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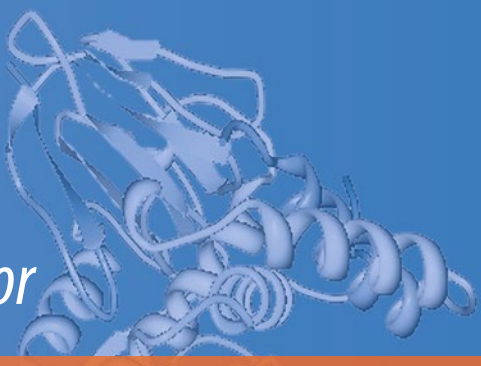

Luis A. del Río *Editor*

Peroxisomes and their Key Role in Cellular Signaling and Metabolism

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Luis A. del Río
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Peroxisomes and their Key Role in Cellular Signaling and Metabolism

Springer

Editor
Luis A. del Río
Depto. Bioquímica, Biología Celular y
Molecular de Plantas
Estación Experimental del Zaidín
CSIC
Profesor Albareda 1
Granada
Spain

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This book is dedicated to Prof. Christian de Duve who first isolated and characterized peroxisomes from mammalian tissues in the early 1960s and coined the name of these organelles, motivating much of the actual research activity in this field, and also to the memory of Prof. Harry Beevers who discovered the β-oxidation system in plant peroxisomes (glyoxysomes) in 1969, 7 years before that in animal peroxisomes, paving the way to our actual understanding of the metabolism and function of peroxisomes.

Preface

Peroxisomes are a class of ubiquitous and dynamic single membrane-bounded cell organelles, devoid of DNA, with an essentially oxidative type of metabolism. When these organelles were first isolated and characterized from mammalian tissues in the beginning of the 1960s by Christian de Duve, it was thought that their main function was the removal by catalase of toxic hydrogen peroxide generated in the peroxisomal respiratory pathway by different oxidases (De Duve and Baudhuin 1966). However, in recent years it has become increasingly clear that peroxisomes are involved in a range of important cellular functions in almost all eukaryotic cells (Wanders and Waterham 2006; Waterham and Wanders 2012; Islinger et al. 2010, 2012; Terlecky and Titorenko 2009; Baker and Graham 2002; Hu et al. 2012).

Today, it is known that fatty acid β-oxidation is a general feature of virtually all types of peroxisomes, but in higher eukaryotes, including humans, peroxisomes catalyze ether phospholipids biosynthesis, fatty acid α-oxidation, and glyoxylate detoxification, and in humans, peroxisomes are associated with several important genetic diseases (Wanders and Waterham 2006; Kunau 2006; Waterham and Wanders 2012). Among the different new roles for human peroxisomes discovered in recent years are antiviral innate immunity (Dixit et al. 2010), peptide hormone metabolism (Höftberger et al., 2010), brain aging and Alzheimer's disease (Kou et al. 2011), and age-related diseases (Fransen et al. 2012). In fungi, new findings have broadened the number of secondary metabolites that are synthesized in peroxisomes, such as antibiotics and several toxins, and have evidenced their involvement in biotin biosynthesis, fungal development, and plant pathogenesis (Kunau 2006; Terlecky and Titorenko 2009; Islinger et al. 2012; Bartoszewska et al. 2011).

In plants, peroxisomes carry out different functions, apart from fatty acid β-oxidation (Baker et al. 2006), mainly including: photorespiration; metabolism of reactive oxygen, nitrogen, and sulfur species (ROS, RNS, and RSS, respectively); photomorphogenesis; biosynthesis of phytohormones; senescence; and defense against pathogens and hervibores (Corpas et al. 2001; Baker and Graham 2002; del Río et al. 2006; Nyathi and Baker 2006; Corpas et al. 2009; del Río 2011; Hu et al. 2012). Different studies of peroxisome biogenesis have allowed to

obtain considerable information on the peroxisomal targeting signals (PTSs) and receptors responsible for the import of peroxisomal matrix and membrane proteins (Islinger et al. 2010; Hu et al. 2012). *In silico* predictions and proteome analysis of peroxisomes have confirmed the presence of many proteins which had been previously described in peroxisomes by cell biology and biochemical methods, and have also identified new proteins, so increasing the knowledge of peroxisome functions (Palma et al. 2009; Islinger et al. 2010; Hu et al. 2012).

Two important characteristics of peroxisomes are their metabolic plasticity and their capacity of sharing metabolic pathways with other cell compartments. This latter property was proposed in the 1980s by Nathan Edward Tolbert in peroxisomes from plant leaves as a result of the important pool of new metabolites found in peroxisomes, and he demonstrated that leaf peroxisomes carry out the photorespiration process in conjunction with chloroplasts and mitochondria (Tolbert et al. 1987).

However, to my knowledge, the first report postulating an overall function of the peroxisome in the cellular-signaling apparatus was made in 1996 by Colin J. Masters in mammalian organisms (Masters 1996). He postulated that peroxisomes were "capable of imprinting a characteristic influence on the regulatory network in the cell" and indicated that the importance of the peroxisomal contribution to the cellular-signaling systems should be recognized. In cellular-signaling studies, it is very important to know the cellular loci where the different secondary messengers are produced. In this respect, the diverse key physiological functions that have been demonstrated in recent years for peroxisomes from different origins (human, animal, fungal, and plant) strongly indicate the interest of studying the role of peroxisomes as a cellular source of different signaling molecules. Although in higher plants the function of peroxisomes as a source of ROS, RNS, and other signaling molecules derived from β-oxidation has been proposed in the last two decades (del Río and Donaldson 1995; del Río et al. 1996, 2002, 2006; Pastori and del Río 1997; Corpas et al. 2001; Baker et al. 2006; Nyathi and Baker 2006; del Río 2011), this important role has never been covered comprehensively in a study on peroxisomes from different origins.

In peroxisome biology, like in other fields of biological sciences, there is a tendency to parcel research and its development according to the different kingdoms of life. This makes very difficult the osmosis of experimental results among the different kingdoms that is necessary to obtain important advances in our common goal: the biochemical and molecular studies of physiological functions and signaling properties of peroxisomes. As it is stated by Prof. Sigrun Reumann in Chap. 12, "it is time to pull down the walls of kingdom-specific organelle research and to jointly explore, collaborate and enjoy this exciting era of peroxisome research." It is with this spirit of inter-kingdom collaboration, overcoming artificial differences, that this book was conceived, trying to exchange information and ideas on the biology of peroxisomes from very different origins. This adventure would not have been possible without the enthusiastic collaboration of colleagues from 12 countries and three continents whom I would like to sincerely thank for contributing their time and keen interest to this project.

In this volume, an updated view of the capacity and function of peroxisomes from human, animal, fungal, and plant origin as cell generators of different signaling molecules involved in distinct processes of high physiological importance is presented.

Feb 2013 Luis A. del Río
Granada

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Contents

Chapter 1
Peroxisome Interactions and Cross-Talk with Other Subcellular Compartments in Animal Cells

Michael Schrader, Sandra Grille, H. Dariush Fahimi, and Markus Islinger

Contents

Abstract Peroxisomes are remarkably plastic and dynamic organelles, which fulfil important functions in hydrogen peroxide and lipid metabolism rendering them essential for human health and development. Despite great advances in the identification and characterization of essential components and molecular mechanisms associated with the biogenesis and function of peroxisomes, our understanding of how peroxisomes are incorporated into metabolic pathways and

M. Schrader (✉)
College of Life and Environmental Sciences, Biosciences, University of Exeter, Geoffrey Pope Building, Stocker Road, Exeter EX4 4QD, UK

Centre for Cell Biology & Department of Biology, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
e-mail: m.schrader@exeter.ac.uk

S. Grille • M. Islinger
Centre for Cell Biology & Department of Biology, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

H.D. Fahimi
Department of Anatomy and Cell Biology, Division of Medical Cell Biology, University of Heidelberg, 69120 Heidelberg, Germany

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cellular communication networks is just beginning to emerge. Here we address the interaction of peroxisomes with other subcellular compartments including the relationship with the endoplasmic reticulum, the peroxisome-mitochondria connection and the association with lipid droplets. We highlight metabolic cooperations and potential cross-talk and summarize recent findings on peroxisome-peroxisome interactions and the interaction of peroxisomes with microtubules in mammalian cells.

Keywords Peroxisomes • Mitochondria • Endoplasmic reticulum • Lipid droplet • Cytoskeleton • Organelle interplay • Organelle cross-talk

1.1 Introduction

Peroxisomes are highly dynamic, multifunctional organelles in eukaryotic cells. They contribute to several anabolic and catabolic cellular pathways, which renders them essential for human health and development (Islinger and Schrader 2011). A remarkable feature of peroxisomes is their ability to respond to cellular and/or environmental changes and stimuli with alterations in their biogenesis, morphology, number, protein composition and metabolic activity. The metabolism of H_2O_2 and the oxidation of fatty acids are their most common functions in animals, plants and fungi. However, the spectrum of peroxisomal tasks is far from being complete, and due to extensive research in the last years, is constantly widening. Several specialized and novel peroxisomal functions have been discovered, including a new biological role in anti-viral defence (see Chap. 4), H_2O_2 signalling in hypothalamic neurons (Diano et al. 2011), the synthesis of hormonal signal molecules in plants (see Chaps. 14 and 16), Ca^{++} signalling (see Chap. 7), or the synthesis of secondary metabolites in fungi (see Chaps. 8 and 9). It is obvious that peroxisomes do not represent isolated entities within the cell, but are functionally integrated into a complex network of communicating subcellular compartments. In this respect, a physical and metabolic interaction with the endoplasmic reticulum (ER) has long been known (see Sect. 1.3; Fig. 1.1a–c), and it became obvious in the last years that the ER can also contribute to peroxisome formation, although the degree of this contribution is debated and may vary among species (see Sect. 1.3). In addition, a closer connection between peroxisomes and mitochondria was revealed (see Sect. 1.4) (Schrader and Yoon 2007; Camoes et al. 2009; Schrader et al. 2012). Despite great advances in the identification and characterization of essential proteins (e.g., peroxins) and molecular mechanisms associated with the biogenesis and function of peroxisomes, our understanding of how peroxisomes are incorporated into metabolic pathways and cellular communication networks is just beginning to emerge. Here we address the interaction of peroxisomes with other subcellular compartments and highlight metabolic cooperations and potential cross-talk. We particularly focus on peroxisome interactions in animals, but where appropriate also refer to recent discoveries in plants and fungi.

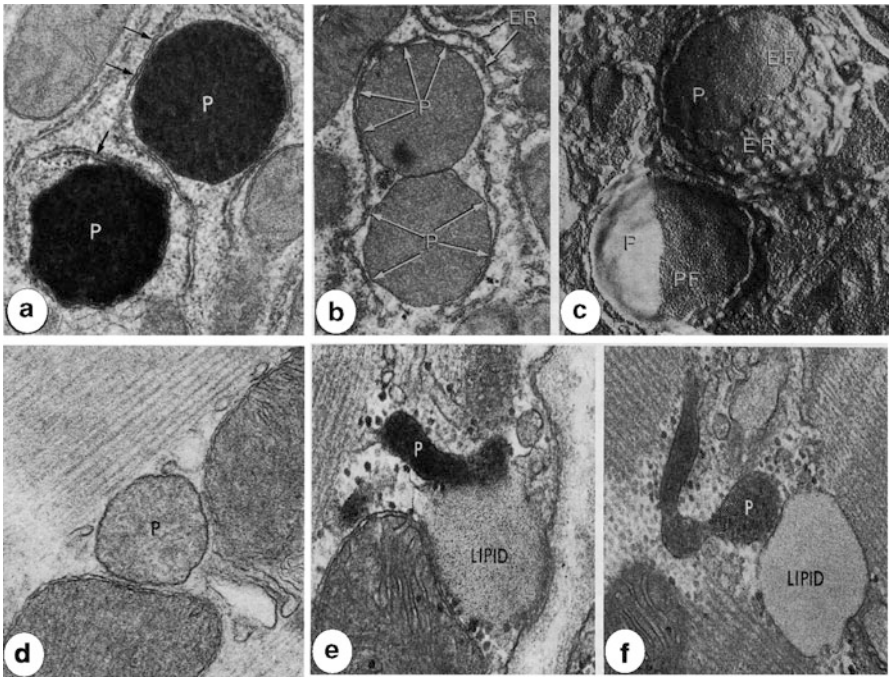

Fig. 1.1 (**a**–**b**) Physical interaction of peroxisomes (P) with the endoplasmic reticulum (ER). (**a**) Cytochemical localization of catalase in rat hepatic peroxisomes stained with the alkaline diamino-benzidine technique. Note the close association of the limiting membrane of peroxisomes with the ER (*arrows*). Magnification, ×33,600 (from Fahimi and Yokota 1981). (**b**) Electron micrograph from the liver of a rat treated with the peroxisome proliferator clofibrate for 7 days illustrating two peroxisomes with their limiting membranes touching each other, giving rise to a junction-like appearance. Note the segments of ER which surround the peroxisomes, forming close contacts with their limiting membranes (*arrows*). Magnification, ×38,000 (from Fahimi et al. 1980). (**c**) The association of ER and peroxisomes is well demonstrated in freeze-etch preparations from rat liver, revealing that peroxisomes are almost entirely engulfed by fenestrated ER. Note also the two fracture faces of the peroxisomal membrane, with the P-face (PF) containing more membrane associated particles then the E-face (EF). Magnification, ×40,000 (from Fahimi et al. 1980). (**d**) Contact of a peroxisome with two mitochondria in mouse myocardium (from Herzog and Fahimi 1976). (**e**, **f**) Intimate physical contacts of elongated peroxisomes with lipid droplets in primate (*Macaca java*) myocardium (from Hicks and Fahimi 1977). Peroxisomes were stained with the alkaline diamino-benzidine technique for catalase

1.2 Peroxisomes and the Nucleus

Unlike mitochondria, peroxisomes do not contain DNA. Thus, all peroxisomal genes are encoded in the nucleus, and their transcription and translation as well as protein import requires coordinated regulation. It is well known that the number and size of peroxisomes as well as their protein/enzyme concentration and composition can be modulated by nutritional factors and environmental stimuli. This so called

peroxisome proliferation is mediated by transcription factors which are activated by endogenous ligands such as fatty acids or synthethic peroxisome proliferators (e.g., hypolipidemic compounds). In mammals, particularly in rodents, peroxisome proliferation is mediated by PPARα, the peroxisome proliferator-activated receptor α, a member of the nuclear receptor superfamily. Upon ligand activation, PPARα forms heterodimers with the retinoid X receptor, which enables binding to PPAR responsive elements on the DNA, thereby activating peroxisomal genes involved in fatty acid β-oxidation and peroxisome proliferation. Similar signalling pathways involving transcription factors such as Oaf1, Pip2, or FarA and FarB have been evolved in yeast and fungi (reviewed in Schrader et al. 2012). Upon withdrawal of the proliferative stimulus or altered nutritional conditions, excess peroxisomes are degraded by autophagic processes (Till et al. 2012). Degradation requires an interaction of peroxisomes with autophagic membranes and/or the endosomal-lysosomal compartment, which involves certain peroxins such as Pex14 and autophagy-related proteins. A specific "eat me" signal on peroxisomes prone for degradation has been predicted, but not yet been identified.

1.3 Relationship of Peroxisomes with the Endoplasmic Reticulum

In ultrastructural studies, peroxisomes have been found in close proximity to the ER, frequently virtually wrapped in ER cisternae implying a close relationship between both organelles (Fig. 1.1a–c) (Novikoff and Novikoff 1972; Zaar et al. 1987; Grabenbauer et al. 2000). Already in the early years of peroxisome research this striking spatial coincidence led to the hypothesis that peroxisomes are formed from terminal ER cisternae (Novikoff and Shin 1964), representing a specialized compartment of the cellular endomembrane system. This view was seriously challenged by the discovery that peroxisomal enzymes are synthesized on free polyribosomes and directly imported from the cytosol supporting the view of an autonomous, self-replicating organelle (Lazarow and Fujiki 1985). During recent years, however, the ER came back into focus as the place of peroxisome origin: deletion of the peroxins Pex3, Pex19 or Pex16 results in total loss of peroxisomal structures as these proteins are required for the maintenance of the peroxisomal membrane (South and Gould 1999; Hettema et al. 2000). Their reintroduction into correspondent deletion mutants leads to the *de novo* generation of peroxisomes, indicating that peroxisomes emerge from another cellular compartment. During this process, Pex3 and Pex19 have been observed to initially localize to the ER before maturing into import-competent peroxisomes (e.g., Hoepfner et al. 2005). That peroxisomes are not exceptionally formed from the ER under the somewhat artificial conditions of a peroxisome-free cell was subsequently suggested (Geuze et al. 2003; Kim et al. 2006; Karnik and Trelease 2007), and ER proteins involved in the secretory pathway such as Sec16B, Sec20, Sec29, Sec61 and Dsl1 have been proposed to contribute to this process (Perry et al. 2009; Yonekawa et al. 2011; Thoms et al.

2012). Recent *in vitro* studies reported the formation of vesicular structures from the ER carrying a specialized set of peroxins (Lam et al. 2010; Agrawal et al. 2011). As initially proposed (Titorenko and Rachubinski 2001), in yeast two biochemically different vesicle pools bearing individual sets of peroxisomal membrane proteins were described using GFP-split (BiFC) assays and are supposed to bud from specialized ER regions (van der Zand et al. 2012). They are reported to subsequently fuse with each other forming import-competent pre-peroxisomes. Those then mature into functional peroxisomes by importing further matrix components but do not fuse with pre-existing organelles. The authors consequently conclude that peroxisomes are exclusively formed by *de novo* synthesis from the ER. A vesicular ER-to-peroxisome protein transport would likely request a reciprocal sorting system in order to retarget misrouted ER-resident proteins and/or components of the sorting machinery to their original location. A study in plants described the routing of the tomato bushy stunt virus replication protein p33 from peroxisomes to the ER in concert with endogenous peroxisomal proteins pointing to a retrograde sorting system (McCartney et al. 2005). Under non-pathogenic conditions, however, the existence of peroxisome-to-ER sorting has yet not been convincingly proven. Other studies contradict the "ER-only" hypothesis for peroxisome biogenesis: expression of a mitochondria-targeted Pex3 variant in a correspondent ΔPex3 *Saccharomyces cerevisiae* mutant was recently reported to induce peroxisome reformation, thus questioning an obligate involvement of the ER (Rucktäschel et al. 2010). Morphologically documented by sequential steps of membrane elongation, constriction and final fission, peroxisomes are also observed to form by growth and division from pre-existing organelles using a division machinery shared with mitochondria (Schrader et al. 2012) (see Sect. 1.4). Furthermore, the dominating pathway for peroxisome formation in *S. cerevisiae* appears to be fission from pre-existing organelles as *de novo* formation is a significantly more time consuming process (Motley and Hettema 2007). Similar conclusions were drawn from studies using *Hansenula polymorpha* (Nagotu et al. 2008) and mammalian cells (Delille et al. 2010). Therefore, both pathways may contribute in parallel to the maintenance and proliferation of peroxisomes (Saraya et al. 2011). Their proportional contribution to total peroxisome numbers has still to be determined and may vary among species. Remarkably, in both processes peroxisomes are not formed as *per se* functional entities but involve a maturation pathway, where peroxisomal pre-compartments are subsequently supplied with proteins performing metabolic functions (Islinger et al. 2012). In either case, phospholipids are required to promote growth of pre-peroxisomal membrane structures and have thus to be delivered from the ER where they are generated. The peroxin-bearing vesicles described above, provided that they contribute to both *de novo* formation and growth and division, could deliver phospholipids as well as proteins to the nascent organelles, which would spare a specialized transport system for phospholipids. However, a non-vesicular transfer of ER-derived phospholipids to peroxisomes has been described (Raychaudhuri and Prinz 2008) suggesting that peroxisomal membrane growth can be independent from the vesicular transfer of peroxisomal membrane proteins from the ER when peroxisomes multiply by growth and division. As the transfer of lipids between the

ER and peroxisomes is suggested to occur bidirectionally, an important function may also be to alter the lipid composition of the peroxisome membrane. Such alterations may modify the organelle's mechanical properties thereby supporting membrane bending or elongation during peroxisome proliferation.

Disregarding the ongoing discussion about the role of the ER in peroxisome biogenesis, it is important to note that the relationship between both organelles includes other aspects of cooperation, in particular to facilitate various metabolic pathways shared by both compartments. This metabolic interplay is *inter alia* documented by the organelle alterations observed in conditional hepatic Pex5 knockout mice which cannot form functional peroxisomes (Dirkx et al. 2005). The knockout mice display severe morphological alterations of mitochondria, an accumulation of lipid droplets and a significant proliferation of the smooth ER. The most familiar pathway of the peroxisome-ER connection may be the biosynthesis of ether-phospholipids, which is initiated in peroxisomes and completed in the ER (Braverman and Moser 2012). Ether glycerolipids constitute about 15–20% of total cellular membranes and are especially enriched in brain, heart and immune cells of the blood. Briefly, their synthesis requires acylation of dihydroxyacetone phosphate by dihydroxyacetone phosphate acyltransferase (DHAPAT). The acyl group is then substituted with an alkyl group by alkyldihydroxyacetone phosphate synthase (alkyl-DHAP synthase or ADHAPS). Both enzymes form a heterotrimeric complex at the inner site of the peroxisomal membrane. Additional reactions associated with the cytosolic site of the peroxisomal membrane include the generation of a long chain alcohol for the substitution reaction described above performed by acyl-CoA reductase, and the reduction of alkyl-DHAP to alkylglycerol-3-phosphate by alkyl-DHAP reductase. All further reactions (acylation in position 2 of the glycerol, dephosphorylation and addition of ethanolamine/cholin in position 3) are carried out in the ER. The function of ether lipids is still unknown, but they are supposed to contribute to a reduction in membrane fluidity (e.g., in lipid rafts) and to act as scavengers for reactive oxygen species to prevent the oxidation of other vulnerable membrane lipids. A defect in ether lipid synthesis represented by the genetic disorder Rhizomelic Chondrodysplasia Punctata (RCDP) results in skeletal dysplasia, severe abnormalities in the central nervous system and cortical cataracts usually leading to death before adulthood (Braverman and Moser 2012). On the cellular level ether lipid deficiency is characterized by impaired membrane traffic and cholesterol distribution affecting the integrity of the plasma membrane (Thai et al. 2001; Gorgas et al. 2006). Recently, the peroxisomal part in ether lipid synthesis has been shown to be crucial for the formation of glycosyl phosphatidyl inositol (GPI)-anchored proteins in the ER, since in mammals, GPI-anchored proteins usually possess mainly 1-alkyl-2-acyl phosphatidyl inositol (Kanzawa et al. 2012). As GPI-anchored proteins fulfil important functions in cell–cell and cell–environment interactions and are prominent constituents of lipid microdomains, the disruption of their synthesis may significantly contribute to the severity of RCDP.

The production of polyunsaturated fatty acids is another biosynthetic process requiring cooperation between ER and peroxisomes. Usually the ER performs the synthesis of such metabolites by desaturation of correspondent saturated fatty acids

but lacks an acyl-CoA-dependent 4 desaturase (Voss et al. 1991). To introduce a double bond at this position, 24 carbon n-6 and n-3 fatty acids synthesized in the ER are transferred to peroxisomes where they are partially degraded by β-oxidation until a double bond at position 4 of the carbonate chain is reached (Sprecher and Chen 1999; Su et al. 2001). Instead of further degradation, such fatty acids are exported and re-imported into the ER to be used for membrane lipid biosynthesis (Sprecher and Chen 1999). This process is of major significance for the production of docosahexaenoic acid, which is supposed to play an important role in neuronal migration during development, maintenance of synaptic plasticity in hippocampal neurons and the attenuation of neuroinflammation. Interestingly, the incorporation of docosahexaenoic acid into the peroxisomal membrane was found to stimulate peroxisome proliferation representing a positive feedback-loop for the coordination of peroxisome function and organelle abundance (Itoyama et al. 2012).

The synthesis of cholesterol and isoprenoids from acetyl-CoA was originally associated with the ER and the cytoplasm until a peroxisomal localization of 3-hydroxymethyl glutaryl-CoA reductase (HMG-CoA reductase) – the key regulatory enzyme in cholesterol biosynthesis – was reported (Keller et al. 1985). Gradually, the whole pre-squalene segment of the pathway up to the step of farnesylpyrophosphate (FPP) synthesis was partially or exclusively associated with peroxisomes whereas the incorporation of FPP into squalene was supposed to occur in the ER (Aboushadi et al. 1999). A peroxisomal contribution to further steps in cholesterol biosynthesis has been proposed (Appelkvist et al. 1990), but the peroxisomal localisation of the correspondent proteins has not been confirmed yet. The contribution of peroxisomes to isoprenoid/cholesterol biosynthesis in mammals remains under debate and is not ultimately clarified (Wanders and Waterham 2006; Kovacs et al. 2007). In plants, by contrast, new studies provide evidence that the final four steps of the pre-squalene segment (up to the generation of FPP) are localized to peroxisomes (Clastre et al. 2011; Simkin et al. 2011). As peroxisomal targeting sequences have also been described for the mammalian homologues (Kovacs et al. 2007), from an evolutionary standpoint this pathway was shared by both compartments in the eukaryotic ancestor and different localizations for some of the enzymes may have been evolved during evolution.

By contrast, the peroxisomal role in bile acid synthesis/cholesterol degradation is well established. The conversion of cholesterol into the bile acid precursor trihydroxycholestanoic acid (THCA) is an extraperoxisomal process and enzymes from the ER, mitochondria and the cytosol participate in two alternative pathways (Russell 2003). THCA is then imported into peroxisomes as THCA-CoA and its side chain is shortened by peroxisomal β-oxidation to form cholic acyl-CoA. Finally, the CoA group of cholic acyl-CoA is substituted with taurine/glycine for excretion by the peroxisomal enzyme bile acid-CoA: amino acid N-acyltransferase (Ferdinandusse et al. 2009). For the conjugation of THCA with CoA, a prerequisite for peroxisome import, two enzymes exist – bile acid-CoA synthetase and very long-chain acyl-CoA synthetase (VLACS) (Mihalik et al. 2002). Both are residents of the ER-membrane, however, VLACS has also been localized to peroxisomes. Nevertheless, peroxisomes show no THCA conjugating activity

(Schepers et al. 1989), which is in line with the observation that VLACS localizes only at the inner site of the peroxisomal membrane (Smith et al. 2000). Thus, import of THCA into peroxisomes seems to rely on efficient metabolite activation at the ER and subsequent transport to peroxisomes.

As exemplified above, peroxisomal and ER metabolism are significantly intermingled requesting sophisticated regulation systems to link corresponding enzyme amounts and enzymatic activities. However, our current knowledge about those processes remains still fragmentary. An interesting example of a peroxisomal impact on ER physiology has been described recently: disruption of peroxisome functions in mice by Pex2 knockout reduced hepatic cholesterol levels to significant amounts which was paralleled by a pronounced up-regulation of sterol regulatory element binding proteins (SREBPs) (Kovacs et al. 2004) – transcription factors controlling cholesterol metabolism. Furthermore, the authors reported that ER stress pathways are activated in the Pex2 knockout mice, which in turn disturb the expression of SREBP-2, SREBP-1c and Insig-2a, leading to further deregulation of endogenous sterol response pathways (Kovacs et al. 2009). Even if not yet profoundly understood, metabolic connections between peroxisomes and the ER seem to give rise to reaction chains leading to a disruption of cholesterol metabolism in a peroxisome-deficient animal model. Likewise, further regulatory networks involved in interorganellar communication may be involved in the development of the complex phenotypes of peroxisomal disorders. With regard to the metabolic cooperations connecting peroxisomes and the ER, the intimate contacts observed in ultrastructural studies (Fig. 1.1a–c) may facilitate the efficient exchange of metabolites. How this organelle proximity is achieved and if it involves physical protein attachment sites is currently unclear. For mitochondria-ER contact sites, however, an involvement of the mitofusin Mfn2 has been reported (de Brito and Scorrano 2008).

Extending our view on the peroxisome-ER connection, new non-metabolism-related cooperative phenomena have been recently reported. MAVS are membrane-associated proteins involved in antiviral signalling and localize at the mitochondrial outer membrane, peroxisomes, and the MAM (mitochondrial-associated membrane) of the ER network (see Chap. 4). In this signalling network, the MAM was suggested to act as a synapse regulating innate immune signalling by MAVS from MAM, peroxisomes, and mitochondria, extending our view on organellar interactions to a dynamic, multiorganellar perspective.

1.4 The Peroxisome-Mitochondria Connection

It became evident in the last years that peroxisomes and mitochondria maintain a much closer interrelationship than previously anticipated (Schrader and Yoon 2007; Camoes et al. 2009; Islinger et al. 2012). The “peroxisome-mitochondria connection” (Fig. 1.2) includes metabolic cooperation in fatty acid β-oxidation to maintain lipid homeostasis (see Sect. 1.5), coordinated biogenesis by sharing key proteins

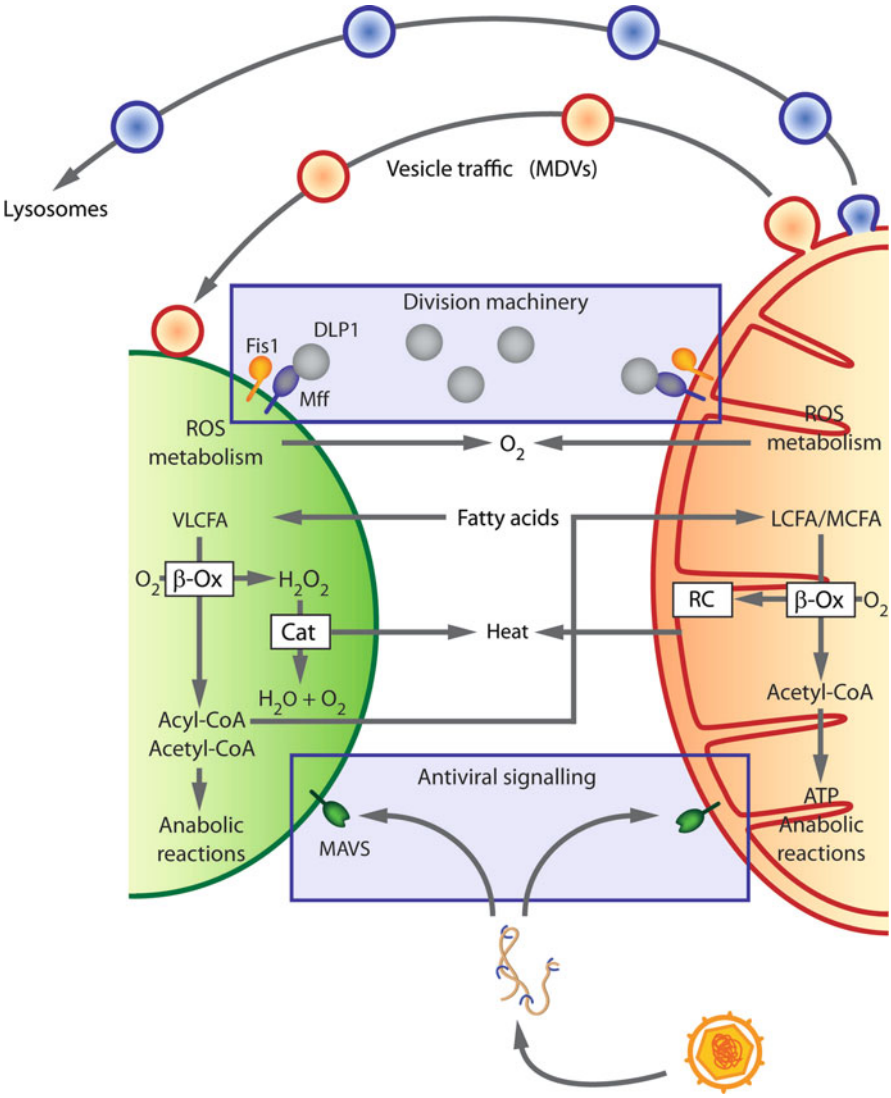

Fig. 1.2 Schematic view of the peroxisome-mitochondria connection. Peroxisomes (*left*) and mitochondria (*right*) in mammals show metabolic cooperation in fatty acid β-oxidation (β-Ox), contribute to heat production, and have a redox-sensitive relationship. They share key components of their division machinery (e.g., DLP1, Mff, Fis1) and contribute to antiviral signalling via MAVS (mitochondrial antiviral-signalling protein). Moreover, novel trafficking pathways from mitochondria to peroxisomes (and lysosomes) involving mitochondria-derived vesicles (MDVs) have been reported (see Sect. 1.4 for details). Cat, peroxisomal catalase; VLCFA, LCFA, MCFA, very long-chain, long-chain and medium-chain fatty acids; RC respiratory chain (from Islinger et al. 2012)

of the organelle division machinery (Schrader et al. 2012), potential exchange by a novel vesicular trafficking pathway from mitochondria to peroxisomes (Neuspiel et al. 2008), a redox-sensitive relationship (Fransen et al. 2012) (see Chap. 3), and cooperation in anti-viral signalling and defence (see Chap. 4).

In animals, peroxisomes and mitochondria cooperate in the degradation of fatty acids. This is different in many yeast species and plants, where fatty acid β-oxidation is solely peroxisomal (Poirier et al. 2006). Although the biochemical/enzymatic steps of fatty acid β-oxidation in both organelles are similar, each organelle harbours its specific set of enzymes. Peroxisomal fatty acid β-oxidation usually requires an acyl-CoA oxidase (dehydrogenation step), a bifunctional enzyme (hydration and dehydrogenation steps), and a thiolase (for thiolytic cleavage). These enzymes show substrate specificity; VLCFA for example, can only be degraded in peroxisomes. In addition, peroxisomes also perform α-oxidation of e.g., dietary fatty acids such as phytanic acid from dairy products (Wanders and Waterham 2006). As, in contrast to mitochondria, peroxisomes do not possess the proteins of the respiratory chain as electron acceptors, peroxisomal fatty acid β-oxidation results in the generation of H_2O_2 through the transfer of electrons to O_2 by acyl-CoA oxidase. Hydrogen peroxide, which can be toxic to the cells, but also serves as a signalling molecule, is degraded by catalase, a prominent peroxisomal matrix enzyme (Schrader and Fahimi 2006b; Bonekamp et al. 2009; Fransen et al. 2012).

Furthermore, peroxisomal β-oxidation generates only chain-shortened fatty acids, and unlike in mitochondria, does not result in complete degradation of fatty acids. The medium chain fatty acids obtained in peroxisomes are routed to mitochondria for further oxidation and ATP production. Export of fatty acids from peroxisomes requires a carnitine shuttle system and the involvement of membrane pores, e.g., formed by PMP22 (Antonenkov and Hiltunen 2011). In addition to shuttle systems and proteinaceous pores, a vesicular trafficking pathway from mitochondria to peroxisomes has been suggested (Neuspiel et al. 2008). Mitochondria were observed to generate so called mitochondria-derived vesicles (MDVs). One population of MDVs carried the mitochondrial anchored protein ligase MAPL and was supposed to target to peroxisomes, but the physiological function of this trafficking pathway is still unclear.

More examples for a metabolic cooperation between peroxisomes and mitochondria (and even chloroplasts) are found in fungi and plants. It has recently been reported that mitochondria and peroxisomes cooperate in the synthesis of biotin in fungi (Tanabe et al. 2011).

Evidence for a coordinated biogenesis of peroxisomes and mitochondria under certain environmental conditions may arise from the discovery that both organelles share key proteins of their division machinery (reviewed in Schrader et al. 2012). Sharing division components appears to be an evolutionary conserved strategy among organisms. In mammals, these components include the dynamin-like GTPase DLP1/Drp1, which forms ring-like oligomeric structures around membrane constrictions and severs the peroxisomal and mitochondrial membranes in a GTP-dependent manner. The tail-anchored membrane proteins Fis1 and Mff are supposed to act as receptors for DLP1 at the organelle membranes. Whereas mitochondrial

dynamics are regulated by balanced fusion and fission events, there is growing evidence that peroxisomes generally do not fuse to exchange matrix or membrane proteins (see Sect. 1.6) (Motley and Hettema 2007; Huybrechts et al. 2009; Bonekamp et al. 2012).

Loss of function of the key division proteins blocks peroxisomal and mitochondrial fission and leads to the accumulation of elongated organelles (Schrader et al. 2012). Elongated peroxisomes and mitochondria have been observed in fibroblasts of a patient who turned out to suffer from DLP1 deficiency based on a point mutation in the middle domain of DLP1 (Waterham et al. 2007) (see Sect. 1.7). The heterozygous, dominant-negative missense mutation A395D was shown to inhibit oligomerization of DLP1 (Chang et al. 2010). The resulting disorder combines peroxisomal and mitochondrial defects causing developmental abnormalities (e.g., in the brain) and early death. Similar observations have been made in DLP1 knockout mice (Ishihara et al. 2009; Wakabayashi et al. 2009). Interestingly, mitochondrial alterations have also been observed under conditions of peroxisomal dysfunction, e.g., in Zellweger syndrome, a peroxisome biogenesis disorder and in related knockout mouse models (see Sect. 1.3) (Baumgart et al. 2001; Dirkx et al. 2005). The lack of peroxisomal activity in Pex5 knockout mice compromised mitochondrial ATP production and resulted in the concomitant activation of AMP-activated kinase leading to elevated carbohydrate combustion (Peeters et al. 2011). Consequently, the Pex5 knockout mice loose body weight despite increased food intake.

If the mitochondrial alterations are based on secondary, indirect effects or are directly related to a loss of the peroxisome-mitochondria interplay, is currently unclear (Camoes et al. 2009). In this respect, recent evidence for a redox-sensitive relationship between peroxisomes and mitochondria (Ivashchenko et al. 2011) might point to the latter. The induction of peroxisomal reactive oxygen species (ROS) production by peroxisome-targeted KillerRed caused mitochondrial fragmentation and an altered mitochondrial redox potential. The mitochondrial redox balance is also disturbed in cells lacking functional peroxisomes or catalase. Correspondingly, catalase deficiency was reported to increase mitochondrial ROS in response to fatty acids in a diabetes mouse model (Hwang et al. 2012). Like mitochondria, peroxisomes contribute to cellular ROS metabolism and contain several enzymes which generate but also decompose ROS, among them acyl-CoA oxidases and catalase (Schrader and Fahimi 2006b; Antonenkov et al. 2010; Fransen et al. 2012; Bonekamp et al. 2011). Thus, peroxisomes are supposed to contribute to cellular ROS homeostasis, oxidative stress and ageing, but also to ROS-mediated cellular signalling. These topics are addressed in detail in Chaps. 3, 9, 13, and 15.

Evidence for a physical interaction of peroxisomes and mitochondria in mammalian cells is still scarce. Intimate contacts can be observed in ultrastructural studies (Fig. 1.1d), but their physiological relevance awaits experimental proof. Peroxisomes and mitochondria have been reported to cluster, e.g., after the expression of membrane proteins (Koch et al. 2005), and can be co-isolated from a specific density after gradient centrifugation and subsequently separated into individual fractions by repeating this centrifugation step (Islinger et al. 2006).

As both organelles are in intimate contact with the ER (see Sect. 1.2), potential peroxisome-mitochondria contacts might as well be indirect and mediated by ER membranes. However, in the fission yeast *Schizosaccharomyces pombe* peroxisome movement in association with mitochondria has been observed (Jourdain et al. 2008). Furthermore, in the red algae *Cyanidioschyzon merolae* only one peroxisome and one mitochondrion exist. Their division is coordinated, and the peroxisome interacts with the mitochondrion to partition into the daughter cell (Miyagishima et al. 1999).

1.5 Peroxisomes and Lipid Droplets

Eukaryotic cells have the ability to accumulate neutral lipids such as triacylglycerol and cholesterol ester and to store them into lipid droplets (LD). The view that LDs represent simple lipid storage containers has gradually changed, and evidence for their complex nature and contribution to multiple cellular functions has been provided (Beller et al. 2010). LDs are dynamic and highly motile organelles. They grow through a fusion process mediated by SNARE proteins and move bi-directionally on microtubules. It is suggested that LD movement supports their interaction with other organelles, among them peroxisomes, endosomes, mitochondria and the ER, to distribute neutral lipids and phospholipids. Compelling evidence for an interaction of peroxisomes with LDs has been presented in early ultrastructural studies (Novikoff et al. 1980) (Fig. 1.1e, f). Using live-cell imaging the dynamic nature of the interaction between peroxisomes and LDs has been confirmed (Schrader 2001). In *S. cerevisiae*, intimate physical contacts between peroxisomes and LDs were observed (Binns et al. 2006). In some cases, peroxisomes formed processes that extended into the core of the LDs. This lipid droplet-peroxisome interaction may serve to link lipolysis mediated by LDs to fatty acid β-oxidation within the peroxisomes. Furthermore, lipids generated by peroxisomes might move into LDs. Defects in peroxisomal fatty acid β-oxidation have been linked to enlarged LDs in the nematode *Caenorhabditis elegans* (Zhang et al. 2010). Changes in the number and size of peroxisomes, mitochondria, and LDs have also been reported in morphometric studies after starvation and clofibrate treatment in mouse hepatocytes (Meijer and Afzelius 1989) and in peroxisome-deficient knockout mice (Dirkx et al. 2005). However, the underlying molecular mechanisms of the peroxisome-lipid droplet interaction remain to be elucidated. An interactome map of protein-protein contacts of LDs with mitochondria and peroxisomes in *S. cerevisiae* has recently been generated using a bimolecular fluorescence complementation assay (Pu et al. 2011). The LD proteins Erg6 and Pet10 were found to be involved in 75% of the interactions detected, including most of the peroxins tested. The physiological significance of these interactions awaits further experimental proof. Some of the interactions of LDs and other organelles (e.g., endosomes) may be regulated by Rab GTPases, which have been found on LDs (reviewed in Murphy et al. 2009).

1.6 Peroxisome-Peroxisome Interactions

As peroxisomes – like mitochondria – multiply by fission and even share key fission proteins (e.g., DLP1, Fis1, Mff) (see Sect. 1.4), it was long debated if peroxisomes can fuse to exchange matrix and membrane components and if they as well share key fusion proteins with mitochondria (e.g., Mfn1, Mfn2, Opa1). We recently addressed this question systematically by applying an *in vitro* fusion assay based on the co-cultivation of mammalian CHO cells stably expressing either red or green fluorescent peroxisomal matrix or membrane proteins. The interaction of red and green peroxisomes and a potential exchange of marker proteins were analyzed in hybridoma cells which were formed through cell fusion (Bonekamp et al. 2012). By combining epifluorescence microscopy, spinning disk confocal microscopy and live cell imaging, we demonstrated that mature peroxisomes in mammalian cells do not fuse and exchange matrix or membrane marker proteins in a mechanism analogous to mitochondria. Moreover, mitochondrial fusion proteins (e.g., Mfn1, Mfn2, Opa1) do not localize or target to peroxisomes and do not contribute to peroxisome dynamics (Bonekamp et al. 2012). These findings are in agreement with previous observations in yeast, plant and mammalian cells (Arimura et al. 2004; Motley and Hettema 2007; Huybrechts et al. 2009). However, our life cell studies revealed that peroxisomes in mammalian cells are engaged in several transient, but vivid and long term contacts (Bonekamp et al. 2012). Similar interactions were reported by real time imaging of GFP-labelled peroxisomes (Schrader et al. 2000). In many cases, peroxisomes moving along microtubules were observed to interact with other peroxisomes. Mathematical analysis showed that the transient interactions display so-called power law behaviour. Power law distributions in biological processes point to intricate dynamics which originate from diverse and yet specific mechanisms (Clauset et al. 2009). Thus, peroxisome interactions are more complex than previously assumed and represent a new dynamic behaviour of peroxisomes. By applying a simple computational model, we demonstrated that when combined with ATP-driven peroxisome movement along microtubules (see Sect. 1.7), the subsequent formation of inter-peroxisomal contacts can potentially contribute to the equilibration of cellular peroxisome pools. Peroxisomes are very heterogeneous in terms of density, protein composition and import competence (Heinemann and Just 1992; Luers et al. 1993; Islinger et al. 2010), thus an exchange of metabolic information might occur. However, an increase in heterogeneity among different peroxisome populations by manipulating ROS and fatty acid levels did not promote peroxisome interactions (Bonekamp et al. 2012), and questions the exchange of metabolites by inter-peroxisomal contacts. Although the physiological role of the transient complex peroxisomal interactions is unclear, they might contribute to a "signalling system" monitoring the state and/or distribution of peroxisome populations within the cell. Interestingly, the formation of small peroxisome groups with close apposition has been documented in ultrastructural studies (Stier et al. 1998; Zaar et al. 1984) (Fig. 1.3a). Peroxisomes which are attached to each other can also be isolated by gradient centrifugation from rat liver after treatment with

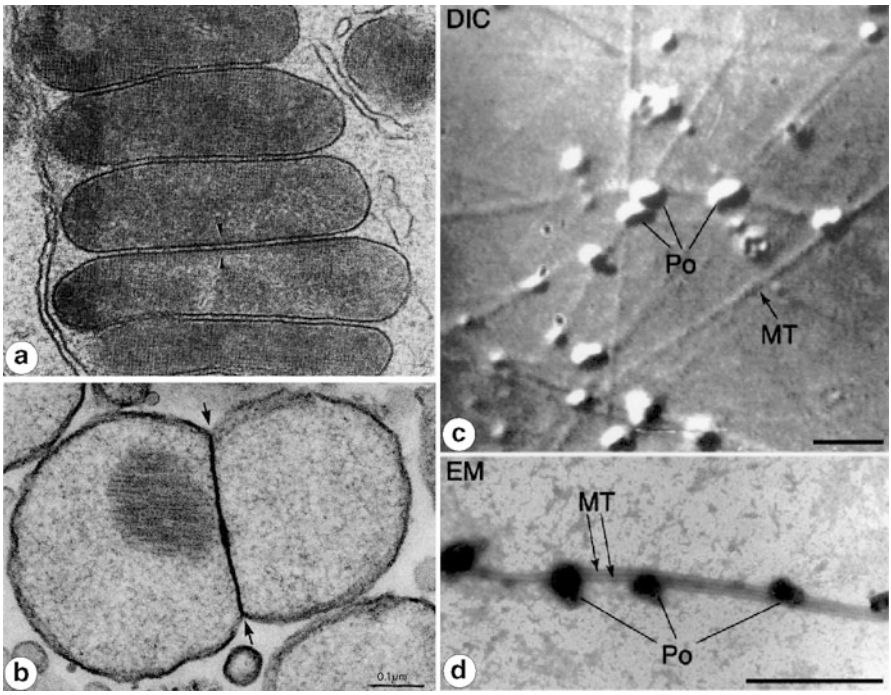

Fig. 1.3 (**a**, **b**) Peroxisome-peroxisome interactions. (**a**) Electron micrograph of ordered stacks of peroxisomes in canine kidney. Magnification, ×120,000 (from Zaar et al. 1984). (**b**) Electron micrograph of closely associated peroxisomes in a purified peroxisome fraction isolated from bezafibrate-treated rat liver by gradient centrifugation. (**c**, **d**) Association of purified rat liver peroxisomes (Po) with microtubules (MT) in an *in vitro* assay. (**c**) Video-enhanced contrast microscopy (VECM) of microtubules and peroxisomes using a Zeiss Axiovert microscope equipped with differential interference contrast (DIC) optics and a Hamamatsu video camera (from Schrader et al. 2003). (**d**) Negative staining electron microscopy (EM) of isolated peroxisomes bound to microtubules (from Thiemann et al. 2000). Bars, 0.2 µm (**c**), 1 µm (**d**)

peroxisome proliferators (Fig. 1.3b). These groups may represent functional units of peroxisomes which interact and cooperate via close but transient contacts. In addition, interactions with other organelles might occur.

1.7 Peroxisomes and the Cytoskeleton

An association of mammalian peroxisomes with cytoskeletal elements was predicted from early ultrastructural observations (Gorgas 1985; Yamamoto and Fahimi 1987), and their direct interaction with and movement along microtubules has been well documented *in vivo* and *in vitro* (reviewed in Schrader et al. 2003; Schrader and Fahimi 2006a) (Fig. 1.3c, d). The peroxisome-microtubule interaction is crucial

for long-range, (bi)-directional motility of peroxisomes and for the positioning of the organelle within the cell (e.g., to maintain a uniform intracellular distribution). Microtubule-based peroxisome movement involves the motor proteins dynein, kinesin and the dynein activator complex dynactin (Schrader et al. 2000; Kural et al. 2005). Whereas dynein can interact with organelle membranes via the dynactin complex, the recruitment of the microtubule plus-end kinesin motors often requires adaptor proteins. However, the role of different kinesins in peroxisome motility is poorly studied. Recently, a role for the peroxin Pex14 in microtubule-binding and peroxisome motility has been suggested (Bharti et al. 2011), but information on potential linker proteins and peroxisomal receptors for motor proteins is generally scarce. This is in part due to the fact that peroxisome motility and distribution in classical genetically accessible model organisms such as bakers' yeast or plant cells depend on the actin cytoskeleton and type-V myosin motors, but not (or only partially) on microtubules. In plants, peroxisome motility can be modulated by ROS and Ca^{++} and may serve to protect the cell against oxidative stress (Rodríguez-Serrano et al. 2009). Peroxisome motility and positioning in *S. cerevisiae* is crucial for partitioning and inheritance of peroxisomes from the mother to the daughter cell (the forming bud), but also to retain peroxisomes in the mother. These processes are highly regulated and depend on actin dynamics, the myosin motor Myo2, and the peroxisomal inheritance proteins Inp1 and Inp2. Inp1 is crucial for retaining peroxisomes in the mother, whereas Inp2 recruits Myo2 to peroxisomes (Fagarasanu et al. 2010). Interestingly, *S. cerevisiae* harbours such a complex and tightly regulated inheritance machinery to guarantee proper distribution of peroxisomes to the bud although peroxisomes in the daughter can form *de novo* from the ER in case they are lost. An explanation might be that *de novo* formation is more energy-consuming, and thus represents a back-up system. Homologues of Inp1 and Inp2 have not been identified in mammals. In contrast to yeast cells which usually harbour 3–5 peroxisomes under non-proliferative conditions, mammalian cells contain hundred or more peroxisomes. Thus, complete loss of peroxisomes in the daughter cell is unlikely, and distribution during mitosis is supposed to be at random (Wiemer et al. 1997).

The analysis of peroxisome motility in cultured mammalian cells revealed, that only 10–15% of the peroxisome population is performing long-range, fast movements in a microtubule-dependent manner (Koch et al. 2003). We recently obtained evidence for a relationship between the percentage of fast moving peroxisomes, energy consumption and the mixing time of different peroxisome populations within a cell (Bonekamp et al. 2012). Our computational model indicates that approx. 15% of ATP-driven peroxisome movement represents an optimum allowing proper mixing/homogenisation of the peroxisomal compartment at minimal energy costs in a physiological time frame (see Sect. 1.6).

Microtubules are not required for matrix protein import into peroxisomes (Brocard et al. 2005), but may facilitate efficient and regulated sorting of proteins to peroxisomes (Chuong et al. 2005). Microtubules and dynein may as well contribute to the early stages of peroxisome biogenesis, as Pex16-mutant cells failed to restore peroxisome formation after microinjection complementation, when microtubules

were depolymerised prior to microinjection, or when a dominant-negative CC1 subunit of the dynein/dynactin motor complex was co-expressed (Brocard et al. 2005). However, the membrane-deforming protein Pex11β was properly targeted to peroxisomes and induced membrane elongation in the absence of microtubules (our unpublished results).

In yeast, the actin cytoskeleton and the Myo2 motor have been suggested to exert pulling forces on the peroxisome membrane thus assisting in peroxisome fission. Indeed, Myo2 and Pex11 are required for the formation of tubular membrane extensions prior to peroxisome fission (Nagotu et al. 2008; Fagarasanu et al. 2010). In mammalian cells microtubules are not essential for membrane elongation of peroxisomes or for organelle division (Schrader et al. 2000). Mammalian peroxisomes can elongate in the complete absence of microtubules (Schrader et al. 1998), and remarkably, membrane elongation is promoted by microtubule-depolymerizing (but not stabilizing) agents (Schrader et al. 1996). Furthermore, the constriction and final fission of peroxisomes precedes independent of microtubules. However, microtubules are crucial for the proper intracellular distribution of peroxisomes after division, for fast and directed peroxisome motility, for their positioning and the maintenance of their uniform distribution within the cell (Schrader et al. 2003). In this respect, a mechanistic link between peroxisome multiplication (proliferation) and trafficking along microtubules has been proposed (Nguyen et al. 2006), suggesting that peroxisome division may trigger binding and transport of newly formed peroxisomes along microtubules.

It can be assumed that a loss of peroxisome motility and defects in the proper trafficking and distribution of peroxisomes result in a regional loss of essential peroxisomal functions and subsequently to cell damage and degeneration, especially in neurons. Correspondingly, loss of DLP1, which is essential for the division of peroxisomes and mitochondria (Koch et al. 2003, 2005; Schrader et al. 2012), has been linked to neurodegeneration and developmental defects in brain (Waterham et al. 2007; Ishihara et al. 2009; Wakabayashi et al. 2009). It is suggested that the block in peroxisomal and mitochondrial division, which results in elongated and enlarged organelles, inhibits their proper distribution within neurons and in the axon thus contributing to cell damage.

1.8 Concluding Remarks

There is compelling evidence that peroxisomes are significantly more dynamic and interactive than previously expected. They do not function as isolated entities, but are integrated into a complex network of communicating endomembranes that is only beginning to emerge. Peroxisomes interact and cooperate with other organelles such as the ER, mitochondria, and lipid droplets to facilitate metabolic processes. Remarkably, they also act as signalling platforms, and contribute to the fine-tuning of cellular processes. The interaction of mammalian peroxisomes with microtubules likely serves to direct peroxisomal movement and to facilitate interactions with

other endomembranes. However, how physical contacts between peroxisomes and other organelles are mediated, what components are transferred or exchanged, and how this transfer is mediated, is largely unknown. It is a great challenge for future investigations to identify and characterize the underlying molecular mechanisms and the physiological relevance of these interactions, and to elucidate their importance for health and disease.

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Chapter 2
Peroxisomes in Human Health and Disease: Metabolic Pathways, Metabolite Transport, Interplay with Other Organelles and Signal Transduction

Ronald J.A. Wanders

Contents

Abstract Peroxisomes play a key role in human physiology as exemplified by the devastating consequences of a defect in peroxisome biogenesis as observed in patients affected by Zellweger syndrome. The main metabolic functions of peroxisomes in humans include: (1) fatty acid beta-oxidation; (2) etherphospholipid

R.J.A. Wanders (✉)
Laboratory Genetic Metabolic Diseases, Room F0-226, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands
e-mail: r.j.wanders@amc.uva.nl

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synthesis; (3) bile acid synthesis; (4) fatty acid alpha-oxidation, and (5) glyoxylate detoxification. Since peroxisomes lack a citric acid cycle and respiratory chain like mitochondria do, metabolism in peroxisomes requires continued cross-talk with other organelles, notably mitochondria and the endoplasmic reticulum in order to allow continued metabolism of the products generated by peroxisomes. Many of the metabolites which require peroxisomes for homeostasis, are involved in signal transduction pathways. These include the primary bile acids; platelet activating factor; plasmalogens, N-acylglycines and N-acyltaurines; docosahexaenoic acid as well as multiple prostanoids. The current state of knowledge in this area will be discussed in this review.

Keywords Peroxisomes • Fatty acids • Signal transduction • Zellweger syndrome • Mitochondria

Abbreviations

Acnat	Acyl-CoA: amino acid N-acyltransferase
ACOX1	Acyl-CoA oxidase 1
ACYL-DHAP	Acyldihydroxyacetone phosphate
ADHAPS	Alkyldihydroxyacetone phosphate synthase
AGT	Alanine glyoxylate aminotransferase
ALD	Adrenoleukodystrophy
BAAT	Bile acid-CoA: amino acid N-acetyltransferase
BCOX	Branched-chain acyl-CoA oxidase
BSEP	Bile salt export pump
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CPT1	Carnitine palmityltransferase 1
CrAT	Carnitine acetyltransferase
CrOT	Carnitine octanoyltransferase
CYP7A1	Cholesterol 7-Alpha-hydroxylase
DBP	D-bifunctional protein
DHA	Docosahexaenoic acid
DHCA	Dihydroxycholestanoic acid
DHAPAT	Dihydroxyacetone phosphate acyltransferase
EPL	Etherphospholipid
ER	Endoplasmic reticulum
FA	Fatty acid
FGF	Fibroblasts growth factor
FXR	Farnesoid-X receptor
LBP	L-bifunctional protein
LRH-1	Liver receptor homologous Protein-1
LRM	Lipid raft microdomains

LTE4	Cysteinyl leukotriene-4
PAF	Platelet activating factor
PD	Peroxisomal disorder
ROS	reactive oxygen species
SCPx	Sterol-carrier-protein X
TH	Thiolase
THCA	Trihydroxycholestanoic acid
VLCFA	Very long-chain fatty acids
ZS	Zellweger syndrome

2.1 Introduction

Peroxisomes are present in multiple eukaryotic organisms including humans. Much of our current knowledge on the physiological role of peroxisomes in humans has come from studies on a rare genetic disease in humans called the cerebro-hepato-renal syndrome, in short named Zellweger syndrome (ZS). In its classical form, ZS patients show multiple, congenital aberrations including craniofacial dysmorphia, neurological aberrations, liver disease and other abnormalities and usually die very early in life. Already in 1973, peroxisomes were found to be lacking in hepatocytes and kidney tubule cells as concluded from immunohistochemical analyses of liver and kidney biopsies of patients affected by ZS (Goldfischer et al. 1973). At that moment in time not too much was known about peroxisomes at least in humans which explains why this in retrospect crucial finding was initially left unnoticed. This all changed in the early 1980s when two key observations were published describing: (1) the accumulation of very long-chain fatty acids (VLCFAs) in plasma from ZS patients (Brown et al. 1982) and (2) the deficiency of plasmalogens – a specific class of etherphospholipids – in erythrocytes and tissues from ZS patients (Heymans et al. 1983). These findings led to a renewed interest in peroxisomes and inspired many researchers around the globe to study the physiological role of peroxisomes and their biogenesis.

2.2 Metabolic Functions of Peroxisomes

The finding of elevated VLCFAs in plasma of Zellweger patients and the deficiency of etherphospholipids including plasmalogens in erythrocytes and tissues of Zellweger patients was soon followed by the identification of additional metabolic abnormalities. This immediately suggested that peroxisomes were not just involved in the homeostasis of VLCFAs and plasmalogens but would also exert other metabolic functions. Below the major metabolic functions of peroxisomes in humans will be discussed.

