-oxidation defects of *pxa1*, suggesting that rice ACBP6 can
779 contribute to the import of FA into peroxisomes for degradation (Meng *et al.*, 2014).
780 Whether this divergence in ACBP function between Arabidopsis and rice is due to
781 different metabolic features of monocot and dicot species remains to be determined.

782

783 Arabidopsis PNC (PNC1 and PNC2) and PXN proteins are peroxisomal ATP and
784 NAD^+ carriers, respectively. PNC1 and PNC2 can catalyze the counter-exchange of
785 ATP with ADP or AMP and complement yeast mutants deficient in peroxisomal ATP
786 import. The *pnc* mutants are impaired in β–oxidation, suggesting that the proteins are
787 essential for supplying peroxisomes with ATP (Linka *et al.,* 2008). PXN can transport
788 many substrates *in vitro*, including NAD^+, NADH, AMP, ADP, CoA and acetyl-CoA
789 (Bernhardt *et al.*, 2012; Agrimi *et al.*, 2012), and contributes to optimal fatty acid
790 degradation during seedling establishment, and photorespiration under fluctuating and
791 high light conditions (Bernhardt *et al.*, 2012; Li *et al.*, 2018a). However, exogenously
792 expressed AtPXN in yeast strains can import NAD^+ into peroxisomes in exchange of
793 AMP but cannot transport CoA or mediate NAD^+/NADH exchange (van Roermund *et*
794 *al.*, 2016).

795

796 Non-selective peroxisomal membrane channels may allow the passage of small
797 solutes, such as organic acids. Yeast PEX11 has pore-forming function, but whether
798 plant PEX11s also possess this function has not been reported (Mindthoff *et al.*, 2016).
799 Several additional peroxisomal membrane proteins, such as PMP22 (peroxisomal
800 membrane protein of 22 kDa), CDC (Ca^{2+}-dependent carrier) and SMP2 (short
801 membrane protein 2), also have potential pore-forming activities (summarized in Pan
802 & Hu, 2018a).

803

804 **VIII. Signaling events that involve Ca^{2+} and protein phosphorylation**

805 Peroxisomes house many potential signaling elements, such as Ca^{2+}, protein kinases
806 and phosphatases, which may contribute to the regulation of metabolic functions of
807 peroxisomes by physiological and environmental cues.

808

809 Ca^{2+} is one of the most prominent second messengers in the cell. In plant peroxisomes,
810 Ca^{2+} and calmodulin (CaM) are important for protein import and function of
811 peroxisomal enzymes involved in detoxification, photorespiration and NO production
812 (Corpas & Barroso, 2018). Arabidopsis calcium-dependent protein kinase 1 (CPK1) is
813 involved in SA signaling and pathogen resistance (Coca & San Segundo, 2010), and
814 the calmodulin-like protein CML3 is involved in DEG15 dimerization and
815 peroxisomal protein import (Dolze *et al.*, 2013). In *Petunia inflata,* peroxisomal
816 Ca^{2+}-dependent protein kinase 2 (CDPK2) and small CDPK-interacting protein 1
817 (SCP1) regulate pollen tube growth (Guo *et al.*, 2013). Cotton peroxisomal CPK33
818 negatively regulates plant resistance to verticillium wilt, a destructive fungal disease,
819 as CPK33-mediated GhOPR3 phosphorylation destabilizes GhOPR3 and reduces JA
820 biosynthesis (Hu *et al.*, 2018). Other peroxisomal protein kinases such as
821 glyoxysomal protein kinase 1 (GPK1), receptor-like protein kinase 1 (RPK1) and PPK
822 (a Protein kinase superfamily protein/Peroxisomal protein kinase), are still unknown
823 in function.

824

825 Arabidopsis peroxisomes also contain protein phosphatase 2A B'h subunit
826 (PP2A-B'h), PP2A-C5, PP2A-A2, PP2A-C2, POL like phosphatase 2 (PLL2), PLL3,
827 purple acid phosphatase 7 (PAP7), PAP5, and MAP kinase phosphatases 1 (MKP1)
828 (Kataya *et al.*, 2019). PP2A is involved in β-oxidation (Kataya *et al.*, 2015a), whereas
829 MKP1, which targets to peroxisomes in a stress dependent manner (Kataya *et al.*,
830 2015b), negatively regulates the production of ROS and SA in stress response (Bartels
831 *et al.*, 2009; Anderson *et al.*, 2011). Maintaining the optimal level of PAP5, which
832 acts upstream of SA accumulation, is necessary for the complete basal resistance to

Pseudomonas syringae (Ravichandran *et al.*, 2013, 2015).

Taken together, protein phosphorylation/dephosphorylation is apparently an important regulatory mechanism of peroxisome proteins and probably widely involved in peroxisome function, which will need to be further elucidated through in-depth and systematic studies.

IX. Conclusions

In the last decade, many milestones were reached in plant peroxisomal research. Hundreds of proteins and numerous metabolic pathways have been identified in this essential organelle, which is highly versatile and contains many functions unique to plants. Peroxisomes are highly dynamic in number, appearance and protein content in a regulated manner, and function collaboratively with other organelles in various metabolic pathways.

Despite these significant advances, there are still many knowledge gaps. We have very limited knowledge about the transcriptional and post-translational mechanisms that link peroxisome dynamics and metabolism to developmental and environmental cues, and the degradation of peroxisomal proteins. Key peroxisomal proteins in pexophagy and interaction with other organelles remain to be determined. Developmental defects caused by the disruption of peroxisomal proteins, such as defects in germination and male-female gametophyte interaction for some β-oxidation mutants, have yet been clearly explained at the molecular level. The presence and physiological roles of novel RNS and RSS await elucidation, and our understanding of the functional relations between different types of antioxidant enzymes needs to be further investigated. Finally, peroxisomes in monocotyledon species, especially cereal crops, are poorly studied. Given the significant differences in development and metabolism between monocots and dicots, peroxisomes very likely perform monocot-specific functions that could be applicable to agriculture to improve crop performance and vigor.

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Author contributions

RP and JH jointly conceptualized this review and co-wrote the manuscript. JL and SW contributed to figure generation. All authors read and approved of the manuscript content.

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Figure Legends

Figure 1. A model for peroxisomal protein import in Arabidopsis.

PEX19 is a chaperone for peroxisomal membrane proteins (PMPs), PEX3 is the membrane anchor for PEX19, and PEX16 is involved in recruiting PEX3 to the membrane. Matrix proteins containing PTS1 and PTS2 are recognized by the receptors PEX5 and PEX7, respectively. PEX13 and PEX14 form the docking complex for the receptor-cargo and allow cargo import. After cargo release, PEX5 is presumably ubiquitinated and recycled, facilitated by the RING proteins PEX2, PEX10 and PEX12 and the ubiquitin conjugating enzyme PEX4. Ubiquitinated PEX5 is removed from the peroxisome by the PEX1-PEX6 AAA ATPase complex. PEX22 is the membrane anchor for PEX4, and PEX26 is the membrane anchor for PEX1 and PEX6. The RING-type E3 ubiquitin ligase SP1 negatively regulates matrix protein import by destabilizing PEX13 and probably other peroxins, while SPL1 and SP1 destabilize each other.

Figure 2. A model for peroxisomal elongation and fission in Arabidopsis.

PEX11a through PEX11e are involved in peroxisomal elongation. PEX11b is transcriptionally activated by HY5 homolog (HYH) in response to light and through phytochrome A (phyA), and repressed by Forkhead-associated domain protein 3 (FHA3). Fission is mediated mainly by dynamin-related proteins DRP3A and DRP3B, and their probable membrane anchors Fission 1A (FIS1A) and FIS1B. Peroxisomal and mitochondrial division 1 (PMD1) and DRP5B are two plant-specific peroxisome fission factors. PMD1 binds to actin and functions genetically downstream of MAP kinase 17 (MPK17), a putative regulator of salt-induced peroxisome proliferation.

Figure 3. A model for peroxisome quality control and proteome remodeling in plants.

DEG15 cleaves PTS2 peptide from PTS2-containing matrix proteins in its dimeric form, and functions as a general protease in its monomeric form. LON2 mediates

protein degradation during peroxisome remodeling and is involved in sustained protein import and pexophagy. RDL1, PXM16 and SCPL20 are putative proteases with undetermined functions. ROS can be eliminated by CAT, but ROS burst can also damage CAT. ROS and CAT deficiency are linked to pexophagy. In pexophagy, ATG8 is the key protein to recruit the autophagic machinery to the peroxisome, and has been found to physically interact with PXA1 and PEX10. During cadmium-induced pexophagy, ATG8 co-localizes with catalase and NBR1 in the electron dense peroxisomal core. Peroxisome-specific receptor for the phagophore has not been determined.

Figure 4. A model for fatty acid degradation in Arabidopsis.
Seed storage oil is hydrolyzed by the lipase SUGAR-DEPENDENT (SDP) into fatty acids (FAs). FA or FA-CoA is imported by PXA1 into peroxisomes, activated by long-chain acyl-CoA synthetase (LACS) and degraded by the β-oxidation pathway that consists of three enzymes: acyl-CoA oxidase (ACX), multifunctional protein (MFP) and 3-ketoacyl-CoA thiolase (KAT). Each β-oxidation cycle generates one molecule of acetyl-CoA, which is utilized by the glyoxylate cycle that consists of peroxisomal enzymes isocitrate lyase (ICL), malate synthase (MLS) and citrate synthase (CSY), and presumably non-peroxisomal enzymes malate dehydrogenase (MDH) and aconitase (ACO). Succinate, isocitrate and citrate produced can enter the mitochondrial TCA cycle for cellular energy consumption. ACN1 activates acetate to produce acetyl-CoA, which can be converted into malate by MLS, thus forming an acetate-malate shunt.