2.2.1 Fatty Acid Beta-Oxidation

Following earlier work (Cooper and Beevers 1969) in glyoxysomes – organelles closely related to peroxisomes – Lazarow and De Duve identified the presence of a fatty acid beta-oxidation system in peroxisomes (Lazarow and De Duve 1976). The importance of a second beta-oxidation system next to that in mitochondria, was not immediately clear. In fact, it was first thought that the peroxisomal beta-oxidation system would constitute an auxiliary system assisting the mitochondrial beta-oxidation pathway in times of fatty acid overload. This assumption was based on the notion that fibrates induce the expression of peroxisomal beta-oxidation enzymes much more than those of the corresponding mitochondrial enzymes. The finding by Moser and co-workers (Brown et al. 1982) of elevated VLCFA levels in plasma of Zellweger patients in contrast to the normal levels of fatty acids like palmitic, oleic and linolenic acid, suggested that peroxisomes have their own unique role in whole cell fatty acid oxidation. Indeed, it is now clear that the substrate specificities of the mitochondrial and peroxisomal beta-oxidation systems are different with some FAs handled exclusively by the peroxisomal beta-oxidation system. These include.

2.2.1.1 VLCFAs

It is generally agreed that certain VLCFAs, notably C24:0 and C26:0 can only be oxidized in peroxisomes and not in mitochondria. At least one reason for this phenomenon is that C24:0 and C26:0 are no substrates for carnitine palmitoyl-transferase 1 (CPT1). Since peroxisomes lack a citric acid cycle and respiratory chain, the end products of beta-oxidation in peroxisomes which include acetyl-CoA, propionyl-CoA and other acyl-CoAs, but also NADH, need to be shuttled from peroxisomes to mitochondria for full oxidation to CO_2 and H_2O in case of acetyl-CoA, propionyl-CoA and the other acyl-CoAs and reoxidation of NADH back to NAD^+ (see Fig. 2.1). This will be discussed later in this review.

2.2.1.2 Pristanic Acid (2,6,10,14-Tetramethylpentadecanoic Acid)

This FA is primarily derived from phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) after one round of alpha-oxidation but is also derived directly from dietary sources. Work by Verhoeven et al. has shown that pristanic acid undergoes three rounds of beta-oxidation in peroxisomes to produce 4,8-dimethylnonanoyl-CoA plus two units of propionyl-CoA and one unit of acetyl-CoA which are transported from the peroxisome as carnitine ester or in their free acid form followed by uptake into mitochondria for full oxidation to CO_2 and H_2O (Verhoeven et al. 1998) (see Fig. 2.2).

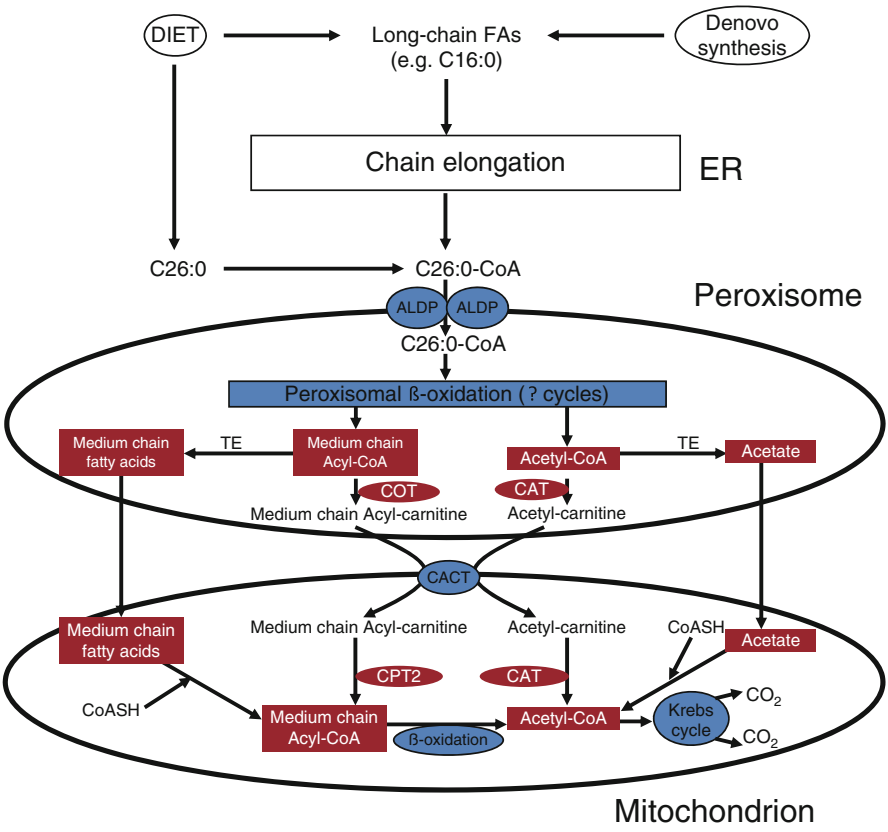

Fig. 2.1 Interplay between the endoplastic reticulum (ER), peroxisomes and mitochondria with respect to the biosynthesis and oxidation of very long-chain fatty acids, notably C26:0. Abbreviations used: ALDP AdrenoLeukoDystrophy Protein, TE Acyl-CoA thioesterase

2.2.1.3 Di- and Trihydroxycholestanoic Acid (DHCA and THCA)

These FAs are produced from cholesterol in the liver and are the immediate precursors of the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA). Formation of CA and CDCA first involves formation of the CoA esters of DHCA and THCA at the endoplasmic reticulum membrane after which the CoA esters enter the peroxisome, possibly mediated by the peroxisomal half-ABC transporter PMP70, to undergo beta-oxidation in peroxisomes to produce choloyl-CoA and chenodeoxycholoyl-CoA. Peroxisomes also contain an enzyme named BAAT (bile acid-CoA: amino acid N-acyltransferase) which can convert the CoA esters of CA and CDCA into the taurine and glycine conjugates which are then exported out of the peroxisome into the cytosol and subsequently out of the hepatocytes across the canalicular membrane as mediated by the bile salt export pump (BSEP) encoded by *ABCD11* (see Fig. 2.3).

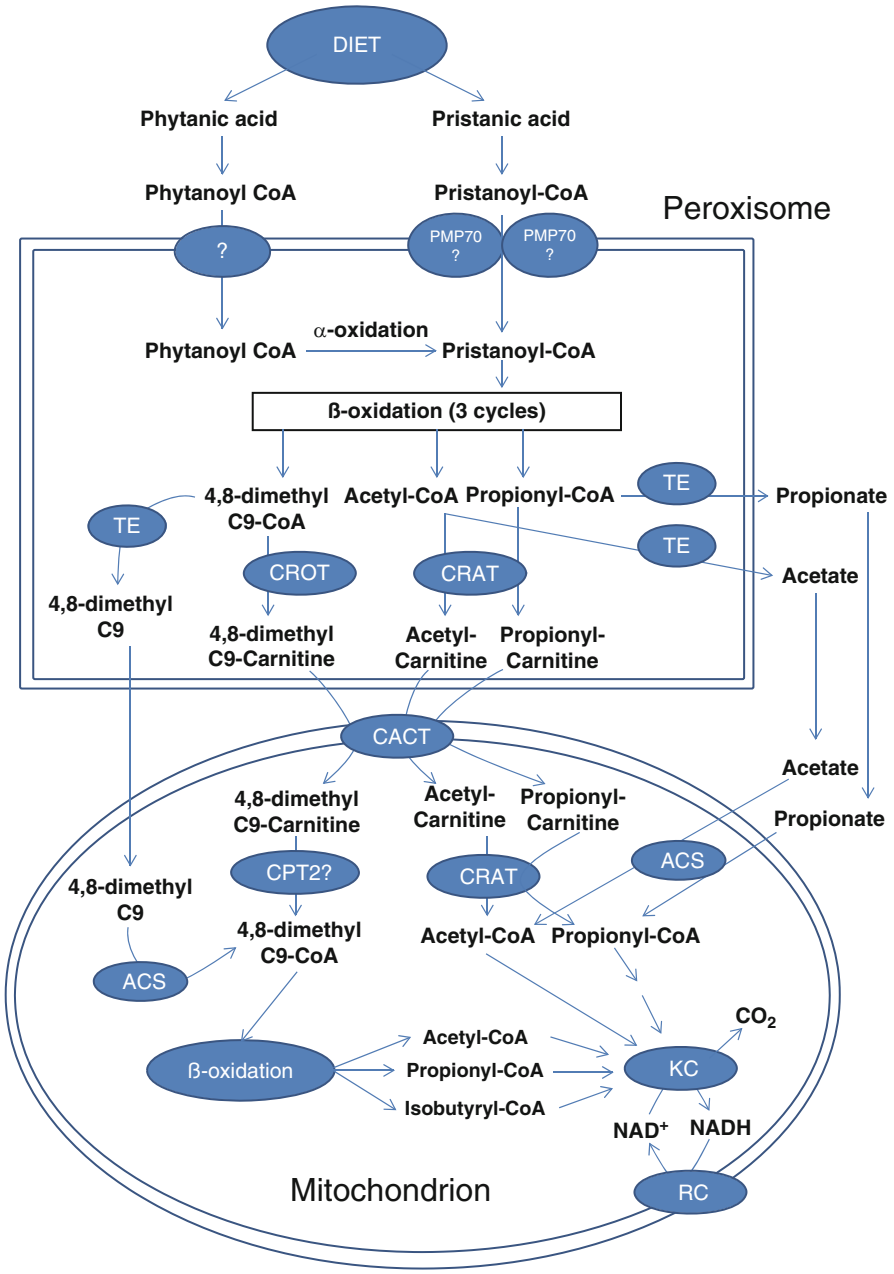

Fig. 2.2 Degradation of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) and pristanic acid (2,6,10,14-tetramethylpentanoic acid) and the interplay between peroxisomes and mitochondria. Abbreviations used: TE Acyl-CoA thioesterase, ACS Acyl-CoA synthetase, CRAT Carnitine Acetyltransferase, CROT Carnitine Octanoyltransferase, CPT2 Carnitine Palmitoyltransferase 2, RC Respiratory chain, and KC Krebs (citric acid) cycle

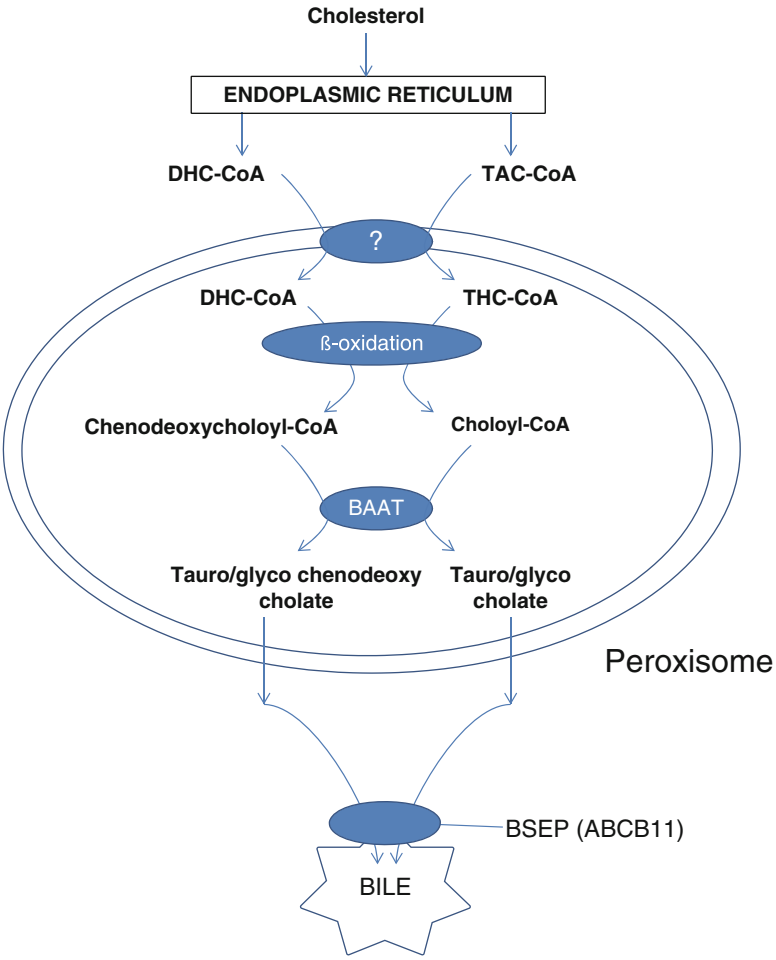

Fig. 2.3 Synthesis of the primary bile acids tauro/glycochenodeoxycholate and taura/glycocholate from cholesterol and the involvement of both the endoplasmic reticulum and peroxisomes and their extrusion into bile via BSEP (ABCB11)

2.2.1.4 Docosahexaenoic Acid Synthesis

Peroxisomes also play a central role in the formation of docosahexaenoic acid (DHA; C22:6n-3) by catalyzing the beta-oxidation of C24:6n-3 as generated from linolenic acid (C18:3n-3) (see Fig. 2.4).

The four steps of peroxisomal beta-oxidation are catalyzed by two acyl-CoA oxidases, two so-called bifunctional proteins and two thiolases. Although there is some overlap in the substrate specificities of the two acyl-CoA oxidases (Vanhove et al. 1993), it is acyl-CoA oxidase 1 (ACOX1) which catalyzes the dehydrogenation

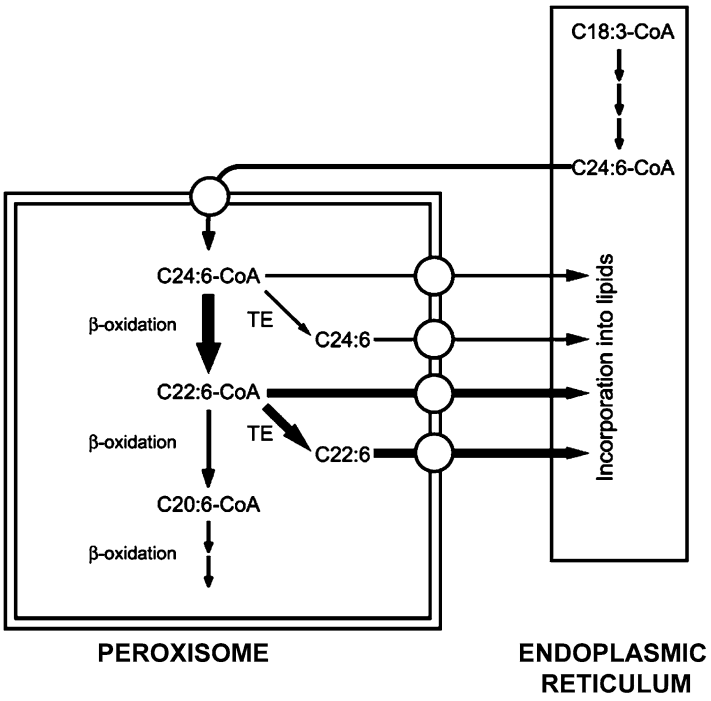

Fig. 2.4 Biosynthesis of C22:6n-3 from C18:3n-3 and the interplay between the endoplasmic reticulum and peroxisomes. Abbreviations used: TE Acyl-CoA thioesterase

of the VLCFAs C24:0- and C26:0-CoA whereas pristanoyl-CoA and di- and trihydroxycholestanoyl-CoA are solely handled by acyl-CoA oxidase 2, also called branched-chain acyl-CoA oxidase (BCOX) (Vanhove et al. 1993). Two subsequent steps of beta-oxidation, i.e. enoyl-CoA hydration and 3-hydroxyacyl-CoA dehydrogenation, may be catalyzed by one of two different peroxisomal bifunctional enzymes. These two bifunctional enzymes have different names including L-and D-bifunctional protein (LBP and DBP) which refers to the fact that LBP has an L-3-hydroxyacyl-CoA as intermediate, whereas a D-3-hydroxyacyl-CoA is formed in the catalytic mechanism of DBP. Available evidence indicates that DBP is involved in the oxidation of C26:0, pristanic acid, and di- and trihydroxycholestanoic acid, whereas LBP plays a key role in the beta-oxidation of dicarboxylic acids (Houten et al. 2012). Finally, the two thiolases in peroxisomes also have different substrate specificities with peroxisomal thiolase 2 (pTH2), better known as SCPx, as the enzyme involved in the beta-oxidation of pristanic acid and di- and tri-hydroxycholestanoic acid. Both thiolases appear to be active in C26:0 beta-oxidation (for review see (Wanders and Waterham 2006)).

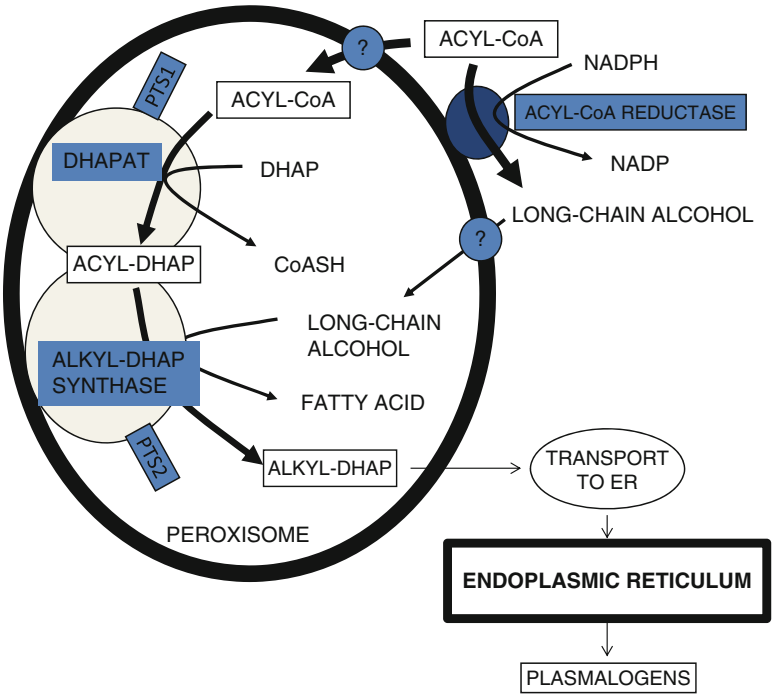

Fig. 2.5 Biosynthesis of plasmalogens and the involvement of both peroxisomes and the endoplasmic reticulum. For abbreviations: see text

2.2.2 Etherphospholipid Biosynthesis

Peroxisomes play a crucial role in the synthesis of etherphospholipids since the first part of the biosynthetic pathway is solely peroxisomal. This includes the enzyme alkyldihydroxyacetone phosphate synthase (ADHAPS) encoded by *AGPS* which is responsible for the generation of the characteristic ether-bond. The two substrates required in the ADHAPS enzyme reaction, i.e. a long chain alcohol and acyldihydroxyacetone phosphate (acyl-DHAP) are also synthesized by peroxisomes via the enzymes acyl-CoA: NADPH oxidoreductase and dihydroxyacetone phosphate acyltransferase (DHAPAT), respectively. DHAPAT and ADHAPS form a complex bound to the inner face of the peroxisomal membrane. The end products of the ADHAPS reaction, i.e. alkyl-DHAP is converted into alkylglycerol-3 phosphate (alkylG3P) either within peroxisomes or at the ER membrane (Brites et al. 2004). All the subsequent steps required for the synthesis of plasmalogens are catalyzed by ER enzymes (see Fig. 2.5).

2.2.3 Fatty Acid Alpha-Oxidation

Some FAs, notably those which contain a methyl group at the 3-position, require alpha-oxidation for breakdown. Alpha-oxidation is completely different from beta-oxidation, generates a one-carbon unit (formyl-CoA) rather than a two-carbon unit (acetyl-CoA), and involves a unique set of enzymes (for review see: Wanders et al. 2011 and Van Veldhoven 2010). FA alpha-oxidation involves a 5-step mechanism which includes: (1) activation to an acyl-CoA; (2) hydroxylation of the acyl-CoA to a 2-hydroxyacyl-CoA; (3) cleavage of the 2-hydroxyacyl-CoA into an (n-1) aldehyde plus formyl-CoA; (4) oxidation of the aldehyde to the corresponding acid, and (5) formation of an acyl-CoA ester. The best substrate known to be alpha-oxidized is phytanic acid which upon alpha-oxidation generates pristanic acid. Although the basic enzymatic machinery involved in alpha-oxidation has been resolved in recent years, the identity of some of the enzymes from the pathway have remained ill-defined. This does not apply to the enzymes catalyzing steps 2 and 3 of the pathway which includes phytanoyl-CoA hydroxylase and hydroxyacyl-CoA lyase which have been well characterized (see (Van Veldhoven 2010) and (Wanders et al. 2011) for review).

2.2.4 Glyoxylate Detoxification

In humans, peroxisomes play a key role in the breakdown of glyoxylate. This is due to the fact that the main enzyme involved in the detoxification of glyoxylate, i.e. alanine glyoxylate aminotransferase (AGT) is solely peroxisomal in humans (Salido et al. 2012).

2.3 Metabolic Interplay with Other Subcellular Organelles

2.3.1 The Peroxisome–Mitochondrion Connection

Unlike mitochondria which can degrade FAs all the way to CO_2 and H_2O, peroxisomes are completely dependent on other organelles, notably mitochondria, for full oxidation to CO_2 and H_2O. Indeed, the end products of peroxisomal beta-oxidation need to be exported from the peroxisome interior into the cytosol and subsequently taken up into mitochondria. The end products of peroxisomal beta-oxidation which include acetyl-CoA, propionyl-CoA and other acyl-CoAs such as 4,8-dimethylnonanoyl-CoA, can be transferred to mitochondria via one of two different mechanisms. The first involves conversion of the acyl-CoAs to the corresponding acylcarnitines via one of the many acyltransferases present in peroxisomes including carnitine acetyltransferase (CrAT) and carnitine octanoyl-

transferase (CrOT) (Hunt et al. 2012). The acylcarnitines are then transported across the peroxisomal membrane into the cytosol followed by the uptake into mitochondria via the mitochondrial carrier protein carnitine-acylcarnitine translocase (CACT), encoded by *SLC25A2*. The other mechanism involves hydrolytic cleavage of the different acyl-CoAs in peroxisomes via one of the many thioesterases identified in peroxisomes followed by release of the FAs from peroxisomes and uptake into the mitochondria, reconversion into the acyl-CoA ester and further metabolism. This is just one example of the interaction between peroxisomes and other subcellular organelles, in this case mitochondria (Fig. 2.2).

2.3.1.1 NADH

During beta-oxidation NAD^+ is reduced in the third step of peroxisomal beta-oxidation to generate NADH. Continued beta-oxidation of FAs in peroxisomes requires the reoxidation of NADH back to NAD^+. This is achieved via so-called redox-shuttles of which the mitochondrial malate/aspartate-shuttle allowing reoxidation of cytosolic NADH to NAD^+, is one of the best examples. In the yeast *S. cerevisiae* we have established that reoxidation of intraperoxisomal NADH is achieved via a redox-shuttle involving cytosolic and peroxisomal malate dehydrogenases (van Roermund et al. 1995). The identity of the redox-shuttle in higher eukaryotes including humans remains to be established definitively although evidence in favour of a lactate dehydrogenase based redox-shuttle has been published (Baumgart et al. 1996).

2.3.1.2 NADPH

Oxidation of (poly)unsaturated fatty acids requires NADPH in the dienoyl-CoA reductase reaction. Reduction of $NADP^+$ back to NADPH, is mediated by a redox-shuttle involving cytosolic and peroxisomal NADP-linked isocitrate dehydrogenases (van Roermund et al. 1998).

2.3.2 The Peroxisome-ER Connection

There is also cross-talk between peroxisomes and other subcellular organelles other than mitochondria. This is especially true for the ER. Indeed, the synthesis of ether-phospholipids (EPLs) is a typical example of the cross-talk between peroxisomes and the ER. Since the first steps of EPL synthesis take place in peroxisomes up to the level or alkyl-DHAP of alkylG3P. All subsequent steps of EPL synthesis take place at the ER (see Fig. 2.5).

Needless to say that there are also other forms of cross-talk between peroxisomes and other subcellular components, notably the nucleus. Indeed, several metabolites

generated within peroxisomes are signalling molecules and affect gene expression by binding to a transcription factor. Examples are the primary bile acids which are known ligands for FXR as discussed later in this review.

2.4 Human Peroxisomal Diseases (PDs)

ZS is the prototype of the group of peroxisomal disorders which now constitutes some 15 different diseases. The PDs are usually subdivided into two groups including (1) the peroxisome biogenesis disorders (PBDs) and (2) the single peroxisomal enzyme deficiencies (PEDs). Although individually rare the incidence of the group of PDs as a whole is substantial with a frequency of at least 1:5,000. Adrenoleukodystrophy (ALD) is the most frequent PD with an estimated incidence of 1:13,000. Recently, two new PDs have been identified in which the defect does not affect peroxisome biogenesis per se or one of the metabolic pathways of peroxisomes but rather the maintenance of peroxisomes with defects at the level of the elongation and fission of peroxisomes respectively (Waterham et al. 2007; Ebberink et al. 2012). In Table 2.1 the individual PDs are listed with information

Table 2.1 The peroxisomal disorders

			Defective mutant		
Disorder	Abbreviation	MIM	Defective protein	Mutant gene	Locus
Disorders of peroxisome biogenesis	PBD				
• ***PBD-group A:***					
Zellweger spectrum disorders	ZSD				
(1.) Zellweger syndrome	ZS	214100	PEX1	*PEX1*	7q21.2
(2.) Neonatal adrenoleukodystrophy	NALD	214110	PEX2	*PEX2*	8q21.1
	IRD	202370	PEX3	*PEX3*	6q24.2
(3.) Infantile Refsum disease			PEX5	*PEX5*	12p13.3
			PEX6	*PEX6*	6p21.1
			PEX10	*PEX10*	1p36.32
			PEX12	*PEX12*	17q12
			PEX13	*PEX13*	2p14-p16
			PEX14	*PEX14*	1p36.22
			PEX16	*PEX16*	11p11.2
			PEX19	*PEX19*	1q22
			PEX26	*PEX26*	22q11.21

(continued)

Table 2.1 (continued)

Disorder	Abbreviation	MIM	Defective mutant		
			Defective protein	Mutant gene	Locus
• **PBD-group B:**					
(4.) Rhizomelic chondrodysplasia Type 1	RCDP-1	215100	PEX7p	*PEX7*	6q21-q22.2
Disorders of peroxisome function					
• ***Fatty acid beta-oxidation***					
(5.) X-linked adrenoleukodystrophy	X-ALD	300100	ALDP	*ABCD1*	Xq28
(6.) Acyl-CoA oxidase deficiency	ACOX-deficiency	264470	ACOX1	*ACOX1*	17q25.1
(7.) D-Bifunctional protein deficiency	DBP-deficiency	261515	DBP/MFP2/ MFEII	*HSD17B4*	5q2
(8.) Sterol-carrier-protein X deficiency	SCPx-deficiency	–	SCPx	*SCP2*	1p32
(9.) 2-Methylacyl-CoA racemase deficiency	AMACR-deficiency	604489	AMACR	*AMACR*	5p13.2-q11.1
• ***Etherphospholipid biosynthesis***					
(10.) Rhizomelic chondrodysplasia puncatata Type 2	RCDP-2	222765	DHAPAT	*GNPAT*	*1q42.1-42.3*
(11.) Rhizomelic chondrodysplasia puncatata Type 3	RCDP-3	600121	ADHAPS	*AGPS*	2q33
• ***Fatty acid alpha-oxidation***					
(12.) Refsum disease	ARD/CRD	266500	PHYH/ PAHX	*PHYH/ PAHX*	10p15-p14
• ***Glyoxylate metabolism***					
(13.) Hyperoxaluria Type 1	PH-1	259900	AGT	*AGTX*	2q37.3
• ***Bile acid synthesis (conjugation)***					
(14.) Bile acid-CoA: amino acid N-acyltransferase deficiency	BAAT-deficiency	607748	BAAT	*BAAT*	9q31.1
• ***H_2O_2-metabolism***					
(15.) Acatalasemia		115500	Catalase	*CAT*	11p13

on the type of (enzyme) protein involved, the encoding gene and other relevant information. The laboratory diagnosis of PDs has been worked out well over the last few years and *bona fide* methods have been developed for the pre- and postnatal diagnosis of these disorders.

One of the areas in which much less progress has been made, concerns the development of treatment strategies for each of the individual PDs. Indeed, for most PDs treatment is only supportive and not curative with only few exceptions. These are: (1) Refsum disease in which a life-long diet to reduce phytanic acid intake as much as possible, is able to stop progression of at least some of the disease manifestations; (2) hyperoxaluria type 1 in which oxalate accumulates as a consequence of the deficient activity of AGT which causes the impaired degradation of glyoxylate and the subsequent accumulation of oxalate. In patients with a full deficiency of AGT, combined liver-kidney transplantation is the only option whereas in patients with residual AGT activity, pyridoxine supplementation may be successful in avoiding development of end-stage kidney disease; (3) X-linked adrenoleukodystrophy in which VLCFAs accumulate as a consequence of mutations in the *ABCD1* gene which codes for the peroxisomal half-ABC transporter ALDP. Available evidence suggests that ALDP catalyses the transport of VLCFAs across the peroxisomal membrane in their CoA-ester form. Hematopoietic stem cell gene therapy which involves the *ex vivo* correction of $CD34^+$ cells from ALD patients via lentiviral-mediated transfer of *ABCD1*, appears to halt disease progression of childhood cerebral ALD (CCALD) patients (Cartier et al. 2009).

2.5 Peroxisomes and Signal Transduction

In the last few years considerable progress has been made with respect to the role of peroxisomes and signal transduction. Peroxisomes may affect signal transduction in various ways which may be different under physiological and pathophysiological conditions. Examples of the importance of peroxisomes for signal transduction under physiological conditions are described below. We will first concentrate on metabolites generated by peroxisomes which are ligands for transcription factors and thereby signal to the nucleus directly.

2.5.1 Tauro Glycocholate and Tauro Glycochenodeoxycholate

As described above, tauro glycocholate and tauro glycochenodeoxycholate are synthesized by peroxisomes via the concerted action of branched-chain acyl-CoA oxidase, D-bifunctional protein, SCPx and the enzyme BAAT. The latter enzyme appears to be fully peroxisomal rather than shared between peroxisomes and other subcellular organelles as believed previously (Pellicoro et al. 2007). The primary

bile acids are ligands for the nuclear hormone receptor FXR which after binding, heterodimerizes with RXR followed by translocation into the nucleus and binding to defined response elements present in the promoters of multiple genes. One of the best known effects of the C_{24}-primary bile acids is down regulation of the de novo bile acid synthesis pathway. The prime target here is *CYP7A1* which codes for the first enzyme involved in bile acid synthesis which is cholesterol 7-alpha-hydroxylase (CYP7A1). FXR inhibits *CYP7A1* transcription via two different mechanisms. The first mechanism involves binding of the bile acids to FXR which then activates transcription of *SHP*(short heterodimeric partner). SHP then inhibits transcription of *LRH1*, the gene coding for the liver receptor homologue 1. LRH-1 normally transactivates *CYP7A1* (as well as *CYP7B1*). The other mechanism is centered around Fibroblasts Growth Factor 19 (FGF19) also called FGF15 in rodents which is an enterokine synthesized and released when bile acids are taken up into the ileum (Beenken and Mohammadi 2009). FGF15/19 is expressed in the distal small intestine where its synthesis is regulated by FXR after the postprandial uptake of bile acids (Holt et al. 2003; Inagaki et al. 2005). Thus, FGF15/19 levels rise in response to feeding (Kurosu et al. 2007). Following transport of FGF15/19 to the liver via the circulation, FGF15/19 signals to the liver via the receptor FGFR4 which forms a complex with beta-Klotho. Both FGFR4 and beta-Klotho are highly expressed in the liver. Binding of FGF15/19 results in the activation of intracellular signalling pathways ultimately giving rise to inhibition of *CYP7A1* transcription. Using intestine-selective FXR knock-out mice, it has recently been shown that the intestinal FXR-FGF15/19 signalling pathway contributes to the regulation of hepatic bile acid synthesis in mice mainly during the dark phase (Stroeve et al. 2010).

2.5.2 N-acylglycines and N-acyltaurines

N-acyl amino acids are emerging as signalling molecules with a wide range of biological activities (Bradshaw and Walker 2005). For instance, arachidonoyl taurine activates the transient receptor potential vanilloid (TRPV) receptor 1 and 4 ion-channels (Saghatelian et al. 2006). Furthermore, different N-acylglycines have been found to be ligands for a number of G-protein coupled receptors such as GPR18 (Kohno et al. 2006), GPR22 (Oh et al. 2008), and GPR 72 (Roy and Hannedouche 2007). Structurely linked to the endocannabinoids, the family of N-acyl amino acids now constitutes fatty acid conjugates with the full complement of the common 20 amino acids (Tan et al. 2010).

Work notably by Alexson and Hunt and collaborators (for review see: Hunt et al. 2012) has revealed that peroxisomes contain the enzymatic machinery to synthesize taurine and glycine conjugated (i.e. amidated) FAs. To start with, the human BAAT enzyme can conjugate long- and very-long chain acyl-CoAs to glycine and taurine although the acyl-CoA:amino acid N-acyltransferase activity of BAAT is only some 10 % of its bile acid-CoA:amino acid N-acyltransferase activity

(O'Byrne et al. 2003). At least in the mouse, two additional acyl-CoA:amino acid N-acyltransferases named Acnat1 and Acnat2 have been identified of which at least Acnat1 seems to be specific for taurine and a range of acyl-CoA esters but not the bile acid-CoA esters (Reilly et al. 2007).

2.5.3 Docosahexaenoic Acid (DHA)

DHA may be derived form exogenous dietary sources but is also synthesized by peroxisomal beta-oxidation of C24:6ω3. DHA is not only a precursor molecule from which multiple biologically active compounds are synthesized including certain resolvins, NPD1, and maresins (Stables and Gilroy 2011; Savary et al. 2012) but is itself also biologically active. Indeed, DHA activates several nuclear receptors and transcription factors including PPAR-alpha, RXR-alpha, HNF4-alpha, and SREBP (Kliewer et al. 1997; Hertz et al. 1998). Furthermore, DHA competes with oxysterols for binding to LXR-alpha thereby antagonizing the effect of sterols and are known to repress SREBP1 expression and proteolytic activation (Yoshikawa et al. 2002) (for review see (Jump et al. 2005; Savary et al. 2012)).

2.5.4 Etherphospholipids Including Platelet Activation Factor (PAF) and Plasmalogens

Synthesis of both PAF and plasmalogens is completely dependent upon peroxisomes since the enzyme which is responsible for the introduction of the characteristic ether-bond present in PAF and plasmalogens, i.e. alkyl-DHAP synthase, is strictly peroxisomal. PAF has been implicated in many pathophysiological conditions and is a ligand for the PAF-receptor (PAFR) which belongs to the large family of G-protein coupled receptors (GPCR). Several relatives of the PAF-receptor (PAFR) branch within the GPCR-family regulate the PI3K/Akt signalling pathway either positively or negatively. These opposing effects on cell survival endow PAF with very potent "double-edged" inflammatory and cytotoxic properties by promoting the survival of inflammatory mediator-producing cells and tissue destruction by directly causing cell death. The mechanisms underlying these contrasting effects are not yet resolved (for review see (Lu et al. 2008)).

Etherphospholipids with plasmalogens as their main representative, at least in higher eukaryotes including humans, are not so much ligands for certain receptors or transcription factors, but do play a major role in signal transduction because the physical properties of plasmalogens are way different as compared to those of the corresponding di-acyl phospholipds. Studies in cells from plasmalogen deficient patients have shown that cellular membranes are more fluid in the absence

of plasmalogens (Wanders and Brites 2010). Importantly, as shown by Rodemer et al. plasmalogens are the dominant glycerolethanolamine species in lipid raft microdomains (LRMs) isolated from myelin (Rodemer et al. 2003). Studies in mice and humans affected by defects in etherphospholipid synthesis, have revealed many pathological abnormalities including defects in spermatogenesis, lens development and CNS myelination. In an effort to try to combine the findings from Rodemer et al. (2003) with these pathological abnormalities, Gorgas and co-workers noted that in all three tissues cell-cell and/or cell-extracellular matrix interactions play an important role in tissue developmental processes (Gorgas et al. 2006). Importantly, many of the junctional proteins involved are glycosylphosphatidyl (GPI)-anchored and sorted to LRMs representing platforms enabling the concentration of junctional, signalling and exo/endocytotic activities.

A defect in etherphospholipid synthesis might interfere with LRM function in various ways. First, plasmalogens are major constituents of LRMs, notably in myelin (Rodemer et al. 2003) and second, plasmalogens may affect the functioning of LRMs in yet another way by changing the intracellular distribution and homeostasis of cholesterol that by itself is an important LRM constituent. Finally, it should be noted that plasmalogens are not the only end point of etherphospholipid biosynthesis. Although grossly overlooked so far, many GPI-anchored proteins contain a GPI-anchor containing an ether-linked alkyl chain. Synthesis of such lipid anchors does require a functional ether-phospholipid biosynthesizing system. Interestingly, such GPI-anchored proteins localize to LRMs. It should be noted that older literature has already pointed to abnormalities in signal transduction in plasmalogen deficient cells (Perichon et al. 1998). Future studies will have to resolve this issue in more detail.

2.5.5 ROS-RNS Metabolism

Peroxisomes generate reactive oxygen species (ROS) and reactive nitrogen species (RNS) as by-products of normal metabolic reactions taking place in peroxisomes but at the same time they possess delicate protective mechanisms to counteract oxidative stress and maintain redox homeostasis (del Río et al. 2002, 2006). Examples of the first group of enzymes, at least in humans, are the many oxidases present in peroxisomes including the two acyl-CoA oxidases, D-amino acid oxidase, D-aspartate oxidase, L-pipecolate oxidase, L-2-hydroxy acid oxidase 1 and 2, and polyamine-oxidase which all generate H_2O_2. Peroxisomes are also believed to contain inducible nitric oxide synthase which generates nitric oxide ($NO^{\cdot}$) and superoxide ($O_2^{\cdot -}$) (del Río 2011; Fransen et al. 2012). On the other hand, peroxisomes contain a number of anti-oxidant enzymes including catalase, superoxide dismutase 1, peroxiredoxin 5, and GST Kappa 1 (for a review see Chap. 13 and (Fransen et al. 2012)). Much of the intricate details which define the precise role of peroxisomes in ROS/RNS-signalling remains to be elucidated in the future (for a review see Chaps. 13 and 15, and Fransen et al. (2012)).

2.5.6 Prostanoids, H(P)ETEs, Lipoxins, Resolvins, Protectins, Maresins and Other Bioactive Lipids

Although long underrated as just membrane constituents and energy storage molecules, lipids are now recognized as potent signalling molecules that regulate a myriad of physiological processes by receptor-mediated pathways affecting key processes like cell growth and cell death, and inflammation/infection (Stables and Gilroy 2011). Although peroxisomes are not so much involved in the biosynthesis of all these different bioactive lipids, they do play an important role in their homeostasis. This is due to the fact that inactivation of these bioactive molecules, which is definitely necessary in the resolution phase of inflammation for instance, involves hydroxylation or reduction of a double-bond but also chain-shortening by beta-oxidation in peroxisomes. Indeed, a series of publications have shown that peroxisomes play an essential role in the beta-oxidation of numerous prostanoids including PGF2-alpha (Diczfalusy et al. 1991), 8-iso-PGF2-alpha (Tsikas et al. 1998), thromboxane-B2 (Diczfalusy et al. 1993; de Waart et al. 1994), monohydroxy-eicosatrienoic acid (12-HETE) (Spector et al. 2004), (15-HETE), leukotriene-B4 (Jedlitschky et al. 1993; Mayatepek and Flock 1999), and the cysteinyl leukotriene LTE4 (Mayatepek et al. 2004) see (Van Veldhoven 2010). Exactly how important peroxisomal beta-oxidation of bioactive lipids is in the resolution phase of inflammation for instance has not been elucidated yet (Fig. 2.6).

2.5.7 Phytanic Acid

Work by Reiser and co-workers has shown that phytanic acid at least at supra-physiological concentrations, affects signal transduction through activation of the free fatty acid receptor GPR40 (Reiser et al. 2006). Earlier work from the same group had shown that phytanic acid and pristanic acid when added to hippocampal neurons, astrocytes and oligodendrocytes elicited a complex array of toxic activities including mitochondrial dysfunction and calcium deregulation which involved the intracellular inositol 1,4,5-triphosphate ($InsP_3$) calcium signalling pathway (Ronicke et al. 2009). These observations suggested the involvement of a membrane receptor coupled to calcium signalling. The authors selected GPR40 as most likely candidate and subsequently showed that phytanic acid and pristanic acid are indeed potent agonists of GPR40 with EC50 values of 34.5 ± 12.9 μmol/L and 7.6 ± 3.1 μmol/L respectively (Kruska and Reiser 2011). Under physiological conditions phytanic acid and pristanic acid levels are <20 and <3 μmol/L, respectively with only a small percentage present in the free acid form which argues against any significant activation of GPR40 under physiological conditions. The situation may be completely different in patients affected by Refsum disease in whom total phytanic acid levels in plasma may reach levels >1000 μmol/L.

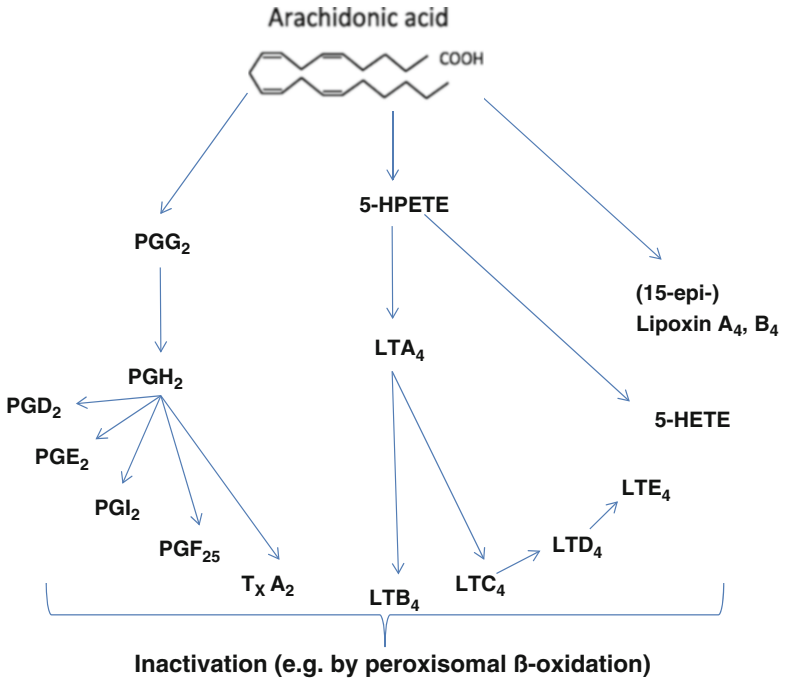

Fig. 2.6 Arachidonic acid and its multiple products and their inactivation via different mechanisms, including peroxisomal beta-oxidation (see text)

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Chapter 3
Aging, Age-Related Diseases and Peroxisomes

Marc Fransen, Marcus Nordgren, Bo Wang, Oksana Apanasets, and Paul P. Van Veldhoven

Contents

Abstract Human aging is considered as one of the biggest risk factors for the development of multiple diseases such as cancer, type-2 diabetes, and neurodegeneration. In addition, it is widely accepted that these age-related diseases result from a combination of various genetic, lifestyle, and environmental factors. As biological aging is a complex and multifactorial phenomenon, the molecular mechanisms underlying disease initiation and progression are not yet fully understood. However, a significant amount of evidence supports the theory that oxidative stress may act as a primary etiologic factor. Indeed, many signaling components like kinases, phosphatases, and transcription factors are exquisitely sensitive to the cellular redox status, and a chronic or severe disturbance in redox homeostasis can promote cell

M. Fransen (✉) • M. Nordgren • B. Wang • O. Apanasets • P.P. Van Veldhoven
Department of Cellular and Molecular Medicine, KU Leuven, Herestraat 49, box 601, B-3000 Leuven, Belgium
e-mail: marc.fransen@med.kuleuven.be

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proliferation or trigger cell death. Now, almost 50 years after their discovery, there is a wealth of evidence that peroxisomes can function as a subcellular source, sink, or target of reactive oxygen and nitrogen molecules. Yet, the possibility that these organelles may act as a signaling platform for a variety of age-related processes has so far been underestimated and largely neglected. In this review, we will critically discuss the possible role of peroxisomes in the human aging process in light of the available data.

Keywords Peroxisomes • Interorganellar crosstalk • Catalase • Oxidative stress • Redox signaling

Abbreviations

αS	α-synuclein
Aβ	Amyloid-β peptide
ACOX1	Acyl-CoA oxidase 1
AD	Alzheimer's disease
AGPS	Alkylglycerone phosphate synthase
AOX	Antioxidants
CAT	Catalase
DAO	D-amino acid oxidase
DHA	Docosahexaenoic acid
ER	Endoplasmic reticulum
(F)ALS	(familial) Amyotrophic lateral sclerosis
GNPAT	Glyceronephosphate O-acyltransferase
MAM	Mitochondrial-associated membrane
NEFAs	Non-esterified fatty acids
NFTs	Neurofibrillary tangles
PBDs	Peroxisome biogenesis disorders
PD	Parkinson's disease
PPAR-α	Peroxisome proliferator activated receptor-alpha
PRDX5	Peroxiredoxin 5
PTS1	C-terminal peroxisomal targeting signal
PUFAs	Polyunsaturated fatty acids
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD1	Cu/Zn superoxide dismutase
TNF-α	Tumor necrosis factor-alpha
UPR	Unfolded protein response
VLCFAs	Very-long-chain fatty acids
X-ALD	X-linked adrenoleukodystrophy

3.1 Aging and Organelle Dysfunction: The Inextricable Link

Over the years, mammalian aging has been linked to a wide range of cellular changes, such as telomere shortening, DNA methylation, organelle dysfunction, a decline in autophagic and proteasomal proteolysis, incorrect protein folding, and alterations in membrane lipid composition (Hamanaka and Chandel 2010; Boya 2012). Considerable efforts have already been made to integrate this information into a unifying model (Terman et al. 2010; Sahin and DePinho 2012). However, although the general consensus is that progressive DNA damage and mitochondrial decline may act as prime instigators of natural aging, it remains to be established how all these factors are interconnected and drive this process (Sahin and DePinho 2012). In the context of this chapter, we will briefly discuss the intricate relationship between organelle (dys)function and aging (Fig. 3.1).

Currently, there is good evidence that the process of physiological aging is associated with changes in organelle composition, morphology and function (Soltow et al. 2010). For example, it has repeatedly been reported that both mitochondrial respiratory function and lysosomal activity decline with age (Gómez and Hagen 2012; Rajawat et al. 2009). These findings, among others, have led to the mitochondrial-lysosomal axis theory of aging (Terman et al. 2010). This theory proposes that defective and enlarged mitochondria, which produce less ATP and generate increased amounts of reactive oxygen species (ROS), progressively accumulate with age as a result of imperfect autophagocytosis. On the other hand, there is also abundant evidence that organelles themselves can contribute to the pathophysiology of age-related diseases (Hamanaka and Chandel 2010; Schon and Przedborski 2011; Boya 2012). This may occur through their normal functions or as a result of damage (e.g. by exposure to harmful substances such as oxygen radicals). For example, changes in nuclear envelope composition that result in more deformable nuclei may contribute to cancer progression (Zwerger et al. 2011), and protein misfolding in the endoplasmic reticulum (ER) may activate the unfolded protein response (UPR), which – in case stress is too severe – can initiate apoptosis via ER-mitochondrial Ca^{2+}-signaling (Decuypere et al. 2011). These examples also nicely illustrate that the reciprocal interplay between organelle activity and aging depends on dynamic and highly interconnected signaling networks. However, the identity of the interorganellar signaling pathways and messenger molecules remain largely to be determined. Nevertheless, it has recently been postulated that aging-associated functional losses are primarily caused by a progressive disruption of redox-regulated signaling mechanisms (Sohal and Orr 2012), and that at least several of these mechanisms may intersect and converge on mitochondria (Sahin and DePinho 2012).

3.2 Peroxisomes and Cellular Aging

During the last decade, mounting evidence has been collected suggesting that peroxisomes may actively contribute to the maintenance of the cellular redox balance (del Río 2011; Fransen et al. 2012). In addition, strong arguments have been

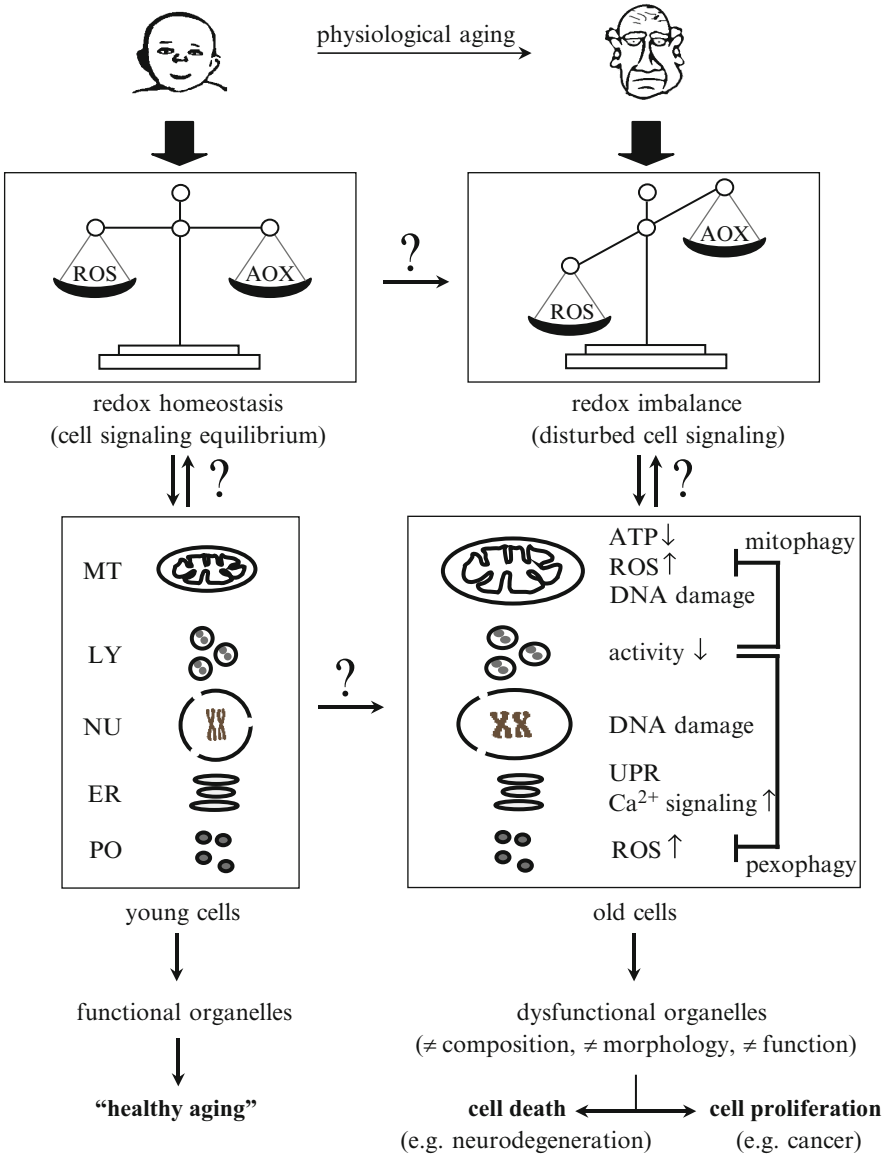

Fig. 3.1 Aging and organelle dysfunction. *AOX* antioxidants, *ER* endoplasmic reticulum, *LY* lysosomes, *MT* mitochondria, *NU* nucleus, *PO* peroxisomes, *ROS* reactive oxygen species, *UPR* unfolded protein response. See text for details

presented that peroxisomal metabolism and cellular aging are closely intertwined (Titorenko and Terlecky 2011), and that these organelles closely cooperate with mitochondria to fulfill their functions (Islinger et al. 2012). Each of these aspects

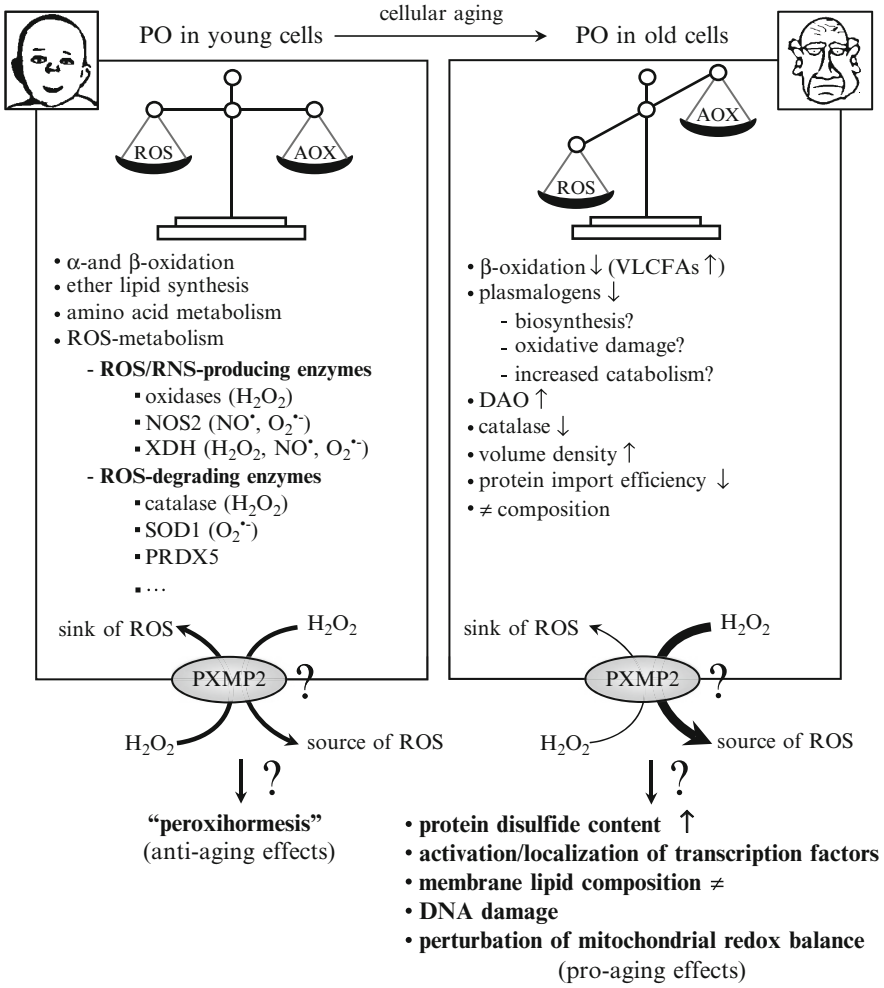

Fig. 3.2 Peroxisomes and cellular aging. *AOX* antioxidants, *DAO* D-amino acid oxidase, *NOS2* inducible nitric oxide synthase, *PO* peroxisomes, *PRDX5* peroxiredoxin 5; *PXMP2* porin-like peroxisomal membrane protein, *RNS* reactive nitrogen species, *ROS* reactive oxygen species, *SOD1* Cu/Zn superoxide dismutase, *VLCFAs* very-long-chain fatty acids, *XDH* xanthine dehydrogenase. See text for details

is discussed in more detail below (Fig. 3.2). Note that, as these sections are mainly focused on the possible role of peroxisomal ROS in the mammalian aging process, we refer to other informative reviews for pioneering research on this topic in other model organisms such as plants (del Río et al. 1992, 2006) and yeasts (Manivannan et al. 2012).

3.2.1 Peroxisomal Metabolism and Oxidative Stress

Mammalian peroxisomes are highly dynamic, multifunctional organelles that play a key role in various metabolic pathways, including fatty acid α- and β-oxidation, amino acid catabolism, and the biosynthesis of docosahexaenoic acid (DHA or 22:6 n-3), bile acids, and plasmalogens (Wanders and Waterham 2006). Many of the enzymes involved in these pathways produce ROS or reactive nitrogen species (RNS) as part of their normal catalytic cycle (see Chaps. 13 and 15). These species include hydrogen peroxide (H_2O_2), superoxide (O_2^-), and nitric oxide (NO). In addition, inside peroxisomes, H_2O_2 can decompose into the highly reactive hydroxyl radical (OH) via the transition metal-catalyzed Fenton reaction, and O_2^- and NO may rapidly combine to form peroxynitrite ($ONOO^-$). To combat the destructive effects of these molecules, peroxisomes also contain various antioxidant enzymes such as catalase (CAT), peroxiredoxin 5 (PRDX5), and Cu/Zn-superoxide dismutase (SOD1) (Antonenkov et al. 2010). For a detailed description of enzymes involved in peroxisomal ROS metabolism, we refer to other excellent reviews (Bonekamp et al. 2009; Antonenkov et al. 2010).

Given that mammalian peroxisomes contain copious amounts of H_2O_2-producing flavin-containing oxidases and the H_2O_2-decomposing enzyme catalase, these organelles can be considered as potentially significant sources or sinks of H_2O_2, a key molecule in cellular redox signaling. This has been nicely illustrated in a number of studies, which showed that (i) in rat liver, peroxisomes may be responsible for as much as 35 % of the total H_2O_2 production (Boveris et al. 1972), (ii) overexpression of the peroxisomal H_2O_2-producing enzyme acyl-CoA oxidase 1 (ACOX1) leads to activation of NFKB1, a pleiotropic redox-sensitive transcription factor that is involved in many biological processes, including cell growth and differentiation, inflammation, tumorigenesis, and apoptosis (Li et al. 2000), and (iii) inhibition of catalase activity by 3-aminotriazole increases the cellular protein disulfide content by 20 % (Yang et al. 2007). Moreover, as peroxisomes play an essential role in cellular lipid metabolism (Van Veldhoven 2010), they may also protect cells against adverse effects of oxidative stress by actively maintaining the optimal membrane lipid composition (Périchon et al. 1998). For example, a decrease in peroxisomal β-oxidation will influence the ability of the cell to shorten very-long-chain fatty acids (VLCFAs) and to synthesize sufficient amounts of DHA (Van Veldhoven 2010). As these VLCFAs may overaccumulate in cellular membranes and DHA is an important membrane phospholipid component, changes in peroxisomal β-oxidation are likely to alter membrane composition, structure, fluidity, and function. In addition, as the first two steps in the biosynthesis of plasmalogens occur exclusively in peroxisomes, a decrease in peroxisome activity will also affect the cell's ability to synthesize sufficient amounts of this class of glycerophospholipids, which normally constitutes up to 18 % of the total phospholipid mass in humans (Braverman and Moser 2012). Importantly, plasmalogens are characterized by the presence of a vinyl ether linkage at the *sn*-1 position, and it has been suggested that this functionality may serve as sacrificial

trap for free radicals and singlet oxygen (Wallner and Schmitz 2011). The finding that plasmalogen-deficient cells from patients with peroxisome biogenesis disorders (PBDs) display an increased sensitivity to UV-induced oxidative stress is in line with this hypothesis (Hoefler et al. 1991). Furthermore, plasmalogens are targets of hypochlorous (HOCl) and hypobromous (HOBr) acids, oxidants produced by myeloperoxidase, an enzyme released by activated leukocytes during inflammation (Pattison and Davies 2006). The generated 2-chloroaldehydes can in turn inhibit nitric oxide biosynthesis (Marsche et al. 2004).

In summary, these data clearly show that peroxisomes can participate, directly or indirectly, in the maintenance of cellular redox homeostasis. Nonetheless, the extent to which these organelles contribute to cellular oxidative stress and redox signaling remain to be established, and – over the years – this topic has generated some controversy. For example, while some studies state that peroxisome proliferation induces oxidative stress (Reddy et al. 1980), others claim that this process may prevent ROS production (Santos et al. 2005; Diano et al. 2011). However, one should always keep in mind that different physiological and environmental variables may predispose whether peroxisomes act as net sources or sinks of ROS within a cell (Fransen et al. 2012).

3.2.2 Peroxisomal Metabolism and Cellular Aging

Over the years, several studies have presented compelling evidence that peroxisomal metabolism and aging are closely intertwined (Titorenko and Terlecky 2011; and references therein). For example, peroxisomal β-oxidation, plasmalogen biosynthesis, and catalase activity have been reported to gradually decrease with age (Périchon et al. 1998; Braverman and Moser 2012). In addition, there are indications that the activities of at least some peroxisomal H_2O_2-producing enzymes (e.g. D-amino acid oxidase) increase with age (Périchon et al. 1998). Furthermore, proteomic analysis studies of peroxisome-enriched fractions from young and old mouse liver and kidney have identified a select set of age-dependent alterations (Mi et al. 2007; Amelina et al. 2011). Finally, it is also known that some peroxisomal enzymes – including catalase – progressively mislocalize to the cytosol as cells age, despite the fact that the volume density of these organelles gradually increases (Beier et al. 1993; Legakis et al. 2002). Whether this increase in peroxisome number is due to increased proliferation or reduced degradation remains to be investigated. However, as the autophagy-lysosomal system is the major degradation pathway for peroxisomes (Ezaki et al. 2011), it sounds reasonable that an age-related decline in lysosome activity may also result in an accumulation of peroxisomes when cells age (Fransen et al. 2012). In this context, it is also interesting to note that a pharmacological inhibition of autophagy raises the percentage of peroxisomes with an abnormally high redox state over time (Ivashchenko et al. 2011). In summary, these findings strongly indicate that cellular aging is likely to have a major impact on peroxisomal lipid and ROS metabolism.

At the moment, it is difficult to provide a definitive assessment of how alterations in peroxisomal pro- and antioxidant enzyme activities may influence the cellular aging process. However, in the light of the available evidence, the self-sustaining peroxisome deterioration spiral model is currently most likely the best working hypothesis (Terlecky et al. 2006). This model places catalase, the most abundant and efficient H_2O_2-metabolizing enzyme, in the centre of an aging spiral and is mainly based on two observations. Firstly, chronically reducing peroxisomal catalase activity induces multiple characteristics of cellular aging, including elevated H_2O_2-associated ROS levels, oxidative protein and DNA damage, the up-regulation of matrix metalloproteases secretion, a slow-down of cell proliferation, and mitochondrial impairment (Wood et al. 2006). Secondly, (extracellular) H_2O_2 causes Pex5p, the import receptor for peroxisomal matrix proteins containing a C-terminal peroxisomal targeting signal (PTS1), to amass on the organelle thereby reducing PTS1 protein import (Legakis et al. 2002). As such, a decrease in peroxisomal catalase activity may result in increased cellular concentrations of H_2O_2, and this may in turn further decrease the efficiency of peroxisomal matrix protein import and result in a self-perpetuating negative spiral. Indeed, as catalase contains a weak PTS1 that interacts poorly with Pex5p, this enzyme will most likely be particularly affected (Legakis et al. 2002). Importantly, the potential validity of this hypothesis has more recently been reinforced by studies showing that catalase-SKL, a molecule which contains a high affinity binding signal for Pex5p, delays the appearance of senescence markers in aging cells (Koepke et al. 2008).

Catalase is known to be one of the most efficient biocatalysts in our cells. Despite this, it has been shown that the enzyme can not completely prevent H_2O_2 release from intact peroxisomes (Mueller et al. 2002). This release most likely occurs through a porin-like channel (Rokka et al. 2009). As H_2O_2, depending on the cell type and its concentration, can have both positive (signaling) and negative (damaging) effects (Veal et al. 2007), it has been postulated that peroxisomal ROS production can play a dual role in determining cell fate: at low levels, peroxisomal ROS may mediate the communication between peroxisomes and other cell compartments and govern anti-aging processes; above a certain threshold, peroxisomal ROS may trigger diverse signaling cascades involved in cell proliferation and apoptosis or even act as a DNA damaging agent (Titorenko and Terlecky 2011). In this context, it is interesting to note that several studies have shown that catalase-overexpressing cells (or animals) are more sensitive to certain types of stressors compared to control cells (or animals) (Chen et al. 2004; Carter et al. 2004). These findings indicate that high levels of catalase activity can dampen H_2O_2-mediated signaling pathways.

3.2.3 The Peroxisome-Mitochondria Connection

Peroxisomes closely cooperate with mitochondria to fulfill their physiological functions (see Chap. 1). The best-known example is perhaps the oxidative metabolism of specific fatty acids, which are initially processed in peroxisomes and then

trafficked to mitochondria where their catabolism is completed (Van Veldhoven 2010). Further evidence that peroxisomes and mitochondria are metabolically linked can be (indirectly) inferred from the observations that both organelles share a redox-sensitive relationship (for more details, see next paragraph) and are linked by division (Islinger et al. 2012). The latter finding reflects the view that the sharing of division proteins may allow the cell to strictly coordinate the number and morphology (and thus activity) of both organelles (Islinger et al. 2012). Finally, peroxisomes and mitochondria have also been reported to cooperate in anti-viral signaling and defense (Dixit et al. 2010).

It is already known for a long time that mitochondrial size, shape, number and functionality is aberrant in human patients and mice with peroxisome biogenesis disorders (Goldfischer et al. 1973; Baes et al. 1997). Although the molecular mechanisms underlying these changes remain unclear, there is considerable evidence that a disturbance in peroxisomal redox homeostasis may affect mitochondrial redox balance, structure and function. For example, it has been shown that brain mitochondria exhibit a decreased respiratory capacity in a catalase-deficient mouse model (Ho et al. 2004). In the meantime, this finding has been indirectly confirmed and extended by others who have shown that a complete or even partial inhibition of catalase activity in intact cells rapidly impairs the mitochondrial redox state, inner membrane potential, and function (Koepke et al. 2008; Ivashchenko et al. 2011; Walton and Pizzitelli 2012; Hwang et al. 2012). In addition, there are data showing that the inner mitochondrial membrane potential is affected in peroxisome-deficient mouse hepatocytes (Dirkx et al. 2005), and that the mitochondrial redox balance is perturbed in peroxisome-deficient fibroblasts (Fransen M. and Apanasets O., unpublished data) and upon generation of excess ROS inside peroxisomes (Ivashchenko et al. 2011). In summary, these findings – among others – suggest that a disturbance in peroxisome function may trigger signaling events that ultimately result in increased mitochondrial stress.

An intriguing question is how peroxisomes and mitochondria can communicate with each other. Potential mechanisms include (i) anaplerotic metabolism, (ii) diffusion of ROS, (iii) transport by vesicular carriers, and (iv) direct physical connections. Regarding the first possibility, one can envisage that – as peroxisomal metabolism slows down – critical metabolic intermediates are not properly produced and trafficked to mitochondria. This in turn may affect mitochondrial metabolism and result in an increase in uncoupled reactions and ROS production (Titorenko and Terlecky 2011). Concerning the second possibility, we have indications that the type of ROS generated inside peroxisomes can differ from the type of ROS causing oxidative stress inside mitochondria (Wang B. and Fransen M., unpublished data). This finding suggests that, at least under the conditions employed, a simple diffusion of ROS from peroxisomes through the cytosol to mitochondria is unlikely. As to the third possibility, it should be noted that – although there is evidence for the existence of a vesicular transport pathway from mitochondria to peroxisomes (Neuspiel et al. 2008) – no data currently exist for such a pathway in the opposite direction. Finally, regarding the last possibility, it is of relevance to point out that a recent confocal microscopy study has visualized a mitochondrial-associated membrane (MAM) that

physically connects peroxisomes to mitochondria (Horner et al. 2011). Whether or not these contact sites provide an axis through which stress stimuli and metabolites are transmitted from peroxisomes to mitochondria remains to be investigated.

3.3 Peroxisomes and Age-Related Diseases

Until about a decade ago, defects in peroxisome function were virtually solely linked with severe congenital neurodevelopmental disorders. Since then, many things have changed, and – from an experimental point of view – there is now abundant evidence for the involvement of these organelles in the etiology and progression of aging and age-related diseases (Fransen et al. 2012). Indeed, as already pointed out above, peroxisomes play an indispensable role in cellular lipid and ROS metabolism, and alterations in these processes have been associated with an age-related decline in cellular, tissue, and organismal function. In this context, it is interesting to note that cellular senescence, a process that can be delayed by restoring the peroxisomal redox balance (see above), has recently been causally implicated in generating age-related phenotypes (Baker et al. 2011). The observation that a compromised catalase activity can be directly correlated with the early onset of some age-related human pathologies, such as psoriasis, hypertension, schizophrenia, type 2-diabetes and cancer (Góth et al. 2004), is in line with these findings. In the following sections, we will focus on the potential role of peroxisomal (dys)function in the age-related pathogenesis of neurodegeneration, cancer, and diabetes (Fig. 3.3).

3.3.1 Peroxisomes and Neurodegenerative Diseases

Plasmalogens and DHA represent essential constituents of neuronal myelin sheaths, which surround and protect nerve fibers. As (i) the synthesis of plasmalogens is critically dependent on peroxisomes, and (ii) the levels of these lipids fall during aging, it is tempting to speculate that alterations in peroxisomal metabolism contribute to the development of neurodegenerative diseases (Lizard et al. 2012). One of the strongest indirect arguments in favor of this hypothesis is that most peroxisomal disorders cause severe neurological dysfunction due to myelin abnormalities, neuronal migration defects, neuroinflammatory reactions, and axonal damage (Wanders and Waterham 2006; Bottelbergs et al. 2012).

One of the proposed causative factors for axonal degeneration in conditions of peroxisome deficiency is oxidative stress. This assumption is mainly based on the observations that (i) excessive levels of oxidative stress can be observed in the spinal cord of a mouse model of X-linked adrenoleukodystrophy (X-ALD; this is the most common single-enzyme defect of the peroxisomal disorders), even long before disease onset (Fourcade et al. 2008), (ii) an *in vivo* antioxidant treatment of these animals prevents oxidation of proteins, axonal damage, and motor disability (López-Erauskin et al. 2011), and (iii) a complete inactivation

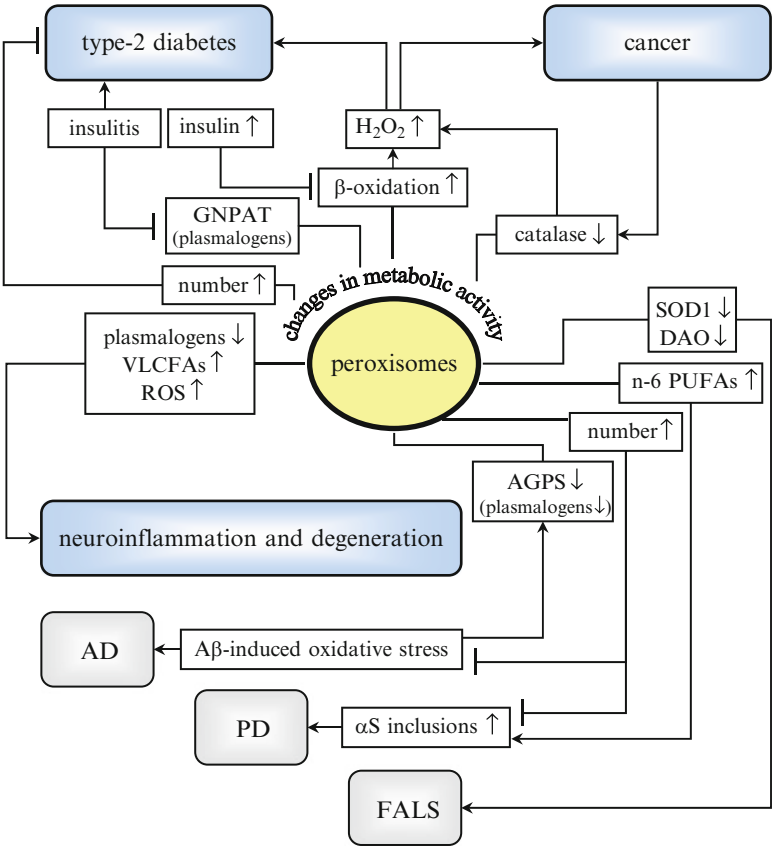

Fig. 3.3 Peroxisomes and age-related diseases. *αS* α-synuclein, *Aβ* amyloid-β peptide, *AD* Alzheimer's disease, *AGPS* alkylglycerone phosphate synthase, *DAO* D-amino acid oxidase, *FALS* familial amyotrophic lateral sclerosis, *GNPAT* glyceronephosphate O-acyltransferase, *PD* Parkinson's disease, *PUFAs* polyunsaturated fatty acids, *ROS* reactive oxygen species, *SOD1* Cu/Zn superoxide dismutase, *VLCFAs* very-long-chain fatty acids. See text for details

of peroxisome function in the brain of Nestin-Cre/Pex13-LoxP mice leads to mitochondria-mediated oxidative stress, neuronal cell death, and impairment of cerebellar development (Müller et al. 2011). However, another study which employed Nestin-Cre/Pex5-LoxP mice could not confirm the role of oxidative stress in neuronal degeneration caused by peroxisome inactivation (Bottelbergs et al. 2012). The reasons for these apparently conflicting data remain unclear.

Another causative factor for axonal degeneration may be neuroinflammation. This postulate is mainly based on the observation that the absence of functional peroxisomes in oligodendrocytes causes a strong proinflammatory environment and the infiltration of B and activated $CD8^+$ T cells into brain lesions (Kassmann et al. 2007). In this context, it should be noted that peroxisomal β-oxidation is also involved in the breakdown of eicosanoids, a class of bioactive lipid mediators that can elicit a broad range of inflammatory reactions (Van Veldhoven 2010;

Terlecky et al. 2012). As (i) a decrease in peroxisomal β-oxidation may lead to an accumulation of VLCFAs (Van Veldhoven 2010), (ii) high concentrations of VLCFAs can stimulate the expression of inflammatory mediators (Singh et al. 2009), and (iii) such molecules are also reported to downregulate peroxisome function (Paintlia et al. 2008), this may eventually result in the activation of a self-perpetuating inflammatory cascade.

Over the years, a vast array of age-related diseases has been associated with changes in lipid metabolism, a disturbance of ROS homeostasis, and chronic neuroinflammation. Somewhat surprisingly, despite the fact that peroxisomes play a critical role in all these processes, the potential involvement of this organelle in the initiation and progression of neurodegenerative disorders has been overlooked for a long time. In the following three paragraphs, we will discuss the data currently available for the potential involvement of peroxisomes in Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis.

Alzheimer's disease (AD) is one of the most common neurological disorders affecting the elderly. It is characterized by progressive impairments in memory and cognition, synaptic loss, and the presence of intracellular neurofibrillary tangles (NFTs) and extracellular neuritic plaques containing, respectively, hyper-phosphorylated Tau proteins and the amyloid-β peptide (Aβ) (Lizard et al. 2012). Another hallmark of AD is mitochondrial dysfunction (Schon and Przedborski 2011). Aβ has been shown to progressively accumulate in mitochondria of brains from AD patients, and – as this is thought to result in a faulty mitochondrial energy metabolism – this may increase mitochondrial ROS production and lead to cellular oxidative stress. Interestingly, on one hand, it has been reported that peroxisome proliferation protects hippocampal neurons from Aβ-induced oxidative stress, neurotoxicity, and cell death (Santos et al. 2005). However, on the other hand, there is also evidence that peroxisomes represent a cellular target for Aβ. This is illustrated by the observation that Aβ-induced ROS production lowers the activity levels of alkylglycerone phosphate synthase (AGPS), a peroxisomal enzyme required for plasmalogen synthesis (Grimm et al. 2011). Intriguingly, a recent longitudinal community-based cohort study focusing on peroxisomes in well-defined autopsy material also revealed substantial peroxisome-related changes in AD brains (Kou et al. 2011). Specifically, this study showed that (i) AD brains contain a marked reduction in plasmalogen content and a significant increase in VLCFAs (C22:0, C24:0, and C26:0) levels, and (ii) at advanced AD stages, peroxisomal volume density is strongly increased and decreased, respectively, in the somata and neurites of neurons in gyrus frontalis. Note that, although the plasmalogen data are in concordance with the findings reported by Grimm et al. (2011), it remains to be investigated whether the observed increase in VLCFA content is due to reduced peroxisomal β-oxidation or enhanced fatty acid elongation (Kou et al. 2011). In addition, as it has already been shown that overexpression of Tau inhibits the kinesin-dependent transport of peroxisomes into neurites (Stamer et al. 2002), the increase in somatic peroxisome density most likely results from abnormally phosphorylated Tau impairing peroxisome trafficking.

Parkinson's disease is a degenerative disorder of the central nervous system which is characterized by the presence of α-synuclein (αS) protein inclusions in the brain and an ongoing loss of dopaminergic neurons in the substantia nigra. Although the etiology and pathogenesis of PD are still elusive, a large body of evidence suggests a prominent role of oxidative stress, inflammation, and mitochondrial dysfunction (Chaturvedi and Beal 2008). Interestingly, it has also been reported that neurons and glia of X-ALD and PBD patients contain increased depositions of αS (Suzuki et al. 2007; Yakunin et al. 2010). In addition, these αS abnormalities are not due to induced oxidative stress and do not correlate with mitochondrial dysfunction; however, they associate with elevated levels of n-6 polyunsaturated fatty acids (PUFAs), a condition associated with PBD mouse models (Yakunin et al. 2010). These findings are in line with other studies showing that peroxisome proliferation abrogates αS toxicity leading to PD (Chaturvedi and Beal 2008).

Amyotrophic lateral sclerosis (ALS) is one of the most common neuromuscular diseases worldwide. The disease is characterized by progressive motor neuron degeneration, skeletal muscle atrophy, paralysis, and death from respiratory failure (Mitchell et al. 2010). The precise molecular mechanisms underlying motor neuron loss remain uncertain. However, several etiological hypotheses have been proposed, including mitochondrial dysfunction and motoneuronal vulnerability to excitotoxicity (Higgins et al. 2003; Sasabe et al. 2012). Interestingly, approximately 5 % of all ALS cases have a family history of ALS (familial ALS; FALS), and two ALS-linked genes have been directly or indirectly linked to peroxisomal dysfunction (Higgins et al. 2003; Mitchell et al. 2010). One of these genes encodes SOD1, an antioxidant enzyme that catalyzes the conversion of O_2^- into O_2 and H_2O_2 and has been found in different cell compartments, including peroxisomes (Islinger et al. 2009). Approximately 20 % of all FALS cases are caused by mutations in SOD1. $SOD1_{G93A}$, the most intensely studied mutant form of SOD1, has been shown to cause mitochondrial vacuolation by expansion of the intermembrane space (Higgins et al. 2003). The same study also reported that peroxisomes actively migrate to or proliferate in the vicinity of abnormal mitochondria and participate in the vacuolation process. Unfortunately, the exact function of these organelles in mitochondrial vacuolation remains to be fully determined. The second ALS-linked gene encodes D-amino acid oxidase (DAO), an exclusively peroxisomal flavoprotein that catalyzes the oxidative deamination of neutral and polar D-amino acids into the corresponding α-keto acids, ammonia, and H_2O_2. Approximately 0.3 % of FALS cases have been shown to be associated with a rare mutation (R199W) in the gene coding for human DAO (Mitchell et al. 2010). This mutation causes almost total loss of enzyme activity, and cell lines expressing DAO_{R199W} show decreased viability and increased levels of ubiquitinated aggregates compared with cells expressing the wild-type protein (Mitchell et al. 2010). Finally, the importance of dysfunctional DAO in FALS has been unequivocally established by studies in DAO knockout mice, which clearly show that loss of DAO activity triggers motor neuron degeneration (Sasabe et al. 2012).

3.3.2 Peroxisomes and Cancer

Over the years, mounting evidence has been collected that peroxisomes are involved, either directly or indirectly, in cancer cell metabolism (see Chap. 5). A classic example is the observation that a prolonged treatment of rodents with peroxisome proliferators causes hepatocellular carcinomas (Reddy et al. 1980). Along the same lines, it has been reported that mice lacking ACOX1, a peroxisomal enzyme which is associated with the degradation of putative endogenous ligands of peroxisome proliferator activated receptor-alpha (PPARα), develop hepatic adenomas and carcinomas (Fan et al. 1998). Another line of evidence is that the activity of catalase, the most abundant protein in mammalian peroxisomes, has been inversely associated with cancer. Indeed, it has been shown that (i) the activity of catalase is strongly decreased in breast, colon, liver, and skin cancers (Lauer et al. 1999; Frederiks et al. 2010; Syed and Mukhtar 2012), (ii) hypocatalasemic mice, with approximately ten-fold less catalase than normal mice, display a higher susceptibility to spontaneous and induced tumorigenesis (Ishii et al. 1996), and (iii) women with a higher catalase activity appear to have a reduced risk of breast cancer and non-melanoma skin cancers (Ahn et al. 2005; Syed and Mukhtar 2012). As a last example, we would like to mention the selective upregulation of peroxisomal branched chain fatty acid β-oxidation in prostate cancers (Zha et al. 2005). Importantly, this finding suggests that a disturbed peroxisomal β-oxidation may elicit metabolic features that would give unique metabolic advantage for prostate transformation (Zha et al. 2005).

The exact mechanisms of how peroxisomal enzymes are up- or downregulated in cancer cells are not yet well understood. One factor that may account for at least some of these changes is tumor necrosis factor-alpha (TNF-α), a pleiotropic inflammatory cytokine that – among other functions – has been shown to downregulate PPAR-α and the mRNAs encoding peroxisomal proteins (Beier et al. 1997). In addition, it is also unknown how a disturbance in peroxisomal metabolism may promote tumor formation. Our leading hypothesis is that alterations in peroxisomal redox metabolism may cause severe imbalances in the cellular redox proteome (see below).

3.3.3 Peroxisomes and Type 2-Diabetes

Type 2-diabetes is a complex age-related metabolic disorder with a continuously growing prevalence worldwide. The disease is characterized by progressive β-cell failure and peripheral insulin resistance. Long-term complications of diabetes include – among others – retinopathy, nephropathy, neuropathy, and cardiomyopathy. Mounting evidence has strengthened the view that oxidative stress may function as the primary component of diabetes pathology. One potential explanation may be that pancreatic β-cells are particularly susceptible to oxidative damage (Lenzen

et al. 1996). This is most likely due to the fact that these cells contain virtually no catalase, since the catalase gene is – together with a handful of other genes – tissue-specifically repressed in islets of Langerhans (Thorrez et al. 2011).

It is already known for some time that elevated levels of long-chain and very-long-chain saturated non-esterified fatty acids (NEFAs) exhibit a strong cytotoxic effect on insulin-producing β-cells (Poitout and Robertson 2002). Recently, it has been shown that this cytotoxic effect is mediated by H_2O_2 derived from peroxisomal β-oxidation, and that overexpression of catalase in the peroxisomes and the cytosol – but not in the mitochondria – can protect the cells against palmitic acid-induced toxicity (Elsner et al. 2011). Others have shown β-cell protection upon exposure to peroxisome proliferators (Hellemans et al. 2007). On the other hand, it has also been reported that catalase overexpression in pancreatic β-cells sensitizes non-obese diabetic mice to autoimmune diabetes and islet cell death (Li et al. 2006). Combined, these findings suggest that a tight regulation of peroxisomal redox metabolism is crucial for pancreatic β-cell function and viability.

The importance of peroxisomes for diabetes progression was recently further exemplified by a study with catalase-knockout mice (Hwang et al. 2012). This study showed that catalase plays an important role in protecting the kidney from NEFA-induced diabetic stress through maintaining peroxisomal and mitochondrial fitness. Finally, it should be noted that both insulin action and islet inflammation have been reported to have a negative impact on peroxisome function: insulin inhibits peroxisomal fatty acid β-oxidation by a yet unclear mechanism requiring insulin degrading enzyme, a protein found in multiple cell compartments, including peroxisomes (Hamel et al. 2001); and the onset of insulitis results in a significantly reduced activity of glyceronephosphate O-acyltransferase (GNPAT) (Lindfors et al. 2009), a peroxisomal enzyme required for plasmalogen synthesis.

3.4 Open Questions and Perspectives

As reviewed here, a growing body of evidence links peroxisomal dysfunction to aging and age-related diseases. Peroxisomes are intimately involved in cellular lipid and ROS metabolism, and alterations in these processes have been invoked in diverse complex diseases such as AD, PD, ALS, type 2-diabetes, and cancer. However, as peroxisomal metabolism both influences and is influenced by the aging process, it is speculative to what extent these organelles are the causal factor for disease initiation and progression. Nevertheless, despite the multifaceted nature of this bidirectional crosstalk, there is now sufficient information available to warrant the conclusion that peroxisomal metabolism and cellular aging are closely intertwined.

There is still a large gap in our understanding of how peroxisomes contribute to stress responses and metabolic signaling pathways that regulate the aging process. A hypothesis gaining prominence is that low concentrations of peroxisomal ROS can

exert important physiological roles in cellular signaling and proliferation (= concept of 'peroxihormesis'), and that a disturbance of peroxisomal redox homeostasis may trigger signaling/communication events that ultimately result in increased mitochondrial stress and the activation of mitochondrial stress pathways (= the peroxisome/mitochondria axis of aging) (Titorenko and Terlecky 2011; Ivashchenko et al. 2011). Experimental evidence in line with the concept of peroxihormesis includes the observations that (i) inhibition of catalase activity by 3-aminotriazole increases the cellular protein disulfide content by 20 % (Yang et al. 2007), (ii) the intracellular localization and activity of many proteins (e.g. kinases, phosphatases, transcription factors, ...) are reversibly controlled by the oxidation status of redox-sensitive cysteine residues (Hekimi et al. 2011), and (iii) peroxisomal H_2O_2 can function as an important modulator of NFKB1, a pleiotropic transcription factor involved in many biological processes, including cell growth and differentiation, inflammation, tumorigenesis, and apoptosis (Li et al. 2000). Unfortunately, virtually nothing else is known about how peroxisomal ROS may effect the cellular redox proteome, neither in physiological nor in pathological conditions. In addition, albeit it is clear that peroxisomes and mitochondria share a redox-sensitive relationship, it is not known how these organelles communicate with each other. Nevertheless, the idea that peroxisomes may act as upstream initiators of mitochondrial ROS signaling pathways may change much of the way we think about aging and how to manipulate it.

A challenging question is whether peroxisomes represent an interesting therapeutic target to mitigate or even stop age-related neurodegenerative diseases. In this context, it is interesting to note that it has been reported that substances able to activate PPAR-α and/or induce peroxisome proliferation may constitute attractive therapeutic tools against neurodegenerative diseases, such as AD and PD (Santos et al. 2005; Mutez et al. 2009; Cimini et al. 2009). However, as many of these compounds also induce non-peroxisomal responses, it is not always clear whether or not clinical improvements are related to an increase in peroxisomal metabolism.

In summary, this review discusses the potential role of peroxisomes in aging and age-related diseases. From all these data, it is clear that peroxisomes can act as a redox signaling platform. However, as the proximal targets of peroxisomal ROS and the signaling pathways that drive mitochondrial degeneration are not yet known, further work is needed to elucidate the mechanisms of how these organelles are incorporated into (intra)cellular communication networks. This in turn is essential to gain a better insight into the importance of peroxisomes in the pathogenesis of age-related diseases.

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Chapter 4
Peroxisomes and the Antiviral Responses of Mammalian Cells

Charlotte Odendall and Jonathan C. Kagan

Contents

Abstract Cell biology and microbiology are some of the oldest areas of scientific inquiry. Despite the depth of knowledge we now have in these respective fields, much remains unclear about how microorganisms interact with host intracellular organelles. Perhaps nowhere is this statement more accurate than in the role of peroxisomes in microbial infections. Peroxisomes were one of the first organelles discovered by Christian De Duve over 50 years ago (de Duve Ann N Y Acad Sci 386:1–4, 1982). These organelles are ubiquitously found in eukaryotic cells, where they serve several well-defined functions in lipid and oxygen homeostasis (Waterham and Wanders Biochim Biophys Acta 1822:1325, 2012). This chapter will discuss the emerging evidence that indicates that in addition to their functions in cellular metabolism, peroxisomes play an important role in viral infections.

Keywords Peroxisomes • Mitochondria • MAM • MAVS • RLRs • Antiviral immunity

C. Odendall • J.C. Kagan (✉)
Harvard Medical School and Division of Gastroenterology, Boston Children's Hospital, Boston, MA 02115, USA
e-mail: jonathan.kagan@childrens.harvard.edu

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4.1 Peroxisomes and Their Interactions with Intracellular Pathogens

Peroxisomes interact functionally and morphologically with other organelles, such as the endoplasmic reticulum (ER), mitochondria and lipid droplets (Hettema and Motley 2009; Schrader 2006; Waterham and Wanders 2012). All of these organelles are involved in interactions between the host cell and virus (Fig. 4.1). For example, poliovirus infection results in massive reorganization of intracellular membranes, mainly ER membranes, into vesicles that harbor replication complexes at their surface (Bienz et al. 1987). These vesicles are found in close proximity to remnants of the ER (Bienz et al. 1987) and the poliovirus viroporin 2B was shown to localize to the ER. Similarly, the polymerase (Protein A) of the insect pathogen flock house virus (FHV) associates with the mitochondrial outer membrane (Miller et al. 2001). This results in viral replication on mitochondria, which serve as important sites

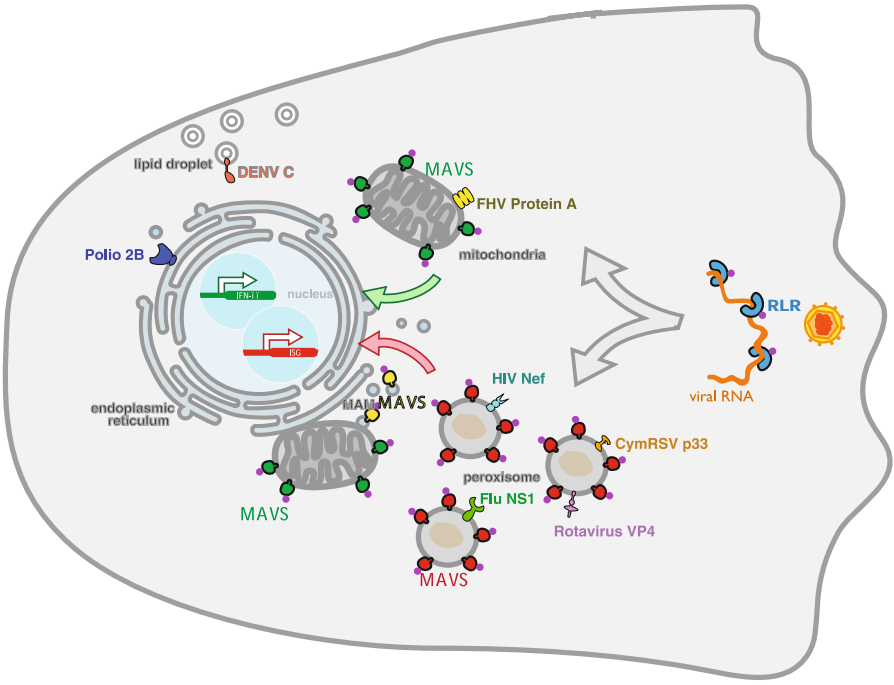

Fig. 4.1 Localization of viral proteins and components of the antiviral innate immune machinery. Proteins encoded by viruses localize to the organelles that interact with peroxisomes. Poliovirus 2B is localized on the endoplasmic reticulum, Flock House virus (*FHV*) protein A is found on mitochondria and Dengue virus (*DENV*) C protein is on lipid droplets. Peroxisomes are also a localization site of viral proteins: VP4 from Rotavirus, NS1 from Influenza (*Flu*), HIV Nef and Cymbidium ringspot virus (*CymRSV*) p33 are found on peroxisomes. The antiviral adaptor MAVS also localizes to mitochondria, peroxisomes and mitochondria-associated membranes (*MAM*). From these locations, MAVS transduces signals that originate from the recognition of viral RNA by RIG-I like receptors (*RLR*)

of innate immune signal transduction to fight viral infections (Seth et al. 2005). Finally, proteins derived from Hepatitis C virus (HCV), rotavirus or the C protein of Dengue virus (DENV) are located on lipid droplets (Cheung et al. 2010; Moradpour et al. 1996; Samsa et al. 2009). Thus, organelles that interact with peroxisomes play important roles in the lifecycles of diverse viruses.

Peroxisomes themselves were shown to be sites of viral protein localization as well as assembly of replication complexes. A member of the replicase complex of some viruses of the tombusvirus family, p33, was shown to associate with plant peroxisomes, and viral replication was shown to occur on the peroxisomal membrane (McCartney et al. 2005; Panavas et al. 2005). A member of this virus family, Cymbidium ringspot virus (CymRSV) induces profound changes to peroxisome morphology, forming small vesicles in the periphery of peroxisomes (Russo et al. 1983). As the infection proceeds, these vesicles fill the peroxisomes, leading to the disappearance of the matrix. Strikingly, these intra-peroxisomal vesicles were shown to contain dsRNA, most probably replicative forms of viral RNA (Russo et al. 1983). In mammals, the Nef protein from HIV, the VP4 protein from rotavirus and the NS1 protein from influenza have been detected on peroxisomes (Cohen et al. 2000; Lazarow 2011; Mohan et al. 2002). Bioinformatic approaches have identified several other viral proteins with putative peroxisomal targeting sequences (Mohan and Atreya 2003), but most of these predicted targeting sequences have yet to be examined experimentally. Overall, these data indicate that like the organelles they interact with, peroxisomes interact with viral components. While the precise role of peroxisomes in the life cycle of most viruses is unclear, recent data on the innate immune responses that fight these infections suggests a critical role for these organelles in host defense.

4.2 Subcellular Localization of Mammalian Sensors of Microbial Infection

The innate immune system of mammals is comprised of a series of structurally diverse but functionally related families of receptors that detect the presence of microorganisms (Akira et al. 2006). These receptor families are the Toll-like Receptors (TLRs), NOD-like Receptors (NLRs), RIG-I like Receptors (RLRs) and the C-type Lectin Receptors of the Dectin family (Brennan and Bowie 2010; Goodridge et al. 2012). These receptors are classically called Pattern Recognition Receptors (PRRs), because they evolved to recognize Pathogen Associated Molecular Patterns (PAMPs) (Janeway 1989), such as bacterial lipopolysaccharides, flagellin, lipoproteins and double-stranded RNA, among others. Microbial detection by PRRs leads to the activation of signal transduction pathways that activate several transcription factors (Medzhitov and Horng 2009). These factors then induce major changes in the host transcriptional response, as hundreds of proinflammatory and immunomodulatory factors are expressed, including the cytokines interleukin-1, TNF and Type I and III interferons (IFNs) (Medzhitov and Horng 2009).

Cell biological analyses of PRRs revealed that each receptors family surveys a distinct subcellular compartment for the presence of microbial products (Kagan 2012). For example, the TLRs and the Dectin family are type I transmembrane proteins that contain an extracellular ligand-binding domain and an intracellular signaling domain (Akira et al. 2006). These receptors survey the extracellular and lumenal compartments of endosomes, and as such are located in these locations. In contrast, the NLRs and the RLRs do not contain transmembrane domains and are rather found in the cytosol (Fig. 4.1), where they survey this compartment for the presence of microbes (Kagan 2012).

During a host-microbe encounter, pathogenic and non-pathogenic microbes will be found in the extracellular space. Therefore, PRRs located at the cell surface (TLRs and Dectins) have the ability to detect all microbes. Microbial pathogens encode sophisticated activities that manipulate the function of host cells, often enabling them to survive and replicate intracellularly (Vance et al. 2009). The outcome of these encounters will almost always results in interactions between the pathogen and components of the host cell cytosol (Vance et al. 2009). For example, bacteria encode secretion systems or secreted toxins that can deliver proteins to the cytosol (Cambronne and Roy 2006). Likewise, a necessary step in the pathogenesis of all viruses is the delivery of their genetic material and proteins in the cytosol of host cells, where they can access the various metabolic activities necessary to complete their lifecycles. Therefore, since the cytosol is accessed only by pathogens, the PRRs located in the cytosol (RLRs and NLRs) can be considered legitimate pathogen detection receptors.

4.3 Activation of Antiviral Innate Immune Responses by RLRs

With the exception of the NLRs, all other PRR families enlist the aid of a transmembrane protein at the receptor-proximal level to activate innate immune responses upon microbial infection. In the case of the plasma membrane localized PRRs (TLRs and Dectins), the transmembrane proteins are the receptors themselves. By contrast, RLRs are cytoplasmic receptors. Upon viral infection, they engage a downstream transmembrane domain-containing adaptor protein to induce innate immune responses. This adaptor is called MAVS (also known as IPS-1, VISA or Cardif, Fig. 4.1) (Kawai et al. 2005; Meylan et al. 2005; Seth et al. 2005; Xu et al. 2005). MAVS contains a C-terminal "tail anchor" transmembrane domain that was originally identified to target this protein to the mitochondrial outer membrane (Seth et al. 2005). In resting cells, the best characterized RLR, RIG-I, is phosphorylated (Nistal-Villan et al. 2010). During an infection, RIG-I binds to viral RNA that displays one or more features, such as a short double-stranded region, a polyU-rich 3′ end, and a 5′ end with a triphosphate group (Kowalinski et al. 2011). RIG-I binding to viral RNA results in its dephosphorylation by unknown phosphatases and its ubiquitination by the E3 ubiquitin ligase TRIM25 (Gack et al. 2007; Nistal-Villan

et al. 2010). A chaperone called 14-3-3ε is then able to engage this modified form of RIG-I and together they translocate to the MAVS adaptor on mitochondria (Liu et al. 2012). MAVS binding by active RIG-I results in the oligomerization of this adaptor into a prion-like state, the signaling competent form of this protein (Liu et al. 2012). Oligomerized MAVS then activates a series of signal transduction pathways that involve ubiquitin ligases (e.g. TRAF3 and TRAF6) and kinases (e.g. TBK1, IKKi) to activate NF-κB and IRF3 transcription factors (Liu et al. 2012). These transcription factors are responsible for upregulating the expression of numerous genes involved in antiviral immunity such as type I and III IFNs, chemokines and IFN-stimulated genes (ISGs) (Belgnaoui et al. 2011). The importance of MAVS localization to mitochondria for RLR signaling was originally revealed through the study of the HCV protease NS3/4a. When overexpressed in mammalian cells, NS3/4a cleaves MAVS near its transmembrane domain, resulting in its release from membranes (Li et al. 2005; Meylan et al. 2005). The release of MAVS into the cytosol renders the RLR signaling pathway inactive. Thus, membrane localization of MAVS is important for its signaling functions.

4.4 Role of Peroxisomes in RLR Signal Transduction

Detailed cell biological analysis of the MAVS protein revealed a more complex integration of the RLR signaling pathway into the infrastructure of the cell. As discussed above, MAVS contains a C-terminal transmembrane domain that anchors it to the mitochondrial outer membrane. This type of localization motif is found in other outer membrane proteins of the mitochondria, such as Fis1 and Mff that control the dynamic changes that occur in mitochondrial morphology during cellular homeostasis (Gandre-Babbe and van der Bliek 2008; Koch and Brocard 2012). Subsequent work revealed that in addition to localizing to mitochondria, Fis1 and Mff are located on peroxisomes and that these proteins regulate the morphology of both organelles (Gandre-Babbe and van der Bliek 2008; Koch and Brocard 2012).

Based on the similarities between the localization domains of MAVS, Fis1 and Mff, we recently examined the localization of MAVS to peroxisomes (Dixit et al. 2010). MAVS is localized to peroxisomes in human hepatocytes, murine fibroblasts and macrophages (Fig. 4.1). Like the mitochondria, the localization of MAVS to peroxisomes is dependent on the C-terminal tail anchor transmembrane domain (Dixit et al. 2010). Using mutant alleles of MAVS that encode variants that target this protein to either mitochondria, peroxisomes or the cytosol, we found that RLR signaling can occur from multiple subcellular locations (Dixit et al. 2010). For example, during infection of fibroblasts with mammalian reovirus, RLR signaling through mitochondrial MAVS induces the expression of all the genes that have been identified as targets of this signaling pathway (e.g. type I IFNs, ISGs and chemokines), albeit with delayed kinetics when compared to wild-type MAVS. In contrast, peroxisomal MAVS induces chemokines and ISGs, but not Type I IFNs.

Also in contrast to mitochondrial MAVS, peroxisomal MAVS induces antiviral gene expression with kinetics similar to that observed in cells expressing wild-type MAVS. As expected from prior studies on MAVS cleavage from membranes by the HCV NS3/4a protease (Li et al. 2005; Loo et al. 2006), MAVS engineered to localize to the cytosol is incapable of participating in RLR signaling (Dixit et al. 2010). Perhaps most notably, the signaling outputs from MAVS located in distinct subcellular compartments correlates with the cell's ability to control viral infections. Fibroblasts expressing wild-type MAVS readily control the replication of vesicular stomatitis virus (VSV) while MAVS-deficient cells or cells expressing cytosolic MAVS cannot (Dixit et al. 2010). Cells expressing MAVS located on peroxisomes also control VSV replication, but not as efficiently as wild type MAVS. In contrast mitochondrial MAVS cannot control VSV replication, despite the fact that cells expressing mitochondrial MAVS induce IFNs and ISGs. These data suggest that the speed at which antiviral responses are induced during an infection is just as important as the type of response that is generated. Thus, the delay in IFN and ISG expression observed in cells expressing mitochondrial MAVS may be responsible for the inability of these cells to control infections.

4.5 Coordinating the Role of Peroxisomal and Mitochondrial MAVS in RLR Signal Transduction Around Mitochondria-Associated Membranes

Recent work has revealed that MAVS is also located on a subdomain of the ER called mitochondria-associated membranes (MAM, Fig. 4.1) (Horner et al. 2011). The MAM are defined as contact sites between the ER and mitochondria, and are thought to play important roles in lipid metabolism and calcium signaling (Bononi et al. 2012; Vance and Shiao 1996). A role for the MAM in activation of the inflammasome, a protein complex that regulates the secretion of a subset of inflammatory cytokines, has also been reported (Raturi and Simmen 2013). Detailed subcellular fractionation studies revealed that in addition to peroxisomes and mitochondria, MAVS is located on the MAM (Horner et al. 2011). A striking observation was made by the Gale group. They found that within hepatocytes encoding HCV replicons, the viral NS3/4a protease preferentially cleaves MAVS from the MAM, as opposed to mitochondria (Horner et al. 2011). Whether NS3/4a cleaves MAVS from peroxisomes is unknown. Gale and colleagues also found that during viral infections of hepatocytes, peroxisomes and mitochondria interact with one another at the MAM (Horner et al. 2011). These contacts have been proposed to form an "innate immune signaling synapse", which coordinates the signaling functions of MAVS on peroxisomes and mitochondria (Horner et al. 2011). These studies also revealed an intriguing link between the MAM and MAVS distribution on mitochondria and peroxisomes. Cells deficient for mitofusin-2, which cannot form mitochondria-ER contacts efficiently, exhibit higher levels of MAVS on peroxisomes than wild-type cells (Horner et al. 2011). These results suggest that

MAVS is likely "cycling" between the MAM, mitochondria and peroxisomes. This cycling may be altered to enrich this protein in one or more compartments by altering the cell biological interactions between these organelles.

4.6 Perspectives on the Future of Peroxisome Research

For many years, the study of basic cell biological processes has occurred separately from the study of host-pathogen interactions and innate immunity. Despite this statement, it is very clear that intracellular pathogens are excellent cell biologists. In fact, in several areas of research, our knowledge of given biological processes has been greatly influenced by the study of pathogens that manipulate said process (Mostowy and Cossart 2009). As such, it is imperative that we continue to consider all aspects of the host-pathogen interaction when studies of pathogenesis are performed. Similarly, it is imperative that basic cell biologists consider infectious models of other disease models to better inform us of the importance of the process they are studying. An excellent example of this statement comes from the recent work (highlighted above) on the role of peroxisomes in antiviral immunity. For many years, peroxisomes were considered metabolic organelles, with no other cell function. Many other organelles (e.g. plasma membrane, endosomes, lysosomes, mitochondria, ER) were also first defined through their metabolic activities, but have since emerged as critical regulators of innate immune signal transduction. Peroxisomes therefore are not unique in their dual role in metabolism and infection. Rather, a common theme is emerging whereby most (perhaps all) organelles will have a metabolic and immune function. A challenge that now faces the community is understanding how the dynamics of peroxisome morphology and inter-organelle interactions influences the operation of the antiviral pathways that operates from these locations. Human patients with peroxisomal biogenesis disorders such as Zellweger Syndrome may prove useful in this regard, as would further characterization of cells deficient in mitochondria/peroxisome/MAM interactions. Additionally, although many viral proteins are predicted to be localized to peroxisomes, whether they are actually present on these organelles is unclear. If they are present, we need to determine the role of peroxisomes, and peroxisomal localization in the infectious cycle of these viruses. One possibility is that like the HCV protease NS3/4a, which cleaves and inactivates MAVS, some of these purported viral peroxisomal proteins may act to interfere with RLR signaling from this organelle. Finally, it remains unclear if the RLR network is the only signaling pathway that operates from peroxisomes. Other signaling pathways (involved in immunity or otherwise) may also operate from this location.

In summary, the years of work invested in understanding basic properties of peroxisomes and antiviral immunity has created an opportunity where these two areas can be integrated rapidly. The peroxisome may therefore emerge as the model of choice to understand the coordination of protein localization and signal transduction. This work may also have the wonderful outcome as revealing novel means of diagnosing and/or treating patients with peroxisomal disorders.

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Chapter 5
Peroxisome Proliferator-Activated Receptor-α Signaling in Hepatocarcinogenesis

Parimal Misra, Navin Viswakarma, and Janardan K. Reddy

Contents

P. Misra (✉)
Department of Biology, Dr. Reddy's Institute of Life Sciences, An Associate Institute of University of Hyderabad, Gachibowli, Hyderabad 500046, India
e-mail: parimalm@drils.org; parimalmils@gmail.com

N. Viswakarma
Department of Molecular Pharmacology and Therapeutics, Loyola University Chicago, Maywood, IL 60153, USA

J.K. Reddy
Department of Pathology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611-3008, USA
e-mail: jkreddy@northwestern.edu

L.A. del Río (ed.), *Peroxisomes and their Key Role in Cellular Signaling and Metabolism*, Subcellular Biochemistry 69, DOI 10.1007/978-94-007-6889-5_5,

Abstract Peroxisomes are subcellular organelles that are found in the cytoplasm of most animal cells. They perform diverse metabolic functions, including H_2O_2-derived respiration, β-oxidation of fatty acids, and cholesterol metabolism. Peroxisome proliferators are a large class of structurally dissimilar industrial and pharmaceutical chemicals that were originally identified as inducers of both the size and the number of peroxisomes in rat and mouse livers or hepatocytes in vitro. Exposure to peroxisome proliferators leads to a stereotypical orchestration of adaptations consisting of hepatocellular hypertrophy and hyperplasia, and transcriptional induction of fatty acid metabolizing enzymes regulated in parallel with peroxisome proliferation. Chronic exposure to peroxisome proliferators causes liver tumors in both male and female mice and rats. Evidence indicates a pivotal role for a subset of nuclear receptor superfamily members, called peroxisome proliferator–activated receptors (PPARs), in mediating energy metabolism. Upon activation, PPARs regulate the expression of genes involved in lipid metabolism and peroxisome proliferation, as well as genes involved in cell growth. In this review, we describe the molecular mode of action of PPAR transcription factors, including ligand binding, interaction with specific DNA response elements, transcriptional activation, and cross talk with other signaling pathways. We discuss the evidence that suggests that PPARα and transcriptional coactivator Med1/PBP, a key subunit of the Mediator complex play a central role in mediating hepatic steatosis to hepatocarcinogenesis. Disproportionate increases in H_2O_2-generating enzymes generates excess reactive oxygen species resulting in sustained oxidative stress and progressive endoplasmic reticulum (ER) stress with activation of unfolded protein response signaling. Thus, these major contributors coupled with hepatocellular proliferation are the key players of peroxisome proliferators-induced hepatocarcinogenesis.

Keywords Peroxisomes • Peroxisome proliferators • PPARα signaling • Med1 subunit of Mediator complex • Fatty acid oxidation • Oxidative stress • Hepatocellular proliferation • Hepatocarcinogenesis

Abbreviations

ACOX1	Acyl –CoA oxidase
CAR	Constitutive androstane receptor
CRPC	Castration-resistant prostate cancer
CBP	CREB binding protein
CARM1	Coactivator-associated arginine methyltransferase-1
CRE	cAMP response element
CREB	cAMP response element-binding
DEHA	Di-(2-ethylhexyl) adipate
DEHP	Di-(2-ethylhexyl)-phthalate

DENA	Diethylnitrosamine
DRIP	Vitamin D receptor-interacting protein(s)
ER	Endoplasmic reticulum
GR	Glucocorticoid receptor
L-PBE	L-bifunctional peroxisomal enzyme
HAT	Histone acetyl transferase
MAPK	Mitogen activated protein kinase
Med1	Mediator complex subunit 1
MFP	Multifunctional protein
NCoA6IP	Nuclear receptor coactivator 6 interacting protein
PBP	PPAR-binding protein
PIMT	PRIP interacting protein with methyltransferase domain
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator response element
PRIP	PPAR-interacting protein
RXR	Retinoid-X-receptor
SBP2	Selenium binding protein-2
SRC-1	p160/steroid receptor coactivator-1
VLCF	Very long chain fatty acid
Tibric Acid	2-chloro-5-(3 5-dimethylpiperidinosulfonyl) benzoic acid
Wy-14, 643	[4-chloro-6-(2, 3-xylidino)-2-pyrimidinylthio] acetic acid

5.1 Peroxisomes: Structure and Functions

Peroxisomes are single membrane-limited cytoplasmic organelles with a finely granular matrix present in a wide variety of cells in animals and plants (De Duve and Baudhuin 1966). In liver parenchymal cells of most animals except human and huminoid primates, peroxisomes contain a dense core with a regular crystalloid structure representing urate oxidase. More than 60 proteins are present in peroxisomes that perform a variety of biological functions (Reddy and Mannaerts 1994). These organelles are required for lipid metabolism and also for the synthesis of bile acids and membrane phospholipids, synthesis of cholesterol, and degradation of uric acid, purines, polyamines and amino acids (De Duve and Baudhuin 1966; Tolbert 1981). Peroxisomes contain an array of H_2O_2-generating oxidases, such as flavin oxidase, urate oxidase, D-amino acid oxidase, L α-hydroxyacid oxidase A and B, polyamine oxidase, glutaryl-CoA oxidase, pipecolic acid oxidase, oxalate oxidase, acyl-CoA oxidase, trihydroxycholestanoyl-CoA oxidase, and pristanoyl-CoA oxidase together with the H_2O_2-degrading catalase (von den Bosch et al. 1992). Because of the presence of these oxidases, the peroxisomes are responsible for ∼20 % of oxygen consumption in liver (De Duve and Baudhuin 1966).

5.2 Peroxisome Proliferators and Peroxisome Proliferation

In liver parenchymal cells, peroxisomes measure ~0.2–1 μm in diameter and are few in number, accounting for less than 2 % of cytoplasmic volume under physiological conditions. In livers with peroxisome proliferation, these organelles can occupy up to 25 % of hepatocyte cytoplasmic volume. Peroxisome proliferation is a unique phenomenon generated by a broad spectrum of compounds of industrial, pharmaceutical and agricultural importance and include certain phthalate ester plasticizers, herbicides, leukotriene D4 receptor antagonists and lipid-lowering drugs, such as Wy-14,643 and ciprofibrate, among others (Reddy and Krishnakantha 1975; Reddy and Lalwani 1983a). These structurally diverse compounds designated peroxisome proliferators induce qualitatively predictable pleiotropic responses, including hepatomegaly, and peroxisome proliferation in liver parenchymal cells of rats and mice (Reddy and Krishnakantha 1975; Reddy and Lalwani 1983a; Ashby et al. 1994; Reddy and Chu 1996). Prolonged exposure to peroxisome proliferators in liver leads to the development of hepatocellular carcinomas in rats and mice (Reddy et al. 1976).

Peroxisome Proliferators are classified depending on their different chemical structures/moieties:

- **Fibrate class**: Several of the structural analogues of clofibrate are extremely potent inducers of hepatic peroxisome proliferation in rats and mice, and also in higher plants (Palma et al. 1991; Nila et al. 2006; Castillo et al. 2008). These included methyl clofenapate, and nafenopin, which are several orders of magnitude more potent than the prototype compound, clofibrate, in inducing hepatic peroxisome proliferation. Several other clofibrate analogues, such as fenofibrate, gemfibrozil, and ciprofibrate were also identified as potent peroxisome proliferators.
- **Acid class**: Two novel compounds, [4-chloro-6-(2, 3-xylidino)-2-pyrimidinylthio] acetic acid (Wy-14, 643) and 2-chloro-5-(3, 5-dimethylpiperidinosulfonyl) benzoic acid (tibric acid) induce peroxisome proliferation and exhibit a lipid lowering property.
- **Phthalate class**: This includes certain phthalate-ester plasticizers, such as di-(2-ethylhexyl)-phthalate (DEHP), and di-(2-ethylhexyl) adipate (DEHA), used in the manufacture of polyvinyl chloride plastics, also induce peroxisome proliferation and exhibit a lipid lowering property (Reddy and Lalwani 1983a; Reddy and Chu 1996; Lalwani et al. 1983).

5.3 Function of Peroxisome Proliferators

Peroxisome proliferators induce qualitatively predictable pleiotropic responses including hepatomegaly, proliferation of peroxisomes in hepatic parenchymal cells and several hepatic enzymes associated with lipid metabolism (Reddy and

Krishnakantha 1975; Reddy and Lalwani 1983a; Ashby et al. 1994; Reddy and Chu 1996). Prolonged exposure to peroxisome proliferators leads to the development of hepatocellular carcinomas in rats and mice (Reddy et al. 1976, 1982).

Peroxisome proliferator-induced pleiotropic responses could be demarcated into two phases: Immediate (short term) and delayed (prolonged or carcinogenic). Short term treatment of peroxisome proliferators results in the ~30 fold increased activities of the enzymes responsible for the peroxisomal β-oxidation namely, fatty acyl –CoA oxidase (ACOX1), enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (L-PBE) and 3-Ketoacyl-CoA thiolase (Reddy et al. 1986). The increased enzyme activities are due to rapid coordinated transcriptional activation of genes encoding these enzymes. The delayed response to chronic exposure to peroxisome proliferators is the development of hepatocellular carcinomas (Reddy and Rao 1977; Reddy et al. 1979; Reddy and Qureshi 1979). The incidence of liver tumors depends on the effectiveness of the different classes of peroxisome proliferators in inducing pleiotropic effects. Potent compounds like Wy-14,643, ciprofibrate, nafenopin, BR-931, and tibric acid induce liver tumors in nearly 100 % of rats and or mice within 50–60 weeks when administered in the diet at concentrations ranging from 0.025 % to 0.2 % (Reddy et al. 1980) whereas less potent peroxisome proliferators like clofibrate and DEHP induce liver tumor between 70 and 104 weeks when fed with diet in much more higher concentrations ranging from 0.50 % to 1.2 % (w/w) (Ashby et al. 1994; Kluwe et al. 1982; David et al. 1999). Evidence suggests that though all the peroxisome proliferators are carcinogenic in rats and mice but the incidence of liver tumors induced by these agents depends on the effective dose of the compound. Results show that when peroxisome volume density reaches or exceeds 20 % of the cytoplasmic volume of hepatocytes in rats and mice induced by peroxisome proliferators, the liver tumor incidences can be expected to reach nearly 100 % level (Ashby et al. 1994; Reddy et al. 1980). These results demonstrate the following important facts: (1) peroxisome proliferators are hepatocarcinogenic in mice and rats, (2) peroxisome proliferation is a biological marker for hepatocarcinogenesis in rats and mice and (3) peroxisome proliferators exert their effects mainly by rapid transcriptional activation of H_2O_2-generating fatty acid oxidation system enzymes (Reddy 2004; Yeldandi et al. 2000).

5.4 PPAR Family of Nuclear Receptors and Signaling

Are the effects exhibited by peroxisome proliferators liver specific? Is there any involvement of a receptor mediated mechanism? How does a peroxisome proliferator transduce the signal?

Experimental facts reveal the following:

- Similarity of biological/pleiotropic responses in liver exerted by structurally different peroxisome proliferators is reflected by composition in the livers of rats/mice treated with structurally dissimilar peroxisome proliferators (Reddy 2004).