Figure 5. Peroxisomal hormone biosynthesis in Arabidopsis. Precursors like 12-oxo-phytodien oic acid (OPDA), dinor-OPDA (dnOPDA), cinnamic acid (CA) and indole-3-butyric acid (IBA) are imported into the peroxisome by PXA1. Before entering the β-oxidation cycle, OPDA and dnOPDA are reduced and activated, while CA and IBA are simply activated into their CoA esters. Benzoic acid (BA) synthesis lacks the dehydrogenation step of β-oxidation. CoA esters produced by β-oxidation

1522 are hydrolyzed to form free jasmonic acid (JA), BA and indole-3-acetic acid (IAA).
1523 OPC6-CoA and OPC8-CoA respectively undergo two and three rounds of
1524 β-oxidation.
1525
1526 **Figure 6.** Biosynthesis of phylloquinone, biotin and ubiquinone in Arabidopsis.
1527 Phylloquinone biosynthesis begins in plastids, producing o-succinylbenzoate (OSB)
1528 that is subsequently activated to OSB-CoA by AAE14, an enzyme dual targeted to
1529 plastids and peroxisomes. OSB and/or OSB-CoA enter the peroxisome. OSB-CoA
1530 undergoes (1) ring cyclization by naphthoate synthase (NS) to form
1531 1,4-dihydroxy-2-naphthoyl-CoA (DHNA-CoA), and (2) DHNA-CoA hydrolysis by
1532 the DHNA-CoA thioesterases (DHNAT1 and DHNAT2) to form DHNA. DHNA is
1533 transported back to the plastids to complete phylloquinone biosynthesis. In biotin
1534 biosynthesis, pimeloyl-CoA is speculated to form in peroxisomes. Biotin F catalyzes
1535 the first committed step of biotin synthesis to convert pimeloyl-CoA to
1536 7-keto-8-aminopelargonic acid (KAPA), which is then transported to mitochondria for
1537 the final steps. In ubiquinone biosynthesis, *p*-coumaric acid is imported into
1538 peroxisomes by PXA1, after which 4-coumarate:CoA ligase 5 (4CL5) activates the
1539 propyl side chain of *p*-coumaric acid for the subsequent chain-shortening by
1540 β-oxidative to form 4-hydroxybenzoic acid (4-HB), which is then transported into
1541 mitochondria for the final steps.
1542
1543 **Figure 7.** The mevalonic acid (MVA) pathway.
1544 The first step is catalyzed by acetoacetyl-CoA thiolase (AACT) to generate
1545 acetoacetyl-CoA. In Arabidopsis, a splicing isoform of acetoacetyl-CoA thiolase 1
1546 (AACT1.3) is peroxisomal, while other AACT1 isoforms and AACT2 are cytosolic.
1547 The subsequent steps are catalyzed by the ER hydroxymethylglutaryl-CoA synthase
1548 (HMGS) to generate 3-hydroxy-3-methylglutaryl CoA (HMG-CoA), and two
1549 cytosolic proteins hydroxymethylglutaryl-CoA reductase (HMGR) and mevalonate
1550 kinase (MVK) to generate MVA and 5-phosphomevalonate (MVP), respectively.
1551 MVP is transported to peroxisomes for the final steps of the MVA pathway, in which

5-phosphomevalonate kinase (PMK) generates 5-diphosphomevalonate (MVPP), mevalonate 5-diphosphate decarboxylase (MVD) generates isopentenyl diphosphate (IPP), and isopentenyl diphosphate isomerase (IDI) reversibly isomerizes IPP to form dimethylallyl diphosphate (DMAPP). IPP and DMAPP can then be used for the biosynthesis of isoprenoids, including the synthesis of sterols in the ER via farnesyl diphosphate (FPP). G3P, glycerol-3-phosphate.

Figure 8. Catabolism of polyamines, urate and sulfite in Arabidopsis.
Polyamines, including spermine, spermidine and putrescine, are oxidatively deaminated in peroxisomes by flavin-containing polyamine oxidases (PAOs) and copper-containing amine oxidases (CuAOs), generating aminobutanal (ABAL). Betaine aldehyde dehydrogenase (BADH), also named aldehyde dehydrogenase 10A9 (ALDH10A9), catalyzes the conversion of ABAL to 4-aminobutyrate (GABA). Urate is generated in purine catabolism and transported to peroxisomes, in which it is catalyzed by urate oxidase (UOX) to form 5-hydroxyisourate (HIU) and then by the dual-functional enzyme allantoin synthase (ALNS) to form 2-oxo-4-hy-droxy-4-carboxy-5-ureidoimidazoline (OHCU) and subsequently *S*-allantoin. *S*-allantoin is transported to the ER to be converted to the end product glyoxylate. Sulfite is a toxic by-product of sulfur assimilation in plastids and can enter peroxisomes to be oxidized to sulfate by the peroxisomal sulfite oxidase (SO).