- Detection of a specific peroxisome proliferator binding moiety in liver cytosol and identification of several peroxisome proliferators binding proteins including a ~55 kDa protein, heat shock protein and others in the liver cells (Reddy 2004; Lalwani et al. 1987; Reddy and Rao 1988).
- Most importantly, the evidence that liver cells possess a mechanism to recognize and respond to peroxisome proliferators irrespective of their location in the body (Reddy 2004).
- In addition to that rapid coordinated transcriptional activation of lipid metabolism genes in the peroxisome proliferators treated livers demonstrate the involvement of a receptor- mediated signal transduction mechanism (Reddy et al. 1986), which is later on confirmed by the cloning of Peroxisome Proliferator Activated Receptor (PPAR) from liver (Issemann and Green 1990). PPARα is highly expressed in liver tissue and targeted disruption of the α- isoform of the peroxisome proliferators-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators including liver cancers (Lee et al. 1995; Peters et al. 1997) and thus, confirming the receptor mediated signal transduction concept in recognizing and sensing xenobiotics (peroxisome proliferators) in liver (Reddy and Lalwani 1983a; Reddy and Rao 1986; Rao and Reddy 1987).

Further studies establish that PPAR family consists of three subtypes namely PPARα, PPAR β/δ and PPARγ (Desvergne and Wahli 1999). PPARα is highly expressed in liver tissue but to a lesser extent in kidney, intestine, heart and the organs and mainly responsible for energy combustion (Reddy et al. 1986; Issemann and Green 1990; Desvergne and Wahli 1999). PPAR β/δ is expressed broadly in many tissues and appears to participate in wide spread fat burning, macrophage VLDL sensor and other functions (Desvergne and Wahli 1999; Peters et al. 2000; Wang et al. 2003). PPARγ is mainly responsible for adipogenesis and energy conservation (Tontonoz et al. 1994).

Each PPAR isotype is encoded by separate gene. PPARγ has two isoforms designated as PPARγ1 and PPARγ2 (Zhu et al. 1995). PPARγ2 is 30 amino acids longer at N-terminal region than PPARγ1. PPARs possess highly conserved DNA binding domains with two zinc fingers that recognize peroxisome proliferator responsive elements (PPRE) in the promoter region of the selected gene (Reddy and Chu 1996; Desvergne and Wahli 1999). Each PPAR also contains two transactivation domains. N-terminal region contains ligand independent transactivation domain known as AF-1 domain whereas, C-terminal region has ligand dependent transactivation domain known as AF-2 domain. Liganded PPAR heterodimerizes with another nuclear receptor, the retinoid-X receptor (RXR) by interacting with specific DNA sequence called peroxisome proliferator responsive element (PPRE) in the promoter regions of target genes (Kliewer et al. 1992). Transcription factors are bound with co-repressors and ligand binding induces change in conformation in the receptor resulting in the replacement of co-repressors and recruitment of a plethora of transcriptional co-activators that enhance transcription of target genes (reviewed in Kornberg 2007; Pyper et al. 2010; Viswakarma et al. 2010; Zhu et al. 2001).

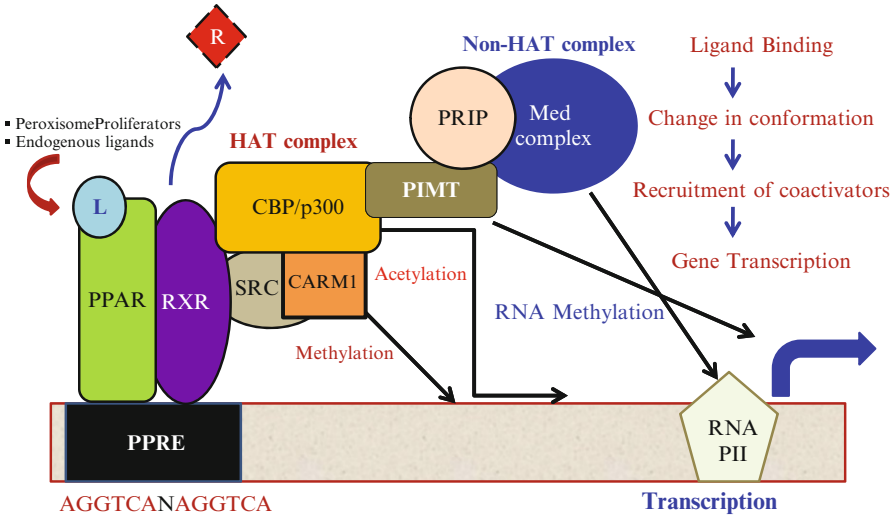

Fig. 5.1 A proposed model of PPAR-mediated transcription: Ligand (L) binding induces change in conformation of the receptor displacing co-repressor (R) and recruiting co-activator. CBP/p300 and other proteins in this complex, such as members of the SRC family, possess intrinsic histone acetyltransferase (HAT) activity, and CARM1 contains histone methyltransferase activity. The TRAP/DRIP/ARC complex anchored by MED1 and other proteins such as PRIP with no known enzymatic activities facilitate the recruitment of general transcription factors (GTFs) and RNA polymerase II holoenzyme to initiate transcription of the ligand-bound nuclear receptors. PIMT, a co-activator binding protein having RNA methyl transferase activity makes a bridge between HAT and non-HAT coactivator complexes facilitating to form a mega complex

Coactivators that have been cloned in recent years include the p160/steroid receptor coactivator-1 (SRC-1) family with three members (SRC-1, TIF2/GRIP1/SRC2, and pCIP/ACTR/AIB1/RAC3/TRAM1/SRC-3) (Pyper et al. 2010; Viswakarma et al. 2010), cAMP-response element binding protein-binding protein (CBP) (Pyper et al. 2010; Viswakarma et al. 2010), and adenovirus E1A-binding protein p300 (Pyper et al. 2010; Viswakarma et al. 2010). Depending on the enzymatic activity, co-activators are classified into two different complexes: (1) complex containing histone acetyltransferase (HAT) activity and (2) complex having no-HAT activity (Fig. 5.1). The CBP/p300 and p160 family of coactivators possess intrinsic HAT activity and modify histone tails by acetylation post-translationally (Pyper et al. 2010; Viswakarma et al. 2010) and CARM1, an important component of this complex methylates histone resulting remodeling of the chromatin structure (Pyper et al. 2010; Viswakarma et al. 2010). Non-HAT co-activators of key importance include ~30 subunits of the Mediator complex (Kornberg 2007; Pyper et al. 2010; Viswakarma et al. 2010). The Mediator complex, with Med1/PBP/TRAP220/DRIP205/ARC (Kornberg 2007; Pyper et al. 2010; Viswakarma et al. 2010) as a key component, links with the general transcription machinery and RNA polymerase II (Kornberg 2007; Pyper et al. 2010;

Viswakarma et al. 2010). Taken together, these two important complexes coordinate with each other to stabilise the pre-initiation complex to modulate transcription (Fig. 5.1).

Other co-activators of importance include PRIP (NCoA6) and PRIP interacting protein, designated PIMT (NCoA6IP) that enhances the PRIP coactivator function (Zhu et al. 2001). PIMT, which has an invariant GXXGXXI segment found in K-homology motifs and many RNA-binding proteins, binds RNA (Kornberg 2007). PIMT also has a nine amino acid VVDAFCGVG methyltransferase motif I and binds S-adenosyl-L-methionine, the methyl donor for the methyltransfer reaction, suggesting that it may be a putative RNA methyl transferase (Zhu et al. 2001). Hypermethylation of the cap structure of both yeast snRNA and snoRNA requires a conserved methyltransferase that is localized to the nucleolus (Mouaikel et al. 2002). PIMT is essential coactivator for embryonic development as knockout of PIMT gene in mice results in early embryonic lethality at blastocyst stage (Jia et al. 2012). PIMT bridges the CBP/p300-anchored coactivator complex with the Med1 anchored coactivator complex resulting in the formation of a mega-complex but differentially modulates coactivator function such that inhibition of the CBP/p300 effect may be designed to enhance the activity of Med1 and PRIP (Misra et al. 2002a) (Fig. 5.1).

In essence, like that of other nuclear receptors, PPAR dependent transcription consists of the following steps: (1) ligand binding to the ligand binding domain, (2) ligand induced change in conformation in the receptor, (3) recruitment of coactivator/coactivators upon replacing co-repressor, post transcription/translational modification of transcriptional machinery and formation of mega complex and (4) modulation of gene transcription. PPARs are expressed in all tissues to a variable extent, but peroxisome proliferator-associated hepatocarcinogenesis is tightly linked to PPARα expression (Palma et al. 1991; Reddy 2004). PPARα is activated by synthetic small molecules such as structurally dissimilar different classes of peroxisome proliferators and by natural ligands like polyunsaturated fatty acids and eicosanoids (Reddy and Chu 1996; Kliewer et al. 1992; Pyper et al. 2010; Gottlicher et al. 1992). The question as to how these different types of PPARα ligands exert similar biological effects in tissue and gene specific remains largely unanswered but two major regulators include PPARα and the Med1 subunit of the Mediator complex (Pyper et al. 2010; Viswakarma et al. 2010).

5.5 Fatty Acid Oxidation in Liver, PPARα Target Genes and Signaling

Three subcellular compartments participate in fatty acid metabolism in that fatty acid β-oxidation occurs in both peroxisomes and mitochondria and fatty acid ω-oxidation takes place in the endoplasmic reticulum (Fig. 5.2). While short and medium chain fatty acids are β-oxidized rather exclusively in mitochondria, LCFAs can be oxidized in both mitochondria and peroxisomes (Fig. 5.2). However,

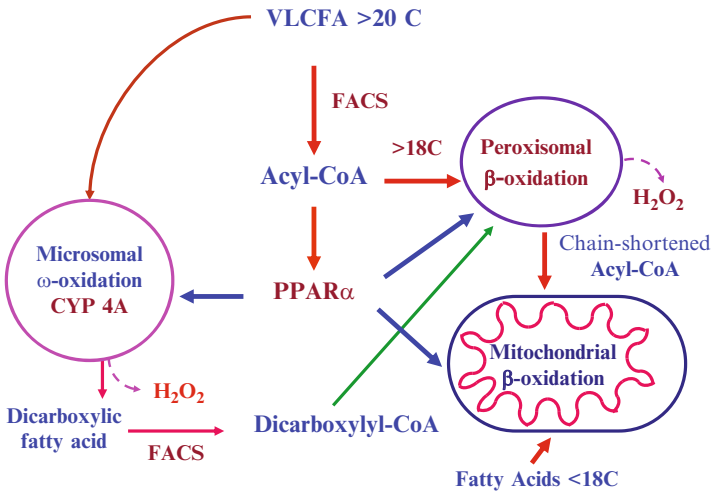

Fig. 5.2 Fatty acid oxidation in liver: PPARα controls the genes involved in peroxisomal and mitochondrial β-oxidation and microsomal ω-oxidation. Peroxisomal β-oxidation is responsible for long chain fatty acid (>C_{18}) whereas, mitochondrial β-oxidation system metabolizes medium and short chain fatty acids. Microsomal ω-oxidation system metabolizes very long chain fatty acid producing dicarboxylic acid which is a ligand of PPARα and thus, regulates the activities of PPARα

mitochondria lack very long-chain fatty acyl-CoA synthetase, hence VLCFAs (>C_{18}) cannot enter these organelles. Peroxisomal membrane on the other hand has at least two acyl-CoA synthetases: a long chain acyl-CoA synthetase and a very long-chain acyl-CoA synthetase capable of activating LCFAs and VLCFAs to acyl-CoAs for β-oxidation (Steinberg et al. 2000). While LCFAs can be oxidized both in mitochondria and peroxisomes, the presence of very long-chain fatty acyl-CoA synthetase on peroxisomal membrane accounts for the exclusively streamlined β-oxidation of VLCFA within the peroxisomes. However, unlike in mitochondria, the β-oxidation does not proceed to completion and the chain-shortened fatty acyl-CoAs are transported to mitochondria for the completion of β-oxidation (Reddy and Mannaerts 1994; Reddy 2004). In peroxisomes, β-oxidation is not coupled to ATP synthesis as peroxisome does not possess any oxidative phosphorylation pathway (Palosaari and Hiltunen 1990). Instead, the high-potential electrons are transferred to O_2 to yield H_2O_2, which is further converted into H_2O and O_2 by peroxisomal catalase. The energy released during peroxisomal fatty acid oxidation is dissipated as heat.

Two separate, but partly interchangeable, β-oxidation systems have been identified in peroxisomes (Mannaerts and van Veldhoven 1996). One system is responsible for the β-oxidation of straight chain fatty acids whereas the second β-oxidation system is designed for β-oxidation of branched chain fatty acids. The first system, which is inducible consists of three enzymes: acyl-CoA oxidase 1 (ACOX1), the first and the rate-limiting enzyme of peroxisomal straight-chain fatty acid oxidation system which produces 2-enoyl-CoA. The second enzyme,

known as multifunctional protein (MFP-1) or L-bifunctional peroxisomal enzyme (L-PBE), contains 2-enoyl-CoA hydratase, dienoyl-CoA isomerase activity and L-3-hydroxyacyl-CoA dehydrogenase activity (Caira et al. 1996; Osumi et al. 1985). This second step in peroxisomal β-oxidation yields ketoacyl-CoA which serves as substrate for 3-ketoacyl-CoA thiolase (PTL), the third enzyme of the peroxisomal β-oxidation system. The enzymatic conversion of ketoacyl-CoA results in acyl-CoA that is two carbon atoms shorter than the original molecule. All three genes of this classical inducible peroxisomal β-oxidation system are tightly regulated by PPARα (Castillo et al. 2008; Reddy 2004).

The second peroxisomal β-oxidation system exerts its function with the help of three enzymes: the first enzyme namely 2-methylacyl-CoA-specific oxidases (such as trihydroxycoprostanoyl-CoA oxidase and pristanoyl-CoA oxidase) process 2-methyl branch chained fatty acids such as pristanic acid and bile acid intermediates; the second multifunctional protein (named MFP-2) contains both hydratase and dehydrogenases activities (Novikov et al. 1994; Dieuaide-Noubhani et al. 1996, 1997; Caira et al. 1998) whereas, third enzyme, a 58 kDa sterol-carrier protein (SCP-2) contains thiolase activity (Mannaerts and van Veldhoven 1996; Seedorf et al. 1994). MFP-1 hydrates 2-trans-enoyl-CoAs into L-3-hydroxyacyl-CoAs and dehydrogenates the L-isomers (Dieuaide-Noubhani et al. 1996, 1997; Osumi and Hashimoto 1980). MFP-2, in contrast, transforms 2-trans-enoyl-CoAs into D-3-hydroxyacyl-CoA and dehydrogenates the D-isomers (Caira et al. 1996; Dieuaide-Noubhani et al. 1996; Qin et al. 1997) and thus, MFP-1and MFP-2 exhibit opposite stereospecificities. Though MFP enzymes are structurally unrelated to each other (Qin et al. 1997) but both MFPs can hydrate 2-methyl-enoyl-CoAs (Dieuaide-Noubhani et al. 1996; Qin et al. 1997; Xu and Cuebas 1996).

In addition to mitochondrial and peroxisomal β-oxidation processes, very long-chain fatty acids are also oxidized to dicarboxylic acids by microsomal ω-oxidation carried out by cytochrome P_{450} CYP4A enzymes that are regulated by PPARα (Reddy and Hashimoto 2001). Both peroxisomal β-oxidation and microsomal omega-oxidation lead to the generation of H_2O_2 (Fig. 5.2). The first step of β-hydroxylation of saturated and unsaturated fatty acids occurs in the endoplasmic reticulum and the resulting β-hydroxy fatty acid is then dehydrogenated to yield highly toxic dicarboxylic acids in the cytosol. Dicarboxylic acids are converted to dicarboxylyl-CoAs and enter peroxisomes for further metabolism by the classical inducible β-oxidative enzymes (Ferdinandusse et al. 2004; Reddy and Rao 2006). Dicarboxylic acids are unique in that they serve as substrates for the peroxisomal β-oxidation system (Fig. 5.2) (Hashimoto et al. 1999). It would appear that dicarboxylic fatty acids streamline their own metabolism by inducing PPARα activation and thus regulating all three fatty acid oxidation enzyme pathways (Reddy and Hashimoto 2001).

Unlike the mitochondrial system, peroxisomal β-oxidation does not go to completion. In the peroxisome, the 2-carbon-shortened acyl-CoA reenters the β-oxidation cycle, and this process repeats for about five cycles, resulting in the removal of ten carbon atoms. Peroxisomal β-oxidation generated chain-shortened acyl- CoAs are shuttled to mitochondria, either as carnitine esters and/or as free fatty acid for the completion of oxidation. Peroxisomes contain carnitine

acetyltransferase and carnitine octanoylated transferase for conjugation and transport of short- and medium-chain acyl-CoAs respectively. Mitochondria on the other hand use carnitine shuttle with carnitine palmitoyl transferase-1 (CPT1) and CPT2 as major players (Reddy and Hashimoto 2001; Brandt et al. 1998).

5.6 Energy Metabolism and Excess Energy Burning in Liver Cancer Development: Lessons Learned from Different Knockout Animal Models

5.6.1 PPARα$^{-/-}$ Mice

PPARα is a master regulator of hepatic combustion of energy. PPARα governs the constitutive and inducible levels of expression of genes involved in all three fatty acid oxidation systems namely peroxisomal, mitochondrial and microsomal fatty acid oxidation system (Lee et al. 1995; Desvergne and Wahli 1999). Livers of PPARα$^{-/-}$ mice develop a mild hepatic steatosis, a decreased constitutive level of mitochondrial fatty acid β-oxidation but the unaltered basal level of expression of enzymes involved in peroxisomal β-oxidation of very long chain fatty acids (VLCFs) indicating that basal peroxisomal β-oxidation of VLCFs is independent of PPARα regulation. Disturbances in peroxisomal energy metabolism lead to death in children especially in the case of pseudo-neonatal adrenoleukodystrophy, a clinical condition linked with deficiency of peroxisomal ACOX1 activity (Wanders et al. 2001; Poll-The et al. 1988). To investigate the impact of the alterations of the peroxisomal fatty acid β-oxidation in depth, mice lacking ACOX1 (ACOX1$^{-/-}$), both PPARα and ACOX1 (PPARα$^{-/-}$ ACOX1$^{-/-}$), L-PBE$^{-/-}$ and both LPBE$^{-/-}$ and D-PBE$^{-/-}$, Thiolase B(Thb$^{-/-}$) and SCP2$^{-/-}$, and whole body and liver specific Med1$^{-/-}$, an important coactivator and other co-activators were generated (Hashimoto et al. 1999; Jia et al. 2003; Fan et al. 1996; Qi et al. 1999; Houten et al. 2012; Baes et al. 2000; Chevillard et al. 2004; Arnauld et al. 2009; Seedorf et al. 1988; Fan et al. 1998). The genetically altered mouse models highlight the criticality of PPARα and its regulated genes in fatty acid oxidation in liver.

5.6.2 ACOX1$^{-/-}$ Mice

ACOX1$^{-/-}$ mice develop hepatomegaly with severe microvesicular hepatic steatosis (Fan et al. 1996). These mice exhibit increased intrahepatic H_2O_2 level and hepatocellular regeneration (Fan et al. 1996). The progressive liver cell regeneration leads to the emergence of hepatocytes with hypertrophic granular cytoplasm that ultimately replaces steatotic hepatic cells and show massive peroxisome proliferation. The presence of polymorphonuclear neutrophills, lipogranulomas and cluster of foamy macrophages are the characteristics of inflammatory responses of the liver and ultimately the older mice develop hepatocellular carcinoma due to sustained

hyperactivation of PPARα (Fan et al. 1998). The ACOX1$^{-/-}$ mice also reveal several fold increase in the expression of PPARα target genes especially the activation of hepatic ω-oxidation associated genes (CYP4A10 and CYP4A14) involved in the production of dicarboxylic acids which remain unmetabolized in the absence of ACOX1 activity and are strong inhibitors of mitochondrial β-oxidation but contributes in the increase of oxidative stress by increased production of intra hepatic H_2O_2 (Fan et al. 1998). The ACOX1 null mice establish that branched chain β-oxidation system is incapable of degrading unmetabolized long-chain fatty acyl-CoAs and other substrates of ACOX1 indicating that substrate specificity of branched chain oxidases of the β-oxidation system (Pyper et al. 2010; Fan et al. 1998). The ACOX1 null mice reveal that disturbance in the activity of this enzyme leads to major changes in the liver, including steatohepatitis, inflammatory hepatic steatosis and transcriptional activation of PPARα regulated genes (Fan et al. 1998). Recent evidence also suggests that sustained increase in fatty acid oxidation reduces hepatic lipid accumulation but induces endoplasmic reticulum stress in liver in these ACOX1 null mice that contributes liver cell death and regeneration leading to the development of hepatocellular carcinoma (Huang et al. 2011, 2012; Tyra et al. 2012). Because up-regulation of fatty acid oxidation increases ER stress and suppression of fatty acid oxidation protects against ER stress, it would appear that fatty acid oxidation *vis a vis* the lipid sensor PPARα is a critical determinant in the pathogenesis of hepatocellular carcinoma (Huang et al. 2011, 2012; Tyra et al. 2012).

5.6.3 ACOX1$^{-/-}$/PPARα$^{-/-}$ Double Null Mice

In mice deficient in both ACOX1 and PPARα (ACOX1$^{-/-}$/PPARα$^{-/-}$ mice), microvesicular steatohepatitis, spontaneous peroxisome proliferation and also the induction of PPARα target genes by unmetabolised biological ligands of PPARα that remain in the absence of ACOX1 activity are absent. Importantly, these mice fail to exhibit the generation of dicarboxylic acids as PPARα dependent induction of ω-oxidation is abrogated. β-oxidation is the major process by which fatty acids are oxidized to generate energy, especially when glucose availability is low during periods of starvation. Mice deficient in PPARα and those nullizygous for both PPARα and ACOX1 show a minimal steatotic phenotype under fed conditions but manifest an exaggerated steatotic response to fasting, indicating that defects in PPARα-inducible fatty acid oxidation determine the severity of fatty liver phenotype to conditions reflecting energy-related stress (Hashimoto et al. 1999).

5.6.4 L-PBE$^{-/-}$ Mice

Mice deficient in enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase (L-PBE), the second enzyme of the peroxisomal β-oxidation system, are viable and

fertile and exhibit no detectable gross phenotypic changes. L-PBE−/− mice showed no hepatic steatosis and manifested no spontaneous peroxisome proliferation, unlike that encountered in livers of mice deficient in ACOX1 (Qi et al. 1999). Recent data shows that L-PBE is involved in dicarboxylic acid metabolism and essential for the production of medium-chain dicarboxylic acid and its expression varies with genes involved in the tricarboxylic acid cycle and in mitochondrial and peroxisomal fatty acid oxidation (Houten et al. 2012).

5.6.5 *D-PBE$^{-/-}$ Mice*

To evaluate the physiological role of D-PBE (MFP-2) of the second peroxisomal β-oxidation pathway, D-PBE/MFP-2 knockout mice were generated. D-PBE/MFP-2 deficiency reveals the following characteristics: (1) severe growth retardation during the first weeks of life, resulting in the premature death of one-third of the D-PBE$^{-/-}$/MFP-2$^{-/-}$ mice, (2) accumulation of VLCFA in brain and liver phospholipids, immature C_{27} bile acids in bile, and (3) after supplementation with phytol, pristanic and phytanic acid in liver triacylglycerols, and (4) enhancement of peroxisomal β-oxidation of long straight chain fatty acids (C_{16}) in liver tissue due to the up-regulation of the enzymes of the classical peroxisomal β-oxidation pathway. The data indicate that D-PBE/MFP-2 is not only essential for the degradation of 2-methyl-branched fatty acids and the bile acid intermediates but also for the breakdown of very long chain fatty acids (Baes et al. 2000).

5.6.6 *L-PBE$^{-/-}$D-PBE$^{-/-}$ Double Null Mice*

Disruption of the inducible β-oxidation pathway at the level of ACOX1 in mice, results in spontaneous peroxisome proliferation and sustained activation of PPARα, leading to the development of liver tumors, whereas disruptions at the level of the second enzyme of this classical pathway or of the noninducible system had no such discernible effects suggesting that D-PBE of non-inducible peroxisomal fatty acid oxidation system metabolizes the enoyl-CoAs in the absence of L-PBE (Qi et al. 1999). Mice with complete inactivation of peroxisomal β-oxidation at the level of the second enzyme, enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase (L-PBE) of the inducible pathway and D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl- CoA dehydrogenase (D-PBE) of the non-inducible pathway (L-PBE$^{-/-}$L-PBE$^{-/-}$), exhibit severe growth retardation and postnatal mortality with none surviving beyond weaning (Jia et al. 2003). L-PBE$^{-/-}$D-PBE$^{-/-}$ mice that survived exceptionally beyond the age of 3 weeks exhibited overexpression of PPARα-regulated genes in liver, despite the absence of morphological evidence of hepatic peroxisome proliferation. These studies establish that peroxisome proliferation in rodent liver is highly correlatable with

the induction mostly of the L- and D-PBE genes. Thus, disruption of peroxisomal fatty acid β-oxidation at the level of second enzyme in mice leads to the induction of many of the PPARα target genes independently of peroxisome proliferation in hepatocytes, raising the possibility that intermediate metabolites of very long-chain fatty acids and peroxisomal β-oxidation act as ligands for PPARα (Jia et al. 2003).

5.6.7 Med1/PBP$^{-/-}$ Null Mice

Disruption of the Med1gene in the mouse results in embryonic lethality around E11.5 days, indicating that this pivotal anchoring coactivator of the Mediator complex affects the function of many nuclear receptors and other transcription factors (Zhu et al. 2000). Med1 is critical for the development of placenta and for the normal embryonic development of the heart, eye, vascular, and hematopoietic systems (Crawford et al. 2002). Liver specific Med1 knockout (Med1 $^{\Delta Liv}$) mice generated using Cre-loxP system failed to respond to PPARα ligands in that Med1 null hepatocytes revealed no peroxisome proliferation, and no hepatocellular proliferation (Jia et al. 2004). Induction of several PPARα governed genes was also not present in Med1 null livers. In essence, Med1 null livers resembled PPARα null livers in their responses to PPARα activation and that PPARα signaled transcription requires Med1(Jia et al. 2004). Liver specific Med1 knockout mice also showed impaired liver regeneration with low survival after partial hepatectomy (Matsumoto et al. 2007). In Med1 null livers, an occasional hepatocyte that escaped Cre-mediated deletion of Med1 floxed alleles respond to PPARα ligand induced peroxisome proliferation and these cells exhibit considerable growth advantage (Jia et al. 2004). Med1 liver conditional null mice develop liver tumors on long-term exposure to PPARα ligand, but all tumors developing in Med1$^{\Delta Liv}$ mice reveal Med1 expression and no tumors developed from Med1$^{-/-}$hepatocytes suggesting that Med1 plays a key role in PPARα ligand-induced liver tumor development and that cells deficient in Med1 do not give rise to liver tumors (Matsumoto et al. 2007).

Med1 subunit is essential for the signaling of nuclear receptors PPARγ, PPARα, CAR (constitutive androstane receptor) and GR (glucocorticoid receptor). Conditional ablation of Med1 gene in mouse liver attenuates glucocorticoid receptor agonist dexamethasone-induced hepatic steatosis indicating that transcriptional coactivator mediator subunit Med1/PBP is required for the development of fatty liver in the mouse (Jia et al. 2009). Transcription coactivator Med1 is also required for high-fat diet-induced and PPARγ-stimulated fatty liver development (Bai et al. 2011). Med1 is also required for the transcriptional regulation of the xenobiotic receptor CAR and CAR regulated drug metabolising genes (Jia et al. 2005; Guo et al. 2006).

We have shown that Med1/PBP contains six different phosphorylation sites specific for PKA, different subtypes of PKC including two sites of the mitogen-activated protein kinase (MAPK) family located at the C-terminal at threonine 1017 and 1444 and Med1/PBP phosphorylation by Raf/MEK/MAPK cascade exert positive effect on PBP mediated coactivator function (Misra et al. 2002b). The UBE2C oncogene is overexpressed in many types of solid tumours including the lethal castration-resistant prostate cancer (CRPC). Recent report shows that phospho-MED1-enhanced UBE2C locus looping drives castration-resistant prostate cancer growth showing the importance of Med1 in receptor mediated signal transduction (Chen et al. 2011).

5.6.8 ACOX1$^{-/-}$ob/ob Mice

PPARα activation in *ob/ob* mice leads to reduction in obesity but increases excess energy burning that increased fatty acid oxidation leads to ER stress and thus, excess energy burning in liver causes liver tumor development (Huang et al. 2012).

5.7 Mechanism of Hepatocarcinogenesis

Prolonged exposure to peroxisome proliferators *vis a vis* sustained activation of PPARα signaling leads to increased fatty acid oxidation contributes to sustained oxidative stress and increased ER stress in liver. These changes lead to liver cell damage and proliferation which contribute to the development of hepatocellular carcinomas (Reddy et al. 1976, 1982). Peroxisomes proliferators as a class has been shown to be nonmutagenic establishing the tenet that peroxisome proliferators cause liver tumor development by nonmutagenic or nongenotoxic mechanism (Reddy and Lalwani 1983a; Reddy and Rao 1986, 1988; Warren et al. 1980). Peroxisome proliferators do not induce mutations or DNA damage in short-term mutagenesis or DNA-repair assays (Warren et al. 1980). They do not cause any mutagenic activity in standard *Salmonella* based microsomal assay (Warren et al. 1980), or produce any chromosomal aberration (Weisburger and Williams 2000) or PP-DNA adducts (Reddy and Rao 1977; Reddy et al. 1979). Further studies showed that hepatocellular carcinoma induced by peroxisomes proliferators do not express classical liver cancinogenic markers like γ-glutamyltranspeptidase and placental form of glutathione-S-transferase (GST-π) establishing that peroxisome proliferator-induced liver tumors exhibit unique phenotype (Rao et al. 1982). Furthermore, gene expression profiles in the liver tumors of mice treated separately with genotoxic carcinogen diethylnitrosamine (DENA), nongenotoxic carcinogen and peroxisome proliferator ciprofibrate and ACOX1$^{-/-}$ and showed that liver tumors developing

in ACOX1$^{-/-}$ mice and those induced by ciprofibrate modulate genes differently compared to that of DENA-induced liver tumors indicating that this information may be useful in establishing the molecular mechanism of nongenotoxic liver carcinogenesis (Meyer et al. 2003). Further study in animals treated with Wy-14643 suggests that DNA-damaging oxidants are generated by enzymes that are induced after activation of PPARα, such as those involved in lipid metabolism in peroxisomes supporting the role of oxidative stress in DNA damage (Rusyn et al. 2004). Expression of base excision DNA repair genes is a sensitive biomarker for in vivo detection of chemical-induced chronic oxidative stress: identification of the molecular source of radicals responsible for DNA damage by peroxisome proliferators (Rusyn et al. 2004).

In normal liver, peroxisomes appear to account for ∼20 % of oxygen consumption and the level of O_2 consumption will be higher in livers when exposed to peroxisome proliferators due to sustained PPARα activation and the resulting increases in the expression of H_2O_2-generating enzymes, namely ACOX1 and CYP4A that enhance fatty acid oxidation (Fig. 5.3). Thus, it has been proposed that disproportionate increases in H_2O_2-generating enzymes and reductions in H_2O_2-degrading enzymes catalase and glutathione peroxidase activity lead to sustained oxidative stress in liver (Reddy 2004). These changes result in progressive accumulation of lipofuscin in liver cells, a byproduct of reactive oxygen species-induced lipid peroxidation, ∼18-fold reduction in selenium binding protein-2(SBP2), an important protein played crucial role in growth inhibition and anti-carcinogenic effects and formation of 8-hydroxydeoxyguanosine adducts in liver DNA (Issemann and Green 1990; Meyer et al. 2003; Rusyn et al. 2004; Reddy and Lalwani 1983b; Rao and Reddy 1987; Reddy et al. 1982). Concurrent administration of antioxidants such as ethoxyquin with peroxisome proliferators retard liver tumorigenesis (Rao et al. 1984) establishing oxidative stress is an important contributor of peroxisome proliferators induced hetpatocarcinogenesis (Fig. 5.3). Recent evidence indicates that reduction in fatty acid oxidation causes a decrease in ER response (Fan et al. 1998) and increased fatty acid oxidation enhances ER stress (Huang et al. 2011, 2012). Thus, sustained activation of PPARα in liver by endogenous or exogenous activators leads to transcriptional activation of genes regulated by this nuclear receptor. The PPARα regulated genes play a major role in energy combustion by increasing hepatic fatty acid oxidation. Sustained increase in fatty acid oxidation adds to oxidative and endoplasmic stress in liver, factors that contribute to hepatocarcinogenesis (Fig. 5.3).

Sustained activation of PPARα by peroxisome proliferators represses the expression of let-7C miRNA, which fails to target the 3′ untranslated region of c-myc mRNA, leading to up regulation of c-myc expression and induction of the oncogenic mir-17-92 cluster and thus, resulting hepatocellular proliferation (Shah et al. 2007). Activation of all three PPAR isoforms causes physiological changes which make them good targets for the treatment and prevention of cancer (Peters et al. 2012). Metabolic syndrome is associated with increased cancer risk which suggests that PPAR agonists should be potential candidates for treating and preventing cancer.

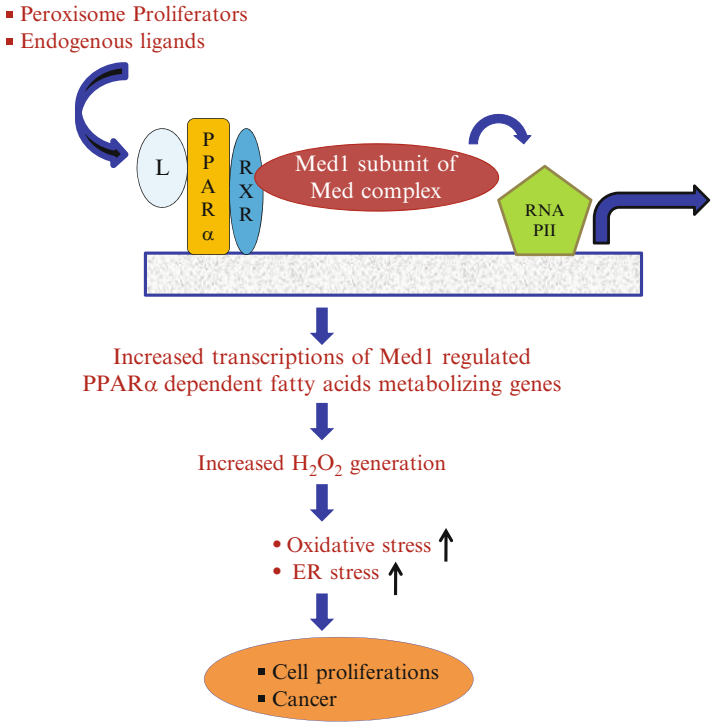

Fig. 5.3 A schematic depicting PPARα activation by peroxisome proliferators and endogenous ligands leads to increased transcription of fatty acid metabolizing genes that requires coactivator Med1 and that FA oxidation generates H_2O_2 and ER stress that adds to cell injury/cell proliferation and cancer

5.8 Conclusion

PPARα modulates the activities of all three fatty acid oxidation systems, namely mitochondrial and peroxisomal β-oxidation and microsomal ω-oxidation as an interlinked systems to burn energy and in the process generate oxidative stress (Fig. 5.2). Endogenous activation of PPARα in liver has also been observed in certain gene knockout mouse models of lipid metabolism, implying the existence of enzymes that either generate (synthesize) or degrade endogenous PPARα agonists (Fig. 5.3). For example, substrates involved in fatty acid oxidation can function as PPARα ligands. PPARα requires transcription coactivator PPAR-binding protein (PBP)/mediator subunit 1(MED1) for its transcriptional activity. Chronic peroxisome proliferation manifest high levels of lipid peroxidation and lipofuscin pigment, a hallmark of chronic oxidative damage to macromolecules. Fatty acid oxidation contributes to ER stress resulting cell death and cell proliferation.

In summary, evidence implicates that hyperactivation of PPARα induced either by peroxisome proliferators or endogeneous ligands plays a central role in hepatocarcinogenesis by disproportionate increases in H_2O_2-generating enzymes and reductions in H_2O_2-degrading enzymes and thus, generating excess reactive oxygen species resulting in sustained oxidative stress and progressive ER stress with activation of unfolded protein response signaling and these ingradients coupled with hepatocellular proliferation are the fundamental causes of peroxisome proliferators-induced hepatocarcinogenesis. Basic PPAR signaling mechanisms are intact in all mammals and it is the relative levels of activation of this mechanism account in part to species sensitivity and relative resistance. The idea that PPARα signaling is not relevant and the risk to humans is minimal is erroneous, simplistic and misleading. PPARα signalling mechanism is very complex and well coordinated functions exerted by various players including transcription factors, co-activators, coactivator binding protein and external stimuli among others. Drug discovery efforts are on to validate some of these players as effective therapeutic targets with emphasis on important and versatile transcriptional coactivator, Med1 for the invention of the drug to treat hepatocarcinogenesis and other cancers.

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Chapter 6
Involvement of Human Peroxisomes in Biosynthesis and Signaling of Steroid and Peptide Hormones

Isabelle Weinhofer, Markus Kunze, Sonja Forss-Petter, and Johannes Berger

Contents

Abstract Although peroxisomes exert essential biological functions, cell type-specific features of this important organelle are still only superficially characterized. An intriguing new aspect of peroxisomal function was recently uncovered by the observation that the peptide hormones β-lipotropin (β-LPH) and β-endorphin are localized to peroxisomes in various human tissues. This suggests a functional link between peptide hormone metabolism and peroxisomes. In addition, because endocrine manifestations that affect steroid hormones are often found in patients suffering from inherited peroxisomal disorders, the question has been raised whether peroxisomes are also involved in steroidogenesis. With this chapter, we will review several crucial aspects concerning peroxisomes and hormone metabolism.

Keywords Peroxisome • Peptide Hormones • β-lipotropin • β-endorphin • Steroid Hormones

I. Weinhofer (✉) • M. Kunze • S. Forss-Petter • J. Berger
Center for Brain Research, Medical University of Vienna, Spitalgasse 4, 1090 Vienna, Austria
e-mail: isabelle.weinhofer@meduniwien.ac.at

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Abbreviations

ABC-transporter	ATP binding cassette transporter
ACTH	Adrenocorticotropic Hormone
ALDP	Adrenoleukodystrophy protein
DHEA	Dehydroepiandrostenedione
IBA	Indole-3-butyric acid
PC	Prohormone convertase
POMC	Proopiomelanocortin
TGN	*Trans*-Golgi network
VLCFA	Very long-chain fatty acids
X-ALD	X-linked adrenoleukodystrophy
α-MSH	α-Melanocyte-Stimulating Hormone
β-LPH	β-Lipotropin

6.1 Introduction

Peroxisomes are ubiquitous, single membrane-bound organelles that participate in a wide variety of metabolic processes, many of which are related to the metabolism of lipids and reactive oxygen species. In humans and other mammals, these include degradation of pipecolic, phytanic, and very long-chain fatty acids (VLCFA; ≥ 22 carbon atoms), the synthesis of plasmalogens, bile acids and docosahexaenoic acid or the detoxification of hydrogen peroxide (Wanders and Komen 2007). Indeed, one of the most rapidly developing areas of organellar biology is that of peroxisomal function. It was recently demonstrated that peroxisomes are important sites of antiviral signal transduction (Dixit et al. 2010), and that they play a role in invariant natural killer T cell stimulation and maturation (Facciotti et al. 2012), in brain aging and Alzheimer's disease (Kou et al. 2011) as well as in peptide hormone metabolism (Höftberger et al. 2010). The latter work of Höftberger and coworkers demonstrated for the first time an unexpected link between hormone metabolism and peroxisomes.

In general, hormones are defined as chemical substances, which are released by a cell or a gland and are conveyed by the bloodstream to another part of the body to effect physiological activities. In mammals, hormones are derived from amino acids, cholesterol or phospholipids and fall into three chemical classes: peptide hormones, lipid- and phospholipid-derived hormones and monoamines. Intriguingly, a role of peroxisomes in hormone metabolism has already been demonstrated in plants: In the model plant *Arabidopsis thaliana*, indole-3-butyric acid (IBA), a storage precursor of the important morphogenetic plant hormone auxin, is activated in the peroxisome by fatty acid β-oxidation (Zolman et al. 2000, 2007). Here, we will review the current knowledge concerning a role of peroxisomes in mammalian peptide and steroid hormone metabolism.

6.2 The Peptide Hormones β-Lipotropin and β-Endorphin are Localized to Peroxisomes

The peptide hormones β-lipotropin (β-LPH) and β-endorphin are produced by post-translational cleavage from the 31-kDa prohormone precursor protein, proopiomelanocortin (POMC), which is synthesized in the pituitary, in the arcuate nucleus of the hypothalamus, in the adrenal gland and in several other peripheral tissues. The POMC precursor protein is produced in the endoplasmic reticulum and moves to the Golgi complex, where it is sorted for delivery to secretory granules by an N-terminal sequence acting as sorting signal (Fig. 6.1). During the trafficking process, POMC is proteolytically cleaved by the endopeptidases prohormone convertase (PC) 1/3 and 2, resulting in a number of biologically active peptides including β-LPH and β-endorphin. PC1/3 is synthesized as an inactive precursor that does not become

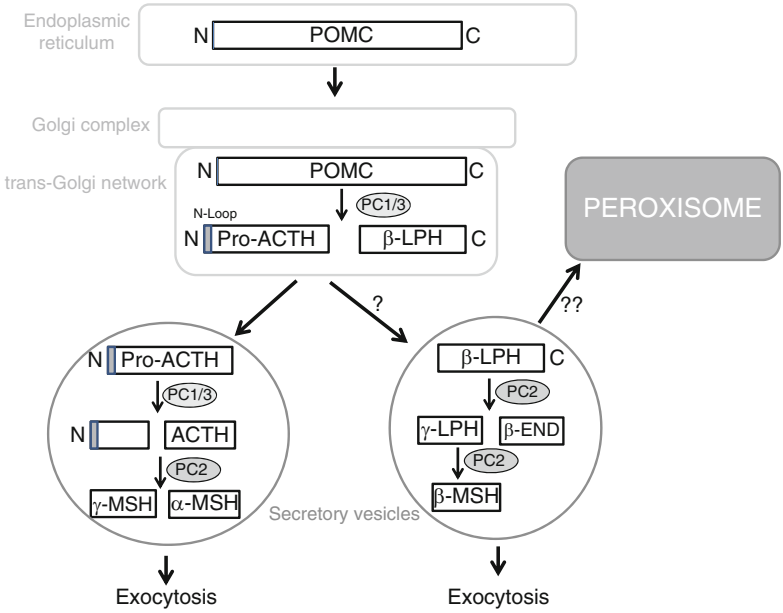

Fig. 6.1 Processing of POMC in the secretory pathway and suggested rerouting of β-LPH and β-endorphin to peroxisomes. In the *trans*-Golgi network, POMC is processed to pro-ACTH and β-LPH by the enzyme prohormone convertase (PC) 1/3. Pro-ACTH and β-LPH are then sorted into secretory granules, possibly into different subpopulations of secretory vesicles, where PC1/3 and PC2 become fully activated and generate ACTH, α-MSH, γ-MSH, β-MSH and β-endorphin. In tissues and cell types that predominantly secrete peptide hormones derived from the N-terminal part of POMC (ACTH, α-MSH or γ-MSH), a localization of the C-terminal peptides β-LPH and β-endorphin to peroxisomes has been observed. Thus, it could be speculated that when predominantly products from the N-terminal part of POMC are secreted, the by-products β-LPH and β-endorphin are prevented from secretion by rerouting them to peroxisomes, possibly resulting in their degradation in this organelle

fully activated until it reaches the immature secretory granules and undergoes an autocatalytic cleavage event (Zhou and Lindberg 1993). However, PC1/3 is partially active before autolysis, resulting in partial processing of POMC to pro-ACTH, the precursor of Adrenocorticotropic Hormone (ACTH) and α-Melanocyte-Stimulating Hormone (MSH), and β-LPH, the precursor of β-MSH and β-endorphin, in the *trans*-Golgi network (TGN) (Jutras et al. 1997). By a mechanism that is not fully understood, pro-ACTH and, presumably, β-LPH are sorted from the TGN into secretory granules. As the N-terminal sorting signal is present on pro-ACTH but not on β-LPH, the sorting of β-LPH apparently occurs through a different mechanism, possibly targeting a different subpopulations of secretory vesicles (Pritchard and White 2007). In the secretory granules, PC1/3 and PC2 become fully activated and generate ACTH, α-MSH, β-MSH γ-MSH and β-endorphin (Benjannet et al. 1991).

There is considerable complexity in this processing pathway with tissue-specific processing of POMC as an important factor conferring selectivity on the production of distinct peptides. In the anterior pituitary, POMC is processed predominantly to ACTH, β-LPH and a 16 kDa N-terminal fragment. Whereas ACTH is critical for the maintenance of adrenocortical function, the biological role of β-LPH is not fully understood, although a role in lipid mobilization such as lipolysis and steroidogenesis was demonstrated (O'Connell et al. 1996; Halabe Bucay 2008). In the hypothalamus and in the intermediate lobe of the pituitary, POMC is more extensively processed: ACTH is further cleaved to produce α-MSH, which influences skin-darkening reactions next to controlling body weight and CNS functions such as memory and learning; β-LPH is processed to γ-LPH and the opioid peptide neurotransmitter β-endorphin, which has a role in modulating pain sensation (Akil et al. 1998). Thus, POMC-expressing neuronal systems cosynthesize, costore and corelease a variety of biologically active peptides of which at least two, α-MSH and β-endorphin, may exert opposing effects upon reaching a common postsynaptic target. The mechanisms involved in regulating these antagonistic activities are unclear. N-terminal acetylation of α-MSH and β-endorphin in the secretory granules, resulting in increased biological activity of α-MSH and reduced binding of β-endorphin to opioid receptors, thus attenuating opiate-mediated analgesia, is proposed as one mechanism for shifting the predominant effects of secreted POMC peptides from endorphinergic to melanocortinergic actions.

In this context of regulating antagonistic hormone activities, the findings of Höftberger and coworkers are of particular interest: Using confocal laser microscopy and immunoelectron microscopy, a peroxisomal localization of β-LPH and β-endorphin could be demonstrated in the anterior pituitary gland (Höftberger et al. 2010). The presence of β-LPH and β-endorphin in peroxisomes was confirmed by colocalization using antibodies against several different peroxisomal proteins, such as catalase and adrenoleukodystrophy protein (ALDP). In the study, peroxisomes were shown to be distinctly separated from secretory vesicles, as ALDP did not colocalize with secretogranin, a typical marker for secretory versicles. Interestingly, the peroxisomal localization of β-LPH and β-endorphin was not restricted to the anterior pituitary gland but could be observed in several specific

cell types and tissues throughout the human body including dorsal root ganglia, adrenal cortex, distal tubules of kidney, and skin.

Intriguingly, an association of β-LPH and β-endorphin with peroxisomes was found only in tissues and cell types that predominantly secrete peptide hormones derived from the N-terminal part of POMC, such as ACTH in the pituitary, γ-MSH in the adrenal cortex or kidney and α-MSH in the skin (Höftberger et al. 2010). In contrast, in tissues like the adrenal medulla that secrete peptide hormones derived from the C-terminal part of POMC, such as β-endorphin, no localization of the peptide hormone in peroxisomes could be detected (Höftberger et al. 2010). Thus, it appears that when predominantly products from the N-terminal part of POMC are to be secreted, the arising by-products β-LPH and β-endorphin are retained by rerouting to peroxisomes, possibly resulting in their degradation in this organelle (Fig. 6.1). In contrast, cell types and tissues destined to secrete C-terminal POMC products, namely β-LPH and β-endorphin, do not show a peroxisomal localization of these peptide hormones.

Alternatively, it could be argued that β-LPH and β-endorphin are addressed to peroxisomes to serve as intracellular messengers to modify peroxisomal functions in response to specific cellular conditions. Several other neuropeptide and hormone products encoded by a single gene, e.g. growth hormone, proenkephalin or insulin, have previously been demonstrated to locate to more than one intracellular compartment including nucleus and mitochondria (Morel 1994; Mertani et al. 1996). The physiological implications of these findings remain unclear.

Interestingly, the peroxisomal localization of β-LPH and β-endorphin is restricted to cells that express the peroxisomal ATP-binding cassette (ABC) transporter ALDP, suggesting a link between peroxisomal β-LPH and β-endorphin and the function of ALDP. Defects in ALDP due to mutations in the *ABCD1* gene lead to X-linked adrenoleukodystrophy (X-ALD), the most common peroxisomal disorder, primarily affecting the adrenal cortex, testes and the central nervous system (Berger and Gärtner 2006). The biochemical hallmark of X-ALD is an abnormal accumulation of VLCFA in tissues, plasma and body fluids due to alterations in peroxisomal β-oxidation as well as in fatty acid chain elongation (Berger and Gärtner 2006). However, since β-LPH and β-endorphin are also found in peroxisomes of tissues from X-ALD patients lacking functional ALDP (Höftberger et al. 2010), ALDP does not seem to be involved in the transport of these peptide hormones into peroxisomes. Thus, further experiments are necessary to establish whether there is a yet unknown, direct link between ALDP and the peroxisomal localization of the peptide hormones or whether the restricted colocalisaztion of β-LPH and β-endorphin to peroxisomes of cell types expressing ALDP is a metabolic coincidence of ALDP function and peptide hormone secretion.

In summary, the peptide hormones β-LPH and β-endorphin, which were classically thought to be destined for secretion to act on distant target cells via specific receptors can also be translocated to another cellular site, namely the peroxisome, within the cells in which they are synthesized. The physiological relevance of these findings which possibly expand the physiological actions of these peptide hormones to peroxisomal function, remains to be elucidated.

6.3 A Possible Role of Peroxisomes in Steroid Hormone Metabolism

In mammalian species, there are seven families of steroid hormones that are classified on both structural and biological (hormonal) basis: estrogens (female sex steroids), androgens (male sex steroids), progestins, mineralcorticoids, glucocorticoids, vitamin D and bile acids. In contrast to peptide hormones, steroid hormones are synthesized in the mitochondria and rough ER and require the presence of specific enzymes that convert cholesterol into the appropriate steroid. The "classical" steroid-producing endocrine glands are the adrenal cortex and the gonads. In addition, the kidneys produce the active steroid metabolite of vitamin D.

In vertebrates and other higher organisms, cholesterol, which is an important component of many cellular membranes, is also the obligatory precursor for synthesis of steroid hormones and bile acids. Cholesterol is either obtained from the diet or synthesized de novo from acetate in a multi-step process involving nearly 30 enzymes. It has been debated for a long time, whether the pre-squalene segment of the cholesterol biosynthetic pathway is, at least under certain conditions, localized to peroxisomes and whether acetyl-CoA derived from peroxisomal β-oxidation of VLCFA and dicarboxylic acids is channeled preferentially to cholesterol synthesis inside the peroxisomes (Kovacs et al. 2007; Hogenboom et al. 2004a, b). As the outcome of these studies was inconclusive, a role of peroxisomes in synthesizing the steroid hormone precursor cholesterol has not yet been clarified.

6.3.1 Peroxisomes and Bile Acid Synthesis

Bile acids have long been known to facilitate digestion and absorption of lipids. Only recently, bile acids were demonstrated to function also as hormones that bind to specific nuclear receptor transcription factors to modulate expression of genes involved in cholesterol homeostasis (Chiang 2004). The conversion of cholesterol into the principal mammalian bile acids cholic acid and chenodeoxycholic acid takes place largely in the liver by a sequence of enzymatic modifications involving several enzymes and multiple subcellular compartments. One step involves side chain shortening by β-oxidation and subsequent conjugation with the amino acids taurine or glycine; both reactions are accepted to take place in peroxisomes. Accordingly, patients with generalized peroxisome deficiency disorders accumulate bile acid intermediates; and the peroxisomal enzymes involved in bile acid biosynthesis have all been identified, for a review see (Ferdinandusse et al. 2009). Only the transporters required for transfer of the C27-bile acid intermediates into the peroxisome and for the conjugated C24-bile acids out of the peroxisome remain unidentified, although a role of the *Abcd3*-encoded protein PMP70 was discussed in this context (Ferdinandusse et al. 2009).

6.3.2 *Peroxisomes and Steroid Hormone Synthesis in the Adrenal Cortex*

The cortex of the adrenal glands is, next to the gonads, the major steroid hormone producing site. The main secretory products of the adrenal cortex are the glucocorticoid cortisol; the mineralcorticoid aldosterone; and the androgens, androstenedione and dehydroepiandrostenedione (DHEA). Whereas cortisol is an important metabolic hormone and aldosterone has a role in salt and water homeostasis, the androgens produced by the adrenal cortex are regarded to have little physiological significance when gonadal function is normal. As discussed above, synthesis of steroid hormones begins with cholesterol. Circulating plasma lipoproteins derived from liver cholesterol synthesis are the major source of adrenal cholesterol, although de novo synthesis from acetate also occurs within the adrenal gland. In addition, cholesterol stored as cholesterol-esters in lipid droplets within adrenocortical cells is also used for steroidogenesis.

Impaired function of the adrenal gland to produce steroid hormones is known as Addison disease and is characterized by the loss of more than 90 % of both adrenal cortices. Intriguingly, many peroxisomal disorders are accompanied by adrenocortical dysfunction, with X-ALD being the most frequent genetic disorder leading to adrenal insufficiency. Histopathological examinations of the adrenal cortex from patients with X-ALD revealed ballooned adrenocortical cells with lamellar inclusions rich in VLCFA esterified with cholesterol (Powers 1980). In addition to entrapping cholesterol in VLCFA-esters, and thus limiting intracellular cholesterol for steroid synthesis, VLCFA accumulation is thought to have a direct toxic effect on intracellular membranes and enzymes, resulting in gradual adrenocortical destruction (Powers 1980). In the initial phase, basal steroid secretion is still within a normal range but cortisol secretion fails to increase in response to stress (ACTH challenge). With further loss of cortical tissue, also basal steroid secretion becomes deficient with the consequence of increased ACTH plasma levels because of decreased negative feedback inhibition due to the lack of cortisol. Accordingly, lowering of VLFCA levels by administration of Lorenzo's oil, a combination of erucic acid and oleic acid, to X-ALD patients with subclinical adrenal failure normalized elevated ACTH levels within 6 months, thus rendering steroid replacement therapy unnecessary (Cappa et al. 2011). An issue that has not been adequately explored, is why X-ALD mice, which also accumulate high levels of VLCFA in the adrenal gland, do not display signs of adrenal atrophy or clinical hypocortisolism (Lu et al. 2007). Also intriguing, and not yet understood, is the finding that very low plasma levels of DHEA(S), the sulfate ester of DHEA, which is the major secretory steroidal product of the adrenal glands, are also found in patients with no apparent adrenal dysfunction and normal plasma cortisol and ACTH levels (Wichers-Rother et al. 2005; Assies et al. 2003). Together, these observations point to a role of peroxisomes in adrenocortical steroidogenesis and forms the foundation for an interesting but still speculative hypothesis.

6.3.3 *Peroxisomes and Steroid Hormone Synthesis in the Gonads*

The ovary and the testis, like the adrenal gland, secrete cholesterol-derived steroid hormones under the control of the stimulatory releasing hormones of the hypothalamo-pituitary axis, mainly through actions of the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), produced by the pituitary gland. Just like the action of ACTH on adrenal cortical cells, LH and FSH increase intracellular concentrations of free cholesterol and its intracellular transport. The biosynthetic pathways to sex hormones in both male and female gonads include the production of the androgens androstenedione and DHEA. Testes and ovaries contain an additional enzyme, 17-β-hydroxysteroid-dehydrogenase (17βHSD), which enables the conversion of androgens to testosterone.

A role of peroxisomes in androgen metabolism has been discussed because patients suffering from the X-ALD phenotypic variant, adrenomyeloneuropathy, present hypogonadism, impairment of Leydig cells and testosterone levels that are in the lower part of the normal range (Stradomska et al. 2012; Brennemann et al. 1997). Additional evidence for a potential involvement of ALDP and peroxisomes in androgen metabolism was also provided by the observation that skin fibroblasts from X-ALD patients less actively convert testosterone to the more potent androgen dihydrotestosterone (DHT) than do fibroblasts derived from healthy control subjects (Petroni et al. 2000). The metabolic reaction of testosterone conversion to DHT is catalyzed by 5alpha-reductase isoform 2 (5α-R2), a key enzyme of steroid hormone metabolism. Intriguingly, 5α-R2 expression in X-ALD fibroblasts dissociates from the observed reduced enzymatic activity as increased 5α-R2 mRNA levels were reported (Petroni et al. 2000).

6.4 Conclusion

The POMC-derived peptide hormones β-LPH and β-endorphin are localized in peroxisomes of various cell types of the human body. Intriguingly, this association of β-LPH and β-endorphin with peroxisomes was found only in tissues and cell types that predominantly secrete peptide hormones derived from the N-terminal part of POMC, such as ACTH, α-MSH or γ-MSH and not from the C-terminal part, such as β-LPH or β-endorphin. As some of these products like α-MSH and β-endorphin have antagonistic activities in target tissues, it could be speculated that whenever predominantly products from the N-terminal part of POMC are to be secreted, the arising by-products β-LPH and β-endorphin are retained by rerouting to peroxisomes, possibly resulting in their degradation in this organelle. Thus, peroxisomes seem to play a role in regulating peptide hormone signaling by preventing the action of β-LPH and β-endorphin in specific situations.

Concerning steroid hormone metabolism, peroxisomal disorders are generally associated with alterations in steroidogenic tisssues, however, a role for peroxisomes in steroidogenesis and steroid signaling is still speculative. Taken together, a role of peroxisomes in hormone metabolism is implied by several findings but is a largely unexplored field that needs to be pursued. No effective treatment is available for most patients with peroxisomal disorders and the development of novel therapeutic strategies requires a detailed knowledge about peroxisomal function. Therefore, it is of major significance to address the cell biological questions about the role of peroxisomes in hormone metabolism.

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Chapter 7
Peroxisome Ca^{2+} Homeostasis in Animal and Plant Cells

Alex Costa, Ilaria Drago, Michela Zottini, Paola Pizzo, and Tullio Pozzan

Contents

Abstract Ca^{2+} homeostasis in peroxisomes has been an unsolved problem for many years. Recently novel probes to monitor Ca^{2+} levels in the lumen of peroxisomes in living cells of both animal and plant cells have been developed. Here we

A. Costa
Department of Biosciences, University of Milan, Milan, Italy

I. Drago • P. Pizzo
Department of Biomedical Sciences, University of Padua, Viale Giuseppe Colombo 3, 35121 Padua, Italy

M. Zottini
Department of Biology, University of Padua, Padua, Italy

T. Pozzan (✉)
Department of Biomedical Sciences, University of Padua, Viale Giuseppe Colombo 3, 35121 Padua, Italy

Institute of Neuroscience, National Research Council (CNR), Padua Section, Italy

Venetian Institute of Molecular Medicine (VIMM), Padua, Italy
e-mail: tullio.pozzan@unipd.it

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discuss the contrasting results obtained in mammalian cells with chemiluminecsent (aequorin) and fluorescent (cameleon) probes targeted to peroxisomes. We briefly discuss the different characteristics of these probes and the possible pitfalls of the two approaches. We conclude that the contrasting results obtained with the two probes may reflect a heterogeneity among peroxisomes in mammalian cells. We also discuss the results obtained in plant peroxisomes. In particular we demonstrate that Ca^{2+} increases in the cytoplasm are mirrored by similar rises of Ca^{2+} concentration the lumen of peroxisomes. The increases in peroxisome Ca^{2+} level results in the activation of a catalase isoform, CAT3. Other functional roles of peroxisomal Ca^{2+} changes in plant physiology are briefly discussed.

Keywords Peroxisomes • Ca^{2+} • Fluorescence • FRET • Catalase

Abbreviations

Aeq	Aequorin
CFP	Cyan Fluorescent Protein
FCCP	Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
FRET	Fluorescence Resonance Energy Transfer
GFP	Green Fluorescent Protein
PPAR	Peroxisome Proliferator Activated Receptor
SPCA	Secretory Pathway Ca^{2+}/Mn^{2+}-ATPases
YFP	Yellow Fluorescent Protein

7.1 Introduction

Peroxisomes are organelles enclosed by a single membrane, with a high matrix protein concentration that includes at least 50 different enzymes involved in several pathways; among these, the best known are fatty acid β-oxidation, scavenging of hydrogen peroxide (functions that in animal cells are partially shared by mitochondria), and the synthesis of etherphospholipids (Platta and Erdmann 2007; Wanders 2004). In particular, peroxisomes are endowed with several enzymes capable of disposing of hydrogen peroxide, e.g., catalase, glutathione peroxidase and peroxiredoxin V (for an exhaustive review see (Wanders and Waterham 2006)). Specialized peroxisomal functions are found in some cells, e.g., plants and fungi, such as fatty acid degradation and synthesis of phytohormones (Goepfert and Poirier 2007) (see also Chaps. 14 and 16 of this book).

These organelles are numerous and dispersed throughout the entire cytosol and can rapidly adapt to cellular demand by increasing their size or numbers; the increase in the number of peroxisomes is mainly triggered by lipids, through the activation of a ligand-dependent transcription factor (Peroxisome Proliferator

Activated Receptor, PPARα), mostly mediated by a fission process similar to that described for mitochondria (Platta and Erdmann 2007).

The peroxisomal membrane is impermeable to high MW molecules (>1,000 Da) and specific carriers are expressed in the organelles for taking up different metabolites ((Wanders 2004) and see Chaps. 2 and 10 of this book), thus suggesting a strictly regulated activity of peroxisomal pathways, acting in concert with cytosolic metabolism (Rottensteiner and Theodoulou 2006; Theodoulou et al. 2006; Visser et al. 2007).

Apart from their metabolic functions, peroxisomes have emerged as organelles important in determining cell fate, contributing to regulate cell differentiation, embryo development and morphogenesis (Titorenko and Rachubinski 2004). Indeed, the lipids synthesized by peroxisomes can be targeted to the nucleus by specific binding proteins, where they activate lipid-dependent transcription factors (including PPARs) that in turn regulate the expression of several genes involved in differentiation and development (Tan et al. 2002). The cellular importance of peroxisomes has been recently reinforced by the fact that impairment of peroxisomal activity or biogenesis is linked to different genetic disorders in humans as the Zellweger syndrome spectrum (Shimozawa 2007).

Given that any enzymatic activity is highly sensitive to the ionic composition of the environment where they operate, it is surprising that information on the luminal ion content of peroxisomes, as well as the permeability characteristics of their membrane to ions, is scarce and contradictory. The luminal pH of peroxisomes has been reported to be indistinguishable, more alkaline or more acidic than that of the cytoplasm. In particular, using a targeted GFP-based pH indicator, Drago et al. and Jankowski et al. concluded that no appreciable pH gradient exists in mammalian cells across the peroxisomal membrane (Jankowski et al. 2001; Drago et al. 2008), while Dansen et al. (using another pH probe) reported that the peroxisomal pH in human fibroblasts is slightly alkaline. A similar conclusion was reached in yeast (Dansen et al. 2000; Waterham et al. 1990; van Roermund et al. 2004). On the contrary, also in yeast, Lasorsa et al. found that the peroxisomal pH is slightly acidic (Lasorsa et al. 2004). The reason for these discrepancies and the mechanisms for the possible generation of the pH gradient remain unsolved. To the best of our knowledge, no direct measurement of intra-peroxisomal monovalent cation or anion concentration has yet been performed, although, based on indirect evidence, (Drago et al. 2008) concluded that the Na^+ concentration is indistinguishable between peroxisomes and cytosol.

Although it is an issue potentially of crucial importance in the understanding of possible regulatory signals for their metabolic activity, little and contrasting information is available (see below) about the Ca^{2+} concentration in the peroxisome lumen, $[Ca^{2+}]_p$, and peroxisomal Ca^{2+} handling. In this chapter we focus on the mechanism regulating peroxisomal Ca^{2+} homeostasis in mammalian and plant cells. We also discuss recent data concerning the functional role of the changes in $[Ca^{2+}]_p$ occurring in living cells. As to this latter aspect, we discuss only data obtained in plants, as no information is presently available concerning the functional role of $[Ca^{2+}]_p$ in mammalian cells.

7.2 Peroxisomal Ca^{2+} Homeostasis

7.2.1 General Characteristics of Peroxisomal Ca^{2+} Homeostasis in Animal Cells

A first attempt to characterize peroxisomal Ca^{2+} homeostasis was made in 2006 in partially purified peroxisomes (Raychaudhury et al. 2006): according to these authors, the free Ca^{2+} concentration within the organelles is ~50 nM; in addition, verapamil-sensitive Ca^{2+} channels and a vanadate-sensitive Ca^{2+} ATPase were reported to be present in their membranes. This study, however, was not performed in living cells and the purity of the peroxisomal fraction was not thoroughly analyzed. In the same year, a peroxisomal isoform of a Ca^{2+}/Mn^{2+}-ATPase was described in a peroxisomal fraction from *Drosophila* (SPoCk-C; (Southall et al. 2006)). This enzyme is homologous to the mammalian Secretory Pathway Ca^{2+}/Mn^{2+}-ATPases (SPCAs), the main Ca^{2+} pump present in both the Golgi apparatus and other vesicles of the secretory pathway (for a review, see (Pizzo et al. 2011)). The authors speculated that this protein was responsible for maintaining high levels of Ca^{2+} and/or Mn^{2+} in the peroxisomal lumen (essential for the correct function of resident enzymes, such as Mn-dependent superoxide dismutase). This pump was found to be highly expressed in Malpighian (renal) tubules and thus it was suggested that it could have a role in maintaining sufficient levels of luminal Ca^{2+} to precipitate oxalates or phosphates, to form the Ca^{2+} spherites that are found in the initial segment of the anterior tubules of the fly (Southall et al. 2006). Again, however, no direct measurement of peroxisomal Ca^{2+} concentration was carried out.

In 2008, two groups independently generated two different Ca^{2+} probes targeted specifically to the peroxisome lumen. These probes allowed the first direct Ca^{2+} measurements within the organelles in intact live cells. By employing two new GFP-based probes, the first group (Drago et al. 2008) carried out single cell analysis of $[Ca^{2+}]_p$ dynamics in two mammalian cell lines (HeLa and GH3 cells). This group showed that, in resting cells, the $[Ca^{2+}]_p$ is similar to that of the cytosol and that only large cytosolic Ca^{2+} increases can be mirrored by concomitant peroxisomal Ca^{2+} increases. These GFP-based Ca^{2+} probes were later also used to measure peroxisomal Ca^{2+} handling in different plant cells (see below), and the results obtained confirmed the conclusions reached in mammalian cell lines.

The other group (Lasorsa et al. 2008) generated a peroxisome-targeted aequorin (a luminescent Ca^{2+} probe) that allows to monitor the $[Ca^{2+}]_p$ dynamics in a population of living cells. Using different mammalian cell lines, these authors obtained substantially different results compared to those of Drago and co-workers (2008). In particular, they suggested that peroxisomes have a luminal resting $[Ca^{2+}]$ much higher than that of the cytosol (about 20–50 fold) and, most importantly, the organelles appear to accumulate Ca^{2+} in their lumen (up to 100 μM) upon Ca^{2+} mobilization from internal stores. The functional characteristics of peroxisomal Ca^{2+} homeostasis, according to (Lasorsa et al. 2008), closely resemble, but are distinct from, those of mammalian mitochondria (see below).

7.2.2 Methods for Peroxisomal Ca^{2+} Measurements in Living Cells

Over the last 10 years, Ca^{2+} measurements with aequorin- and GFP-based probes have been carried out for many different cell compartments and the results obtained by the two types of probe have been qualitatively similar, though some quantitative differences have sometimes been reported. Peroxisomes represent the first case in which two different types of Ca^{2+} probes have provided qualitatively different results. We suspect that a possible reason for this discrepancy lies in the very different characteristics of these probes and the measuring approach. Accordingly, a brief description of the main characteristics of these two families of Ca^{2+} sensors (aequorins and GFP-based indicators) appear necessary in the attempt to explain the contradictory findings obtained in peroxisomes.

7.2.3 Luminescent Protein Ca^{2+} Sensors

Aequorin (Aeq), the first protein indicator used for Ca^{2+} measurements in living cells, (Shimomura et al. 1962, 1963) is a 21 kDa protein, remarkably non-toxic in living cells, capable of reversibly binding Ca^{2+}. Natural Aeq covalently binds a coenzyme (coelenterazine) and apoaequorin can be reconstituted in vitro spontaneously upon incubation of the protein with the coenzyme in a reducing environment and in the absence of Ca^{2+}. Ca^{2+} binding to Aeq causes the destabilisation of the Aeq–coelenterazine complex and the breakdown of the covalent bond between the two molecules results in the production of CO_2, release of the oxidized coenzyme and emission of a blue photon (Fig. 7.1a). The reaction is irreversible and once the photon has been emitted Aeq is "consumed". The rate of photon emission is a complex function of the Ca^{2+} concentration where Aeq is located, and algorithms have been developed that correlate the rate of photon emission to the Ca^{2+} concentration (Fig. 7.1b; for review see (Robert et al. 2000)). The identification of the Aeq gene and of its coding sequence (Prasher et al. 1985) allowed the protein to be expressed recombinantly in cells and tissues from different organisms (for a review see (Robert et al. 2000; Rizzuto and Pozzan 2006)).

A further development of the Aeq technology resulted from its targeting to intracellular compartments (Rizzuto et al. 1992). For this purpose, Aeq chimeras were generated containing targeting sequences for different organelles, see for example (Lasorsa et al. 2008; Montero et al. 1995; Rizzuto et al. 1992). Two major problems are encountered when using Aeq in living cells: (i) the low amount of photons that a single cell can emit even under strong stimulation and (ii) the non linearity of the rate of Aeq light emission as a function of the Ca^{2+} concentration.

The first problem depends on the nature of the light emission mechanism by the protein: the maximum theoretical amount of photons releasable by one molecule of

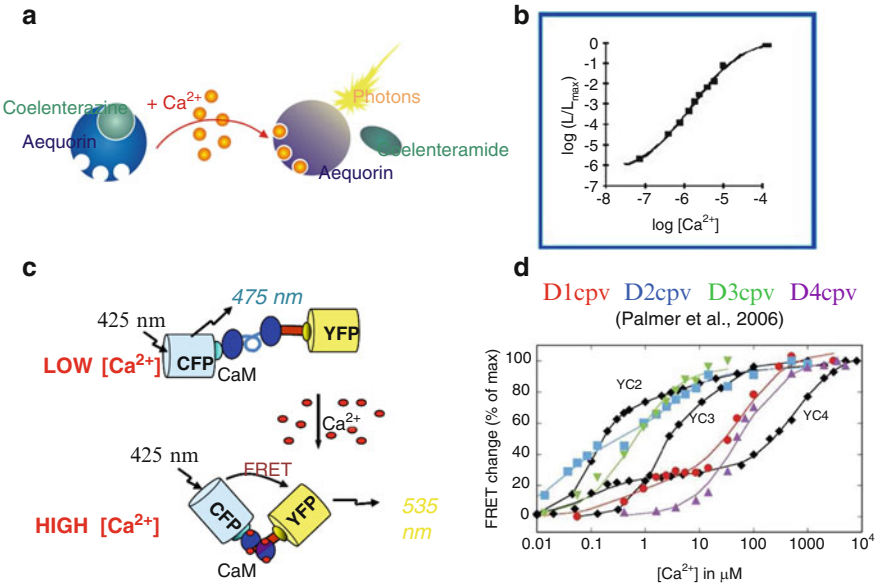

Fig. 7.1 Aequorin and cameleon Ca^{2+} probes. Schematic representation of the mechanism of Ca^{2+} activation of luminescence (aequorin, **a**) or of FRET change (cameleon, **c**). (**b**) in vitro relationship between the rate of aequorin photon emission (L/Lmax) and the Ca^{2+} concentration (see refs. Robert et al. (2000), Rizzuto and Pozzan (2006) for details). (**d**) in vitro FRET changes as a function of the Ca^{2+} concentration of the D family of cameleons. This latter panel was reproduced from refs. Palmer et al. (2006), Palmer and Tsien (2006)

Aeq is 1 (in practice it is much lower, about 0.3 photons/molecule). Accordingly, given the amount of Aeq molecules that can be expressed recombinantly by a cell (in the order of 10^4/cell), the amount of photons that are emitted from a single stimulated cell is in the order of 10–100 photons/s (for review, see (Robert et al. 2000)).

The second problem, the strong non linearity of the relationship between Aeq light emission rate and Ca^{2+} concentration, is more subtle and often not appreciated enough by non experts. In particular, within the physiological range of Ca^{2+} concentration (10^{-7}–10^{-5} M) the rate of photon emission increases by more than 100 fold for a change of ten times in Ca^{2+} concentration. In practical terms this means that for a change in $[Ca^{2+}]$ from 0.1 μM (typical of the cytoplasm of a resting cell) to 3–4 μM (typical of the peak in an activated cell), the rate of Aeq photon emission increases over 1.000 fold. This means, however, that the signal coming from 1 activated cell will be the same as that of 1.000 cells at resting Ca^{2+}. In practical terms, if $[Ca^{2+}]$ is not homogeneous in the population of cells (or organelles), the overall Aeq light emission is dominated by the most responding sub-population.

7.2.4 *Fluorescent Protein Ca^{2+} Sensors*

The majority of genetically encoded fluorescent Ca^{2+} probes are based on mutants of the green fluorescent protein (GFP) originally discovered in the above mentioned jellyfish, *A. victoria*; more recently, fluorescent Ca^{2+} reporters have also been developed from fluorescent proteins produced by other organisms including Anthozoa, e.g., dsRed (Verkhusha and Lukyanov 2004).

At present, three main types of GFP-based sensor are in use: the so-called cameleons (Miyawaki et al. 1997), the camgaroos (Baird et al. 1999) and the pericams (Nagai et al. 2001). In the first type, Ca^{2+}-responsive elements alter the efficiency of fluorescence resonance energy transfer (FRET) between two GFP mutants, while in camgaroos, pericams (and GCaMPs), Ca^{2+}-responsive elements are inserted into a single fluorescent protein to modulate the protonation state of its chromophore. Here we will limit ourselves to a brief discussion of cameleons, as these were the only ones used in peroxisomes.

The first cameleon Ca^{2+} probe was made up of two GFP variants: a blue fluorescent protein (BFP) and green fluorescent protein (GFP), linked by a peptide comprising calmodulin (CaM) and the CaM-binding peptide of smooth muscle myosin light chain kinase, M13 (Miyawaki et al. 1997). This probe has been then modified and improved, including replacement of BFP and GFP with the cyan (CFP) and yellow (YFP) mutants (Fig. 7.1c), introduction of mutations in the CaM domain and replacement of M13 with a different CaM-binding peptide, derived from troponin C. In addition, in some recent variants, YFP has been substituted with the less pH and Cl^- sensitive YFP analogues Citrine and Venus (Nagai et al. 2002) or circular permuted Venus (cpv) (Nagai et al. 2004). Presently a number of cameleons with different Ca^{2+} sensitivities (targeted to most sub-cellular compartments) are available (Jaconi et al. 2000; Palmer et al. 2006; Palmer and Tsien 2006; Drago et al. 2008; Giacomello et al. 2010; Lissandron et al. 2010). Cameleons have been successfully used also in vivo (Rudolf et al. 2004, 2006; Pozzan and Rudolf 2009; Reiff et al. 2005; Pologruto et al. 2004; Hasan et al. 2004).

In the more recent family of cameleon probes, the Dcpv family (Palmer et al. 2006; Palmer and Tsien 2006), the cameleon was redesigned to generate selective and specific Ca^{2+} binding pairs that could not be perturbed by wild-type CaM. In addition, steric bumps in the target peptide and complementary holes in CaM were computationally designed in order to generate a series of indicators (D1, D2, D3 and D4; Fig. 7.1d) with varying Ca^{2+} affinities. This forms the basis of the probe used by Drago and co-workers (2008) to measure peroxisomal Ca^{2+} handling (Drago et al. 2008).

Cameleons have been extensively used in the last decade and are endowed with a number of extremely useful physico-chemical characteristics. In particular, single cell and single organelle analysis is routine with cameleons and the strong non-linearity of the Aeq signal, as a function of $[Ca^{2+}]$ (see above), does not plague the cameleon signal.

7.3 Peroxisomal Ca^{2+} Dynamics in Living Mammalian Cells: An Unsettled Question

As mentioned above, two papers were published in 2008 (Drago et al. 2008; Lasorsa et al. 2008), in which the dynamics of peroxisomal luminal Ca^{2+} was for the first time investigated in intact living cells. In the first study, Drago et al. targeted a cameleon Ca^{2+} sensor (D3cpv) to the peroxisomal lumen by a modified version of the classical peroxisomal targeting sequence Ser-Lys-Leu (Gould et al. 1989). The modification was necessary since addition of only the classical targeting sequence for peroxisomes (SKL) to the cameleon resulted in a partial mis-targeting of the Ca^{2+} probe to the cytosol. Peroxisome targeting was strongly improved by adding a Lys-Val-Lys tripeptide (KVK) before the SKL sequence, as shown previously by (Neuberger et al. 2003) (Fig. 7.2a–f). To rule out the possibility that the probe was localized on the peroxisomal membrane facing (and thus measuring) the cytoplasmic Ca^{2+} concentration, the authors treated digitonin-permeabilized cells with proteinase K. In this condition, the enzyme digests every protein that is membrane bound but facing the cytosol: indeed, in cells expressing an outer mitochondrial membrane-bound GFP (TOM20-GFP), proteinase K treatment caused the disappearance of the GFP signal. On the contrary, upon proteinase K treatment, the peroxisomal Ca^{2+} probe fluorescence was unaffected (Fig. 7.2g) (Drago et al. 2008).

Using this probe, it was concluded that: (i) the resting value of $[Ca^{2+}]_p$ in different cell types, such as HeLa, GH3 and SH-SY5Y is indistinguishable from that of the cytoplasm, i.e., about 0.1 μM; (ii) peroxisomal Ca^{2+} rises both upon stimulation with an IP_3-generating agonist (that causes the release of Ca^{2+} from internal stores) and upon opening of voltage-gated plasma membrane Ca^{2+} channels. In particular, the higher the cytosolic Ca^{2+} peaks the higher the peroxisomal Ca^{2+} rises (Fig. 7.3a–d). Of interest, confirming that the peroxisomal membrane represents a significant barrier to Ca^{2+} diffusion into the organelle lumen, the kinetics of Ca^{2+} increases in the two compartments were different: peroxisomes exhibit significantly slower kinetics than observed in the cytosol. However, no evidence for active Ca^{2+} accumulation in peroxisomes was obtained: the existence of an ATP-dependent pump or gradient (either of H^+ or Na^+) that drives Ca^{2+} entry into peroxisomes was ruled out. Indeed, neither the absence of ATP, nor the addition of the Na^+/H^+ ionophore monensin, of the uncloupler FCCP or cytosolic alkalinization by NH_4Cl, were able to affect peroxisomal Ca^{2+} entry upon cell stimulation (Fig. 7.3e, f). By applying an in situ calibration of the FRET signal, it was concluded that Ca^{2+} concentration within the organelles essentially equilibrates with that in the cytosol.

The other work, published a few weeks after that of Drago et al., reached very different conclusions, as was already mentioned above. Lasorsa and colleagues targeted the Ca^{2+}-sensitive photoprotein Aeq to the peroxisomal lumen through the classical SKL peroxisomal targeting sequence. They verified the targeting efficiency by Aeq co-localization with a peroxisomal-targeted DsRed (Lasorsa et al. 2008) and ran a series of controls to rule out the possibility that the intra-peroxisomal

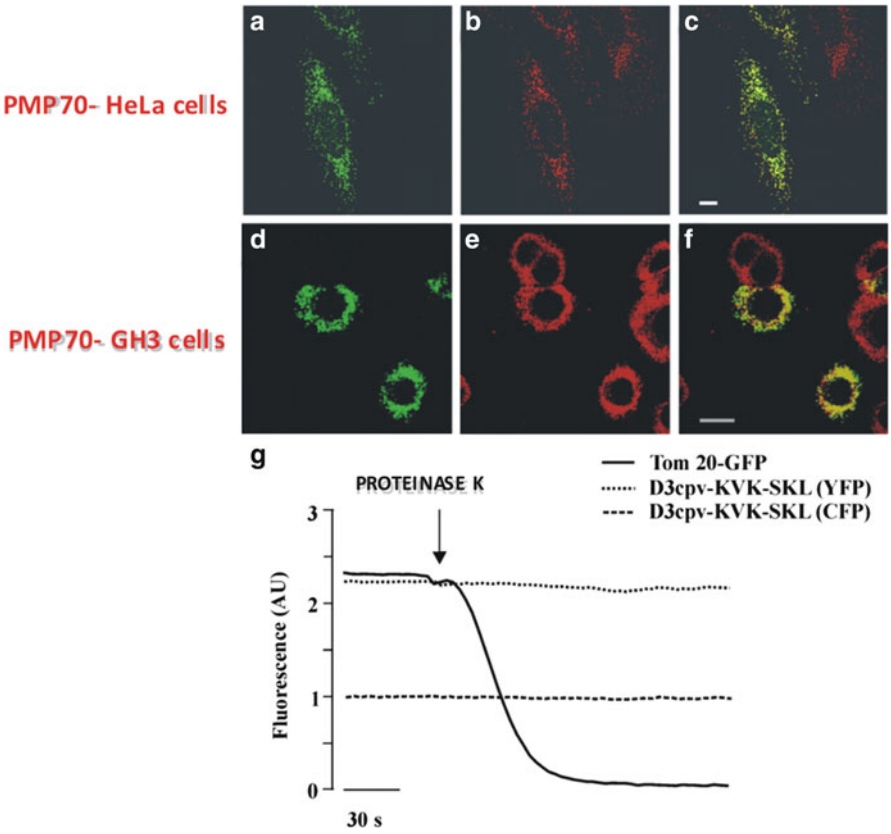

Fig. 7.2 Sub-cellular localization of the peroxisomal cameleon probe D3cpv-KVK-SKL in HeLa and GH3 cells. Confocal images of HeLa (**a**–**c**) and GH3 cells (**d**–**f**) transiently expressing the D3cpv-KVK-SKL (**a** and **d**) and immuno-labeled with antibody against the peroxisome membrane protein PMP70 (**b** and **e**). In **c** and **f**, co-localization of the two signals is coded as *yellow*. Scale bar 10 μm. (**g**) HeLa cells transiently expressing either D3cpv-KVK-SKL or TOM20-GFP, a fluorescent protein linked to the outer mitochondrial membrane and facing the cytosol, were permeabilized with digitonin, in an intracellular-like medium, and treated with proteinase K. GFP fluorescence (*continuous trace*), YFP fluorescence (*dotted trace*) and CFP fluorescence (*dashed trace*) are presented (*AU* arbitrary units). The *arrow* indicates Proteinase K addition. Reproduced from Drago et al. (2008)

environment affected the Aeq signal. In particular: (i) they measured the peroxisomal pH using a pH-sensitive protein, pHluorin, and they didn't find, at rest, any significant difference between the pH of this organelle and that of the cytosol; (ii) since modification of Aeq C-terminus has been reported to modify its Ca^{2+}-dependent luminescence characteristics (Kendall et al. 1992; Montero et al. 1995), the authors performed an in vitro calibration of their new probe and concluded that the SKL C-terminus modification did not changed the probe's Ca^{2+} sensitivity. After these careful controls, they then challenged cell types such as CHO and

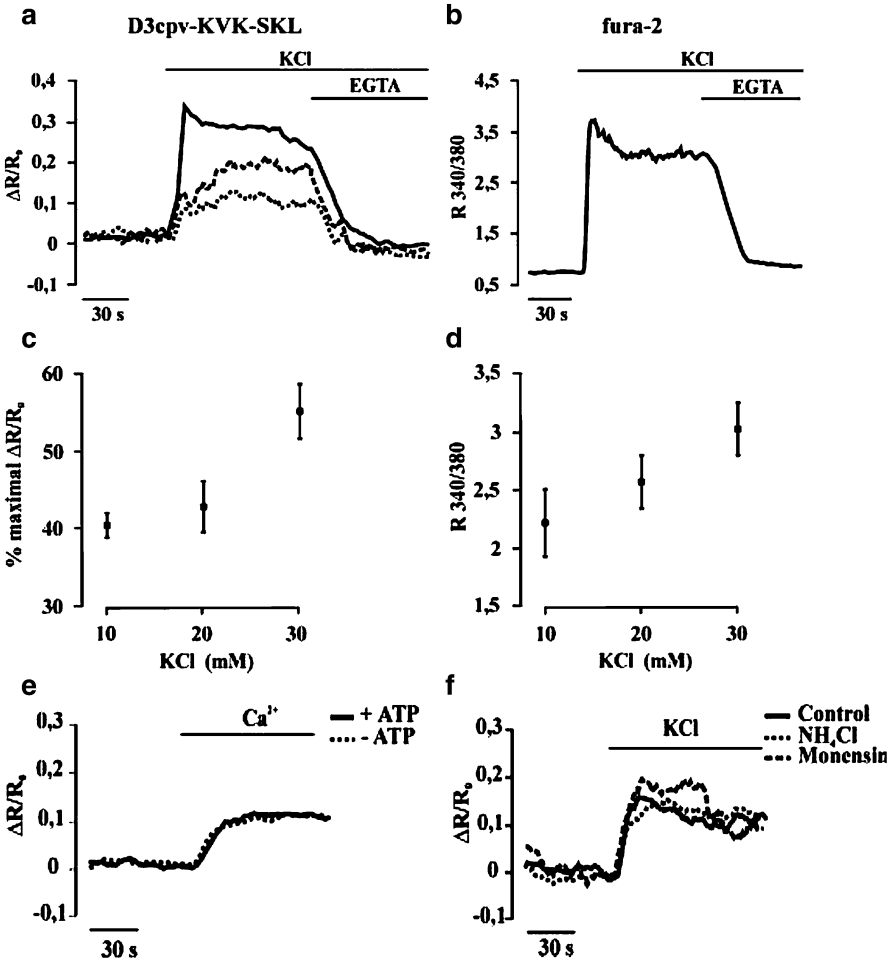

Fig. 7.3 Peroxisomal Ca^{2+} handling in GH3 cells measured with the cameleon Ca^{2+} probe D3cpv-KVK-SKL. (**a**–**b**) Fluorescence changes of typical GH3 cells transiently expressing D3cpv-KVK-SKL, selectively within peroxisomes (**a**, 2 cells, *dashed* and *dotted traces*), mistargeted to the cytosol (**a**, *continuous trace*) or loaded with fura-2 (**b**). Where indicated KCl (30 mM) and EGTA (2 mM) were added, in a Ca^{2+}-containing medium. Data are plotted as $\Delta R/R_0$, where R_0 is the fluorescence emission ratio (R 540/480 nm) at time 0 and ΔR is the increase in fluorescence emission ratio at any point. For fura-2 measurements, the ratio of the light intensity emitted at 505 nm upon dye excitation at the two wavelengths (R 340/380) is a function of the cytosolic [Ca^{2+}] and is displayed on the *left* side of the panel. (**c**–**d**) Mean rises in peroxisome Ca^{2+} level (**c**), expressed as % of the 540/480 maximal $\Delta R/R_0$, or R 340/380 fluorescence excitation ratio of the fura-2 signal (D), as a function of KCl concentration. Mean of 15 (**c**) or 19 (**d**) experiments ± s.e.m. (**e**) GH3 cells transiently expressing D3cpv-KVK-SKL were permeabilized with digitonin in an intracellular-like medium with (*continuous trace*) or without (*dotted trace*) ATP (200 μM) and succinate (2 mM). After digitonin washout, the cells were superfused with medium whose [Ca^{2+}] was buffered at 500 nM. (**f**) Intact GH3 cells expressing the peroxisomal D3cpv-KVK-SKL were treated with NH_4Cl (10 mM; *dotted trace*) or monensin (5 μM; *dashed trace*) 30 s before inducing the depolarization with KCl (30 mM). Reproduced from Drago et al. (2008)

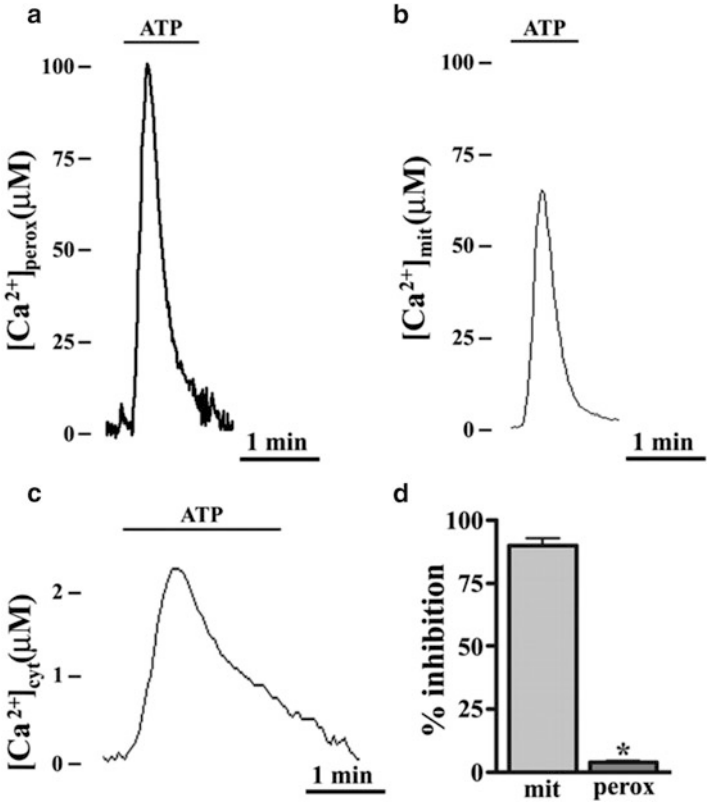

Fig. 7.4 Peroxisomal Ca^{2+} handling in CHO cells measured by a selective Aeq Ca^{2+} probe: the agonist-stimulated Ca^{2+} uptake is higher than in mitochondria and cytosol and is not inhibited by Ruthenium red. (**a**–**c**) CHO cells expressing either peroxisomal AEQ (**a**), mitochondrial AEQ (**b**) or cytosolic AEQ (**c**) were perfused with the same medium and triggered with ATP (100 μM). (**d**) CHO cells expressing either mitochondrial AEQ or peroxisomal AEQ were permeabilized by 1-min incubation with digitonin (20 μM) and perfused in an intracellular-like medium. Mitochondrial and peroxisomal Ca^{2+} uptakes were measured upon addition of IP3 (5 μM) in the presence or absence of Ruthenium red (4 μM). Aequorin luminescence was calibrated and measured into $[Ca^{2+}]$ values, and presented as mean ± s.e.m. from four replicates express the inhibition percentage of Ca^{2+} uptake as compared to control cells (*, $p < 0.001$, one-way analysis of variance followed by Bonferroni *t* test). Reproduced from Lasorsa et al. (2008)

HeLa with IP_3-generating agonists and found that a fast peroxisomal Ca^{2+} uptake, reaching $[Ca^{2+}]_p$ as high as 100 μM, followed Ca^{2+} mobilization from internal stores (Fig. 7.4). Not only, but according to their measurements, peroxisomes appear to have a resting steady-state $[Ca^{2+}]$ 20–50 fold higher than the cytosolic one. Finally, they also investigated the mechanism that allows peroxisomes to maintain this Ca^{2+} gradient and to accumulate Ca^{2+} during stimulation. According to their data, bafilomycin (a H^+ pump inhibitor) and FCCP (a mitochondrial uncoupler) caused a decrease in peroxisomal refilling after Ca^{2+} depletion, and they thus

concluded that the steady state $[Ca^{2+}]_p$ is somehow dependent on a V-type ATPase. They, on the contrary, suggested that the high Ca^{2+} peaks reached upon cellular stimulation depend on the combination of Ca^{2+}/H^+ and Ca^{2+}/Na^+ exchangers (based on the effects of ionophores that dissipate either H^+, e.g., FCCP, or Na^+, e.g., monensin). Surprisingly, however, no evidence for either Na^+ or H^+ gradients was provided. They however showed that, following Ca^{2+} accumulation, there was a transient pH acidification of the peroxisome lumen (Lasorsa et al. 2008).

At present, it is extremely difficult to explain the very different data sets obtained utilizing the two Ca^{2+} sensors. It can be excluded that such differences could be due to an altered Ca^{2+} sensitivity of the cameleon employed: (Drago et al. 2008) indeed repeated all the experiments with a peroxisome-targeted lower Ca^{2+} affinity FRET-based probe, such as D1cpv (Kd $\sim$ 60 μM; (Palmer et al. 2006)) and no differences were found neither in the amplitude of the $[Ca^{2+}]_p$ peaks nor in their kinetics (Drago et al. unpublished observations). Similarly, cell-specific peroxisomal Ca^{2+} characteristics can be ruled out: experiments with D3cpv-KVK-SKL have been performed also in CHO cells, the cell model chosen by (Lasorsa et al. 2008): also in this case, no difference was observed, compared to HeLa cells, i.e., a slow equilibration of peroxisomal lumen with cytosolic Ca^{2+} upon cellular stimulations was found (Drago et al. unpublished observations).

The only plausible explanations that we could envisage to account for the discrepancies observed rely, on the one hand, on the existence of different peroxisomal sub-populations, and, on the other, on the characteristics of the Aeq response to $[Ca^{2+}]$ changes (briefly mentioned above). As to peroxisome heterogeneity, evidence for it was provided initially over a decade ago (Volkl et al. 1999). More recently, (Neuspiel et al. 2008) demonstrated the existence of MAPL-positive mitochondria-derived vesicles that detach from mitochondria and fuse with a subpopulation of peroxisomes (Neuspiel et al. 2008; Andrade-Navarro et al. 2009). It is tempting to speculate that this latter sub-population of peroxisomes could retain at least in part the functional characteristics of mitochondria (i.e., the capacity to accumulate Ca^{2+} in an energy dependent way). If this is the case, the unique characteristics of the Aeq response to heterogeneous Ca^{2+} levels could offer an explanation for the contradictory findings: in particular, as discussed above, the average Aeq light emission would be dramatically dominated by the small fraction of molecules entrapped in an environment with a high Ca^{2+} concentration while the signal coming from the large majority of peroxisomes (i.e., those that dominate the signal revealed by the GFP-based Ca^{2+} probe) would be dramatically underestimated. Whether or not this is the explanation for the contradictory results remains to be established and further experiments are required. For example, a detailed single peroxisome analysis of the Ca^{2+} levels is still missing. The fluorescence of large single organelles (or small clusters of vesicles) has been individually investigated in cells expressing the peroxisome-targeted cameleon (Drago et al. 2008) and no evidence for significant heterogeneity in Ca^{2+} handling was obtained; however, smaller organelles are below the spatial resolution of classical confocal microscopy and other approaches (e.g., super-resolution by STED microscopy) may be necessary to solve the issue. This is obviously our biased interpretation and may not be shared by other investigators.

7.4 General Characteristics of Peroxisomal Ca^{2+} Homeostasis in Plants

Plant peroxisomes are fundamental in a plethora of processes crucial to plant growth and development. Peroxisomes are the site of fatty acid β-oxidation in plant cells and are involved in the generation of phytohormones such as the auxin IAA, jasmonic acid (JA) and salicylic acid (SA) [28; see Chaps. 14 and 16]. In conjunction with mitochondria and chloroplasts, peroxisomes participate in the photorespiration mechanism. In addition to these processes, plant peroxisomes are also involved in other metabolic and signalling pathways, including detoxification, generation of signalling molecules, the glyoxylate cycle, and biosynthesis of polyamines and branched-chain amino acids (reviewed in (Kaur et al. 2009)). Recent studies have also revealed roles for peroxisomes in the plant immune response (Lipka et al. 2005; Lipka and Panstruga 2005; Coca and San Segundo 2010; Rojas et al. 2012).

The first, and so far only paper addressing the mechanism of peroxisomal Ca^{2+} handling in living plant cells is that published by Costa and co-workers (2010). In this contribution, the dynamics of $[Ca^{2+}]_p$ in plant cells was investigated through the generation of *Arabidopsis* transgenic lines expressing the peroxisomal targeted Ca^{2+} cameleon probe D3cpv-KVK-SKL (Fig. 7.5) (Costa et al. 2010). The use of this probe allowed Costa and co-workers (2010) to monitor Ca^{2+} dynamics in single cells and also in single peroxisomes of guard cells (Costa et al. 2010). The in vivo experiments performed in *Arabidopsis* guard cells of D3cpv-KVK-SKL transgenic plants revealed that rises in $[Ca^{2+}]_p$ occurred after stimuli capable of evoking cytoplasmic Ca^{2+} increases, such as plasma membrane hyperpolarization (Fig. 7.6a, b). The kinetics and quantitative analysis of intra-peroxisomal Ca^{2+} dynamics revealed that: (i) the maximum Ca^{2+} accumulation was reached more slowly compared to the cytoplasm, confirming the behaviour of these organelles initially observed in mammalian cell lines; (ii) the peak of $[Ca^{2+}]$ reached within the peroxisome lumen is similar or lower than that observed in the cytoplasm. Thus, in peroxisomes from plant cells, Ca^{2+} diffuses passively from the cytosol. Importantly, no evidence for active accumulation of Ca^{2+} could be obtained.

More recently, Costa and co-workers (Costa et al. unpublished) extended this observation to *Arabidopsis* root tip cells challenged with external ATP (exATP). The exATP administration induced a rapid increase in both cytoplasmic and peroxisomal $[Ca^{2+}]$ (Fig. 7.6c, d); again, the peroxisomal increase was slightly slower compared to that occurring in the cytoplasm. Of interest, the decrease in $[Ca^{2+}]_p$ was kinetically indistinguishable from that of the cytoplasm, except for the fact that no intra-peroxisomal Ca^{2+} oscillations were observed in parallel with those occurring in the cytosol. It is unclear at the moment why $[Ca^{2+}]_p$ oscillations could not be observed in these experiments. Two not mutually exclusive possibilities could be proposed. First, the rate of diffusion of cytosolic Ca^{2+} into peroxisomes may not be sufficiently fast to allow equilibration of the relatively rapid and small cytosolic Ca^{2+} oscillations. Second, the root tip response shown in Fig. 7.5 does not reflect the dynamics of $[Ca^{2+}]$ of a single cell, but rather that of a population of cells and of a population of peroxisomes within them (Dodd et al. 2006). Accordingly,

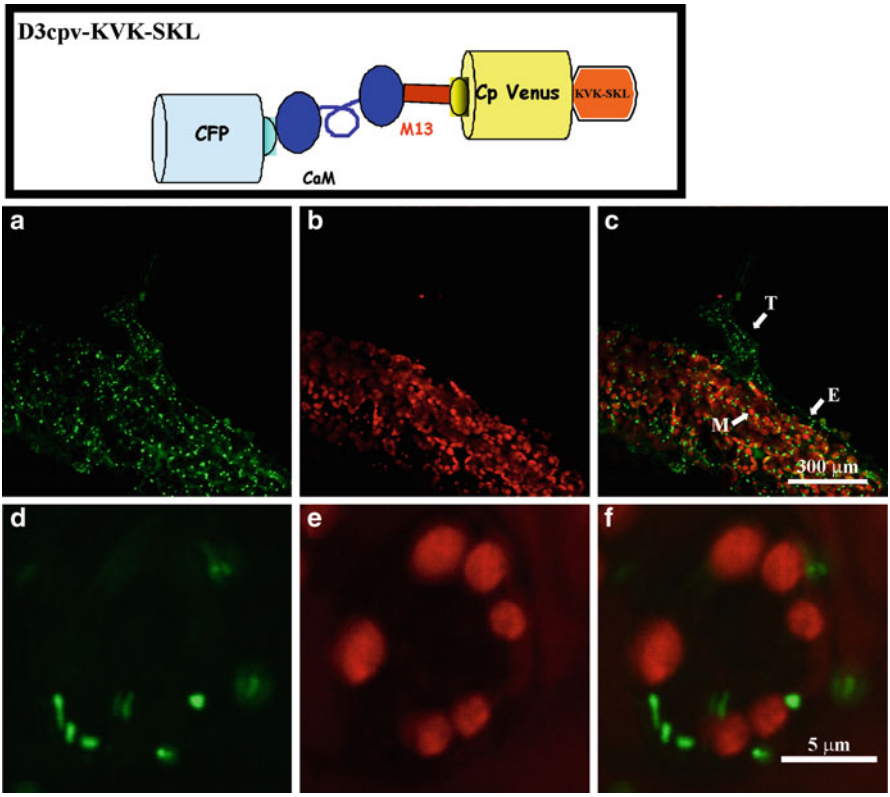

Fig. 7.5 Subcellular distribution of D3cpv-KVK-SKL in stable transgenic *Arabidopsis plants. Top* panel. Schematic structure of the D3cpv-KVK-SKL cameleon probe. (**a**–**f**) Confocal images of stable transgenic *Arabidopsis* plants transformed with D3cpv-KVK-SKL. (**a**) Cameleon cpVenus fluorescence in *Arabidopsis* leaf epidermal cells. (**b**) Chlorophyll fluorescence of the same leaf shown in (**a**). (**c**) Overlay image of (**a**) and (**b**). Mesophyll (M), epidermis (E), trichome (T). (**d**) Cameleon cpVenus fluorescence in stomata guard cells. (**e**) Chlorophyll fluorescence of the same stomata guard cell shown in (**d**). (**f**) Overlay image of (**d**) and (**e**). Reproduced from Costa et al. (2010)

one can speculate that while the initial Ca^{2+} transient increases of all peroxisomes is synchronized, the second more sustained $[Ca^{2+}]_p$ increase may represent the merging of asynchronous $[Ca^{2+}]_p$ oscillations that occur in different cells. Due to the much larger Ca^{2+} peaks observable in the cytosol, asynchronous oscillations could still be appreciated at the population level, while the much smaller ones occurring in peroxisomes might be masked by the trace noise. The use of confocal microscopy with a faster temporal resolution (resonant scanner or spinning disk) will help to optimize peroxisomal Ca^{2+} analyses in single cells of root, as already reported for mitochondria (Loro et al. 2012), to better understand their Ca^{2+} handling in complex tissues.

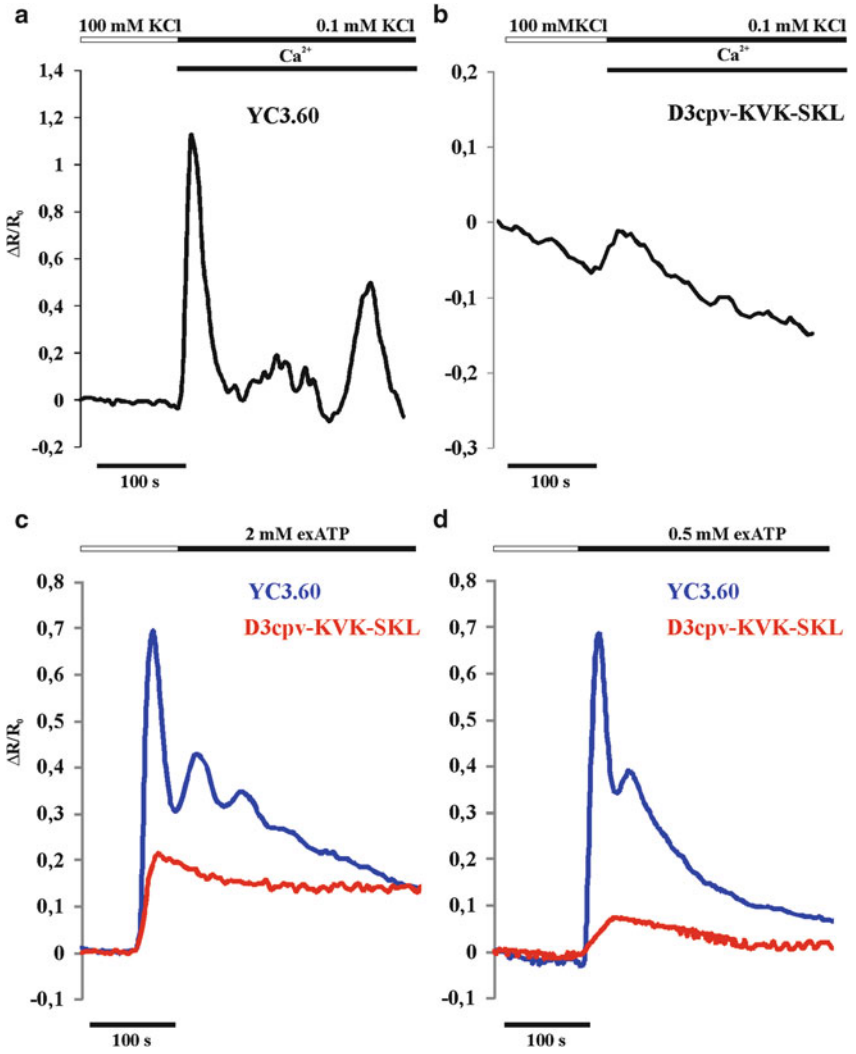

Fig. 7.6 $[Ca^{2+}]_c$ and $[Ca^{2+}]_p$ monitoring in *Arabidopsis* guard and root tip cells. (**a**–**b**) $[Ca^{2+}]_c$ and $[Ca^{2+}]_p$ dynamics monitored in *Arabidopsis* guard cells expressing cameleon YC3.60 and D3cpv-KVK-SKL respectively: *Arabidopsis* guard cells were bathed in depolarization buffer (*white bar*) and perfused with the hyperpolarization buffer (*black bar*) and Ca^{2+} was added at the same time of hyperpolarization. The $[Ca^{2+}]$ variations are reported as normalized cpVenus/CFP ratio ($\Delta R/R_0$). (**a**) $[Ca^{2+}]_c$ dynamics monitored in *Arabidopsis* guard cells expressing cameleon YC3.6 in response to plasma membrane hyperpolarization. (**b**) $[Ca^{2+}]_p$ dynamics monitored in *Arabidopsis* guard cells expressing Cameleon D3cpv-KVK-SKL in response to plasma membrane hyperpolarization. (**c**–**d**) $[Ca^{2+}]_c$ and $[Ca^{2+}]_p$ dynamics monitored in *Arabidopsis* root tip cells. Root tips of 12-day-old seedlings of YC3.6, and D3cpv-KVK-SKL were bathed in 5 mM KCl, 10 mM Mes, 10 mM Ca^{2+} pH 5.8 and at the indicated time 2 or 0.5 mM of ATP (exATP) were added to the chamber. The $[Ca^{2+}]$ variations are reported as normalized cpVenus/CFP ratio ($\Delta R/R_0$). (**c**) $[Ca^{2+}]_c$ (*blue trace*) and $[Ca^{2+}]_p$ (*red trace*) in response to 2 mM of exATP. (**d**) $[Ca^{2+}]_c$ (*blue trace*) and $[Ca^{2+}]_p$ (*red trace*) in response to 0.5 mM of exATP. Panel 7.6a and b are reproduced from Costa et al. (2010)

7.5 Functional Role of Peroxisomal Ca^{2+}

The physiological role of Ca^{2+} within peroxisomes is a key question, still unresolved. To date, no evidence exists to indicate the existence of Ca^{2+}-modulated enzyme activities in mammalian peroxisomes, while the presence of a number of Ca^{2+}-binding proteins in plant peroxisome lumen is an established fact (Yang and Poovaiah 2002; Reumann et al. 2007; Chigri et al. 2012) (see also below). The first indirect evidence suggesting that, in plants, intra-peroxisomal Ca^{2+} levels could regulate key H_2O_2 scavenging enzymes, like catalases, was provided about a decade ago (Yang and Poovaiah 2002). In particular, Yang and Poovaiah demonstrated in vitro that the purified tobacco catalase activity is stimulated by Ca^{2+} and CaM, with the prediction that, among the different *Arabidopsis* catalase isoforms, CAT3 (highly expressed in plant peroxisomes) is the target of this activation. The same authors demonstrated that *Arabidopsis* CAT3 in vitro can bind CaM in a Ca^{2+}-dependent way and that the peptide corresponding to the predicted CAT3 CaM binding region is able to competitively inhibit in vitro the Ca^{2+}-dependent stimulation of tobacco catalase activity (Yang and Poovaiah 2002). The demonstration that significant changes in $[Ca^{2+}]$ occur in plant peroxisomes under physiological conditions prompted researchers to investigate whether indeed this Ca^{2+} regulation of CAT3 occurs in intact cells. In order to understand the physiological relevance of this study, it should be considered that CAT3 is annotated as a senescence-associated gene (*SEN2*), and indeed it shows high expression in senescent leaves, where its activity increases with age and where it represents the main H_2O_2 scavenging system (Zimmermann et al. 2006; Costa et al. 2010). Costa and co-workers (2010) thus expressed a new genetically encoded probe HyPer, sensitive to H_2O_2 (Belousov et al. 2006), in the peroxisomal lumen of *Arabidopsis* guard cells. They showed that, in parallel to the Ca^{2+} increases in the peroxisome lumen, there is a fast acceleration in H_2O_2 catabolism. Most relevant, the stimulating effect of intra-peroxisomal Ca^{2+} on the catabolism of H_2O_2 strictly correlated with plant age (Fig. 7.7a, (Costa et al. 2010)). In particular, upon imposing a Ca^{2+} increase caused by plasma membrane hyperpolarization, an increase in the rate of H_2O_2 scavenging was observed in guard cell peroxisomes of 6- and 4-week-old plant leaves. However, in leaves of older plants, the effect of Ca^{2+} entry was much stronger than in younger plants, indicating that Ca^{2+}-activated peroxisomal H_2O_2 scavenging correlates with CAT3 expression and activity levels. These data thus demonstrated a strict correlation between CAT3 expression levels and efficiency of the Ca^{2+}-dependent H_2O_2 scavenging system. This correlation was also supported by experiments of transient expression of CAT3 in tobacco leaves. Tobacco leaves were agro-infiltrated with *HyPer–KSRM* alone or together with a *CaMV35S–CAT3* construct for its overexpression, and the capacity of epidermal cells (controls and CAT3-overexpressing) to metabolize H_2O_2 was compared. While in the absence of a Ca^{2+} rise the rate of H_2O_2 catabolism (measured by

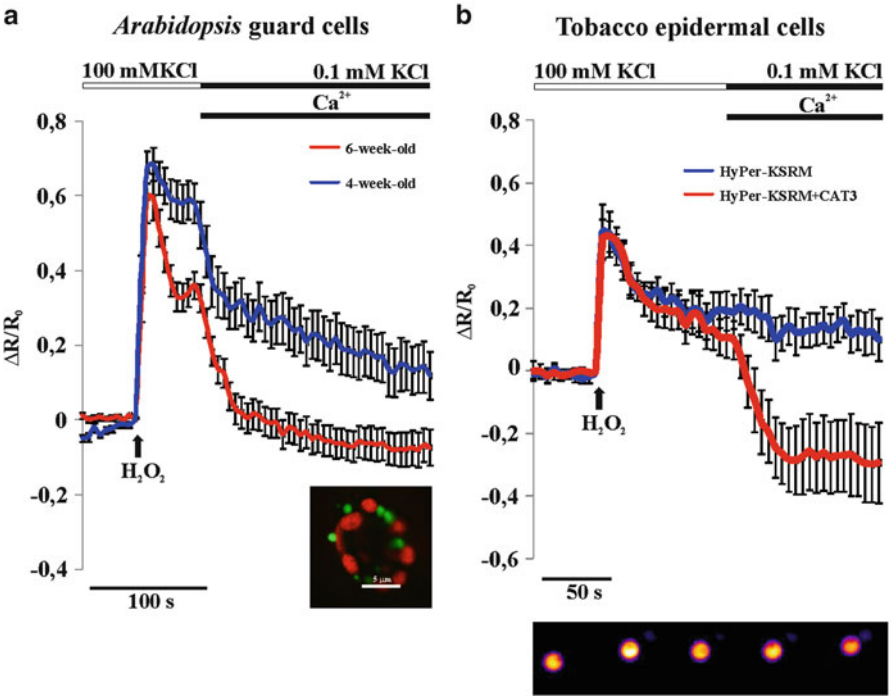

Fig. 7.7 Ca^{2+}-dependent peroxisomal H_2O_2 scavenging efficiency correlates with changes of *CAT3* expression. (**a**) Leaves from 4-to 6-week-old plants were bathed in depolarization buffer and H_2O_2 (100 μM) was added at the indicated time point. The perfusion of the guard cells with the hyperpolarization solution in presence of external Ca^{2+} induced a different response in the leaf guard cells of the different plant ages. In the guard cells peroxisomes of 6-week-old plants the Ca^{2+}-dependent HyPer fluorescence ratio drop was more consistent (*red trace*), compared with the responses observed in guard cells of 4- (*blue trace*) plants. (**b**) Tobacco epidermal cells expressing HyPer-KSRM were subjected to H_2O_2 (1 mM) administration and afterwards hyperpolarization in presence of Ca^{2+} was applied. A steep and prolonged Ca^{2+}-dependent H_2O_2 scavenging occurred only in cells of tobacco leaves co-transformed with the *HyPer-KSRM* and the *CaMV35S-CAT3*. *Bottom* panel. An example of HyPer emitted fluorescence (ex 480 nm, em 530 nm), in a tobacco epidermal cell, subjected to H_2O_2 addition. Reproduced from Costa et al. (2010)

HyPer–KSRM) was indistinguishable in control and CAT3-overexpressing cells, a Ca^{2+}-dependent fast H_2O_2 catabolism was observed in the cells overexpressing CAT3 (Fig. 7.7b). These results demonstrate that CAT3 can be responsible for, at least, part of the Ca^{2+}-dependent H_2O_2 scavenging, though they do not exclude the existence of other H_2O_2 scavenging systems, whose activity could be stimulated by Ca^{2+}.

7.6 Other Ca^{2+}-Dependent Functions in Plant Peroxisomes

Plant peroxisomes, as their mammalian and yeast counterparts, are organelles surrounded by single membranes, whose main function is, as reported above, to host oxidative metabolism (van den Bosch et al. 1992). Among the very different functions played by peroxisomes, our knowledge of the contribution played by intra-peroxisomal Ca^{2+} is still very scarce. The Ca^{2+} activation of CAT3 activity, demonstrated both in vitro and in living cells, represents the first well characterized effect of intra-peroxisomal Ca^{2+} in an important physiological plant process. Most likely, this event plays a key role during senescence, as CAT3 is mainly expressed during this period of the plant life (Zimmermann et al. 2006). Recent findings suggest that peroxisomal Ca^{2+} could play other important roles in the regulation of other catalases or different enzymes within the organelles (see below).

An important contribution for the discovery of new peroxisomal Ca^{2+}-dependent activities might come from the identification of intra-peroxisomal CaM-like proteins that could act as sensors of the Ca^{2+} levels and relay signals to other enzymes (Perochon et al. 2011). Yang and Poovaiah already demonstrated the presence of CaM (or a CaM cross-reacting protein) in pea peroxisomes but without providing any information regarding the protein sequence or the locus gene coding for it (Yang and Poovaiah 2002). Very recently, Chigri and co-workers (2012) succeed in the identification of an *Arabidopsis* gene (At3g07490) encoding a peroxisome-localized CaM-like protein (CML), named AtCML3. AtCML3 shows the typical structure of CaMs, with two pairs of EF-hand motifs separated by a short linker domain (Chigri et al. 2012). The role of AtCML3 has not been clarified, since no obvious phenotypic effects were observed in knock-out *Arabidopsis* plants (Hanada et al. 2009). CMLs are found exclusively in the plant kingdom and a complete study of the AtCML3 physiological role is still missing. However, we can try to speculate on the possible role(s) played by AtCML3, or its homologues, in peroxisome physiology; in particular, this protein could be the missing link between Ca^{2+} and the activity of two key processes: (i) the Ca^{2+}-CaM dependent peroxisomal nitric-oxide synthase (NOS) (Barroso et al. 1999; Corpas et al. 2004; del Río 2011); and (ii) the Ca^{2+}-stimulated catalase activity in tobacco and *Arabidopsis* plants (Yang and Poovaiah 2002; Costa et al. 2010). In both mechanisms, the presence of a CaM-like protein is indeed required. Although still indirect, the evidence for a role of AtCML3 is particularly compelling as far as NO synthase is concerned. In particular, Prado and co-workers (2004) demonstrated that pollen tube peroxisomes are the sites of NO synthesis, and the latter messenger is required for the proper pollen tube growth and re-orientation (Prado et al. 2004). Noteworthy, in *Arabidopsis*, during pollen germination and pollen tube growth, the expression of AtCML3 is up-regulated (Wang et al. 2008). We could therefore hypothesize that AtCML3 is the CML required for regulating the NOS-like Ca^{2+}-dependent activity identified by Barroso and co-workers (1999). In this specific case, a direct role of intra-peroxisomal Ca^{2+} on NO production is still missing, but could represent an interesting experiment to

perform, considering also the importance of Ca^{2+} in the regulation of pollen tube growth (Feijo et al. 2001).

Concerning the role of *AtCML3* in regulating the catalase activity, it is interesting to note that it is down-regulated in *RDH2 Arabidopsis* mutant (Torres et al. 1998; Foreman et al. 2003). This mutant lacks RBOH, a member of the respiratory burst oxidase family (Torres et al. 1998; Foreman et al. 2003), and is characterized by reduced levels of H_2O_2 and, consequently, short root hairs. It can be speculated that the expression level of *AtCML3* in these mutant plants is lower than in wt because catalase, that it is putatively regulated (Yang and Poovaiah 2002; Costa et al. 2010), is not necessary. Moreover, all RBOH proteins contain two EF-hand motifs for Ca^{2+} binding and peroxisomes have an important function in ROS signalling [see Chap. 13]. Taken as a whole, these data might point towards a function of AtCML3 in Ca^{2+} regulation in correlation with ROS signalling (Chigri et al. 2012).

Finally, another very interesting Ca^{2+}-modulated protein has been found in the plant peroxisome: AtCPK1, a member of the *Arabidopsis* Ca^{2+}-dependent protein kinase family. However, AtCPK1 has been reported to bind to the external surface of peroxisomes (Dammann et al. 2003) and, accordingly, its activity should be modulated by the cytoplasmic and not by intra-peroxisomal [Ca^{2+}]. AtCPK1 has been also found associated with lipid bodies (Coca and San Segundo 2010). The AtCPK1 peroxisomal localization is likely due to an N-terminal myristoylation signal typically present in many Ca^{2+}-dependent protein kinases (CDPKs) and other kinases (Lu and Hrabak 2002; Benetka et al. 2008; Stael et al. 2012). From a functional point of view, the AtCPK1 activity has been demonstrated to mediate pathogen resistance (Coca and San Segundo 2010).

In the last few years, strong efforts have been made, mainly through proteomic analyses, in order to discover new roles played by plant peroxisomes (Reumann et al. 2004, 2007; Kaur et al. 2009; Reumann et al. 2009; Reumann 2011). The rationale was the identification of "new peroxisomal proteins" in the hope of discovering additional functions where they could be involved. This approach can be adopted for the identification of other proteins whose activity is regulated by, or dependent on, intra-peroxisomal Ca^{2+}. Only one protein, in addition to those mentioned above, has been so far identified which shows a potential connection with "Ca^{2+} signalling". In *Arabidopsis*, this protein is encoded in the At1g64850 locus and annotated as "Calcium-binding EF-hand family protein" but no functional information is yet available. It is easy to predict that, now that tools to directly investigate peroxisomal Ca^{2+} homeostasis in intact plants are available, the search for potential Ca^{2+} targets in the organelles will be intensified and new developments in the field are therefore expected.

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Chapter 8
The Versatility of Peroxisome Function in Filamentous Fungi

Ida J. van der Klei and Marten Veenhuis

Contents

Abstract Peroxisomes are ubiquitous and versatile cell organelles. They consist of a single membrane that encloses a proteinaceous matrix. Conserved functions are fatty acid β-oxidation and hydrogen peroxide metabolism. In filamentous fungi, many other metabolic functions have been identified. Also, they contain highly specialized peroxisome-derived structures termed Woronin bodies, which have a structural function in plugging septal pores in order to prevent cytoplasmic bleeding of damaged hyphae.

In filamentous fungi peroxisomes play key roles in the production of a range of secondary metabolites such as antibiotics. Most likely the atlas of fungal

I.J. van der Klei (✉) • M. Veenhuis
Molecular Cell Biology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, P.O. Box 11103, 9700CC Groningen, The Netherlands
e-mail: i.j.van.der.klei@rug.nl; m.veenhuis@rug.nl

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peroxisomal metabolic pathways is still far from complete. Relative recently discovered functions include their role in biotin biosynthesis as well as in the production of several toxins, among which polyketides. Finally, in filamentous fungi peroxisomes are important for development and pathogenesis.

In this contribution we present an overview of our current knowledge on fungal peroxisome formation as well as on their functional diversity.

Keywords Filamentous fungus • Peroxisome • Woronin body • *PEX* genes • Secondary metabolites • Toxins • Pathogenicity • Development

8.1 Introduction

Peroxisomes occur in almost all eukaryotes. They are without exception very simple of architecture and consist of a single membrane, which encloses an enzyme rich matrix. Peroxisomes display an unprecedented versatility of functions dependent on organism, cell type and developmental stage. Because of this, peroxisomes characteristically proliferate in response to environmental stimuli and metabolic needs.

Peroxisomal enzymes are involved in both catabolic and anabolic pathways. As indicated by their name, catabolic processes often are catalyzed by hydrogen peroxide producing oxidases. Conserved peroxisomal processes are the β-oxidation of fatty acids and hydrogen peroxide metabolism. In filamentous fungi, peroxisomes play a predominant role in the metabolism of specific carbon sources used for growth and in secondary metabolism. As a consequence, defects in peroxisome biogenesis affect these metabolic processes. However, peroxisome biogenesis defects also disturb processes related to fungal development (e.g. sporulation) and pathogenesis.

Several filamentous fungi can grow on oleic acid as sole carbon source (e.g. *Aspergillus nidulans, Penicillium chrysogenum*). The metabolism of this compound involves peroxisomal enzymes of the β-oxidation pathway (Hynes et al. 2006; Valenciano et al. 1998). Mutants of these organisms that are defective in the biogenesis of peroxisomes (*pex* mutants) are unable to grow on oleic acid, stressing the importance of the compartmentalization of the β-oxidation enzymes in peroxisomes (Valenciano et al. 1998; Meijer et al. 2010; De Lucas et al. 1997). Both during growth on fatty acids and C2 compounds, such as acetate, peroxisomes harbor enzymes of the glyoxylate cycle (Valenciano et al. 1998; Szewczyk et al. 2001).

Peroxisomes are of major importance for the biosynthesis of several commercially important secondary metabolites. For instance, in *Aspergillus nidulans* (Valenciano et al. 1998) and *P. chrysogenum* (Meijer et al. 2010) peroxisomes are required for penicillin biosynthesis. A highly specialized peroxisome-derived organelle, called Woronin body, occurs in filamentous ascomycetes. This organelle plays an important role in closing septal pores to prevent cytoplasmic leakage when hyphae are damaged. Hence, this organelle plays a structural role, which is unique for filamentous fungi.

In this contribution we present an overview of the current knowledge on peroxisome in filamentous fungi, focusing on the molecular mechanisms involved in their formation as well as on their highly diverse functions in metabolism, pathogenicity and development.

8.2 Biogenesis of Fungal Peroxisomes and Woronin Bodies

8.2.1 Peroxisomal Matrix Protein Import

Peroxisomal matrix proteins are post-translationally translocated across the peroxisomal membrane, a process that involves peroxisomal targeting signals (PTSs). Two peroxisomal targeting signals (termed PTS1 and PTS2) are known that are recognized by their respective receptors Pex5p and Pex7p (for a detailed review see (Ma et al. 2011)). Most peroxisomal proteins contain a PTS1. This signal resides in the last 12 residues and ends with the tripeptide –SKL or conserved variants (Brocard and Hartig 2006).

Recent findings indicated that in fungi a portion of specific glycolytic enzymes, which are normally localized to the cytosol, is localized to peroxisomes. These enzymes contain PTS1 signals that are formed upon alternative spicing or stop codon read through (Freitag et al. 2012). For instance, in *Ustilago maydis* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 3-phosphoglycerate kinase (PGK) have a dual localization in the cytosol and peroxisomes. At the C-terminus of PGK a PTS1 is generated via ribosomal read-through, whereas alternative splicing generates a variant containing a PTS1 in case of GAPDH. The physiological relevance of the partial peroxisomal localization of these two glycolytic enzymes is evident from the observation that *U. maydis* mutants, unable to form the PTS1 containing isoforms of GAPDH or PGK1, show reduced virulence. A similar phenomenon has been observed in *Candida albicans*. In this organism alternative splicing was shown to result in partial peroxisomal localization of cytosolic 6-phosphogluconate dehydrogenase, an enzyme of the pentose phosphate pathway (Strijbis et al. 2012). In both *U. maydis* and *C. albicans* the partial peroxisomal localization was suggested to be important for peroxisomal redox homeostasis.

The PTS2 is present at the N-terminus of peroxisomal matrix proteins. Its consensus sequence is (R/K)(L/V/I)X_5(Q/H)(L/A/I). Relatively few proteins contain a PTS2, which is sometimes cleaved upon import.

The PTS1 and PTS2 receptors, Pex5 and Pex7, are predominantly cytosolic proteins. Upon binding their newly synthesized cargo protein, they associate with the peroxisomal membrane at a receptor docking site consisting of Pex13, Pex14 and Pex14/17 (see below). A set of 8 other peroxisomal membrane bound peroxins (Pex1, Pex2, Pex4, Pex6, Pex8, Pex10, Pex12, Pex15) is proposed to be involved in cargo release and receptor recycling. How the cargo exactly crosses the peroxisomal

membrane and which proteins are involved in this process is still topic of debate. For PTS1 import, it has been proposed that the receptor protein Pex5 itself may transiently form a pore in the peroxisomal membrane (Meinecke et al. 2010) (for a recent review see (Nuttall et al. 2011)).

Pex5 consists of two domains, an N-terminal domain that is required for the interaction of the protein with other peroxins and a C-terminal domain that recognizes the PTS1. In the PTS2 protein import pathway, Pex7 binds the PTS2, whereas a co-receptor is required to associate Pex7 with other peroxins. In most fungi, the co-receptor of Pex7 is Pex20 (Sichting et al. 2003). The C-terminus of Pex20 contains a Pex7 binding site. Interestingly, the N-terminal region of Pex20 highly resembles the Pex5 N-terminus. The structural similarity might explain why the basidiomycetes *U. maydis* and *C. neoformans* lack a separate Pex20. In these species, a Pex5/Pex20 fusion protein is present, the N-terminus of which resembles the N-termini of Pex20 and Pex5(Kiel et al. 2006).

Most *PEX* genes encode proteins involved in matrix protein import. Most were initially identified by functional complementation of yeast peroxisome deficient (*pex*) strains and extensively studied in yeast model systems. Analyses of genome databases has strongly contributed to the identification of *PEX* genes of filamentous fungi (Kiel et al. 2006; Kiel and van der Klei 2009).

The major differences between filamentous fungi and other species appears to be the presence of Pex14/17 (later also designated Pex33) (Kiel and van der Klei 2009). In yeast, the receptor docking site consists of three peroxins, Pex13, Pex14, and Pex17. Analyses of genome databases revealed that Pex13 and Pex14 are highly conserved in filamentous fungi, whereas Pex17 is absent in these species. However, in addition to Pex14, filamentous fungi contain the Pex14/17 protein. This protein consists of an N-terminal domain with high similarity to a conserved region present in the N-terminus of Pex14, followed by a putative coiled-coil region showing weak similarity to yeast Pex17. Functional characterization of this proteins in different species (*P. chrysogenum* (Opalinski et al. 2010), *N. crassa* (Managadze et al. 2010a) and *Podospora anserina* (Peraza-Reyes et al. 2011)), revealed that Pex14/17 is a genuine peroxin, whose absence results in (partial) mislocalization of PTS1 and PTS2 proteins. The N-terminal domain of the protein, constituting the region of high similarity to Pex14, was shown not to be required for peroxisome biogenesis in *P. chrysogenum*, implying that Pex14/17 is a member of the Pex17 family (Opalinski et al. 2010).

8.2.2 Formation of the Peroxisomal Membrane

So far, three peroxins have been implicated to play an essential role in the formation of the peroxisomal membrane, namely Pex3, Pex19 and (in mammals) Pex16, because in the absence of these proteins peroxisomes are generally completely absent. The functions of these proteins are still highly debated. Pex3 is a peroxisomal membrane protein which has been proposed to serve as docking site for

Pex19, a soluble cytosolic peroxin which may act as receptor/chaperone for newly synthesized peroxisomal membrane proteins (PMPs). In this model PMPs insert directly in the peroxisomal membrane (Schliebs and Kunau 2004). In an alternative model PMPs traffic to peroxisomes via the ER. Because in *PEX3* and *PEX19* deficient yeast strains PMPs were observed to accumulate at the ER, these proteins have also been proposed to play a role in the exit of PMPs from the ER (van der Zand et al. 2006). Further studies are required to elucidate the exact function of both proteins.

Although the absence of Pex3 and Pex19 invariably leads to peroxisome deficiency, this is not always the case for Pex16. Mutations in the human *PEX16* gene are the cause of Zellweger syndrome, a lethal disorder caused by the complete absence of peroxisomal membrane structures in cells of these patients (Honsho et al. 1998). However, most yeast species in fact lack a *PEX16* ortholog indicating that the function of the protein is not generally conserved. Moreover, in *Y. lipolytica*, a yeast species that does have a *PEX16* gene, deletion of this gene does not result in complete peroxisome deficiency (Eitzen et al. 1997). Similarly, we recently showed that deletion of *PEX16* in *P. chrysogenum* does not result in a *pex* phenotype, as peroxisomal structures were still present in the cells although, as in *Y. lipolytica*, a portion of the matrix proteins were mislocalized to the cytosol (Opalinski et al. 2012). The observed strongly reduced levels of the PMP Pex11 in *P. chrysogenum pex16* cells suggest that Pex16 may be important, but not essential for membrane protein insertion/assembly in peroxisomes (Opalinski et al. 2012).

Interestingly, also in *Drosophila* and *Arabidopsis* disruption of *PEX16* does not result in the complete absence of peroxisomes (Nakayama et al. 2011; Kondo et al. 2007). Hence, the current data suggest that *PEX16* is only essential for peroxisome formation in mammals, not required for peroxisome biogenesis in almost all yeast species, whereas a partial defect in peroxisome formation is observed in filamentous fungi, plant and *Drosophila* when Pex16 is absent.

8.2.3 Peroxisome Proliferation

Two modes of peroxisome proliferation exist namely multiplication of pre-existing organelles by fission and formation of new organelles from the endoplasmic reticulum (ER; the so called de novo formation). The contribution of both processes to peroxisome proliferation in wild-type cells is currently debated and might be dependent on species, tissue and their developmental stage (Ma et al. 2011; Nuttall et al. 2011).

Several proteins involved in peroxisome fission have been characterized. The first protein identified to be involved in peroxisome fission is the peroxisomal membrane protein Pex11. In all eukaryotes studied so far deletion of *PEX11* leads to a strong reduction of peroxisome numbers concomitant with an increase in organelle size (Figs. 8.1 and 8.2), whereas *PEX11* overexpression results in an increase in organelle number in conjunction with a reduction in organelle size.

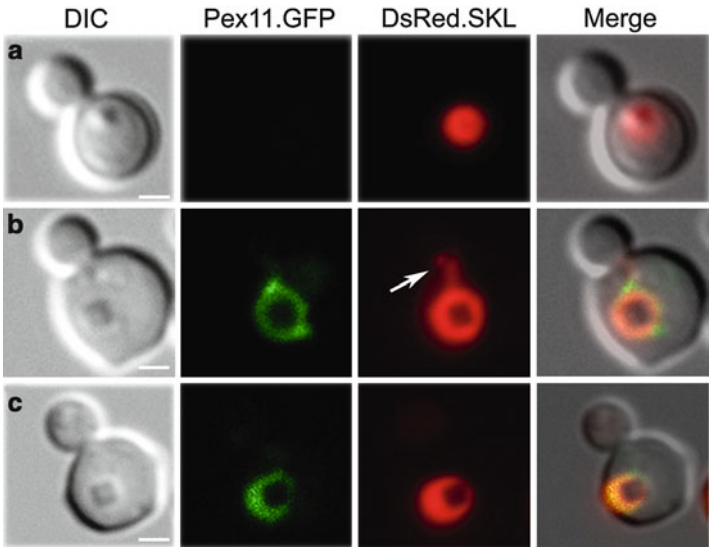

Fig. 8.1 The importance of the N-terminal amphipathic α-helical domain of *P. chrysogenum* Pex11 for the function of the protein in peroxisome fission shown by fluorescence microscopy. The *P. chrysogenum* Pex11 protein containing a C-terminal GFP was produced in a *Hansenula polymorpha pex11 dnm1* double deletion strain (**b**). During budding of the cells a tubular extension of the peroxisomes protrude into the daughter cell (evident from the peroxisomal matrix marker DsRed-SKL). This extension was not observed in the *H. polymorpha pex11 dnm1* host strain (**a**). This demonstrates that PcPex11 is functional in peroxisomal membrane tubulation in the heterologous host *H. polymorpha*. However, upon introduction of a mutant variant of PcPex11 in which the amphipathic nature of the helix was disturbed (reduction of the hydrophobic surface), no tubular structures were observed (**c**). DIC – differential interference contrast. Adapted from Opalinski et al. (2011)

The first Pex11 protein of a filamentous fungus was identified in *P. chrysogenum*, as the most abundant peroxisomal membrane protein (Kiel et al. 2005). Like in other species, this protein is involved in peroxisome proliferation. Interestingly, overexpression of *P. chrysogenum PEX11* did not only result in enhanced organelle numbers but also in enhanced penicillin production (for details see below) (Kiel et al. 2005).

Analysis of the genomes of eukaryotes suggest that all species contain multiple members of the Pex11 protein family. In *P. chrysogenum* three members have been identified: Pex11, Pex11B and Pex11C. Pex11 and Pex11C are constituents of the peroxisomal membrane, whereas Pex11B is localized to the ER (Opalinski et al. 2012) (Fig. 8.2). As in yeast, the absence of Pex11, but not of Pex11B or Pex11C, leads to decreased numbers of peroxisomes of increased size. Hence, Pex11 is the prominent factor controlling peroxisome numbers in this fungus. However, because overexpression of *PEX11C* also resulted in peroxisome proliferation, Pex11C most likely is also important to control peroxisome numbers. Pex11B represents the first Pex11 family member that is uniquely localized to an organelle other than the

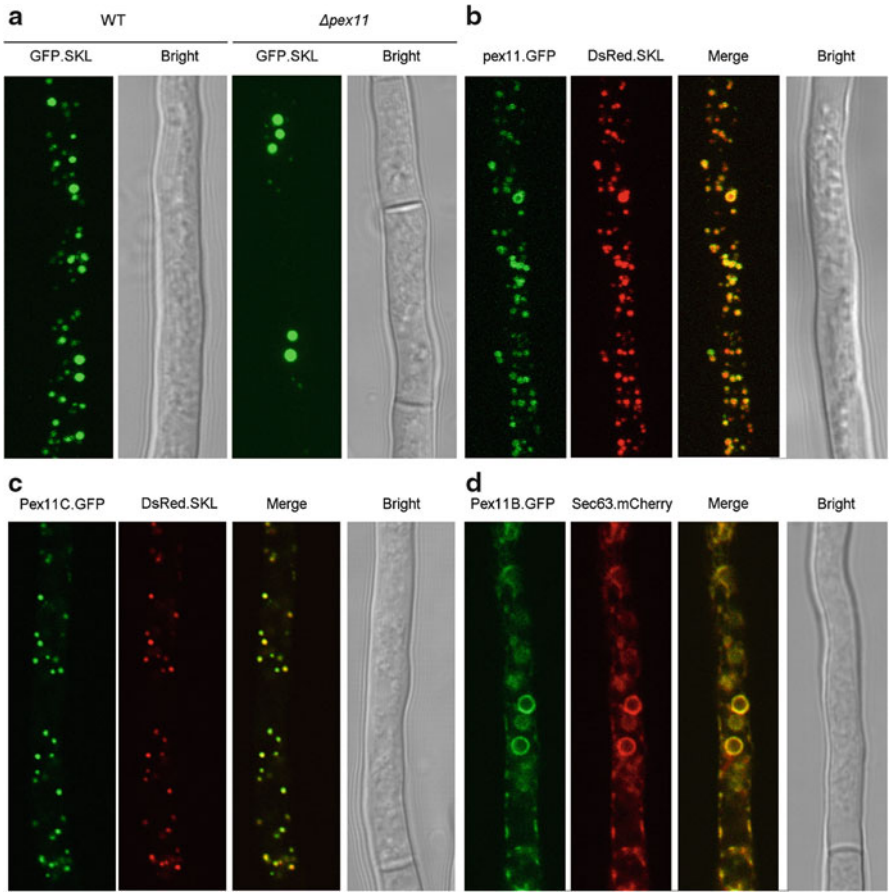

Fig. 8.2 The *P. chrysogenum* proteins of the Pex11 protein family. Deletion of the *PEX11* gene in *P. chrysogenum* results in a strong reduction of peroxisome numbers, together with an increase in their size (**a**). The peroxisomal matrix is marked with GFP. Subcellular localization of Pex11 (**b**), Pex11C (**c**) and Pex11B (**d**). The GFP fusion proteins of Pex11 and Pex11C are localized to peroxisomes (*green*) and co-localize with the peroxisomal marker protein DsRed.SKL (*red*). Pex11B is localized to the ER, where it colocalizes with the ER marker protein Sec63

peroxisome. Deletion of *PEX11B* did not affect peroxisomal profiles significantly. Most likely, the protein is involved in peroxisome proliferation from the peroxisome reticulum in the hyphal tip. However, other data suggest that Pex11B is not involved in de novo peroxisome formation (see below). Hence, unraveling the exact function of Pex11B in *P. chrysogenum* requires further investigation.

Recently, the first mechanistic insight in the function of Pex11 in peroxisome fission has been elucidated for *P. chrysogenum* Pex11. This protein contains a conserved sequence at the N-terminus that can adopt the structure of an amphipathic helix which mediates peroxisome membrane remodeling (tubulation) as the first

step in organelle fission (Opalinski et al. 2011). Point mutations that affect the alpha helical amphipathic structure block the function of the protein in peroxisome fission (Fig. 8.1). This membrane remodeling capacity of Pex11 appeared to be conserved from yeast to man.

The actual peroxisome fission process requires the activity of the dynamin-related proteins (DRP) (e.g. Dnm1 and Vps1 in *S. cerevisiae*) (Hoepfner et al. 2001; Motley and Hettema 2007; Nagotu et al. 2008). DRPs are large GTPases that are involved in multiple membrane fission and fusion events. Vps1 was initially identified to be involved in vacuolar protein sorting in *S. cerevisiae*, whereas Dnm1 is required for mitochondrial fission in this organism. Hence, the peroxisomal fission machinery is not unique for this organelle but shares components with other membrane fission/fusion processes. Studies in both lower and higher eukaryotes indicate that in most species peroxisome fission requires the DRP that also mediates mitochondrial fission. Association of this protein to the organelles involves the tail anchored membrane protein Fis1, which is localized in the mitochondrial outer membrane and in the peroxisomal membrane. Studies in *P. chrysogenum* confirmed that also in this organism Dnm1 is involved in peroxisome fission (Meijer et al. 2010).

Studies in *H. polymorpha* and *S. cerevisiae* revealed that one of the Pex11 family members (HpPex25 and ScPex25) is involved in de novo peroxisome formation (Saraya et al. 2011). In mutants lacking Pex11 and Pex25 both peroxisome fission and de novo peroxisome formation is blocked. As a consequence all processes of peroxisome formation are defective, resulting in a *pex* phenotype. Recent data obtained in *P. chrysogenum* indicated that deletion of all genes encoding Pex11 family members did not result in a *pex* phenotype in this species, suggesting that de novo peroxisome formation is independent of all Pex11 family members in this species, including ER-borne Pex11B (Opalinski et al. 2012). Hence, the de novo peroxisome biogenesis pathway in *P. chrysogenum* most likely differs from that in yeast.

8.3 The Biogenesis of Woronin Bodies

Woronin bodies are unique to filamentous ascomycetes (Jedd and Chua 2000). They consist of a single membrane that encloses a dense core of Hex1 protein (Yuan et al. 2003) (Fig. 8.3). These organelles originate from peroxisomes and seal septal pores in response to cellular wounding. Woronin bodies almost exclusively consist of Hex1 protein as evident from a recent proteomic study where only Hex1 was unambiguously identified in purified *N. crassa* Woronin bodies whereas almost 200 proteins were identified when highly purified peroxisomes of the same organism were analyzed (Managadze et al. 2010b) The Hex1 dense core is essential for the function of Woronin bodies. Deletion of the *HEX1* gene in *N. crassa* results in

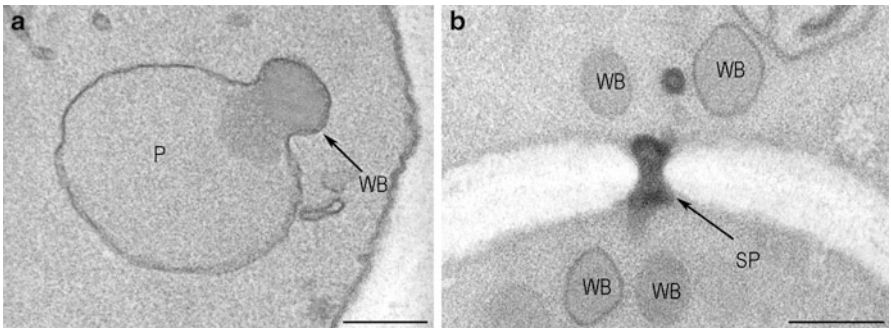

Fig. 8.3 Woronin bodies in *P. chrysogenum*. Electron micrographs of Woronin body formation in *P. chrysogenum*. (**a**) The dense core of Hex1 buds off from the mother peroxisome. (**b**) Woronin bodies present in the vicinity of and plugging the septal pore (*arrow*). *P* peroxisome, *SP* septal pore, *WB* Woronin body. The bar represents 200 nm

hyphae that lack Woronin bodies and bleeding of cytoplasm after hyphal damage (Jedd and Chua 2000; Tenney et al. 2000). Core formation is also essential because a mutant Hex1 variant which is defective in forming the dense core also leads to a non-functional organelle (Yuan et al. 2003). These mutants also show defects in sporulation in *N. crassa* (Jedd and Chua 2000; Tenney et al. 2000) and affect fungal pathogenicity as well (for *Magnaporthe oryzae* (Soundararajan et al. 2004)).

Hex1 is a PTS1 protein which upon import into peroxisome self-assembles to form a dense core (Jedd and Chua 2000). Subsequently, the peroxisomal membrane-bound Woronin sorting complex protein (WSC) envelops Hex1 cores to facilitate budding off from the mother organelle, a process that – like peroxisome fission – involves the function of Pex11 (Liu et al. 2008; Escano et al. 2009). The newly formed Woronin bodies physically associate with the cell cortex a process that involves the cytosolic Leashin protein. In this way Woronin bodies are inherited to sub-apical compartments, where they are immobilized (Tey et al. 2005; Momany et al. 2002; Ng et al. 2009), a process that ensures that new hyphal compartments are supplied with Woronin bodies.

8.4 The Role of Peroxisomes in the Production of Secondary Metabolites

Although peroxisomes are well known for their metabolic processes generally mediated by hydrogen peroxide producing oxidases, several biosynthetic pathways are also (partially) compartmentalized to these organelles. This is especially the case for many filamentous fungi, where peroxisomes are involved in the production of several secondary metabolites. Representative examples are discussed below.

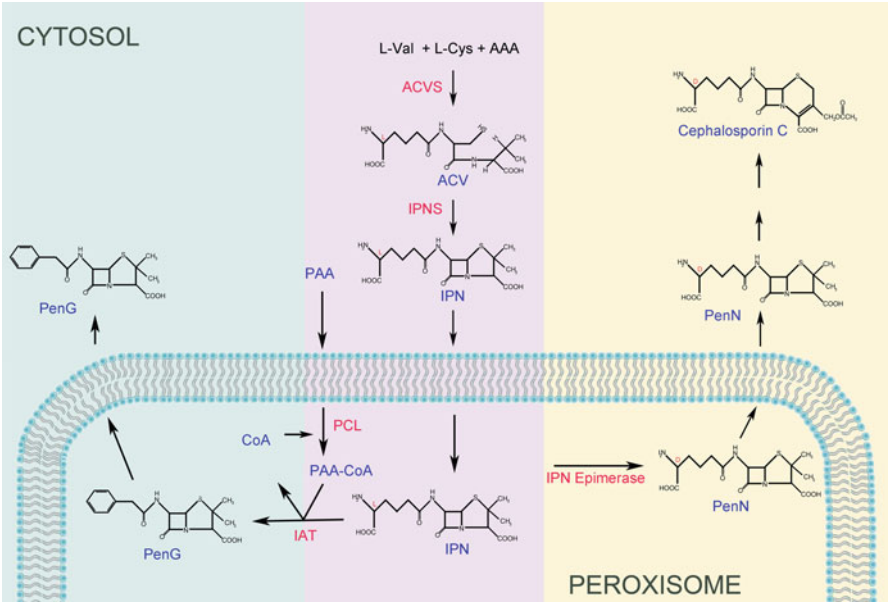

Fig. 8.4 Schematic representation of Penicillin G and Cepohalosporin C biosynthesis. The first two reactions of β-lactam biosynthesis are catalysed by δ-(L-aminoadipoyl)-L-cysteinyl-D-valine synthase (*ACVS*) and isopenicillin N synthase (*IPNS*) and occur in the cytosol. IPN is subsequently transported into peroxisomes. For PenG biosynthesis the enzyme phenylacetyl CoA ligase (*PCL*) activates the side chain precursor phenyl acetic acid (*PAA*), resulting in the formation of PAA-CoA. The peroxisomal enzyme isopenicillin N acyltransferase (*IAT*) exchanges the L-α-AAA side chain of IPN by phenylacetic acid. The product PenG is subsequently exported to the cytosol. In *A. chrysogenum* peroxisomal enzymes of the IPN epimerase system result in the synthesis of PenN, which is exported from the organelle. The last steps of Cephalosporin biosynthesis occur in the cytosol. Adapted from Bartoszewska et al. (2011b)

8.4.1 β-Lactam Antibiotics

Peroxisomal enzymes are crucial for the biosynthesis of β-lactam antibiotics in filamentous fungi (Martin et al. 2010). Penicillins are β-lactam antibiotics that are naturally produced by *P. chrysogenum* and *Aspergillus nidulans*. *Acremonium chrysogenum* produces cephalosporins, which are derivatives of β-lactam compounds. The first two enzymes involved in both pathways are cytosolic and result in the synthesis of isopenicillin N (IPN) (Fig. 8.4). At this stage the penicillin and cephalosporin biosynthesis pathways diverge. For penicillin biosynthesis IPN is imported into peroxisomes (Ullan et al. 2010), where the L-α-AAA side chain of IPN is exchanged by an acyl group (generally phenylacetic acid or phenoxyacetic acid) catalyzed by the peroxisomal enzyme isopenicillin *N*-acyltransferase (IAT) (Muller et al. 1992). Penicillin G and V are synthesized when the medium is supplemented with phenylacetic acid or phenoxyacetic acid, respectively. Activation

of these side chain precursors requires the peroxisomal enzyme phenylacetyl coenzyme A ligase (PCL) (Meijer et al. 2010; Koetsier et al. 2009).

In *A. chrysogenum* IPN-CoA synthetase and IPN-CoA epimerase catalyze the subsequent steps in cephalosporin biosynthesis. These enzymes are components of the IPN epimerase system. Sequence analysis revealed that two proteins of IPN epimerase system contain a putative peroxisomal targeting signal, suggesting that they are peroxisomal (Ullan et al. 2010; Martin et al. 2004). In *P. chrysogenum* the putative homologs of these proteins were experimentally proven to be peroxisome borne, supporting the hypothesis that this step of cephalosporin biosynthesis may occur in peroxisomes (Kiel et al. 2009). Moreover, recent studies revealed the presence of two peroxisomal membrane proteins, CefP and CefM, that play a role in transport of IPN across the peroxisomal membrane (Ullan et al. 2010). The last steps of cephalosporin biosynthesis occur in the cytosol.

The current industrial penicillin producing strains of *P. chrysogenum* are the result of many years of intensive strain improvement programs. Comparison of various strains from the strain improvement lineage revealed that high penicillin producing strains contained increased peroxisome volume fractions relative to low producers. Interestingly, proliferation of peroxisomes is also enhanced in *A. nidulans* grown at penicillin producing conditions, strengthening the significance of peroxisomes in penicillin formation.

The importance of peroxisomes for penicillin production is evident from the fact that in mutants defective in peroxisome biogenesis penicillin productivity is strongly decreased (Meijer et al. 2010; Opalinski et al. 2010). Similarly, also in *A. nidulans* the absence of functional peroxisomes resulted in a significant decrease in penicillin titers (Sprote et al. 2009).

Interestingly, an artificial increase of peroxisome numbers by overproduction of the PMPs Pex11 (Kiel et al. 2005) or Pex14/17 (Opalinski et al. 2010) resulted in increased penicillin production in *P. chrysogenum*. This increase was not due to enhanced levels of peroxisome-borne penicillin biosynthetic enzymes, since these were unaltered upon overproduction of Pex11 or Pex14/17 (Kiel et al. 2005). In line with these observations, also inhibition of autophagy which is responsible for peroxisome degradation, resulted in enhanced peroxisome numbers concomitant with increased penicillin production in *P. chrysogenum* (Bartoszewska et al. 2011a).

8.4.2 Polyketide Biosynthesis

Polyketides are natural products with a wide variety of biological activities including antibiotic, immunosuppressive and anticancer properties. Peroxisomes have been shown to be involved in the biosynthesis of virulence factors (e.g. melanin by *Colletotrichum orbiculare*) and mycotoxins (aflatoxin by *A. flavus* and *A. parasiticus*; sterigmatocystin by *A. nidulans*) (reviewed by (Bhetariya et al. 2011)).

Polyketides consist of multiple $–CH_2–CO–$ ketide groups and are synthesized by repetitive condensation or polymerization reactions. Acetyl-CoA is the key building

block, both as starter moiety and extender unit via malonyl-CoA formation. Hence, metabolic pathways that contribute to the cellular acetyl-CoA pool are important in polyketide biosynthesis.

Peroxisomal fatty acid β-oxidation is an important cellular source of acetyl-CoA. In *A. nidulans* stimulation of this process by the addition of oleic acid to the growth medium also stimulated sterigmatocystin biosynthesis. This strongly suggests an important relationship between peroxisomal β-oxidation and polyketide biosynthesis (Maggio-Hall et al. 2005).

Peroxisome may however play additional roles in polyketide biosynthesis. In aflatoxin and sterigmatocystin biosynthesis, the first stable intermediate is the fluorescent compound norsoloinic acid (NOR). Interestingly, in *A. nidulans* mutants that accumulate NOR, the fluorescent NOR is transiently observed to co-localize with peroxisomes. NOR is produced by a complex of polyketide synthase and fatty acid synthase. Although these enzymes have not yet been demonstrated to localize to peroxisomes, the accumulation of NOR in peroxisomes of specific mutants suggests that these organelles may have additional functions in polyketide biosynthesis.

Peroxisomal β-oxidation has been shown to be important for melanin production by *C. orbiculare*, the causal agent of cucumber anthracnose. During host plant infection, the conidia of *C. orbiculare* germinate and the germ tube forms an infection structure, called appressorium. The presence of melanin in an appressorium is essential for its function. Premature appressoria contain multiple lipid droplets (LDs) which disappear during appressorial maturation, a process that requires peroxisomal β-oxidation. In this process peroxisomes are essential because deletion of *PEX6* (Kimura et al. 2001), *PEX13* (Fujihara et al. 2010) or *MFE1*, encoding the peroxisomal multifunctional enzyme involved in β-oxidation, resulted in non-melanized appressoria with abundant LDs (Asakura et al. 2012).

8.4.3 Biotin

D-Biotin is a water-soluble vitamin that functions as a prosthetic group for many carboxylases. It can be synthesized by many prokaryotes as well as by plants and a number of fungi. Animals depend on the supply of this vitamin in the food. Recent data indicated that the biosynthesis of biotin in *A. oryzae* and *A. nidulans* involves the peroxisomal enzyme AON synthase, which catalyzes the decarboxylative condensation of L-alanine and pimelate thioester to form 8-amino-7-oxononanoate (AON) (Magliano et al. 2011; Tanabe et al. 2011). Both *A. oryzae* and *A. nidulans* ANO synthase contain a C-terminal PTS1 and are localized to peroxisomes. Correct localization of the enzyme in peroxisomes is essential for biotin biosynthesis in both fungi. In addition, peroxisomal β-oxidation is important for biotin biosynthesis. Deletion of genes encoding *A. nidulans* peroxisomal enzymes of the β-oxidation pathway, acyl-CoA oxidase or multifunctional enzyme, resulted in the requirement

of biotin for growth (Magliano et al. 2011). These data indicate that the substrate for ANO synthase, pimeloyl-CoA, is most likely generated via peroxisomal β-oxidation.

8.4.4 The Toxins Paxilline and AK-Toxin

The filamentous fungus *Penicillium paxilli* produces the indole-diterpene toxin paxilline, which is a selective and reversible blocker of the smooth muscle high conductance Ca^{2+}-activated K^+ channels. The initial step in paxillin biosynthesis is the formation of geranylgeranyl diphosphate (GGPP) from isopentenyl diphosphate (IPP) and farnesyl diphosphate (FPP) catalysed by geranylgeranyl diphosphate synthases, PaxG. PaxG contains a PTS1 sequence and is localized to peroxisomes. A strain that produces a mutant PaxG variant that mislocalizes to the cytosol was defective in paxillin production, indicating that paxilline biosynthesis depends on the compartmentation of PaxG in peroxisomes (Saikia and Scott 2009).

The Japanese pear pathotype of the phytopathogenic fungus *Alternaria alternata* produces the host-selective AK-toxin, which is an epoxy-decatrienoic acid ester. Three enzymes (encoded by *AKT1*, *AKT2* and *AKT3*) are required for AK-toxin biosynthesis. Based on sequence analysis, these proteins are predicted to represent a carboxyl-activating enzyme, an esterase-lipase family enzyme and a hydratase-isomerase family enzyme, respectively. All three enzymes contain a PTS1 and are localized to peroxisomes. The finding that deletion of *PEX6* gene resulted in mislocalization of these enzymes to the cytosol, together with a defect in the biosynthesis of AK-toxin, further emphasizes the important role of peroxisomes in the synthesis of AK-toxin. As expected the *PEX6* deletion strain was also defective in plant invasion and tissue colonization (Imazaki et al. 2010).

8.5 Miscellaneous Processes Related to Peroxisomes in Fungi

Mutant strains of filamentous fungi that are defective in peroxisome biogenesis show a variety of phenotypes in addition to deficiencies in the metabolism of specific carbon sources or the production of certain secondary metabolites, which involve peroxisomal enzymes. Striking are the numerous reported defects in pathogenicity or development in peroxisome deficient mutant strains of filamentous fungi.

In some cases it is clear that reduced pathogenicity is directly related to a block in a peroxisomal biosynthetic pathway, such as in *A. alternate* where the reduced pathogenicity of *pex6* cells can be explained by a defect in AK-toxin production. Similarly, peroxisome deficient mutants of C. *orbiculare* produce less

melanin, which is required for their virulence. However, the molecular basis for most phenotypes related to pathogenicity or development of fungal *pex* strains are not yet elucidated.

For instance, in *M. oryzae* functional Woronin bodies are important for pathogenicity. This was demonstrated using a strain producing a mutant Hex1 variant that was unable to form a dense core. This mutant strain showed defects in the formation of appressoria and hence host penetration. Microscopy studies revealed that the mutant strain was unable to penetrate cross-walls within the host plant tissue and to elaborate normal blast disease symptoms. What the role of Woronin bodies is in these process remains to be studied (Soundararajan et al. 2004).

Studies in *P. anserina* revealed that in this fungus the absence of different peroxins involved in matrix protein import or peroxisomal membrane formation does not lead to the same developmental defects. Lack of the docking complex protein Pex13, the RING-finger proteins Pex2, Pex10 or Pex12, or Pex3 and Pex19, proteins involved in peroxisomal membrane formation, block sexual development. Surprisingly however, in the absence of the PTS receptors Pex5 and Pex7 or the docking complex protein Pex14 karyogamy and meiosis normally proceed and sexual spores are formed (Peraza-Reyes et al. 2008, 2011). Unexpectedly, the PTS2 co-receptor Pex20, but not the PTS2 receptor Pex7 itself, is essential for meiocyte differentiation (Peraza-Reyes et al. 2011). A possible explanation for these findings may be that the import of peroxisomal matrix proteins is fully blocked in the first group of mutants, resulting in inhibition of sexual development. In the other mutants import is most likely not completely blocked. In *pex5* cells peroxisomes still contain PTS2 proteins, whereas peroxisomes of *pex7* mutant cells harbor PTS1 proteins. Also, *pex14* mutant strains of the yeast *Hansenula polymorpha* show some residual matrix protein import (Komori et al. 1997; Koek et al. 2007; Salomons et al. 2000), whereas in *pex13* cells of this organism matrix protein import is fully impaired (Koek et al. 2007). In case peroxisomes are important for the production of signaling molecules that are required for development, these may still be synthesized to sufficient levels in those mutants where import defects are less severe. Identification of these biosynthetic pathways is required to further understand these phenomena.

8.6 Concluding Remarks

Peroxisomes of filamentous fungi perform a variety of functions including both metabolic and structural ones (i.e. the Woronin bodies). Recent studies resulted in the identification of several novel and often unexpected peroxisome functions. Most of these are related to the production of antibiotics and toxins as well as developmental processes. Why peroxisomes are important for fungal development is still unclear. Possibly, they are involved in the production of signalling molecules.

Peroxisomes are important for the metabolism of hydrogen peroxide. They invariably contain hydrogen peroxide producing oxidases as well as enzymes (generally catalase) that degrade hydrogen this toxic product. Recent findings in

various organisms indicated that depending on the levels hydrogen peroxide may perform an important role as signalling molecule. In line with these observations peroxisomes are likely to perform important roles in redox signalling, including in filamentous fungi.

The recent identification of several novel fungal peroxisome functions is largely a result of the analysis of fungal genomes (e.g. by searching for proteins that contain a putative PTS1 or PTS2) as well as the analysis of the peroxisome protein content by proteomics studies using isolated organelles (Managadze et al. 2010b; Kiel et al. 2009). Hence, several functions which were thought to be mediated by cytosolic enzymes, in fact are peroxisome borne. Given the steadily expanding list of novel peroxisome functions, our knowledge on their ultimate potential is still far from complete.

Hence, additional studies to further unravel the unprecedented significance of peroxisomes in cellular metabolism, development and signalling is of crucial importance. Because fungal peroxisomes are involved in the biosynthesis of several commercially/pharmaceutically important compounds, the interest in further research on the role of peroxisomes in filamentous fungi is rapidly growing and undoubtedly will have relevance for biotechnology.

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Chapter 9
Essential Roles of Peroxisomally Produced and Metabolized Biomolecules in Regulating Yeast Longevity

Adam Beach and Vladimir I. Titorenko

Contents

Abstract The essential role of the peroxisome in oxidizing fatty acids, maintaining reactive oxygen species homeostasis and replenishing tricarboxylic acid cycle intermediates is well known. Recent findings have broadened a spectrum of biomolecules that are synthesized and metabolized in peroxisomes. Emergent evidence supports the view that, by releasing various biomolecules known to modulate essential cellular processes, the peroxisome not only operates as an organizing platform for several developmental and differentiation programs but is also actively involved in defining the replicative and chronological age of a eukaryotic cell. The scope of this chapter is to summarize the evidence that the peroxisome defines yeast longevity by operating as a system controller that: (1) modulates levels of non-esterified fatty acids and diacylglycerol; (2) replenishes tricarboxylic acid cycle

A. Beach (✉) • V.I. Titorenko (✉)
Department of Biology, Concordia University, 7141 Sherbrooke Street, West, SP Building, Montreal, Quebec H4B 1R6, Canada
e-mail: adam.pb.beach@gmail.com; vladimir.titorenko@concordia.ca

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intermediates destined for mitochondria; and (3) contributes to the synthesis of polyamines. We critically evaluate molecular mechanisms underlying the essential role of peroxisomally produced and metabolized biomolecules in governing cellular aging in yeast.

Keywords Yeast • Peroxisome • Cellular aging • Longevity • Endomembrane system governing cellular aging • Peroxisomal fatty acid oxidation • Reactive oxygen species • Peroxisomal protein import • Lipid bodies • Triacylglycerols • Mitochondria • Apoptosis • Necrosis • Polyamines • Autophagy

Abbreviations

ATG	Autophagy-related genes
CR	Caloric restriction
DAG	Diacylglycerol
ER	Endoplasmic reticulum
FA	Fatty acids
ODC	Ornithine decarboxylase
PTS1	Peroxisomal targeting signal type 1
PTS2	Peroxisomal targeting signal type 2
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RTG	Retrograde signaling pathway
TAG	Triacylglycerols

9.1 Peroxisomes Regulate Intracellular Levels of Diverse Biomolecules that Modulate Essential Cellular Processes

Peroxisomes serve numerous functions and are involved in various metabolic pathways, making these organelles an integral part of the proper development, functioning and health of many organisms. These organelles have long been known for their essential role in β-oxidation of fatty acids (FA) and hydrogen peroxide homeostasis, functions that are conserved between fungi, plants and animals (Wanders and Waterham 2006; Schlüter et al. 2010; Islinger et al. 2012). Recent findings have broadened a spectrum of biomolecules that are synthesized and metabolized in peroxisomes of evolutionarily distant organisms. The array of these biomolecules, and by extension the cellular processes in which they are actively involved, is ever-expanding, leading to the discovery of new peroxisomal functions. These functions are quite broad, as briefly outlined below.

In fungi, peroxisomes are required for efficient biosynthesis of penicillin (Spröte et al. 2009; Meijer et al. 2010). The peroxisomes of pathogenic fungi produce toxins

that are necessary for host invasion and responsible for plant diseases (Imazaki et al. 2010). Fungi and plants utilize peroxisomes for biotin synthesis (Tanabe et al. 2011) and for storing specialized enzymes implicated in secondary metabolite synthesis (Bartoszewska et al. 2011). Synthesis of vitamin K1 and glycine betaine has been associated with plant peroxisomes (Babujee et al. 2010; Mitsuya et al. 2011; see Chap. 12). Animal peroxisomes produce pheromones (Joo et al. 2010; Spiegel et al. 2011), whereas peroxisomes in plants house enzymes involved in the biosynthesis of hormonal signaling molecules (i.e., jasmonate and auxin) (Kienow et al. 2008; see Chaps. 14 and 16). The final four steps of the isoprenoid/cholesterol biosynthesis pathway have been localized to peroxisomes in plants (Thabet et al. 2011). The catabolism of polyamines, small molecules that stabilize nucleic acids and are involved many essential cellular processes, is confined to peroxisomes in yeast, mammals and plants (Nishikawa et al. 2000; Vujcic et al. 2003; Ono et al. 2012). Plant peroxisomes also house the catabolism of purines (Sandalio et al. 1988; del Río et al. 1989; Corpas et al. 1997, 2008; Mateos et al. 2003).

The mechanisms by which some of the peroxisomally synthesized or metabolized biomolecules modulate essential cellular processes are beginning to emerge. Recent findings suggest that these biomolecules are actively involved in coordinating development, differentiation, morphogenesis and aging in organisms that have long since split from one another on the evolutionary tree (reviewed in Titorenko and Rachubinski 2004; Thoms et al. 2009; Titorenko and Terlecky 2011; Beach et al. 2012). Among them are plant and mammalian cells, where ongoing processes within the peroxisome, such as FA metabolism and reactive oxygen and nitrogen species (ROS and RNS, respectively) turnover determine the levels of signaling lipids, ROS and RNS in extraperoxisomal locations (recently reviewed in Titorenko and Terlecky 2011). Outside of peroxisomes, these peroxisomally produced biomolecules serve as potent signaling molecules capable of binding and activating particular transcription factors, which in turn alter gene expression profiles and ultimately, impact development and differentiation processes (reviewed in Titorenko and Rachubinski 2004; Thoms et al. 2009; Beach et al. 2012). Thus, by modulating the intracellular levels of these biomolecules, the peroxisome operates as both an intracellular signaling platform and an organization centre for developmental and differentiation decisions that affect the cell and the entire organism. In fact, numerous developmental and differentiation processes are linked to peroxisomes, including (1) acting as a site for the initial steps of plasmalogen biosynthesis in both nematode and mammalian cells; (2) supplying acetyl-CoA for melanin and glycerol biosynthesis in fungi; and (3) providing the location where very long-chain fatty acids, phytanic acid and pristinic acid are oxidatively decomposed in mammalian cells (Titorenko and Rachubinski 2004; Thoms et al. 2009; Dixit et al. 2010; Ivashchenko et al. 2011; Mast et al. 2011; Titorenko and Terlecky 2011). Recent evidence also suggests that the replicative and chronological age of eukaryotic cells is determined, in part, by the cellular and metabolic processes occurring in peroxisomes, such as FA oxidation, regeneration of tricarboxylic acid (TCA) cycle intermediates and ROS turnover (see below for a detailed discussion of this topic).

9.2 Peroxisomes Are Integrated into an Endomembrane System that Regulates Longevity

There are several mechanisms through which peroxisomes are capable of regulating longevity-defining cellular processes. In the core of these mechanisms is the ability of peroxisomes to establish physical contacts with some organelles and to communicate with other organelles in a contact-independent fashion by coordinating a bi-directional flow of metabolites, lipids and proteins. We therefore recently proposed a model for the dynamic integration of peroxisomes into an endomembrane system governing cellular aging (Beach et al. 2012). Our model envisions that (1) the interplay between peroxisomes and other organelles is dictated by the age-dependent efficiency of protein import into the peroxisome; and (2) certain pathways that regulate longevity are affected by the aforementioned physical and contact-independent interactions of peroxisomes and other cellular compartments (Beach et al. 2012) (Fig. 9.1). The efficiency of peroxisomal protein import is largely governed by functions of Pex5p and Pex7p (the peroxisomal targeting signal type 1 [PTS1] and PTS2 cytosolic shuttling receptors, respectively), which bind to cargo proteins in the cytosol, translocate the receptor-cargo complexes across the peroxisomal membrane and then shuttle back to the cytosol for recycling (Ma et al. 2011; Rucktäschel et al. 2011; Titorenko and Terlecky 2011). It is important to note that over time, as cells age, these processes do not occur as efficiently (Legakis et al. 2002; Terlecky et al. 2006; Titorenko and Terlecky 2011). According to our model for the dynamic integration of peroxisomes into an endomembrane system governing cellular aging, peroxisomes in which protein import is highly functional are capable of promoting anti-aging processes throughout the entire system (Fig. 9.1a). Should peroxisomal protein import be inefficient, however, a pro-aging program would instead be activated (Beach et al. 2012; Titorenko and Terlecky 2011) (Fig. 9.1b). An important function of Pex5p and Pex7p concerns the import of Fox1p, Fox2p and Fox3p into peroxisomes (Hiltunen et al. 2003). As the fundamental enzymes involved in the β-oxidation of FA, their efficient import into peroxisomes of "young" cells enhances the decomposition of FA, which are derived from triacylglycerols (TAG) produced in the endoplasmic reticulum (ER) and later found in lipid bodies (Goodman 2008; Goldberg et al. 2009a, b; Titorenko and Terlecky 2011). This high intensity process in "young" cells reduces the level of FA and diacylglycerol (DAG), thereby enabling these cells (1) to escape necrotic and apoptotic modes of death induced by FA; and (2) to maintain stress resistance by weakening the DAG-activated protein kinase C signaling (Goldberg et al. 2009a, b; Titorenko and Terlecky 2011) (Fig. 9.1a). Finally, the sirtuins Hst3p and Hst4p contribute to the anti-aging process of rapid FA oxidation in "young" cells by activating acyl-CoA synthetases that are necessary for the peroxisomal import and oxidation of short chain FA (Beach et al. 2012). Once again, Pex5p and Pex7p play an integral role in peroxisomal import, this time with respect to Mdh3p and Pnc1p; the former produces a substrate for the sirtuins while the latter breaks down an inhibitor (Beach et al. 2012).

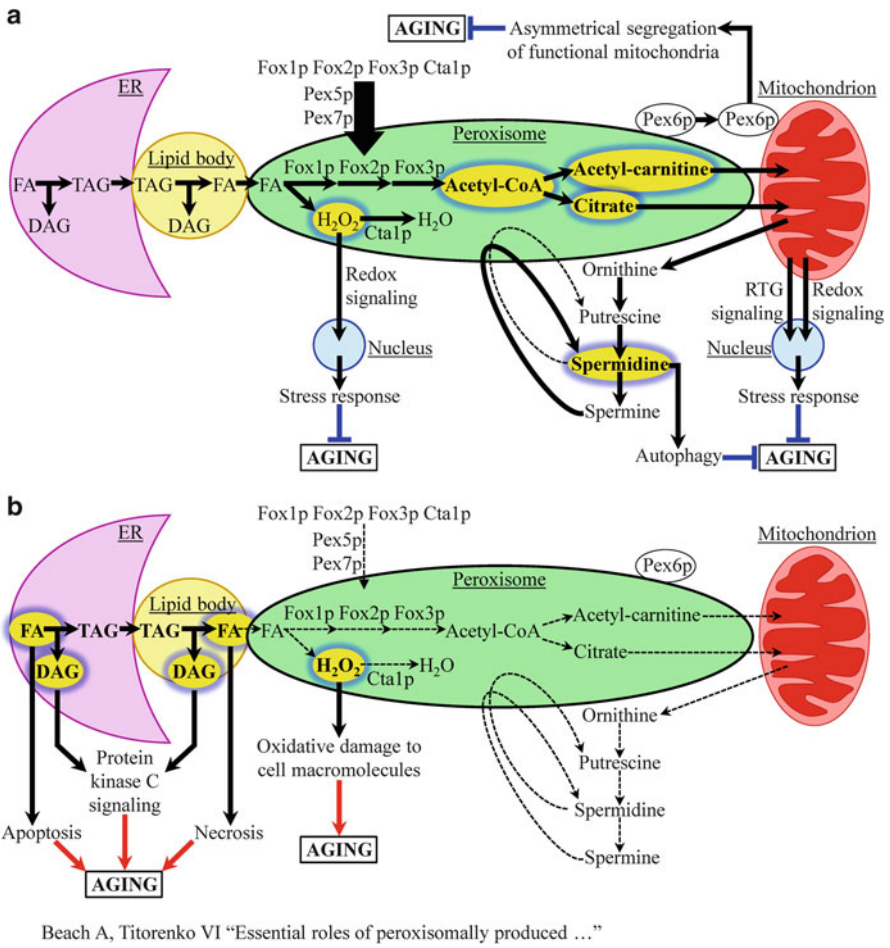

Fig. 9.1 Peroxisomes are integrated into an endomembrane system regulating yeast longevity. The ability of peroxisomes to establish physical contacts with some organelles and to communicate with other organelles in a contact-independent fashion by coordinating a bi-directional flow of metabolites, lipids and proteins underlies several mechanisms through which peroxisomes regulate longevity-defining cellular processes in replicatively and chronologically "young" (**a**) and "old" (**b**) cells. The metabolites accumulated in bulk quantities are shown in *bold and enclosed in highlighted yellow ovals*. The *thickness of arrows* correlates with the rates of processes these arrows symbolize. *Blue T bars* denote inhibition of the process; *red arrows* signify activation of the process. See text for details. Abbreviations: *DAG* diacylglycerol, *ER* endoplasmic reticulum, *FA* fatty acids, *RTG* retrograde signaling pathway, *TAG* triacylglycerols

The retrograde (RTG) signaling pathway (which relies on the peroxisomes-mitochondria, mitochondria-nucleus and nucleus-peroxisomes communications) serves as another longevity-extending mechanism that peroxisomes are able to act upon, again through efficient import of Fox1p, Fox2p, Fox3p, Cit2p and Cat2p

into the peroxisome of "young" cells (Titorenko and Terlecky 2011) (Fig. 9.1a). Yet again, Pex5p and Pex7p are essential for the efficient peroxisomal import of Fox1p, Fox2p and Fox3p needed for the generation of acetyl-CoA from FA; moreover, Pex5p helps import Cit2p and Cat2p for the production of citrate and acetyl-carnitine from acetyl-CoA (Epstein et al. 2001; Traven et al. 2001; Hiltunen et al. 2003; Titorenko and Terlecky 2011; Beach et al. 2012) (Fig. 9.1a). Pex11p also plays a role in longevity extension by intensifying peroxisome biogenesis, thereby providing more sites for both FA oxidation and replenishment of TCA cycle intermediates destined to be delivered to mitochondria (Jazwinski 2005a, b; Titorenko and Terlecky 2011). The interplay between the functional state of mitochondria and the RTG pathway in "young" cells create conditions under which the intensity of information flow through the RTG pathway defines mitochondrial ROS levels (Titorenko and Terlecky 2011). In turn, the efficient maintenance of ROS homeostasis observed in "young" cells results in activation of redox signaling pathways that promote synthesis and/or increase activities of numerous anti-aging and stress-protecting proteins (D'Autréaux and Toledano 2007) (Fig. 9.1a). In addition, through a currently unknown mechanism, the peroxin Pex6p is shuttled from peroxisomes to mitochondria and enables the deliberate distribution of functional mitochondria to "young" daughter cells while "old" mother cells obtain the dysfunctional or damaged mitochondria, again highlighting the important role of the functional state of mitochondria in the complex process of cellular aging (Lai et al. 2002; Seo et al. 2007) (Fig. 9.1a).

As a consequence of replicative and chronological aging, the efficiency of peroxisomal protein import declines over time, as taken into consideration by our model (Beach et al. 2012) (Fig. 9.1b). Accumulation of oxidatively damaged peroxisomal proteins is at the heart of this decreased protein import efficiency, which is likely a result of peroxisomally-produced ROS (see Chap. 13). Of the various peroxisomal processes, the Pex5p-mediated import of catalase into peroxisomes is extremely sensitive to oxidative damage and usually the first indicator of a shift in the functional state of the peroxisome (Legakis et al. 2002; Terlecky et al. 2006; Titorenko and Terlecky 2011). Oxidative damage to proteins and lipids increases due to the reduction in catalase import, since catalase has been shown to be responsible for attenuating oxidative damage of peroxisomal membrane lipids and proteins (Antonenkov et al. 2010; Ivashchenko et al. 2011; Titorenko and Terlecky 2011). This culminates in a "deterioration spiral" that further reduces peroxisomal protein import below a certain threshold (Beach et al. 2012). Eventually, a paradigm shift in the peroxisomal regulation of cellular aging within the endomembrane system is initiated, with reduced peroxisomal function no longer able to support anti-aging processes, but rather to foster a series of pro-aging cellular processes (del Río et al. 1998; Titorenko and Terlecky 2011) (Fig. 9.1b). Various mechanisms that were in place to ensure a high level of peroxisomal function – namely, the receptor accumulation and degradation in the absence of recycling pathway, the insulin degrading enzyme and peroxisomal Lon proteases, the peroxisome-associated protein degradation system, and pexophagy – are now

unable to properly perform their tasks as a direct consequence of accumulated oxidative damage to proteins and lipids in the peroxisome (reviewed in Titorenko and Terlecky 2011; Beach et al. 2012). Dysfunctional peroxisomes are thus associated with oxidative macromolecular damage and cellular aging, no longer being able to support various anti-aging processes. These compromised longevity-extension processes include (1) stress response pathways in peroxisomes and mitochondria; (2) the Hst3p/Hst4p-dependent stimulation of acyl-CoA synthetases; (3) the RTG signaling pathway; and (4) Pex6p-dependent distribution of functional and dysfunctional mitochondria to "young" daughter and "old" mother cells, respectively (Beach et al. 2012) (Fig. 9.1b). A further extension of the model takes into account the accumulation of dysfunctional peroxisomes, pointing to an inability to process FA and DAG appropriately. The resulting accumulation of FA triggers necrotic and apoptotic modes of cell death, while the build-up of DAG lowers stress resistance through DAG-activated protein kinase C signaling (Beach et al. 2012) (Fig. 9.1b).

9.3 Peroxisomes Contribute to Longevity Regulation in Yeast by Modulating Levels of Fatty Acids and Diacylglycerol

Under laboratory conditions, where yeast are grown in glucose-containing media, the primary source of peroxisomally oxidized FA are the neutral lipids TAG and ergosteryl esters (Binns et al. 2006; Thiele and Spandl 2008; Olofsson et al. 2009). The ER serves as the site for synthesis of these neutral lipids, which are then transferred to lipid bodies (Goodman 2008; Olofsson et al. 2009; Walther and Farese 2009). Extensive physical contact between peroxisomes, the ER and lipid bodies constitutes an endomembrane network that facilitates FA oxidation in peroxisomes (Goldberg et al. 2009a, b; Titorenko and Terlecky 2011). Due to the association of lipid bodies with peroxisomes, the latter are able to generate specialized structures known as pexopodia, which invade the lipid core of lipid bodies to access the pool of FA (Binns et al. 2006). Yeast mutants lacking Fox1p, Fox2p or Fox3p, and therefore incapable of oxidizing FA, accumulate gnarls in lipid bodies (Binns et al. 2006). These gnarls consist of electron-dense arrays of FA that have accumulated and are unable to be oxidized, owing to mutations in Fox1p, Fox2p or Fox3p. Furthermore, these Fox mutants also accumulate TAGs in lipid bodies (Binns et al. 2006).

When yeast cells grow in glucose-containing media, they accumulate ethanol as a product of glucose fermentation (Fabrizio et al. 2005; Lin and Sinclair 2008). Ethanol also has the effect of downregulating the synthesis of peroxisomally-targeted proteins, such as the core enzymes of FA oxidation, namely Fox1p, Fox2p and Fox3p (Hiltunen et al. 2003; van der Klei et al. 2006). Thus, an increase in the concentration of ethanol leads to a reduction in FA oxidation, the accumulation of FA and premature cellular aging (Titorenko and Terlecky 2011). As such, the accumulation of ethanol mirrors the dysfunctional peroxisomal FA oxidation seen

in yeast mutants lacking Fox1p, Fox2p or Fox3p. This observation may help to explain the longevity extending mechanism of caloric restriction (CR), a low-calorie diet that reduces the level of ethanol in yeast (Goldberg et al. 2009a, b) and extends lifespan in various organisms (Mair and Dillin 2008).

Incorporation of these ideas points to the ability of the endomembrane system (which includes peroxisomes, ER and lipid bodies) to regulate yeast longevity by altering lipid dynamics in a caloric intake-dependent manner (Goldberg et al. 2009a, b; Titorenko and Terlecky 2011). Lipid bodies play a central role in this model, acting as a bridge for neutral lipid synthesis in the ER and FA oxidation in peroxisomes. Under non-CR conditions, peroxisomal oxidation of FA cannot proceed efficiently, since ethanol accumulation suppresses synthesis of Fox1p, Fox2p and Fox3p, the core enzyme of FA oxidation (Goldberg et al. 2009a, b; Titorenko and Terlecky 2011). The consequence is a build-up of FA arrays, known as gnarls, which activate negative feedback loops associated with TAG metabolism (Goldberg et al. 2009a, b; Titorenko and Terlecky 2011). As a result of these negative feedback loops, TAG accumulates in lipid bodies whereas DAG and FA amass in the ER (Goldberg et al. 2009a, b; Titorenko and Terlecky 2011). Taking the above points into consideration, we arrive at the conclusion that under non-CR conditions, the endomembrane system alters lipid dynamics in yeast cells to cause their premature death. This premature cell death is due to (1) inefficient peroxisomal oxidation of FA leading to necrosis; (2) lipoapoptosis triggered by the accumulation of FA and DAG; and (3) the DAG-activated protein kinase C signal transduction network, which is involved in stress response and longevity regulation (Goldberg et al. 2009a, b; Titorenko and Terlecky 2011) (Fig. 9.1).

9.4 Peroxisomes Govern Cellular Aging in Yeast by Replenishing TCA Cycle Intermediates Destined for Mitochondria

The functional states of both peroxisomes and mitochondria are integral in determining how cellular aging unfolds (Titorenko and Terlecky 2011; Beach et al. 2012). In yeast, the RTG signaling pathway responds in particular to mitochondrial dysfunction in the cell (Jazwinski 2005a; Liu and Butow 2006). Transcriptional activation of nuclear genes is one of the first steps in the RTG pathway, which is mediated by a distinct set of evolutionarily conserved regulatory proteins (Jazwinski and Kriete 2012). These regulatory proteins orchestrate a multitude of changes, including a specific alteration of carbohydrate and nitrogen metabolism, promotion of peroxisome proliferation to intensify FA oxidation and anaplerotic metabolism, stress response, and an increase in the stability of both mitochondrial and nuclear DNA (Epstein et al. 2001; Traven et al. 2001; Liu and Butow 2006; Jazwinski 2012; Jazwinski and Kriete 2012). Taken together, these global alterations to

cell metabolism and physiology are aimed at keeping the cell operational while compensating for the reduced functionality of mitochondria (Jazwinski 2005a, 2012; Liu and Butow 2006).

Mitochondrial dysfunction normally appears as cells age, often signaled by a reduced mitochondrial membrane potential (Ψ_m); in response, the RTG signaling pathway is intensified proportionally to the extent of mitochondrial dysfunction (Lai et al. 2002; Borghouts et al. 2004; Jazwinski 2012). Other ways of activating the retrograde response include changes to mitochondrial DNA, either large deletions or a complete loss, as well as the loss of Cox4p, a subunit of cytochrome c oxidase (Parikh et al. 1987; Liao et al. 1991; Kirchman et al. 1999). The end result is an impairment of oxidative phosphorylation and the TCA cycle (Liu and Butow 2006). The particular step of the TCA cycle that is affected concerns the conversion of succinate to oxaloacetic acid, which is catalyzed by succinate dehydrogenase. It is important to note that a portion of the TCA cycle, upstream of the block, continues to function and provides metabolites for biosynthetic reactions (Liu and Butow 2006; Jazwinski and Kriete 2012). At this point in time, replenishment of acetyl-CoA, oxaloacetate and α-ketoglutarate is essential, since the level of glutamate must be maintained in order for the nitrogen requirements of the cell to be met so that the biosynthesis of amino acids can continue unimpeded (Liu and Butow 2006; Jazwinski and Kriete 2012). The RTG signaling pathway enables this process; upon reaching the nucleus, a dimer of the transcription factors Rtg1p and Rtg3p triggers transcription of a plethora of RTG-target genes (Jazwinski 2005a, b; Liu and Butow 2006; Jazwinski and Kriete 2012). Protein products of these target genes act to ensure a steady supply of TCA cycle intermediates flow to mitochondria from a variety of sources, the peroxisome being a noteworthy one (Titorenko and Terlecky 2011). The list of genes targeted by the RTG signaling pathway includes three enzymes of the TCA cycle, cytosolic forms of enzymes involved in oxaloacetate and acetyl-CoA synthesis, a mitochondrial transporter for carnitine-dependent transfer of acetyl-CoA, mitochondrial transporters for citrate and α-ketoglutarate, and plasma membrane transporters for carnitine and glutamate (Liu and Butow 2006; Jazwinski and Kriete 2012). With respect to peroxisomes, the RTG pathway targets are involved in the proliferation and efficient function of these organelles (Epstein et al. 2001; Traven et al. 2001; Jazwinski 2005a, b; Liu and Butow 2006; Titorenko and Terlecky 2011). Among them are Fox1p, Fox2p and Fox3p (the core enzymes of FA oxidation), Pex11p (a driver of peroxisomal biogenesis), as well as Cit2p and Cat2p (which convert acetyl-CoA, the final product of FA oxidation, to citrate and acetyl-carnitine, respectively) (Epstein et al. 2001; Traven et al. 2001; Liu and Butow 2006; Jazwinski and Kriete 2012). Collectively, these proteins cause an upregulation of FA oxidation and anaplerotic metabolism in peroxisomes and ensure that TCA cycle intermediates are not only replenished but also delivered to mitochondria. Therefore, in the event of mitochondrial dysfunction, peroxisomes and their associated proteins and functions are essential to the ability of retrograde response to regulate yeast longevity (Jazwinski 2005a; Liu and Butow 2006; Titorenko and Terlecky 2011).

9.5 Peroxisomes May Contribute to Longevity Regulation in Yeast by Modulating Levels of Spermidine and Other Polyamines

As polycations, polyamines exhibit an affinity for negatively charged molecules, including proteins, DNA and RNA (Minois et al. 2011). Their ability to bind such a variety of essential macromolecules inherently ties them to several cellular processes, including growth, survival and proliferation (Minois et al. 2011). The three main polyamines of spermidine, spermine and putrescine are synthesized within a precisely regulated metabolic pathway. In yeast, the only source of polyamines is their cellular synthesis de novo; in mammals, both ingestion and microbial synthesis in the gut provide additional polyamine sources (Minois et al. 2011). The first step of polyamine biosynthesis, which is catalyzed by ornithine decarboxylase (ODC), involves the decarboxylation of ornithine to produce putrescine (Morselli et al. 2009; Minois et al. 2011). Spermidine can be made from putrescine through the action of spermidine synthase; this spermidine can then serve as a substrate for spermine synthase to yield spermine (Minois et al. 2011). Both spermidine and spermine can be acetylated by spermidine/spermine N^1-acetyltransferase, which allows them to enter the peroxisome and be oxidized by polyamine oxidase, releasing hydrogen peroxide and acetaminopropanal as by-products (Minois et al. 2011) (Fig. 9.1).

Recent studies revealed the essential role of these polyamines in longevity regulation (Eisenberg et al. 2009; Morselli et al. 2009; Minois et al. 2011). As an organism ages, the level of polyamines appears to decrease, a trend that has been observed in evolutionarily distant organisms (Eisenberg et al. 2009; Minois et al. 2011). The role of the polyamine spermidine in the aging process was investigated by using the *spe1Δ* yeast strain, which lacks a functional ODC (Eisenberg et al. 2009). The shortened longevity of this mutant, as well as longevity of wild-type strain, can be significantly extended by exogenous spermidine (Eisenberg et al. 2009). Importantly, exogenously added spermidine is capable of extending the lifespan of both replicatively and chronologically aging yeast, while increasing stress resistance to both heat and hydrogen peroxide (Eisenberg et al. 2009).

The longevity-extending potency of spermidine is linked to autophagy (Eisenberg et al. 2009; Morselli et al. 2009). Autophagy is the essential cytoprotective mechanism in eukaryotic cells because it enables cells to breakdown aged/dysfunctional proteins and organelles in an effort to recycle nutrients (Galluzzi et al. 2008). The formation of an autophagosome is a key step of autophagy, where a double membrane vesicle is formed around the material intended to be degraded (He and Klionsky 2009; Mizushima and Klionsky 2007). The autophagosome then fuses with a lysosome (a vacuole in yeast), releasing lysosomal (vacuolar in yeast) hydrolases that degrade the contents of the autophagosome, ultimately enabling to return a variety of small molecules to the cytoplasm for use by the cell (Kundu and Thompson 2008; Levine and Kroemer 2008). In yeast, autophagy has been extensively studied to examine the molecular mechanisms underlying the process,

leading to the identification of autophagy-related (ATG) genes (Klionsky et al. 2003; Nakatogawa et al. 2009). Inefficient or compromised autophagy has been implicated in several diseases and disorders, most notably in neurodegenerative disorders, heart failure and hereditary myopathies (Mizushima et al. 2008; Singh et al. 2009). Furthermore, knockout of ATG genes results in greater susceptibility to cellular stresses as well as increased cell death, pointing to the involvement of autophagy in stress response and longevity regulation (Lum et al. 2005; Kroemer and Levine 2008).

Importantly, spermidine induces autophagy in yeast by activating transcription of the autophagy-related genes *ATG7*, *ATG11* and *ATG15* (Eisenberg et al. 2009; Morselli et al. 2009). If the *ATG7* gene is knocked out in yeast, the ability of spermidine to cause longevity extension is abolished, revealing that autophagy is the mechanism through which spermidine (and other polyamines) can activate an anti-aging response (Eisenberg et al. 2009; Morselli et al. 2009). Because of the localization of polyamine oxidase in the peroxisome (Minois et al. 2011), this organelle is integral to the catabolism of polyamines, including spermidine. It is conceivable therefore that, by modulating the intracellular levels of spermidine and other polyamines in an age-related manner, the peroxisome contributes not only to autophagy activation but also to longevity regulation (Fig. 9.1).

9.6 Conclusions

Recent progress in defining essential cellular events governed by the peroxisome has helped to define this organelle as integral in a variety of processes that regulate cellular aging. The incorporation of peroxisomes into an endomembrane system enables crosstalk between several organelles and underlies various mechanisms governing cellular aging. The functional state of the peroxisome plays an important role in determining whether a pro- or anti-aging course is charted at the cellular level. In particular, efficient oxidation of fatty acids in the peroxisome is the essential process governing cellular aging. The extent of mitochondrial dysfunction also serves as a pivotal factor in the peroxisome-mediated modulation of cellular aging. The retrograde signaling pathway – which is activated in response and proportionally to mitochondrial dysfunction – promotes peroxisome biogenesis, stimulates peroxisomal fatty acid oxidation and increases the efficacy with which tricarboxylic citric acid cycle intermediates are replenished by the peroxisome. Moreover, spermidine and other polyamines delay cellular aging by inducing autophagy, which helps to recycle long-lived proteins and organelles that may have been oxidatively damaged. The peroxisome serves as a processing site of polyamines and thus modulates their cellular levels, once again pointing to its pivotal role in defining cellular age. Due to the complex nature of the aging process, further research is required to better understand the cellular and molecular mechanisms underlying the essential roles of various peroxisomally produced and metabolized biomolecules in regulating cellular aging. Future studies are likely to reveal novel

pharmaceuticals and their targets capable of delaying cellular aging, extending longevity and improving health by altering the efficacy with which the peroxisome synthesizes, releases and decomposes biomolecules that define cellular age.

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Chapter 10
Metabolite Transporters of the Plant Peroxisomal Membrane: Known and Unknown

Nicole Linka and Frederica L. Theodoulou

Contents

Abstract Tremendous progress in plant peroxisome research has revealed unexpected metabolic functions for plant peroxisomes. Besides photorespiration and lipid metabolism, plant peroxisomes play a key role in many metabolic and signaling pathways, such as biosynthesis of phytohormones, pathogen defense, senescence-associated processes, biosynthesis of biotin and isoprenoids, and metabolism of urate, polyamines, sulfite, phylloquinone, volatile benzenoids, and

N. Linka (✉)
Department of Plant Biochemistry, Heinrich Heine University Düsseldorf, Universitätsstrasse 1, 40225 Düsseldorf, Germany
e-mail: Nicole.Linka@uni-duesseldorf.de

F.L. Theodoulou
Biological Chemistry and Crop Protection Department, Rothamsted Research, Harpenden AL5 2JQ, UK

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branched chain amino acids. These peroxisomal 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 have to shuttle across peroxisome membranes. However, our knowledge of their membrane passage is still quite limited. This review describes the solute transport processes required to connect peroxisomes with other cell compartments. Furthermore, we discuss the known and yet-to-be-defined transport proteins that mediate these metabolic exchanges across the peroxisomal bilayer.

Keywords Plant • Peroxisomes • Metabolite transport proteins

Abbreviations

AA	Amino acid
AAE	Acyl-activating enzyme
ABC	ATP binding cassette
ABI5	Abscisic acid-insensitive 5
APX	Ascorbate peroxidase
ASC	Ascorbate
ATP	Adenosine triphosphate
BA	Benzoic acid
BCAA	Branched chain amino acid
β-ox	β-oxidation
BZO1	Benzoyl-CoA ligase
CHY1/DBR5	Peroxisomal β-hydroxyisobutyryl-CoA hydrolase
CoA	Coenzyme A
CTS	COMATOSE (peroxisomal ABC transporter)
2,4-D	Dichlorophenoxyacetic acid
DAR	Dehydroascorbate reductase
2,4-DB	2,4-dichlorophenoxybutyrate
D-2-HG	D-2-hydroxyglutarate
DHNA	1,4-dihydroxy-2-naphthoate
DiT2	Glutamate/2-oxoglutarate translocator
DMAPP	Dimethylallyl diphosphate
DOXP	1-deoxy-D-xylulose-5-phosphate
FA	Fatty acid
FFS	Farnesyl diphosphate synthase
GAP	Glycerinaldehyde 3-phosphate
Glu	Glutamate
G6PD	Glucose-6-phosphate dehydrogenase
GR	Glutathione reductase
GSH	Glutathione
GSSH	Glutathione disulfide

H_2O_2	Hydrogen peroxide
IAA	Indole acetic acid (auxin)
IBA	Indole butyric acid
IDH	Isocitrate dehydrogenase
IDI	Isopentenyl diphosphate isomerase
IPP	Isopentenyl diphosphate
JA	Jasmonic acid
KAPA	7-keto-8-amino-pelargonic acid
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide reduced
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
NAT	Nucleobase-ascorbate transport protein
NH_4^+	Ammonium
MDAR	Monodehydroascorbate reductase
MVA	Mevalonate
MVD	Mevalonate diphosphate decarboxylase
OG	2-oxoglutarate
OPC8:0	3-oxo-2-(2′-[Z]-pentenyl)cyclopentane-1-octanoic acid
OPDA	12-oxophytodienoic acid
OPPP	Oxidative pentose phosphate pathway
PA	Polyamine
PAL	Phenylalanine ammonia lyase
PHYLLO	Plastid multifunctional enzyme
6PGD	6-phosphogluconate dehydrogenase
6PGL	6-phosphogluconolactone
PMK	Phosphomevalonate kinase
PMP22	22 kDa peroxisomal membrane protein
PNC	Peroxisomal ATP transport proteins
P_i	Inorganic phosphate
PPase	Pyrophosphatase
PPi	Pyrophosphate
PT	Plastidial phosphate translocator
PTS	Peroxisomal targeting signal
PUT1	Small aliphatic amine transporter
Pxmp2	Peroxisomal membrane protein 2
PXN	Peroxisomal NAD/CoA transport protein
Ru1.5BP	Ribulose-1.5-bisphosphate
RNAi	RNA interference
ROS	Reactive oxygen species
SA	Salicylic acid
SO	Sulfite oxidase
TCA	Tricarboxylic acid cycle
YFP	Yellow fluorescent protein
XDH	Xanthine dehydrogenase

10.1 Introduction

Peroxisomes carry out a wide range of functions and as such, can be hard to define in terms of their metabolic capability. However, a well-established function of peroxisomes in all eukaryotic organisms is their oxidative metabolism. Many reactions within peroxisomes generate reactive oxygen species (ROS), such as hydrogen peroxide and superoxide (reviewed in del Río et al. 2006). Antioxidant systems, including catalase and the glutathione/ascorbate cycle, are available for the efficient detoxification of these by-products in the peroxisomal matrix (del Río et al. 2006). This capability makes peroxisomes highly versatile and therefore they share many metabolic and signaling pathways with other cell compartments. Thus, peroxisomes have been referred to as "organelles at the crossroads".

In plants, peroxisomes play a major role in fatty acid β-oxidation, photorespiration, biosynthesis of phytohormones, pathogen defense, senescence-associated processes, biosynthesis of biotin and isoprenoids, and metabolism of urate, polyamines, sulfite, phylloquinone, volatile benzenoids, and branched chain amino acids (Hu et al. 2012). To date, 163 putative *Arabidopsis* proteins have been discovered to be peroxisomal by proteome and *in silico* studies (Reumann et al. 2004, 2007, 2009; Eubel et al. 2008; Hu et al. 2012). On one hand, each novel protein increases the list of metabolic pathways that occur in plant peroxisomes. On the other hand, the presence of many more proteins is required to fulfill not only the predicted functions but also the experimentally established reactions of plant peroxisomes.

The peroxisomal matrix is surrounded by a single bilayer. Consequently, metabolites and cofactors have to cross the peroxisome membrane (Theodoulou et al. 2011; Linka and Esser 2012). Most of these solutes are unable to diffuse freely through the lipid bilayer to due to their molecular size and physicochemical properties. Thus, specific transport proteins are required for their membrane passage (Theodoulou et al. 2011; Linka and Esser 2012). The activities of these transport proteins control the flux of metabolites and cofactors and furthermore connect peroxisomal metabolism with that of the other cell compartments. Up to now, only a few peroxisomal transport proteins are known at the molecular level in plants, as is the case for other eukaryotes (Theodoulou et al. 2011; Linka and Esser 2012). In this review, we focus on the intermediates which have to be exchanged to fulfill both established and newly identified metabolic functions in plant peroxisomes. Based on recent literature, we speculate about candidates responsible for these solute transfers at the peroxisomal bilayer.

10.2 Fatty Acids

Fatty acids are degraded via β-oxidation, which takes place exclusively in plant peroxisomes. A high flux of fatty acids (FAs) through this pathway plays a major role during seedling establishment of oilseed plants (Theodoulou and Eastmond

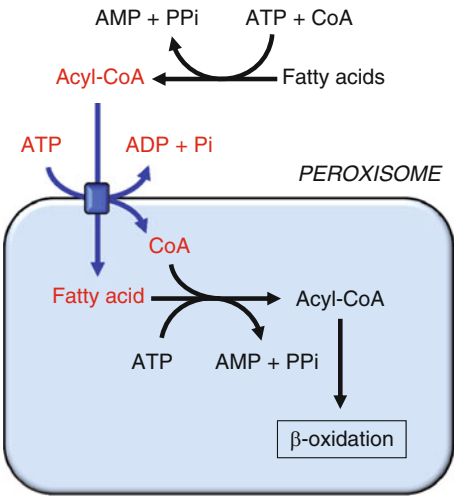

Fig. 10.1 Proposed transport mechanism of the peroxisomal ABC transporter CTS. The CoA moiety is cleaved during transport and re-esterified to free fatty acid on the lumen side of the membrane. *CoA* Coenzyme A, *Pi* inorganic phosphate, *PPi* pyrophosphate. It is also possible that CoA is liberated on the cytosolic side of the membrane

2012). Fatty acid breakdown also plays a role in turnover of membrane lipids in dark-induced senescence (Kunz et al. 2009; Slocombe et al. 2009), although its significance in natural senescence has been questioned (Yang and Ohlrogge 2009). The import of fatty acids into peroxisomes is mediated by the peroxisomal ABC transporter, here referred to as COMATOSE (CTS, also known as AtABCD1, AtPXA1, PED3, ACN2; reviewed in Theodoulou et al. 2011). Complementation of a yeast mutant lacking the orthologous yeast transporter, Pxa1p/Pxa2p suggests that CTS might import fatty acyl-CoA esters into peroxisomes (Nyathi et al. 2010). During this ATP-driven transport process, the CoA moiety is cleaved off either by the ABC transporter (Van Roermund et al. 2012; De Marcos Lousa et al. 2013). Acyl-activating enzymes (AAEs) then re-esterify the FAs with coenzyme A (CoA) inside the peroxisome by peroxisomal acyl-CoA synthetases (Fulda et al. 2004); this activation is a prerequisite for entering β-oxidation (Fig. 10.1).

CTS-dependent import of fatty acids is essential during seedling establishment, indicating that free fatty acids are unable to pass the peroxisome membrane via simple diffusion at a rate sufficient to sustain heterotrophic growth (Theodoulou et al. 2011). Mutations at the *CTS* locus cause a severe block in β-oxidation, resulting in *Arabidopsis* seedlings that are arrested in growth and development (Zolman et al. 2001; Footitt et al. 2002; Hayashi et al. 2002). Due to a defect in FA import, the fatty acids released from storage oil cannot be degraded to provide energy and carbon skeletons necessary for the establishment of a photoautotrophic plant (Zolman et al. 2001; Footitt et al. 2002; Hayashi et al. 2002). Under senescent conditions when the carbon and energy status are low, impaired import of fatty acids, most likely derived from plastidic lipid turnover, causes a rapid lethal phenotype in the absence of CTS which is associated with fatty acid toxicity as well as the inability to respire lipid (Kunz et al. 2009; Slocombe et al. 2009).

10.3 Hormones, Cyclic and Aromatic Compounds

The isolation of CTS in five independent forward genetic screens has extended the range of functions known for this transporter (reviewed in Theodoulou et al. 2011), including roles in metabolism of the auxin, indole butyric acid (IBA; Zolman et al. 2001; *pxa1* allele) and the auxin analogue, 2,4-dichlorophenoxybutyrate (2,4-DB; Hayashi et al. 2002; *ped3* alleles). IBA and 2,4-DB are metabolised via one round of β-oxidation to yield the bioactive compounds: indole acetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) respectively (Fig. 10.2). β-oxidation is also required for biosynthesis of the lipid-derived phytohormone, jasmonic acid (JA) and oxylipin profiling has shown that CTS participates in JA synthesis, suggesting a role in import of the precursors 12-oxophytodienoic acid (OPDA) and dinor OPDA (Theodoulou et al. 2005). However, null *cts* alleles contain residual basal levels of JA (sufficient to confer male fertility) and also synthesise a modest amount of JA in response to wounding, therefore a second pathway for OPDA import exists. The physicochemical properties of OPDA suggest that it could be imported into peroxisomes via passive diffusion and ion trapping, provided the lumen is basic relative to the cytosolic pH, but this does not rule out the possibility of a second transporter (Theodoulou et al. 2005). A more compelling hypothesis, however, is that free OPDA crosses the peroxisome membrane passively and is effectively trapped by activation to the CoA ester catalysed by OPDA: CoA ligase (Kienow et al. 2008). This vectorial acylation step not only prevents exit from the peroxisome but also channels OPDA into β-oxidation.

Impaired OPDA import in *cts* mutants leads to elevated levels of this signal molecule in the cytosol, triggering the up-regulation of the protein abundance of the transcription factor ABSCISIC ACID-INSENSITIVE 5 (ABI5) (Dave et al. 2011). ABI5 in turns induces the expression of polygalacturonase inhibiting proteins. These proteins block the degradation of pectin in the seed coat, and thus prevent seed coat rupture during germination (Kanai et al. 2010). The dormant (non-germinating) phenotype of *cts* seeds led to the name COMATOSE (Russell et al. 2000; Footitt et al. 2002).

As for fatty acid import, the precise molecular species transported by CTS (free acids or CoA esters) remain to be established empirically. Interestingly, peroxisomes contain an acyl activating enzyme with activity towards 2,4-DB (AAE18,Wiszniewski et al. 2009) but the severe 2,4-DB resistance phenotypes of *cts* mutants suggest that a CTS-independent 2,4-DB import pathway involving vectorial acylation does not operate in vivo. Thus the function of AAE18 may be to re-activate 2,4-DB, following CoA cleavage upon import, as has been shown for fatty acid import in yeast and plants (Van Roermund et al. 2012; De Marcos Lousa et al. 2013), or alternatively, CTS may import free 2,4-DB.

β-oxidation has also been proposed to play a role in the biosynthesis of the plant hormone and defence signalling compound, salicylic acid (SA; 2-hyroxybenzoic acid; Reumann et al. 2004). In this pathway, cinnamoyl-CoA derived from phenylalanine via phenylalanine ammonia lyase (PAL) is proposed to undergo one cycle

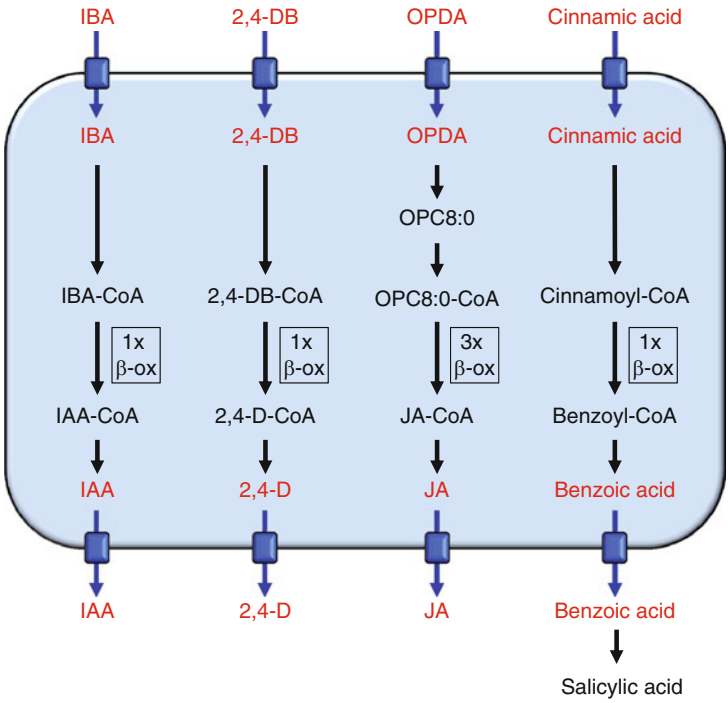

Fig. 10.2 The transport of diverse β-oxidation substrates across the peroxisomal membrane. The import of these compounds is mediated via the peroxisomal ABC transporter CTS, whereas the export of the resulted end products remains elusive. It has not yet been determined whether the transporter accepts free acid substrates or their respective CoA esters. *β-ox* β-oxidation, *IAA* indole acetic acid (auxin), *IBA* indole butyric acid, *2,4-D* 2,4-dichlorophenoxyacetic acid, *2,4-DB* 2,4-dichlorophenoxybutyrate, *JA* jasmonic acid, *OPC8:0* 3-oxo-2-(2′-[Z]-pentenyl)cyclopentane-1-octanoic acid, *OPDA* 12-oxophytodienoic acid

of β-oxidation to yield benzoyl-CoA (Fig. 10.2). Benzoic acid (BA) liberated via the action of a thioesterase would then be converted to SA by BA-2-hydroxylase. However, various studies show that that the predominant biosynthetic route for SA in *Arabidopsis* under both basal and inducing conditions is from isochorismate, although the presence of residual SA in a double isochorismate synthase mutant is consistent with a minor role for PAL-derived SA (Dempsey et al. 2011).

10.4 Benzoyloxyglucosinolates and Volatile Benzenoid Production

In addition to potential roles in SA biosynthesis (see above), cinnamoyl-CoA and benzoyl CoA are important intermediates in the biosynthesis of various plant secondary metabolites. Volatile benzenoids are major components of aroma and scent

in many plant species but the biosynthesis of BA has only recently been elucidated, largely through studies of petunia (*Petunia hybrida*) flowers, which produce large amounts of methyl benzoate. Metabolic flux analysis has shown that benzoyl-CoA is synthesised primarily via the β-oxidative pathway in petunia corollas, with a modest contribution from BA formed via a non-β-oxidative pathway involving conversion of cinnamoyl-CoA to BA by an aldehyde oxidase (Klempien et al. 2012, and refs therein). Gene silencing indicated a role for peroxisomal thiolase in conversation of 3-oxo-3-phenylpropanonyl-CoA to benzoyl-CoA in petunia corollas (Van Moerkercke et al. 2009) and analysis of candidate acyl activating enzymes identified a peroxisomal cinnamate:CoA ligase required for volatile production (Klempien et al. 2012), defining cinnamoyl-CoA formation as the first committed step in BA synthesis. The recent characterisation of a bifunctional cinnamoyl-CoA hydratase-dehydrogenase from petunia completed the elucidation of the core BA β-oxidative biosynthetic route in plants (Qualley et al. 2012).

Benzoyloxyglucosinolates are a prominent class of glucosinolates in *Arabidopsis* seeds. A benzoxyglucosinolate-deficient *Arabidopsis* mutant lacking a functional copy of the Arabidopsis peroxisomal AAE, BZO1 eliminates benzoyl-CoA biosynthesis in *Arabidopsis* seeds (Kliebenstein et al. 2007). It was initially proposed that BZO1 is a benzoyl-CoA ligase but phylogenetic and biochemical analysis indicate that it is cinnamoyl CoA ligase in vivo (Klempien et al. 2012; Lee et al. 2012). This together with the absence of BA-derived compounds in *bzo1* and *chy1* mutants (Ibdah and Pichersky 2009) suggests the BA biosynthesis occurs primarily via the β-oxidative pathway in Arabidopsis seeds, of which formation of cinnamoyl-CoA is the first committed step (Lee et al. 2012). This establishes peroxisomal BA formation as a hub for the biosynthesis of secondary products in different plant organs and species and likely defines a further substrate for CTS – cinnamoyl-CoA – although this remains to be tested experimentally.

10.5 Pathways Producing Propionyl-CoA

During senescence or carbon starvation, branched chain amino acids (BCAAs) leucine, isoleucine and valine can be broken down to generate energy. Although the pathways and compartmentalisation of BCAA catabolism have not been fully elucidated, breakdown is initiated in the mitochondrion, yielding CoA-esterified intermediates, which are further processed in the peroxisome (Lucas et al. 2007 and refs therein), possibly following import by CTS. The involvement of peroxisomes in BCAA degradation was demonstrated by the identification of *chy1* and *dbr5*, allelic *Arabidopsis* mutants, which were isolated on the basis of resistance to IBA and 2,4-DB respectively (Zolman et al. 2001; Lange et al. 2004). *CHY1*/*DBR5* encodes a peroxisomal β-hydroxyisobutyryl-CoA hydrolase, which participates in valine catabolism. The auxin-resistant phenotypes of plants lacking this activity are considered to result from accumulation of a toxic intermediate, methacrylyl-CoA,

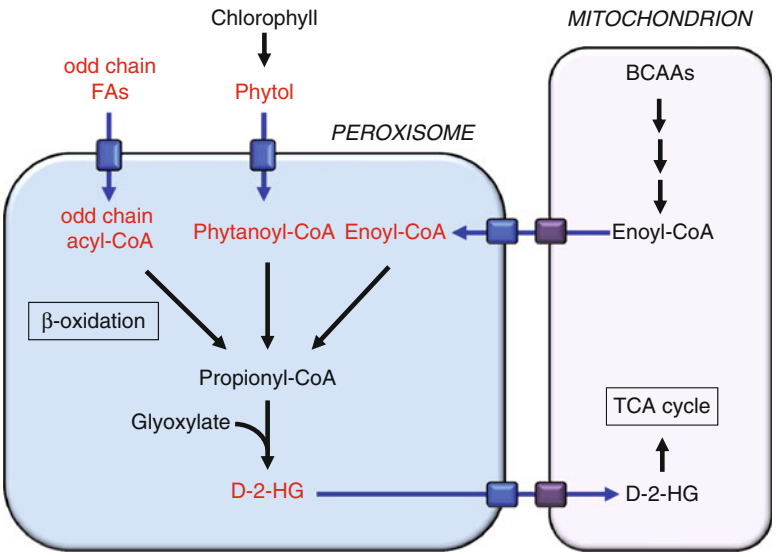

Fig. 10.3 Schematic presentation of the propionyl-CoA metabolism. The breakdown of odd chain fatty acids during β-oxidation, degradation of phytol and the BCAA catabolism produce propionyl-CoA inside peroxisomes. *BCAAs* branched amino acids, *D-2-HG* D-2-hydroxyglutarate, *FAs* fatty acids

which inhibits 3-ketoacyl CoA thiolase (Zolman et al. 2001; Lange et al. 2004). In wild type plants, the proposed end-point of peroxisomal BCAA catabolism is propionyl-CoA, a metabolite which is also produced during β-oxidation of odd chain fatty acids and by breakdown of the methyl branched fatty acid, phytanate, derived from the degradation of phytol (Lucas et al. 2007 and refs therein).

The fate of propionyl-CoA has been enigmatic, since plants do not contain homologues of mammalian or bacterial propionyl-CoA carboxylases. Feeding studies with *Arabidopsis* seedlings demonstrated that exogenously supplied propionate and butyrate are processed by peroxisomal metabolism to produce hydroxypropionate and hydroxybutyrate, respectively (Lucas et al. 2007), however, an alternative route has been proposed for propionyl-CoA produced endogenously during senescence. In a manner analogous to the "export" of acetyl-CoA as citrate (Pracharoenwattana et al. 2005), D-2-hydroxyglutarate (D-2-HG) formed from the condensation of propionyl-CoA and glyoxylate could be exported from the peroxisome to the mitochondrion, where it is processed by D-2-HG dehydrogenase to 2-ketoglutarate which enters the TCA cycle and electrons are donated to the respiratory chain via the ETF/ETFQO complex (Araujo et al. 2010; Engqvist et al. 2011) (Fig. 10.3). Interestingly expression of D-2-HG dehydrogenase is co-expressed with genes involved in β-oxidation, fatty acid respiration and degradation of BCAAs and chlorophyll (Engqvist et al. 2011). This would require a peroxisomal D-2-HG transporter.

10.6 Phylloquinone (Vitamin K_1) Biosynthesis

Phylloquinone (vitamin K_1) is a redox co-factor required for electron transfer in photosystem I and for the formation of protein disulphides and is also an important vitamin in human health. Until recently, phylloquinone was thought to be synthesised in plastids but two independent reports have provided evidence that the biosynthetic pathway is shared between plastids and peroxisomes (Babujee et al. 2010; Widhalm et al. 2012; see Chap. 12). The naphthoquinone ring of phylloquinone is produced from chorismate, by the sequential action of isochorismate synthases and a plastid multifunctional enzyme known as PHYLLO. The product of PHYLLO, *o*-succinylbenzoate is then activated by esterification to CoA and cyclised to produce 1,4-dihydroxy-2-naphthoyl (DHNA)-CoA. A proteomic survey of spinach leaf peroxisomes identified DHNA-CoA synthase as a putative peroxisomal protein and the localisation of the *Arabidopsis* orthologue (which contains a putative PTS2 motif) was confirmed by YFP fusion (Babujee et al. 2010). Moreover, the preceding enzyme in the pathway, succinyl benzoyl CoA:ligase (AAE14) was shown to be dual targeted to plastids and peroxisomes (Babujee et al. 2010; see Chap. 12). Subsequently, the identification of two peroxisomal thioesterases, which hydrolyse DHNA-CoA and are required for phylloquinone biosynthesis in *Arabidopsis* (Widhalm et al. 2012), firmly established a role for peroxisomes in this pathway. This implies that succinyl benzoate or succinyl benzoyl CoA must be exported from the plastid and imported into the peroxisome. The most plausible candidate for peroxisomal import is the ABC transporter CTS, which handles a range of cyclic and aromatic CoA esters, in addition to fatty acyl-CoAs (see above), although *cts* mutants do not exhibit the severe phenotypes observed in phylloquinone deficient mutants (Gross et al. 2006). By analogy with fatty acid import in yeast (Van Roermund et al. 2012), it is likely that the CoA ester is recognised by the transporter, and CoA cleaved off during import is re-esterified by peroxisome-localised AAE14, however direct proof awaits metabolite profiling of *cts* mutants and transport studies. The mechanism by which DHNA is exported for further processing to phylloquinone in the chloroplast is currently unclear.

10.7 Intermediates of Isoprenoid Biosynthesis

Isoprenoids, such as terpenoids, carotenoids, ubiquinone, phytosterols and brassinosteroids, play a vital role in basic plant processes, including photosynthesis, growth and development, reproduction, plant defense, and adaptions to environmental conditions (reviewed in Hemmerlin et al. 2012). For isoprenoid biosynthesis, the mevalonate (MVA) pathway provides the universal C5 precursors isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP) (Clastre et al. 2011; Simkin et al. 2011). As in mammals, the plant MVA enzymes are separated between cytosol and ER, whereas the last steps catalyzed by

phosphomevalonate kinase (PMK), mevalonate diphosphate decarboxylase (MVD), and isopentenyl diphosphate isomerase (IDI) are located to peroxisomes (Sapir-Mir et al. 2008; Simkin et al. 2011). This complex compartmentalization necessitates the transfer of intermediates across these intracellular membranes (Fig. 10.4). For example, mevalonate phosphate has to enter the peroxisomes for its conversion to IPP and DMAPP, which have to be exported for producing the isoprenoid end products (Sapir-Mir et al. 2008; Simkin et al. 2011). The existence of such transport systems across the peroxisomal membrane remains to be tested. Beside the MVA pathway, plants are also able to produce the building blocks for isoprenoids by the plastidic 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway, suggesting that metabolic crosstalk between these two IPP-producing pathways occurs via the exchange of intermediates (Bick and Lange 2003). IPP uptake from the cytosol into plastids has been experimentally demonstrated by in vitro uptake studies using liposomes reconstituted with chloroplast envelopes, indicating the presence of a plastidial IPP transporter (Flügge and Gao 2005). A recent report identified the farnesyl diphosphate synthase (FFS) activity in peroxisomes (Thabet et al. 2011), which catalyzes the condensation of three IPPs to farnesyl diphosphate. Another ABC transporter might be a good candidate for the export of this phosphorylated C15 hydrocarbon chain due to its structural similarity to fatty acids. Moreover, plant members of ABC subfamily G have been shown to transport terpenoids and related molecules (Jasiński et al. 2001).

10.8 Cofactor-Dependent Pathways

Cofactors such as ATP, NAD, and CoA, are essential for numerous enzymatic reactions inside plant peroxisomes, including those of β-oxidation, the photorespiratory C2 cycle, and detoxification of ROS (Hu et al. 2012). The cytosol serves as a pool for cofactors: ATP is exported to the cytosol from mitochondria as a product of oxidative phosphorylation (Haferkamp et al. 2011). For NAD and CoA biosynthesis, the last steps occur in the cytosol (Hashida et al. 2010). Peroxisomes are dependent on the import of these "bulky" molecules; the mechanisms by which this occurs have been a matter of controversy for several decades and it has been suggested either that the membrane is impermeable to these molecules, implying the existence of a pre-formed peroxisomal pool or that specific transport proteins catalyze their diffusion (reviewed in Antonenkov and Hiltunen 2011).

Recently, two peroxisomal ATP transport proteins (PNC1 and PNC2, reviewed in Linka and Esser 2012) have been identified that are able to import ATP in exchange with ADP or AMP. Repressing both PNC genes in *Arabidopsis* via RNA interference (RNAi) resulted in seedlings that were unable to metabolize their storage oil due to a block in the ATP-dependent fatty acid activation required for β-oxidation (Arai et al. 2008a; Linka et al. 2008). As a consequence the mutants were severely arrested in their growth, indicating that (i) the PNC-mediated transport

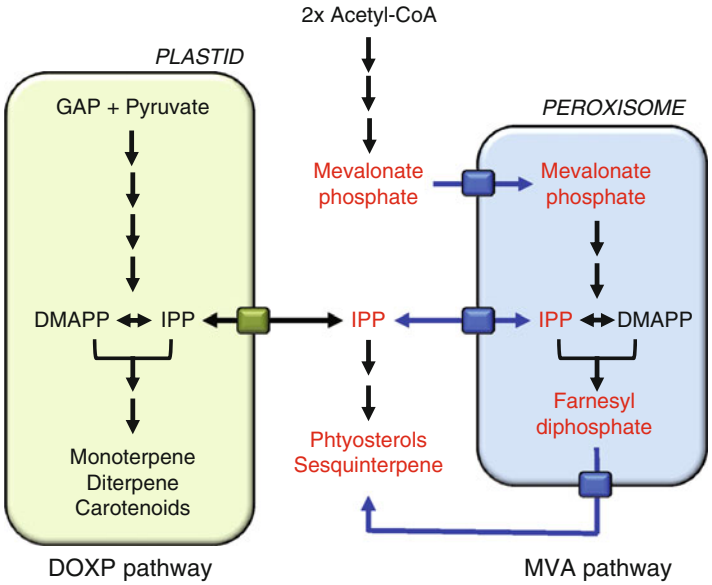

Fig. 10.4 Schematic presentation of the isoprenoid biosynthetic pathways. A few steps of the mevalonate (*MVA*) pathway takes place inside peroxisomes and require the shuttling of intermediates through the peroxisomal membrane. *DOXP* 1-deoxy-D-xylulose-5-phosphate, *DMAPP* dimethylallyl diphosphate, *GAP* glycerinaldehyde 3-phosphate, *IPP* isopentenyl diphosphate

pathway is the primary source for ATP and that (ii) other major ATP-generating systems, such as substrate-level phosphorylation, do not exist in peroxisomes to support fatty acid breakdown (Arai et al. 2008a; Linka et al. 2008).

The import of NAD and CoA into plant peroxisomes is mediated by the peroxisomal NAD carrier, PXN (reviewed in Linka and Esser 2012). Like the NAD carrier proteins from mitochondria and plastids (Palmieri et al. 2009), the recombinant protein mediates the transport of NAD and AMP in vitro. PXN also accepts NADH and CoA as transport substrates (Agrimi et al. 2012; Bernhardt et al. 2012). Based on its broad substrate specificity, the likely physiological role for PXN is to mediate the import of NAD or CoA in exchange for AMP (Agrimi et al. 2012; Bernhardt et al. 2012). Furthermore, as an NAD/NADH antiporter, it might transfer reducing equivalents in addition to the malate/oxaloacetate shuttle (Bernhardt et al. 2012), although studies with multiple *Arabidopsis* knockout lines suggest that peroxisomal malate dehydrogenase and hydroxypyruvate reductase are responsible for the bulk of this activity (Pracharoenwattana et al. 2010). Based on the proposed *in planta* function, it is surprising that the *Arabidopsis* plants lacking PXN are still viable (Bernhardt et al. 2012). *pxn* mutant alleles exhibit only a subtle metabolic phenotype: the rate of the NAD- and CoA-dependent β-oxidation is retarded but not abolished (Bernhardt et al. 2012). This suggests that peroxisomes possess additional transport routes, for example, it is possible that CoA is imported

as a consequence of the unusual transport mechanism of the ABC transporter CTS (Fig. 10.1), although it has not yet been determined on which side of the membrane CoA is liberated (De Marcos Lousa et al. 2013). Alternatively, cofactor-dependent enzymes may bind their cofactor during translation in the cytosol and are then imported as fully folded proteins through the peroxisomal import pore (Bernhardt et al. 2012).

10.9 Carboxylic Anions and Other Small Organic Anions

A peroxisomal pore-forming channel (porin) has been postulated to permit the passive diffusion of a broad spectrum of small solutes (reviewed in Reumann 2010). In spinach leaves and germinating castor beans these channels are anion-selective and facilitate the diffusion of carboxylic anions, such as the intermediates of photorespiration (e.g. glycolate, 2-oxoglutarate, and glycerate, Fig. 10.5) and the glyoxylate cycle (e.g. malate, oxaloacetate, succinate, citrate, isocitrate).

For many years, the molecular identity of porins remained obscure, but the mouse peroxisomal membrane protein 2 (Pxmp2) has been identified recently, which mimics the above described channel activity (Rokka et al. 2009). Pxmp2, when heterologously expressed in insect cells, forms a relatively wide, water-filled pore in an artificial lipid bilayer (Rokka et al. 2009). Despite its weak cation selectivity, this channel facilitates the diffusion of various organic anions, such as glycolate, pyruvate, and 2-ketoglutarate (Rokka et al. 2009). The *Arabidopsis* 22 kDa peroxisomal membrane protein (PMP22, Tugal et al. 1999) displays a high sequence similarity to Pxmp2. Electrophysiological experiments with the recombinant protein will elucidate whether PMP22 represents the proposed peroxisomal channels in plants.

In plant peroxisomes, ascorbate (ASC, vitamin C) plays an important role in the detoxification of hydrogen peroxide (H_2O_2) (del Río et al. 2006). The ascorbate-glutathione cycle, (also known as the Foyer-Halliwell-Asada cycle) efficiently detoxifies H_2O_2 via four enzymatic reactions catalyzed by ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DAR) and glutathione reductase (GR) (Jiménez et al. 1997). The peroxisomal distribution of the four enzymes has been studied in pea, tomato and pumpkin (del Río et al. 2006). Monodehydroascorbate reductase has been shown to be essential for storage oil mobilization during early seedling growth (Eastmond 2007). The oxidation of ASC to MDA is linked with the reduction of H_2O_2, catalyzed by ascorbate peroxidase, to oxygen and water. MDA is then exported to the cytosol, where it is re-oxidized to ASC via peroxisomal MDAR. APX is associated with the peroxisomal membrane. In case of the pumpkin APX its catalytic domain is orientated towards the cytosolic side (Yamaguchi et al. 1995). Thus, this cycle requires the export of MDA and import of ASC. In general, ASC is synthesized in the cytosol via de novo or salvage pathways and is then distributed to different cell compartments (Wheeler et al. 1998). Transport of ASC across plant plasma, mitochondrial and

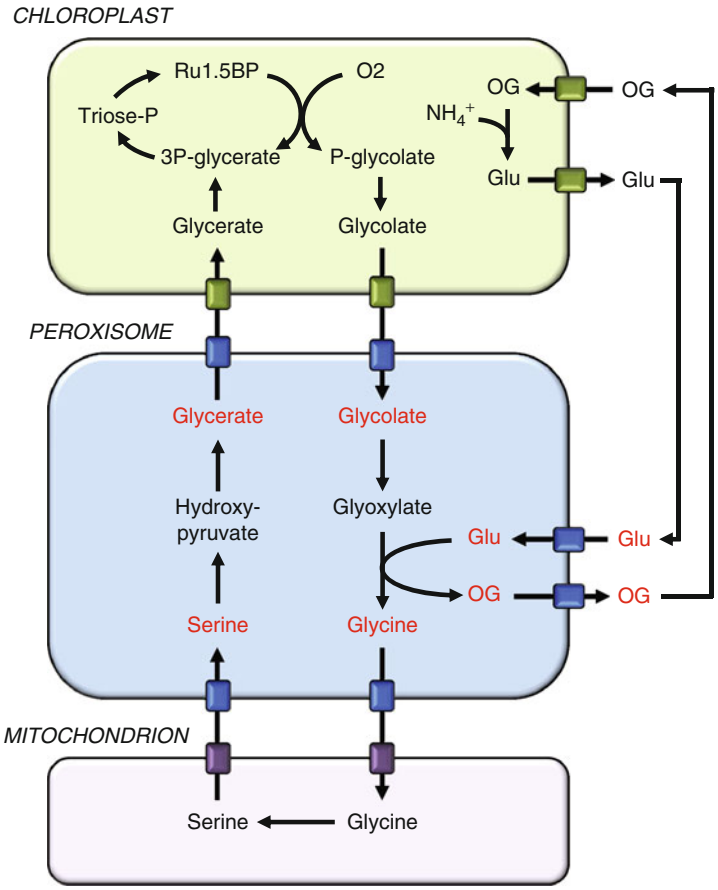

Fig. 10.5 Schematic presentation of the photorespiratory C2 cycle. Chloroplast, peroxisome and mitochondrion contribute to this pathway. The conversion of glycolate to glycine and serine to glycerate take place in the peroxisomal matrix. The shuttling of the small organic compounds glycolate, glycerate and 2-oxoglutarate as well as the exchange of amino acids glycine, serine and glutamate are required across the peroxisomal membrane. *Glu* glutamate, NH_4^+ ammonium, *OG* 2-oxoglutarate, *Ru1.5BP* ribulose-1.5-bisphosphate

plastid membranes has been demonstrated, but the carrier proteins responsible for ASC uptake are still unknown (Horemans et al. 2000). In mammals two plasma membrane-localized nucleobase-ascorbate (NAT) transporters catalyze the sodium-coupled import of ASC (Daruwala et al. 1999; Tsukaguchi et al. 1999). The *Arabidopsis* genome encodes 12 NAT proteins (Maurino et al. 2006), which may include peroxisomal members since most have not been characterized with respect to their subcellular localization and transport properties. Alternatively, the postulated channel-forming protein PMP22 (as mentioned above) might be involved in the exchange of the small organic acids ASC and MDA across the peroxisomal bilayer.

10.10 Redox Shuttles

Core β-oxidation results in the net production of NADH which is converted back to the NAD+ by the action of peroxisomal malate dehydrogenases (MDH) and also potentially, hydroxypyruvate reductase (Pracharoenwattana et al. 2007, 2010). This implies the existence of a redox shuttle involving either cytosolic or mitochondrial MDH. In the former case, malate would be exported from the peroxisome, and converted in the cytosol to oxaloacetate which then re-enters the peroxisome. A malate-aspartate shuttle has also been proposed, in which oxaloacetate is generated from aspartate and 2-oxogluatarate by aspartate amino transferase, with the concomitant formation of glutamate (reviewed in Rottensteiner and Theodoulou 2006). This requires exchange of malate/2-oxoglutarate and glutamate/aspartate across the peroxisomal membrane. It is currently unclear which system operates in plants and by which route metabolites are exchanged.

Several reductive reactions in plant peroxisomes depend on NADPH, such as JA biosynthesis (OPDA reductase), β-oxidation of unsaturated fatty acids (Δ^2-Δ^4-dienoyl-CoA reductase), and H_2O_2 protection systems (glutathione reductase), thus peroxisomes require a route for the regeneration of NADPH. Two possible mechanisms have been proposed. Firstly, early biochemical studies revealed the activities of the glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) in the peroxisomal matrix (Corpas et al. 1998). These two enzymes represent the NADPH-generating part of the oxidative pentose phosphate pathway (OPPP), implying a role in providing plant peroxisomes with reducing equivalents (Corpas et al. 1998). Proteome analyses validated the peroxisomal localization of 6PGD and 6-phosphogluconolactone (6PGL), the missing link for a complete oxidative OPPP in peroxisomes (Reumann et al. 2007, 2009; Eubel et al. 2008). The protein responsible for peroxisomal G6PD activity was not identified by proteomics experiments but a recent report showed that the plastid-localized G6PD isoform is recruited to peroxisomes in *Arabidopsis*, depending on the cytosolic redox state (Meyer et al. 2011). Under oxidative stress conditions, the non-functional isoform G6PD4 binds to G6PD1, masking the plastidic transit peptide and thus as a consequence confers peroxisome localization via the internal PTS1-like motif (Meyer et al. 2011). This alternative peroxisomal targeting mechanism allows a temporary completion of the oxidative OPPP when the peroxisomal metabolism requires additional reducing equivalents (Meyer et al. 2011). The sequential conversion of glucose-6-phosphate to ribulose-5-phosphate via the three-enzyme cascade necessitates the import of the substrate and the export of the end product. Such a transport process is known for plastids, where the plastidial phosphate translocator (PT) family mediates the exchange of sugar phosphates with inorganic phosphate (Linka and Weber 2010). To date, there is no evidence for the existence of a PT member in the peroxisomal bilayer.

An alternative or additional mechanism for NADPH regeneration could be provided by an isocitrate/2-oxoglutarate shuttle. Here, NADPH is regenerated inside peroxisomes via the oxidation of isocitrate to 2-oxoglutarate catalyzed by the

NADP-dependent isocitrate dehydrogenase (IDH). 2-oxoglutarate is exported from the peroxisome and is possibly re-reduced to isocitrate by the cytosolic isocitrate dehydrogenase (Corpas et al. 1999; del Río et al. 2002). The resulting isocitrate is then re-imported into peroxisomes. IDH has been detected in leaf peroxisomes (Corpas et al. 1999) and in yeast, peroxisomal IDH has been shown to be required for metabolism of unsaturated fatty acids with double bonds at even positions (van Roermund et al. 1998). The transport proteins to link the peroxisomal and cytosolic pools of NADPH remain elusive (Rottensteiner and Theodoulou 2006). However, a peroxisomal porin could be responsible for shuttling of isocitrate and 2-oxoglutarate (reviewed in Reumann 2000).

10.11 Amino Acids and Oligopeptides

During photorespiration, glyoxylate is generated via oxidation of glycolate in the peroxisomal matrix (Reumann and Weber 2006; Fig. 10.5). In the next step, the amino group of the imported glutamate is transferred to glyoxylate, resulting in 2-oxoglutarate and glycine. Both products are exported from the peroxisomes (Reumann and Weber 2006). 2-oxoglutarate is transported to the plastids, where it is again transaminated to glutamate and two molecules of glycine are converted to serine in the mitochondria, which re-enters the peroxisomes (Reumann and Weber 2006). The amino group of serine is removed to produce hydroxypyruvate. For this part of the photorespiratory C2 cycle, the amino acids glycine, serine, and glutamate have to be shuttled across the peroxisomal bilayer (Linka and Weber 2010). To date, amino acid (AA) transporters have been identified at the plasma membrane, tonoplast, and mitochondrial membrane but not in peroxisomes (Linka and Weber 2010). Although the peroxisomal porin from spinach leaves allows the diffusion of 2-oxoglutarate, it does not exhibit transport activities for the neutral AAs serine and glycine due to its strong anion selectivity (Reumann et al. 1998). Peroxisomes might possess an antiporter for the exchange of glutamate and 2-oxoglutarate, similar to the plastidic glutamate/2-oxoglutarate translocator (DiT2, Weber et al. 1995). However, the two members of this transporter family in *Arabidopsis* are restricted to the plastids (Linka and Weber 2010).

The tripeptide glutathione (GSH), consisting of glutamic acid, cysteine, and glycine, functions as an antioxidant in the ascorbate-glutathione cycle of plant peroxisomes (Jiménez et al. 1997). H_2O_2 generated by the peroxisomal metabolism is degraded via the oxidation of ASC to dehydroascorbate and also by APX. To regenerate ASC, GSH is oxidized to glutathione disulfide (GSSG), which is subsequently reduced to GSH by NADPH-dependent glutathione reductase (Jiménez et al. 1997). Because biosynthesis of GSH occurs in both plastids and cytosol (Wachter et al. 2005), GSH has to be imported into peroxisomes for this antioxidant cycle. GSH permeases, including oligopeptide transporters, chloroquinone-like transporters, Bcl2-associated anathogene, and ABC transporters

(multidrug resistance-associated protein), are required for GSH uptake into the cell, plastids, mitochondria, nucleus, and vacuole, respectively, but no peroxisomal GSH transporter has yet been reported (reviewed in Noctor et al. 2011).

10.12 Other Nitrogenous Intermediates

During senescence, plants are able to use purine nucleotides as a nitrogen source and purines generated by symbiotic nitrogen fixation are similarly degraded in nonsymbiotic cells. The purine ring system is completely metabolized to glyoxylate, carbon dioxide, and ammonia (reviewed in Werner and Witte 2011) and the end products are then recycled to synthesize organic molecules as building blocks for new growth. The first common intermediate in the degradation pathway of all purines is xanthine. Xanthine is oxidized to urate by xanthine dehydrogenase (XDH), followed by three enzymatic reactions, leading to allantoin. XDH activities have been found in peroxisomes from watermelon cotyledons, pea leaves and pepper fruits (Sandalio et al. 1988; del Río et al. 1989; Mateos et al. 2003; Corpas et al. 1997). Its peroxisomal localization has been demonstrated in pea leaf peroxisomes by immunogold electron microscopy (Corpas et al. 2008). However, in Arabidopsis XDH lacks a typical PTS motif and is probably located to the cytosol. The conversion of urate to allantoin takes place in peroxisomes, possibly because it generates H_2O_2 as a by-product, which can be efficiently detoxified via peroxisomal catalase (Lamberto et al. 2010). To shuttle either xanthine or urate in and allantoin out of plant peroxisomes requires transport proteins. The candidate channel-forming protein, PMP22 (see above) might be responsible for the transport of these heterocyclic nitrogen compounds. The loss of the mouse ortholog led to elevated levels of urate in the blood, implying Pxmp2 catalyzes in vivo the urate uptake in mice peroxisomes (Rokka et al. 2009). However, other putative peroxisomal allantoin and uric acid carriers need to be considered. Several solute transporter families consist of members that mediate the transport of allantoin and urate across the plasma membrane, indicating that homologues might represent the corresponding peroxisomal carrier. For example, a member of the nucleobase transporter family is able to complement an allantoin transport-deficient yeast strain, indicating that it functions as plasma membrane-localized allantoin permease (Desimone et al. 2002). Another type of transporter for these nitrogenous compounds is the nucleobase transporter family (see above, Maurino et al. 2006), which might contain a peroxisome-localized member specific for allantoin and urate (Gillissen et al. 2000). Also, an ABC transporter, ABCG2 has recently been shown to mediate urate transport across the plasma membrane in mammals (Woodward et al. 2009).

Polyamines (PAs) such as spermine, spermidine and putrescine, are essential growth regulators found in all pro- and eukaryotes. In plants, they are involved in the control of cell division, flowering, retardation of senescence, responses to osmotic stress, drought and salt stresses, and plant-pathogen interactions (reviewed in

Wimalasekera et al. 2011). Peroxisomes play a role in the catabolism of polyamines and thus regulate the cellular PA levels. Spermine imported into peroxisomes is degraded to spermidine via oxidative deamination catalyzed by peroxisomal polyamine oxidase (Moschou et al. 2008; Kamada-Nobusada et al. 2008). An *Arabidopsis* mutant deficient in this enzyme activity accumulates spermine in the roots, but lacks a visible phenotype (Kamada-Nobusada et al. 2008). In prokaryotes, polyamine uptake systems belong to the ABC transporter family. Biochemical evidence for polyamine transport in plants has been reported for protoplasts, mitochondria and vacuoles (Igarashi and Kashiwagi 2010) and recently, a transporter gene in rice has been identified for the exchange of these small aliphatic amines across the plasma membrane (PUT1, Mulangi et al. 2012). This carrier belongs to the large amino acid transporter family, to which the peroxisomal polyamine permease might also belong.

Biotin (vitamin H or B8) is an essential cofactor involved in a number of carboxylation and decarboxylation reactions (Pinon et al. 2005). In plants, de novo synthesis is split between cytosol, mitochondria, and peroxisomes (Pinon et al. 2005). The first steps are located in peroxisomes, leading to the biotin precursor 7-keto-8-amino-pelargonic acid (KAPA) via the decarboxylative condensation of pimeloyl-CoA and alanine (Tanabe et al. 2011). The β-ketoacid aldimine KAPA is then exported for further conversion to biotin in mitochondria. It is possible that pimeloyl-CoA is generated intraperoxisomally from pimelic acid, which is derived from long chain fatty acids such as oleic acid (Tanabe et al. 2011). The compartmentation of biotin biosynthesis implies an intracellular trafficking of KAPA between peroxisomes and mitochondria, the details of which are, however, largely unknown.

10.13 Inorganic Compounds

Activation of β-oxidation substrates by esterification to CoA generates pyrophosphate (PPi) (Fulda et al. 2004). To prevent inhibitory accumulation inside peroxisomes, PPi is probably hydrolyzed to two molecules inorganic phosphate (P_i) via a pyrophosphatase (PPase). Although not identified in proteomics experiments and/or lacking a canonical PTS signal, it cannot be excluded that a soluble or proton-pumping PPase is located in peroxisomes. Consequently, either PPi or Pi needs to be exported from the peroxisomes to the cytosol. A Pi/PPi exchange activity has been demonstrated using liposomes reconstituted with membranes from bovine kidney peroxisomes (Visser et al. 2005), but the corresponding gene remains to be defined. An alternative export route for phosphate is via the peroxisomal ATP carrier (Arai et al. 2008a; Linka et al. 2008). Like the plastidial ATP transporter (Trentmann et al. 2008), PNC proteins could export PPi or Pi from the peroxisomal matrix to balance the electrogenic ATP/AMP exchange (Linka and Esser 2012). However, future uptake studies are required to determine whether the PNCs mediate such phosphate-coupled transport.

The presence of sulfite oxidase (SO) in the peroxisomal lumen indicates that peroxisomes are involved in the oxidation of sulfite to sulfate (Nowak et al. 2004). This enzyme uses oxygen as electron acceptor and generates significant amounts of superoxide as a by-product (Byrne et al. 2009). The physiological role of this peroxisomal reaction remains to be elucidated. Because elevated levels of sulfite can be detoxified via a non-enzymatic reaction in the presence of H_2O_2, it is speculated that SO plays a role in the generation of superoxide as signal molecule (Byrne et al. 2009). A sulfite/sulfate exchange across the peroxisomal bilayer would allow the supply the peroxisomal SO with its substrate and the export its end product sulfate for vacuolar storage.

10.14 Conclusions

As outlined above, multiple, distinct transmembrane transport steps are required for a functional peroxisome. Despite substantial efforts, the majority of the participating metabolite transporters are still unknown (Theodoulou et al. 2011; Linka and Esser 2012). Currently, only a relatively small number of carrier proteins have been characterized at the molecular level in plants, fungi, and mammals: CTS, the broad specificity importer for fatty acids and their derivatives, PNC, the ATP carrier, and PXN, the NAD/CoA transporter (Theodoulou et al. 2011; Linka and Esser 2012). Recent reports suggest PMP22 as a strong candidate for the peroxisomal channel-protein which mediates transfer of small anionic solutes in *Arabidopsis*, but the extent of its transport properties need to be investigated (Antonenkov and Hiltunen 2011).

A major challenge for the future will be to identify the genes responsible for the transport processes which are required to execute the great diversity of peroxisomal function in plants. To date, proteome studies have not detected further transmembrane-spanning proteins in the peroxisome membrane, beyond the relatively highly abundant CTS, PNC, PXN, and PMP22 proteins (Reumann et al. 2007, 2009; Eubel et al. 2008). To detect the under-represented proteins of the 'permeome', peroxisomal membranes in sufficient quantity and purity are required, since, unlike soluble proteins, novel peroxisomal membrane proteins cannot be identified *in silico*. Fortunately, new DNA sequencing technologies will enable future proteomics approaches to include non-model species, such as pea, sunflower and pumpkin, which are known to be more suitable for higher peroxisome yields. Early studies were able to successfully purify peroxisomes from different plants (López-Huertas et al. 1995, 1997, 1999; Fukao et al. 2002; Arai et al. 2008b).

Forward genetic approaches using *Arabidopsis* mutants did not reveal additional peroxisomal carriers as might have been expected; only CTS has been identified in this way via five independent screens for mutants defective in peroxisomal metabolism or germination and seedling establishment (reviewed in Theodoulou et al. 2011). This implies either that transporter mutants exhibit lethal phenotypes or that redundant transport routes exist in plant peroxisomes. Other complementary

strategies, including co-expression analyses and systematic studies of membrane protein subcellular localization, will help to discover new transport proteins involved in a particular peroxisomal pathway.

The current state of knowledge is consistent with the 'two transporter' model proposed by Antonenkov and Hiltunen (2011). The permeability of the peroxisomal membrane results from: (i) a non-selective channel with broad substrate specificity, enabling the unrestricted diffusion of small metabolites and (ii) specific transport proteins for 'bulky' molecules, such as fatty acids, aromatics, ATP, NAD, and CoA. This concept implies that the peroxisomal bilayer is freely permeable to all cellular metabolites and thus no additional carriers would be required, which would mirror the low diversity of peroxisomal membrane proteins (Eubel et al. 2008; Reumann et al. 2009). However, transfer of these diverse metabolites and their peroxisomal metabolism needs to be tightly regulated. Future investigations will elucidate whether metabolic trafficking via channel-forming proteins is controlled via yet-to-be-defined mechanisms or whether the peroxisomal membrane contains a wide range of specific transport proteins as key regulators, as is the case for the plastidial and mitochondrial inner membranes.

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Chapter 11
Peroxisomes and Photomorphogenesis

Navneet Kaur, Jiying Li, and Jianping Hu

Contents

Abstract In higher plants, light-grown seedlings exhibit photomorphogenesis, a developmental program controlled by a complex web of interactions between photoreceptors, central repressors, and downstream effectors that leads to changes in gene expression and physiological changes. Light induces peroxisomal proliferation through a phytochrome A-mediated pathway, in which the transcription factor HYH activates the peroxisomal proliferation factor gene *PEX11b*. Microarray analysis revealed that light activates the expression of a number of peroxisomal genes, especially those involved in photorespiration, a process intimately associated with photosynthesis. In contrast, light represses the expression of genes involved in β–oxidation and the glyoxylate cycle, peroxisomal pathways essential for seedling establishment before photosynthesis begins. Furthermore, the peroxisome is a source of signaling molecules, notably nitric oxide, which promotes photomorphogenesis. Lastly, a gain-of-function mutant of the peroxisomal membrane-tethered

N. Kaur • J. Li • J. Hu (✉)
MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA
e-mail: huji@msu.edu

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RING-type E3 ubiquitin ligase PEX2 partially suppresses the phenotype of the photomorphogenic mutant *det1*. Possible mechanisms underlying this phenomenon are discussed.

Keywords Photomorphogenesis • Phytochrome A • HYH • De-etiolated 1 • COP • Peroxisome proliferation • Photorespiration • β-oxidation • Glyoxylate cycle • Nitric oxide • PEX11 • PEX2

11.1 Light-Regulated Plant Development and Photomorphogenesis

Light is an environmental cue with the most profound impact on plant development. The wavelength, intensity, direction and duration of light control a myriad of physiological responses such as seed germination, photomorphogenesis (de-etiolation), shade avoidance, phototropism, movement of organelles (e.g., chloroplasts) and guard cells, circadian rhythm, photoperiodic control of flowering time, and UV acclimation. Light perception and response in plants is orchestrated by a complex network of interactions between photoreceptors, early signaling elements, central integrators, and downstream effectors. These interactions result mostly in the activation of a transcriptional cascade that leads to altered expression of hundreds or even thousands of responsive genes encoding proteins that execute light dependent physiological changes (Chen et al. 2004; Chory 2010; Heijde and Ulm 2012; Kami et al. 2010; Neff et al. 2000).

In higher plants, such as the dicotyledonous reference plant *Arabidopsis thaliana*, seedlings display contrasting developmental programs in the presence or absence of light. When germinating in the darkness of the soil, seedlings undergo skotomorphogenesis (etiolation), where they develop a long primary stem (hypocotyl) and hooked/undeveloped embryonic leaves (cotyledons) to help the seedlings push their way and reach the surface of the soil to capture light for photosynthesis. Upon exposure to light, seedlings exhibit photomorphogenic responses (or de-etiolation) – i.e., inhibition of hypocotyl growth, opening of cotyledons, chloroplast development, and expression of genes involved in photosynthesis and related functions that facilitate autotrophic plant growth (Chen and Chory 2011; Chen et al. 2004; Wang and Deng 2003).

The major photoreceptors responsible for seedling photomorphogenesis are phytochromes, which perceive red and far-red light, and cryptochromes, which capture blue and UV-A light (Chen and Chory 2011; Yu et al. 2010). Upon activation by light, phytochromes translocate to photobodies in the nucleus, mediating the removal of Phytochrome Interacting Factors (PIFs), a group of bHLH transcriptional factors that repress photomorphogenesis, while promoting the stabilization of positive regulators of photomorphogenesis, such as HY5 (Chen and Chory 2011; Leivar and Quail 2011). Plant cryptochromes also function in the nucleus to modulate gene expression (Liu et al. 2011). The central regulators, COSTITUTIVE

PHOTOMORPHOGENIC/DE-ETIOLATED 1/FUSCA (COP/DET1/FUS), are repressors of photomorphogenesis because in the dark their loss-of-function mutants are de-etiolated, showing characteristics of light-grown wild-type seedlings. The COP/DET1/FUS proteins constitute three distinct protein complexes in a ubiquitin (Ub)-proteasome system, the components of which are largely conserved from plants to human. COP1 is a RING type E3 ligase. The COP1-containing CULLIN4-based E3 complex targets several key positive regulators of photomorphogenesis for degradation in the dark. Among the best known targets are phytochrome A and several positively acting transcription factors of photomorphogenesis – i.e., HY5 and its homolog HYH, HFR1, and LAF1, which contain the bZIP, HLH, and MYB DNA-binding domains respectively. The DET1-containing protein complex is another CUL4-based E3 complex, which, together with the third complex – the COP9 signalosome (CSN), enhances the activity of the COP1 complex. In addition, DET1 can also act as a transcriptional repressor and CSN plays roles in regulating CULLIN-RING E3 ligases (CRL) involved in various aspects of plant development (Lau and Deng 2012; Nezames and Deng 2012). Downstream from the core transcription factors are hundreds of genes encoding proteins involved in the numerous physiological responses that occur during photomorphogenesis (Jiao et al. 2007; Leivar et al. 2009).

11.2 Light Induction of Peroxisomal Proliferation During Photomorphogenesis

While going through photomorphogenesis, plant seedlings transition from heterotrophic to autotrophic growth, whereby genes involved in photosynthesis and related functions are actively expressed and pertinent organelles developed. Photorespiration is a process tightly associated with photosynthesis, and the peroxisome is a prominent player in this pathway. In this process, peroxisomes act coordinately with chloroplasts and mitochondria to convert phosphoglycolate produced by the oxygenase activity of Ribulose-1,5-bisphosphate-carboxylase/oxygenase (Rubisco) to glycerate, which re-enters the chloroplastic calvin cycle; ammonia is simultaneously recycled (Foyer et al. 2009; Peterhansel et al. 2010; see Chap. 10). Although photorespiration reduces photosynthetic efficiency by consuming O_2 and causing the loss of carbon, nitrogen and energy, it clearly plays a crucial role in cellular metabolism, especially under high light, high temperature, low CO_2 and water deficiency, as plants defective in photorespiration are compromised in growth under these conditions (Foyer et al. 2009).

Given the development of photosynthetic apparatus and active expression of genes encoding proteins involved in photosynthesis and related processes during photomorphogenesis, peroxisomes, whose functions are intimately linked to photosynthesis, may also increase their abundance during this process. To test this hypothesis, Desai and Hu (2008) treated dark-grown *Arabidopsis* seedlings containing a peroxisomal fluorescent marker protein with light, and observed an

increase in the total number of peroxisomes in cotyledon cells. The expression of *PEX11b*, which encodes a peroxisomal proliferation factor acting at the early rate-limiting peroxisomal elongation/tubulation step, was strongly up-regulated in the same process and far-red light seemed to exert the strongest effect. Consistent with the notion that PEX11b is a crucial mediator in light-induced peroxisome proliferation, the *PEX11b* RNAi lines showed a strong reduction in peroxisome number increase during photomorphogenesis. Genetic analysis showed that the light induction of *PEX11b* expression was strongly decreased in *phyA* and *hyh* mutants. In agreement with this finding, peroxisome abundance in these mutants was markedly reduced and this phenotype was rescued by overexpression of the *PEX11b* gene. Finally, HYH but not its homolog HY5 could directly bind to the promoter of *PEX11b* in gel-shift assays (Desai and Hu 2008; Hu and Desai 2008). These studies revealed a phyA-mediated light signaling pathway that promotes the proliferation of peroxisomes in photomorphogenesis through the bZIP transcription factor HYH and the early peroxisome proliferation factor *PEX11b*. These results corroborated with the postulation that during photomorphogenesis, an increase in the number of peroxisomes is needed in addition to the active expression and import of photorespiratory enzymes.

Environmental and metabolic stress conditions such as ozone, herbicide, clofibrate, salt stress, H_2O_2, high light, UV light, and senescence have been reported to increase peroxisome abundance or induce peroxisomal fission in plant cells (Castillo et al. 2008; de Felipe et al. 1988; del Río et al. 1998; Ferreira et al. 1989; Mitsuya et al. 2010; Nila et al. 2006; Oksanen et al. 2003; Palma et al. 1991; Sinclair et al. 2009). However, little is known about the signaling pathways that control peroxisome abundance at the mechanistic level in response to these stresses. PEX11, which exists as multigene families in diverse eukaryotes and as a highly abundant constituent of the peroxisomal membrane involved in the rate-limiting first steps of peroxisome multiplication, is subjected to metabolic regulation in yeasts and mammals at the transcriptional level (Hu 2009; Schrader et al. 2012). In addition to light, other cues may also exert their regulatory roles on peroxisome proliferation through the *PEX11* genes in plants. For example, salt stress, abscisic acid (ABA), and jasmonic acid (JA) have been found to regulate the expression of *Arabidopsis* and/or rice *PEX11* genes (Mitsuya et al. 2010; Nayidu et al. 2008). The signaling cascades leading to these gene expression changes remain to be determined.

11.3 Light Regulation of Peroxisomal Function During Photomorphogenesis

Early events during germination occur in the dark or very low light, and the seed utilizes its stored resources to fuel germination. Perception of light by germinating seedlings triggers autotrophic growth, during which genes involved in photosynthesis and related functions are activated. To understand the regulatory role of light on peroxisomal functions, we performed online microarray database searches using 114 experimentally confirmed *Arabidopsis* peroxisomal genes to analyze their

expression level under various light conditions. This gene list was primarily based on a summary in our previous study (Kaur and Hu 2011), after omitting genes with unclear functions in the peroxisome and genes whose expression data are unavailable in the microarray database. A total of 25 genes involved in peroxisome biogenesis and 89 genes encoding peroxisomal enzymes with known biochemical functions were subjected to analysis. Microarray data from dark, red, blue and far-red light conditions of *Arabidopsis* germinating seedlings were used to generate heatmaps to display expression changes (fold change in log 2) of biogenesis (Fig. 11.1a) and metabolism (Fig. 11.1b) genes against the level of expression of the respective genes in white light.

11.3.1 Light Regulation of Peroxisome Biogenesis Genes

Consistent with PEX11b's role as a key mediator in the light induction of peroxisome proliferation (Desai and Hu 2008; Hu and Desai 2008), the expression of *PEX11b* is repressed in the dark and strongly up-regulated in the light, especially in far-red light. PEX3A, which encodes a protein involved in early peroxisomal assembly, shows a similar light activation pattern, albeit at a lower degree (Fig. 11.1a).

In contrast to *PEX11b*, two PEX11 isoforms, *PEX11c* and *PEX11e* are induced in the dark (Fig. 11.1a). This seems paradoxical to the observed proliferation of peroxisomes in light (Desai and Hu 2008; Hu and Desai 2008). However, it must be considered that these two genes are highly expressed in seeds and roots (Orth et al. 2007), whereas perception of light occurs in the greening cotyledons. Further, *FIS1B* is also up-regulated in the dark, consistent with previous findings that PEX11c to e and FIS1B act coordinately in cell cycle-associated peroxisome duplication (Lingard et al. 2008). An additional caveat here is that cells in the cotyledons grow by expansion and not cell division (Chandler 2008), indicating that peroxisome replication via the actions of PEX11c to e and FIS1B is probably confined to seed and root tissues and might not occur in green tissues. The peroxisome membrane protein receptor PEX19A is also actively expressed in dark conditions, possibly reflecting its role in ensuring the targeting of PEX11c, PEX11e and FIS1B to the peroxisome.

Taken together, these data suggest that whereas light induces peroxisome proliferation through activation of biogenesis genes such as *PEX11b* and *PEX3A*, in the dark, PEX11c, PEX11e and FIS1B seem to constitute a peroxisome division complex that is limited to tissues that do not perceive light.

11.3.2 Light Regulation of Peroxisomal Metabolic Genes

Heatmap generated from analyzing expression of peroxisome metabolic pathway genes shows two obvious trends, wherein one set of genes is induced in the light and

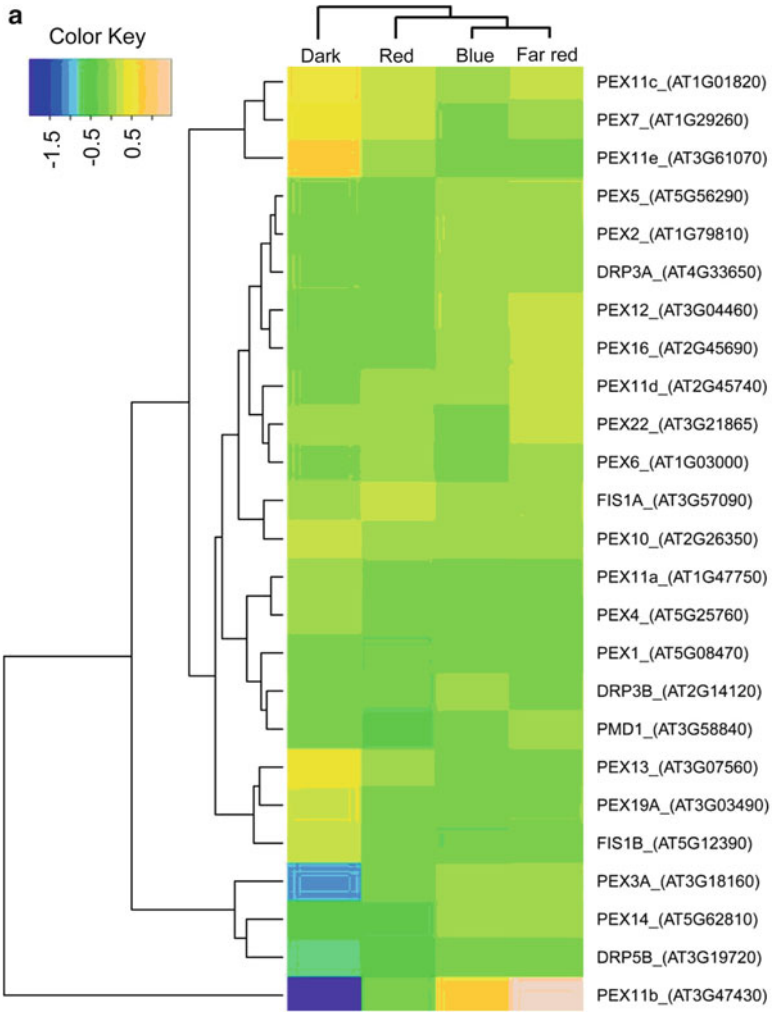

Fig. 11.1 Heatmap of expression changes of *Arabidopsis* peroxisomal genes in dark and various light conditions compared with *white* light conditions. Microarray data set GSE5617 was downloaded from the NCBI (*GEO*) gene expression omnibus database (Barrett et al. 2011; Edgar et al. 2002). GSE5617 contains Affymetrix microarray ATH1 gene expression data from germinating *Arabidopsis* seedlings grown under various light conditions (three biological replicates for each condition). Here, log-2 based gene expression fold changes between four conditions (*dark*, *red*, *blue*, *far-red light*) and *white* light were computated. Analysis was performed using the Bioconductor software (Gentleman et al. 2004) with the statistical computing language R (version 2.15.2). Normalization of gene expression values was carried out with the (*RMA*) robust multi-array average algorithm (Irizarry et al. 2003) implemented in the Affy package of Bioconductor. Statistical significance of the differential expression values were assessed with Linear models for microarray (limma) package (Smyth 2004). Hierarchical clustering of the differentially expressed genes was visualized by creating heatmaps using the color palette package RColorBrewer and the *gplots* package (Warnes et al. 2011). (**a**) Peroxisome biogenesis genes. (**b**) Peroxisomal metabolic genes (with *PEX11b* as a reference)

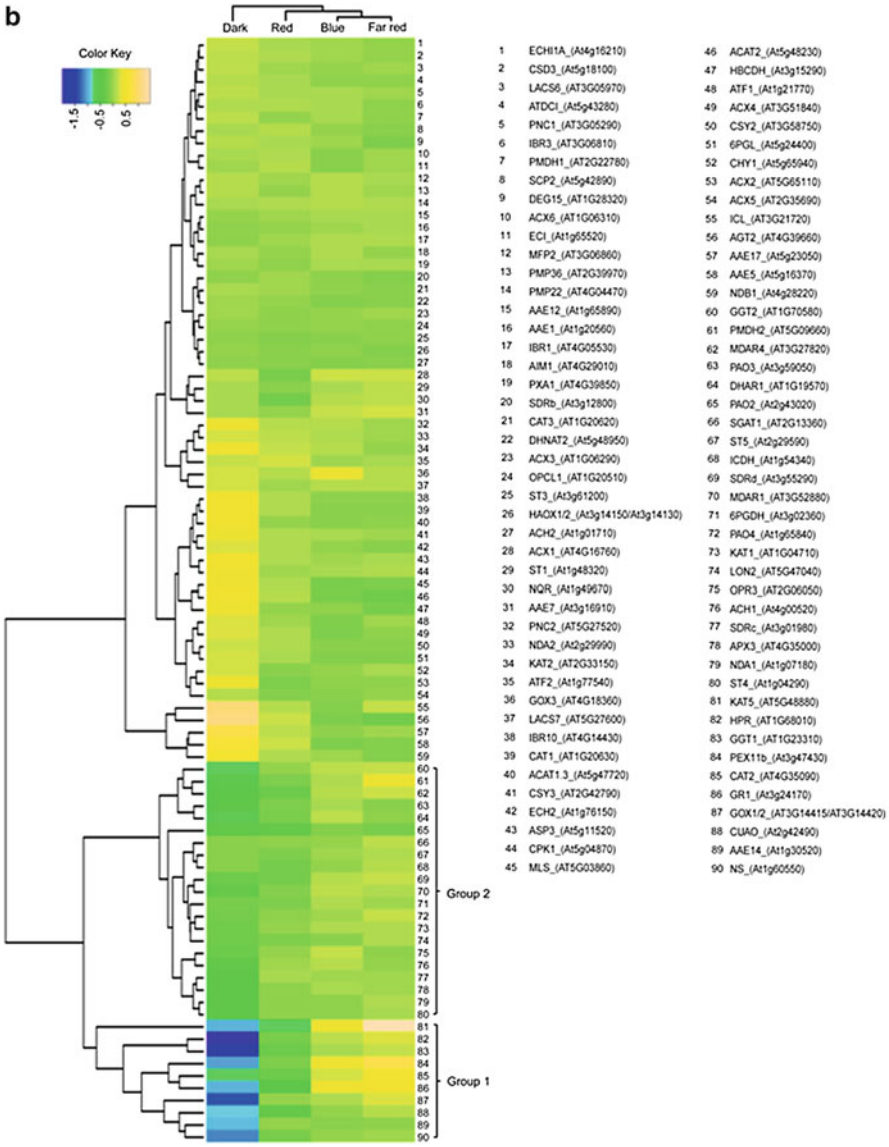

Fig. 11.1 (continued)

the second set is induced in the dark. About 31 peroxisomal genes (Fig. 11.1b, genes 60–90) are up-regulated by light, which can be further divided into two groups: those also repressed in the dark (group one) and those with no expression changes in the dark (group two).

Not surprisingly, virtually all the *Arabidopsis* peroxisomal genes linked to photorespiration show clear induction by light; many of them are also repressed in the dark, thus belonging to group one (Fig. 11.1b). The most prominent role of peroxisomes in photosynthetic tissues is their participation in photorespiration. With the exception of hydroxypyruvate reductase (HPR), peroxisomal proteins involved in photorespiration are mostly encoded by multi-gene families. These enzymes include: glycolate oxidase (GOX1, GOX2, and GOX3), catalase (CAT1, CAT2, CAT3), Ser-glyoxylate aminotransferase (SGAT), Glu-glyoxylate aminotransferase (GGT1 and GGT2), and the peroxisomal malate dehydrogenase (pMDH1 and pMDH2) (Hu et al. 2012). Two genes encoding phylloquinone biosynthesis enzymes (napthoate synthase – *NS*; acyl activating enzyme – *AAE14*), a putative NO producer – copper amine oxidase (*CuAO*), and the β–oxidation enzyme 3-ketoacyl CoA thiolase (*KAT5*) also cluster with group one genes. Notably, these genes exhibit similar patterns of expression with *PEX11b*, prompting the speculation that some genes in group one may also be induced via the phyA–HYH mediated pathway. phyA accumulates in the dark and dominates the regulation of early light responses in *Arabidopsis* (Peschke and Kretsch 2011). Moreover, phyA functions also extend to blue light perception (Casal and Mazzella 1998; Neff and Chory 1998; Poppe et al. 1998). In line with these previous findings, several of group one genes are also up-regulated in blue light. The only group one gene that is not repressed by dark is *CAT2*. This is not surprising as H_2O_2 is produced in both dark and light conditions and catalase, the major enzyme that scavenges H_2O_2, needs to be on irrespective of light conditions.

NS and *AAE14* encode phylloquinone (vitamin K1) biosynthetic enzymes napthoate synthase and acyl activating enzyme respectively. Phylloquinone (PhQ), a compound comprising of a napthoquinone ring and a phytyl chain, is exclusively synthesized in plants and cyanobacteria (Gross et al. 2006). PhQ is an essential cofactor for Photosystem I (PSI)-mediated electron transfer and may also function as a membrane embedded redox sensor (Gross et al. 2006). PhQ biosynthesis is accomplished through partially compartmentalized reactions that take place in chloroplasts and peroxisomes; mutants in PhQ production exhibit compromised PSI activity (see Chap. 12; Babujee et al. 2010; Gross et al. 2006; Kim et al. 2008; Shimada et al. 2005; Widhalm et al. 2012). Given the critical need of PhQ in photosynthesis and consequently autotrophic growth, the up-regulation of *NS* and *AAE14* seems logical.

Group two light-activated genes contain an isoform of *GGT* and *pMDH* as well as the gene encoding the antioxidant enzyme monodehydroascorbate reductase isoform 4 (MDAR4). Although pMDH proteins are not essential for photorespiration, their absence results in altered stoichiometry of CO_2 release and are thus believed to contribute to optimal rates of photorespiration in air (Cousins et al. 2008). Only one isoform of pMDH (pMDH2) is induced in the light, suggesting that pMDH2 may have more vital roles in photorespiration during the initial greening of seedlings.

Seeds germinating in the dark rely on mobilization of stored reserves and activation of metabolic pathways pertaining to gluconeogenesis such as glycolysis and tricarboxylic cycle (TCA), sucrose synthesis, and peroxisomal β-oxidation and glyoxylate cycle (Weitbrecht et al. 2011). Consistent with this notion, many of the peroxisomal genes up-regulated in the dark are involved in β-oxidation and the glyoxylate cycle (Fig. 11.1b, genes 32–59). Proteins encoded by these genes include the peroxisomal adenine nucleotide carrier 2 (PNC2), acyl-activating enzymes AAE5 and AAE17, long-chain acyl-CoA synthetase 7 (LACS7), acyl-CoA oxidases ACX2 and ACX4, enoyl-CoA hydratases ECH2 and IBR10 (indole-3-butyric acid response 10), hydroxybutyryl-CoA dehydrogenase HBCDH, 3-ketoacyl CoA thiolase KAT2, isocitrate lyase (ICL), malate synthase (MLS), citrate synthases CSY2 and CSY3, acetoacetyl-CoA thiolases ACAT2 and ACAT1.3, and 3-hydroxyisobutyryl – CoA hydrolase (CHY1). Two aberrations in this group of peroxisomal genes are aspartate aminotransferase (ASP3) (Schultz and Coruzzi 1995) and the dual-localized alanine aminotransferase AGT2 (Carrie et al. 2009). Seed storage proteins are rich in Asp and Glu residues; several aminotransferases are activated during imbibition, yielding amino acids that can be incorporated into newly synthesized proteins (Weitbrecht et al. 2011). Under hypoxia, these two aminotransferases were postulated to deplete excess pyruvate produced through glycolysis by channeling pyruvate into a modified TCA cycle, resulting in the generation of ATP (Rocha et al. 2010). Peroxisomes contain several precursors/substrates that are part of the TCA cycle and also actively exchange metabolites with the cytosol as well as mitochondria that house all the other enzymes operating in the gluconeogenic pathways. Thus, we hypothesize that peroxisomal isoforms of these two enzymes may have a role in providing metabolic precursors and promoting ATP synthesis during germination.

11.4 Peroxisomes as a Source of Retrograde Signals in Photomorphogenesis

Peroxisomes are known to produce and release reactive oxygen and reactive nitrogen species (ROS and RNS) molecules, such as H_2O_2, superoxide radicals, and nitric oxide (NO), and fatty acid derivatives such as jasmonic acid, to influence gene expression, intracellular redox homeostasis (in combination with a suite of peroxisomal antioxidative proteins), and other cellular functions (del Río 2011; del Río et al. 2006; Foyer et al. 2009; Hu et al. 2012; Kaur et al. 2009). Among these molecules, nitric oxide has been shown to promote photomorphogenesis in *Arabidopsis* (see below Sect. 11.4.1). The identification of a gain-of-function mutant of the peroxisome biogenesis gene *PEX2* as a suppressor of the photomorphogenic mutant *det1* also suggested the role of peroxisomes in photomorphogenesis (see below Sect. 11.4.2).

11.4.1 The Role of Nitric Oxide (NO)

Studies using wheat, lettuce, *Arabidopsis*, and potato revealed that application of NO donors to dark-grown seedlings or seedlings grown in low-intensity light not only promotes seed germination, but also partially induces photomorphogenesis, whereby seedlings display an inhibition of hypocotyl and internode elongation and contain more chlorophylls. In contrast, treating seedlings with chemicals that deplete NO led to opposite phenotypes (Beligni and Lamattina 2000; Tonon et al. 2010). A recent study in *Arabidopsis* (Lozano-Juste and Leon 2011) showed that seedlings deficient of NO have longer hypocotyls than the wild type under red light and increased expression of a few Phytochrome Interacting Factor (PIF) genes, which encode key repressors of photomorphogenesis. In contrast, under conditions in which NO levels are elevated, hypocotyls are shortened and an increase in the level of the DELLA protein, a repressor of gibberellin (GA) signaling and inducer of photomorphogenesis, was observed. Thus, NO is an important element in photomorphogenesis, repressing the expression of PIFs and antagonizing the function of gibberellins (GAs) by up-regulating the level of DELLA to inhibit cell elongation (Lozano-Juste and León 2011). The NO-GA antagonism model also extended to Arabidopsis root, where NO reduces cell elongation through the DELLA protein as well (Fernandez-Marcos et al. 2012).

Enzymatic NO synthesis in *Arabidopsis* has been linked to two distinct enzymes, NOA1 (nitric oxide associated 1) and NIA (nitrate reductase, two isoforms) (Wimalasekera et al. 2011a). Characterization of triple mutants revealed that they still produce residual levels (~10 %) of NO, suggesting that all sources of NO in *Arabidopsis* have yet to be identified (Lozano-Juste and Leon 2010). It has long been debated and finally admitted that higher plants do not have a canonical nitric oxide synthase (NOS) like that in animals (Zemojtel et al. 2006; Corpas et al. 2009; del Río 2011; Hancock 2012). In recent years a body of evidence has accumulated to support the existence of L-arginine-dependent NOS activity in at least 11 different plant species (Corpas et al. 2009; del Río 2011). Furthermore, the existence of a L-arginine-dependent NOS activity and the generation of NO has been demonstrated in peroxisomes isolated from pea leaves by different methods (see Chap. 15), although neither the protein nor the gene responsible for this activity has been identified. Interestingly, it has been hypothesized that polyamine catabolism could result in synthesis of NO (Wimalasekera et al. 2011a). Polyamine catabolism can occur through the activities of either polyamine oxidases (PAO) or copper amine oxidases (CuAO). In this context, an isoform of CuAO was shown to contribute to polyamine-induced NO biosynthesis (Wimalasekera et al. 2011b). Since peroxisomes contain both PAO and CuAO (Eubel et al. 2008; Kamada-Nobusada et al. 2008; Moschou et al. 2008; Reumann et al. 2009), it is tempting to speculate that these enzymes are possible sources of NO synthesis within peroxisomes. Studies on these candidate proteins and identification of other peroxisomal protein responsible for generating NO will be crucial to deciphering the specific contribution of peroxisomes to photomorphogenesis through the NO pathway.

11.4.2 The Role of the Peroxisomal Membrane-Tethered E3 Ubiquitin Ligase PEX2

The peroxisomal membrane protein PEX2 was shown to be involved in DET1-mediated photomorphogenesis in *Arabidopsis* (Hu et al. 2002). The *det1* mutants were among the first identified loss-of-function de-etiolated mutants. They display the typical de-etiolated/constitutively photomorphogenic phenotypes with short hypocotyls, opened cotyledons, developed chloroplasts, and ectopically expressed light-responsive genes in the dark, and other general growth defects including dwarfness, pale-green leaves and seedling lethality in strong alleles (Chory et al. 1989; Pepper et al. 1994). After 20 years of study, it is now known that DET1 forms a protein complex (CDD complex) with DAMAGED DNA BINDING PROTEIN 1 (DDB1) and COP10 to facilitate the activity of the COP1 complex in targeting positive regulators of photomorphogenesis for degradation (Lau and Deng 2012). In addition, DET1 also interacts with the N-terminal tail of histone H2B of the nucleosome (Benvenuto et al. 2002) and was found to be recruited to the promoter of circadian clock genes through interaction with other transcription factors, thus may also function as a transcriptional repressor (Lau et al. 2011).

In the early years of the study of DET1, several extragenic suppressors of the *det1-1* allele were isolated and named *ted*, for reversal of *det* (Pepper and Chory 1997). Among them, *ted3* contains a gain-of-function mutation in the peroxisome biogenesis factor *PEX2*, partially suppressing the *det1* phenotype (and a weak allele of *cop1*) in a dominant manner (Hu et al. 2002). PEX2 is a RING domain-containing peroxisomal membrane protein in all eukaryotes, involved in peroxisome protein import. At least in yeast, PEX2, together with two other RING peroxisomal membrane proteins (or peroxins) PEX10 and PEX12, act as E3 ubiquitin ligases required for the recycling of the matrix protein receptor PEX5 (Rucktaschel et al. 2011). PEX2 and most other plant peroxins are essential to embryogenesis in *Arabidopsis* (Hu et al. 2002). Recently, work from our laboratory demonstrated that all three *Arabidopsis* RING peroxins (PEX2, PEX10, and PEX12) can function as E3 ligases and function together with the ubiquitin receptor protein DSK2 in the peroxisomal membrane-associated protein degradation system (Kaur et al. 2013). Substrates for the RING peroxins in plants have not been identified.

What is the mechanism underlying the partial suppression of *det1* by *ted3*? The *det1-1* mutants show some phenotypes typical for peroxisomal β-oxidation mutants, such as sugar dependence and partial resistance to IBA, a protoauxin that is converted to the active auxin IAA through peroxisomal β–oxidation, suggesting that peroxisomal activities are impaired in *det1*. These physiological deficiencies, the de-etiolated phenotype, and abnormal gene expression were partially reversed in *ted3 det1-1* (Hu et al. 2002). Consistent with the findings that light activates photorespiration and peroxisome proliferation (see Sects. 11.2 and 11.3) and that *det1* behaves like a light-grown plant, genes encoding peroxisomal proliferator PEX11b, β-oxidation enzyme 3-keto-acyl CoA thiolase, and peroxisomal photorespiratory

enzymes HPR and pMDH are strongly up-regulated. In contrast, the expression of isocitrate lyase (ICL), a glyoxylate cycle enzyme that is highly abundant in the seed but gradually disappears after seedlings start photosynthesis, is down-regulated. The expression levels of all these genes were reversed by *ted3* (Hu et al. 2002). A possible conclusion drawn from these results is that DET1 positively regulates the function of peroxisomes in photomorphogenesis and that peroxisomes in *ted3* contain enhanced peroxisomal activities that can suppress the *det1* phenotype. In agreement with this, overexpression of wild-type PEX2 also partially suppressed the phenotype of *det1*. Based on these data, loss-of-function peroxisomal mutants were predicted to show some level of de-etiolation in the dark. However, none of the viable loss-of-function peroxisomal mutants have opened cotyledons in the dark although their hypocotyls are shorter, suggesting that the peroxisome contains some of the events downstream from DET1's regulatory network in development and is not a major executor of photomorphogenesis. In addition, DET1 is a repressor of photomorphogenesis yet peroxisomal photorespiration and proliferation are enhanced by light, which also argues against the idea that peroxisomes function as a major component downstream in the photomorphogenic pathway controlled by DET1.

In addition to being regulated by DET1, peroxisomes may also generate a signaling molecule (chemical or peptide) that feed-back regulates nuclear gene expression or by-passes the function of DET1/COP proteins in the nucleus. Since *ted3* also partially suppressed *cop1* but not *det2*, a de-etiolated mutant defective in brassinosteroid biosynthesis, this mutation seems to specifically affect the photomorphogenic pathway controlled by the DET1/COP proteins. Besides affecting gene expression, the "signal" generated by *ted3* could have an impact on the DET1-controlled pathway through by-passing or substituting DET1/COP's function in the nucleus. The *ted3* mutation contains a transition from G to A, resulting in a substitution of a valine to methionine one amino acid upstream of the first cysteine of the RING domain. One favorable hypothesis is that the creation of methionine just upstream from the RING initiated the translation of a small peptide that only contains the RING domain. This RING domain from PEX2, which has been shown to contain E3 ubiquitin ligase activity (Kaur et al. 2013), could translocate to the nucleus, by-pass the function of the COP1-DET1 E3 ligase complexes, and participate directly in the targeted degradation of some positive regulators of photomorphogenesis such as HY5, thus conferring partial etiolation in the *det1* or *cop1* mutant background. At this point, this hypothesized truncated peptide has not been detected in *ted3 det1* (M Desai and J Hu, unpublished data).

Taken together, more than one mechanism may be accountable for *ted3*'s suppression of *det1*'s phenotype, i.e., enhancement of peroxisomal activity and generation of a chemical or peptide signal that feedback regulates DET1-mediated pathway from the nucleus. What exactly constitutes this "signal" remains to be investigated and may turn out to be a complex issue to address.

11.5 Conclusions

Light governs many aspects of plant development, one of which is photomorphogenesis. Peroxisomes are essential organelles mediating a variety of metabolic processes in plants, including photorespiration, a pathway that is intimately associated with photosynthesis and affecting photosynthetic efficiency. Light induces the proliferation of peroxisomes during photomorphogenesis through activating the peroxisome proliferator gene *PEX11b* via a phyA-dependent pathway. Light upregulates genes encoding enzymes involved in photorespiration and represses the expression of genes involved in β–oxidation and the glyoxylate cycle, pathways essential for seedling establishment before photosynthesis begins. Peroxisomes also emit signals that impact photomorphogenic pathways. Identification of the peroxisomal proteins responsible for generating NO will be crucial to deciphering the specific contribution of peroxisomes to the promotion of photomorphogenesis through this signaling molecule. The major factor that contributes to the suppression of the photomorphogenic mutant *det1* in the *pex2* gain-of-function mutant in *Arabidopsis* will also need to be clarified.

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Chapter 12
Biosynthesis of Vitamin K1 (Phylloquinone) by Plant Peroxisomes and Its Integration into Signaling Molecule Synthesis Pathways

Sigrun Reumann

Contents

Abstract Vitamin K1 (phylloquinone) is a substituted membrane-anchored naphthoquinone that functions as an essential electron carrier in photosystem I in photosynthetic organisms. While plants can synthesize phylloquinone de novo, humans rely on vitamin K1 uptake from green leafy vegetables as a precursor for the synthesis of its structural derivative, menaquinone-4 (vitamin K2). In vertebrates, menaquinone-4 serves as an enzymatic co-factor that is required for posttranslational protein modification, i.e. the γ-carboxylation of glutamate residues in specific proteins involved in blood coagulation, bone metabolism and vascular biology. Comprehensive knowledge of the subcellular compartmentalization of vitamin K biosynthesis in plants, pathway regulation and its integration in cellular metabolic networks is important to design functional food with elevated vitamin levels and health benefits to human consumers. It had long been assumed that plants obtained all enzymes for phylloquinone biosynthesis from the ancient cyanobacterial

S. Reumann (✉)
Centre for Organelle Research, Faculty of Science and Technology, University of Stavanger, N-4036 Stavanger, Norway
e-mail: sigrun.reumann@uis.no

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endosymbiont and that, upon gene transfer to the nucleus, all biosynthetic enzymes were re-directed to the plastid. This view, however, has been recently challenged by the exclusive localization of the 6th pathway enzyme (MenB/NS) to peroxisomes in *Arabidopsis*. Soon afterwards, not only the preceding enzyme, acyl-activating enzyme 14 (MenE/AAE14), but also the succeeding thioesterase (DHNAT) were also shown to be peroxisomal. Phylogenetic analysis revealed a heterogeneous evolutionary origin of the peroxisomal enzymes. Phylloquinone biosynthesis reveals several branching points leading to the synthesis of important defence signalling molecules, such as salicylic acid and benzoic acid derivatives. Recent research data demonstrate that, of the two phenylalanine-dependent pathways for benzoic and salicylic acid biosynthesis, the CoA-dependent β-oxidative pathway, which is peroxisomal, is the major route. Hence, peroxisomes emerge as an important cell compartment for the interconnected networks of phylloquinone, benzoic and salicylic acid biosynthesis. Numerous mechanisms to regulate intermediate flux and the fine-tuned inducible production of secondary metabolites, including signalling molecules, await their characterization at the molecular level.

Keywords Acyl-activating enzyme • Benzoic acid • Defence signalling molecules • Phylloquinone • Vitamin K • Salicylic acid

Abbreviations

AAE14	Acyl-CoA activating enzyme isoform 14
BA	Benzoic acid
BZO1	Benzoate:CoA ligase
CFP	Cyan fluorescent protein
CNL	Cinnamate:CoA ligase
DHNA-CoA	1,4-dihydroxy-2-naphthoyl-CoA
DHNAT	Dihydroxynaphthoate thioesterase
DsRed	*Discosoma* sp. red fluorescent protein
EYFP	Enhanced yellow fluorescent protein
GGCX	Gamma-glutamyl carboxylase
Gla	γ-carboxyglutamate
Men	Menaquinone
MK-4/7	Menaquinone-4/7
NS	Naphthoate synthase
OSB	*o*-succinyl benzoate
PTM	Posttranslational modification
PTS1/2	Peroxisomal targeting signal type 1/2
ROS	Reactive oxygen species
SA	Salicylic acid

12.1 Introduction

The peroxisome is an important cell organelle that is ubiquitous in nearly all eukaryotes and compartmentalizes oxidative metabolic reactions by a single boundary membrane. A characteristic property of peroxisomes is their functional plasticity, as their enzymatic content drastically varies depending on the organism, the type of tissue, and the environmental conditions. Metabolic reactions located in peroxisomes are often part of long metabolic pathways and complex anabolic or catabolic networks that are distributed over different subcellular compartments, including the cytosol, mitochondria, and plastids in plants, for optimal functionality. In many cases, key metabolic reactions are located in peroxisomes, and these are often related to the production of toxic reactive oxygen species (ROS). For instance, the recycling of 2-phosphoglycolate produced in large quantities during photorespiration essentially relies on peroxisomal oxidation of glycolate and the detoxification of stoichiometric quantities of H_2O_2 by peroxisomal catalase. Subcellular compartmentalization of photorespiration involves the co-ordinated functioning of at least 11 chloroplastic, peroxisomal, and mitochondrial enzymes and 14–18 distinct transmembrane transport steps for intermediate transfer between cell compartments (Reumann and Weber 2006). Likewise, central to fatty acid β-oxidation are substrate oxidation and successive chain shortening by C_2 units in peroxisomes. Lipid mobilization is initiated by lipases located on lipid bodies, continues in peroxisomes, and involves mitochondrial reactions (Graham 2008).

Intensive research over the past decade taking advantage of the nuclear *Arabidopsis* genome sequence (The *Arabidopsis* Genome Initiative 2000) and applying state-of-the art strategies such as forward genetic screens and reverse genetic approaches for functional in vivo protein analysis, has revealed an unexpected metabolic complexity and plasticity of plant peroxisomes. Apart from ROS metabolism, photorespiration, and β-oxidation of saturated and unsaturated fatty acids, plant peroxisomes are now known to be involved in catabolism of branched amino acids, and in metabolism of sulfite and polyamines (Hu et al. 2012). Importantly, plant peroxisomes also catalyze an increasing number of biosynthetic reactions. These include, for instance, the biosynthesis of (i) the plant defence signalling molecules, jasmonic and salicylic acid (SA), (ii) the plant hormone indole-acetic acid, (iii) isoprenoids, and (iv) phylloquinone (for review, see Kaur et al. 2009; Reumann 2011; Hu et al. 2012). Moreover, the biosynthesis of benzoic acid (BA) and its derivatives including xanthone phytoalexins and benzoyl glucosinolates via the CoA-dependent β-oxidative pathway have recently been shown to essentially involve plant peroxisomal enzymes (see below, Kliebenstein et al. 2007; Van Moerkercke et al. 2009; Klempien et al. 2012; Qualley et al. 2012; Lee et al. 2012; Gaid et al. 2012; Colquhoun et al. 2012). Accumulating evidence is emerging that peroxisomes have important functions in specific defence mechanisms, conferring resistance against pathogen attack (for review, see Kaur et al. 2009; Kangasjarvi et al. 2012; Sørhagen et al. 2013). The biosynthetic capabilities of plant peroxisomes

in de novo phylloquinone production have been uncovered only recently and will be reviewed in this book chapter and be connected to signalling molecule synthesis networks.

The identification of additional plant peroxisome functions has been made possible by large-scale "omics" approaches, primarily proteomics and bioinformatics, in combination with cell molecular biology, biochemistry, and genetics (for review, see Palma et al. 2009; Reumann 2011). Peroxisomal matrix proteins are nuclear-encoded and imported from the cytosol by conserved PTSs, generally located at the C-terminal end (type 1, PTS1) or in the N-terminal domain (type 2, PTS2). PTS1 sequences consist of the C-terminal approx. 14 amino acids (aa) with the terminal tripeptide being the major targeting determinant (Reumann 2004; Lingner et al. 2011; Chowdhary et al. 2012). A recently published machine learning method to predict PTS1 proteins in spermatophytes (PWM model, position weight matrices) predicted 389 *Arabidopsis* proteins (including transcriptional and translational protein variants) as peroxisome targeted and has been shown to give fairly accurate predictions of novel PTS1 tripeptides and putatively novel *Arabidopsis* PTS1s, including weak non-canonical and evolving PTS1s (Lingner et al. 2011). Approx. 75 % of the predicted *Arabidopsis* proteins were not known to be peroxisomal, indicating that many peroxisomal proteins and novel functions remain to be uncovered. Indeed, several PTS1 protein predictions have been correctly validated up to now and helped to uncover novel peroxisomal enzymes involved in the biosynthesis of phylloquinone and defence signaling molecules such as SA (see below).

12.2 Vitamin K Group Characteristics and Members

Vitamin K is a group of structurally similar, fat-soluble vitamins. Vitamin K members are derivatives of 2-methyl-1,4-naphthoquinone, all of which contain an aliphatic substitution at position 3 of the naphthoquinone ring. Vitamin K1 (phylloquinone, 2-methyl-3-phytyl-1,4-naphthoquinone) contains four isoprenoid residues, one of which is unsaturated (Fig. 12.1). The hydrophobic side chain is attached as an 18-carbon-saturated phytyl tail and tightly anchors the co-factor to the thylakoid membrane. Phylloquinone functions as an essential one-electron carrier cofactor at the A1 site of photosystem I. Hence, phylloquinone is ubiquitous to plants and most cyanobacteria, and de novo phylloquinone biosynthesis is an essential pathway in these organisms.

Phylloquinone is structurally closely related to the second major vitamin K, menaquinone (vitamin K2), which contains the same substituted naphthoquinone ring but an unsaturated isoprenoid side chain, which is variable in length (Fig. 12.1). Menaquinones are designated as MK-n, where n specifies the number of isoprenoids. MK-4 is the major form and mostly needed by and found in animals including humans, while MK-7 is less abundant and mostly found in bacteria (Fig. 12.1). Menaquinones have two important functions in cell metabolism and biology. On the one hand, menaquinone serves as an enzymatic co-factor that

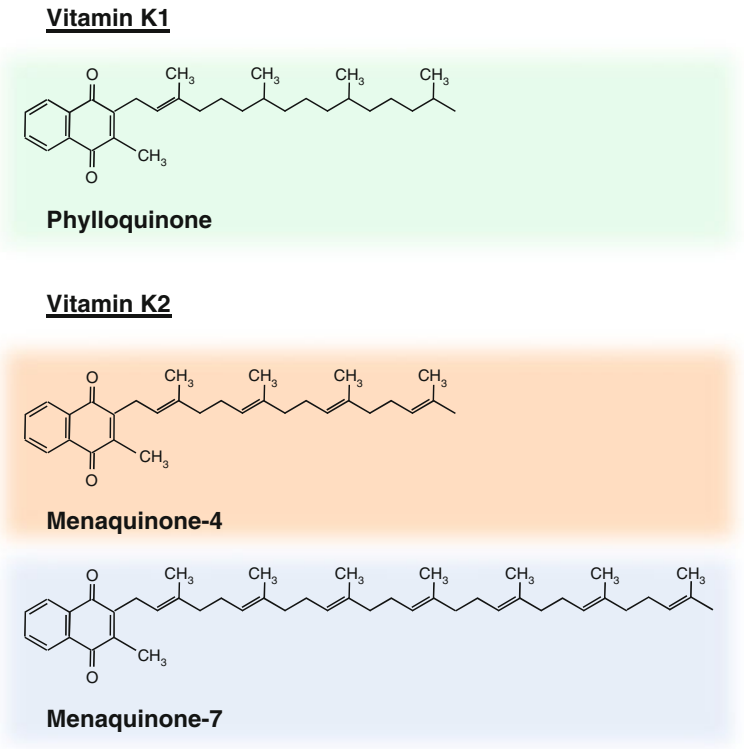

Fig. 12.1 Structure of vitamin K group members

is required for essential posttranslational protein modifications (MK-4). On the other hand, menaquinone functions as a 2-electron carrier in anaerobic respiration (MK-7). Menaquinone can only be synthesized de novo by lower photosynthetic eukaryotes (red algae, diatoms) and most prokaryotes.

12.3 The Significance of Plant Vitamin K1 for Human Health

Even though animals lack photosynthesis, vertebrates including humans require phylloquinone uptake from plant nutrition because they cannot synthesize menaquinone *de novo* but have the ability to convert dietary phylloquinone into MK-4 in specific tissues (Suttie and Booth 2011). Hence, phylloquinone is an important component in human nutrition. The main providers of dietary vitamin K to humans is the phylloquinone of green leafy vegetables such as romaine lettuce, kale, broccoli and spinach, and in vegetable oils, such as that of soybean, sunflower, olive, and canola (Booth 2009; Suttie and Booth 2011). Vitamin K2 is found in liver, milk, cheese, and fermented soy products (Stafford 2005). The

U.S. Dietary Reference Intake for an adequate intake of vitamin K for adults is 90–120 μg/day (Suttie and Booth 2011). The tight binding of phylloquinone to thylakoid membranes in chloroplasts reduces its bioavailability. For example, cooked spinach has a 5 % bioavailability of phylloquinone, which can be increased to 13 % by the addition of fat due to elevated vitamin K solubility (for review see Shearer et al. 2012). The exact mechanism by which vitamin K1 is converted to MK-4 in humans and the tissue site of this conversion presently are active research areas (Suttie and Booth 2011).

Phylloquinone was identified in 1929 by the Danish scientist Henrik Dam (1929, 1935) and referred to as vitamin K for "coagulation vitamin" (in German spelt with K, "Koagulationsvitamin"). Contrary to reasonable expectations, the function of vitamin K in animals does not resemble that of plant phylloquinone as an electron carrier. In animals, vitamin K is needed for an unusual posttranslational modification (PTM) of specific proteins. These PTM targets are primarily proteins required for blood coagulation. The modification comprises carboxylation of certain glutamate residues located in specific protein domains to form γ-carboxyglutamate (Gla) residues. Therefore, the target proteins of vitamin K-dependent PTMs are also referred to as vitamin K-dependent Gla proteins. The two carboxylate groups on the amino acid side chain allow chelation of calcium ions, which often triggers the proteins' functions, such as those of the so-called vitamin K dependent clotting factors (Furie et al. 1999).

To date, more than a dozen Gla proteins have been discovered in humans and have been reported to play key roles in the regulation of three physiological processes, including blood coagulation, bone metabolism and vascular biology (Suttie 1985; Suttie and Booth 2011). For instance, the blood coagulation factors II (prothrombin), VII, IX, and X, are Gla proteins (Booth 2009). An important Gla protein involved in bone metabolism is osteocalcin. The physiological role of several additional human Gla proteins is still unclear. Vitamin K and vitamin K-dependent proteins may also be involved in the regulation of energy metabolism and inflammation (Booth 2009).

The vitamin K-dependent enzyme that catalyzes the posttranslational carboxylation of Glu residues is gamma-glutamyl carboxylase, oxidizing the co-factor vitamin K hydroquinone to vitamin K epoxide (Suttie 1985; Wu et al. 1991; Presnell and Stafford 2002). The latter is reconverted to vitamin K in the so-called vitamin K cycle (Stafford 2005; Tie and Stafford 2008; Tie et al. 2011). This vitamin K recycling reduces the need of phylloquinone uptake. The biochemical function of vitamin K may also extend beyond that of a cofactor for the vitamin K-dependent carboxylation of glutamyl residues in vitamin K-dependent proteins (Booth 2009).

12.4 The Traditional View of Plant Phylloquinone Biosynthesis

For both fundamental knowledge of plant metabolism as well as applied research aimed at increasing the nutritional value including vitamin levels in crops

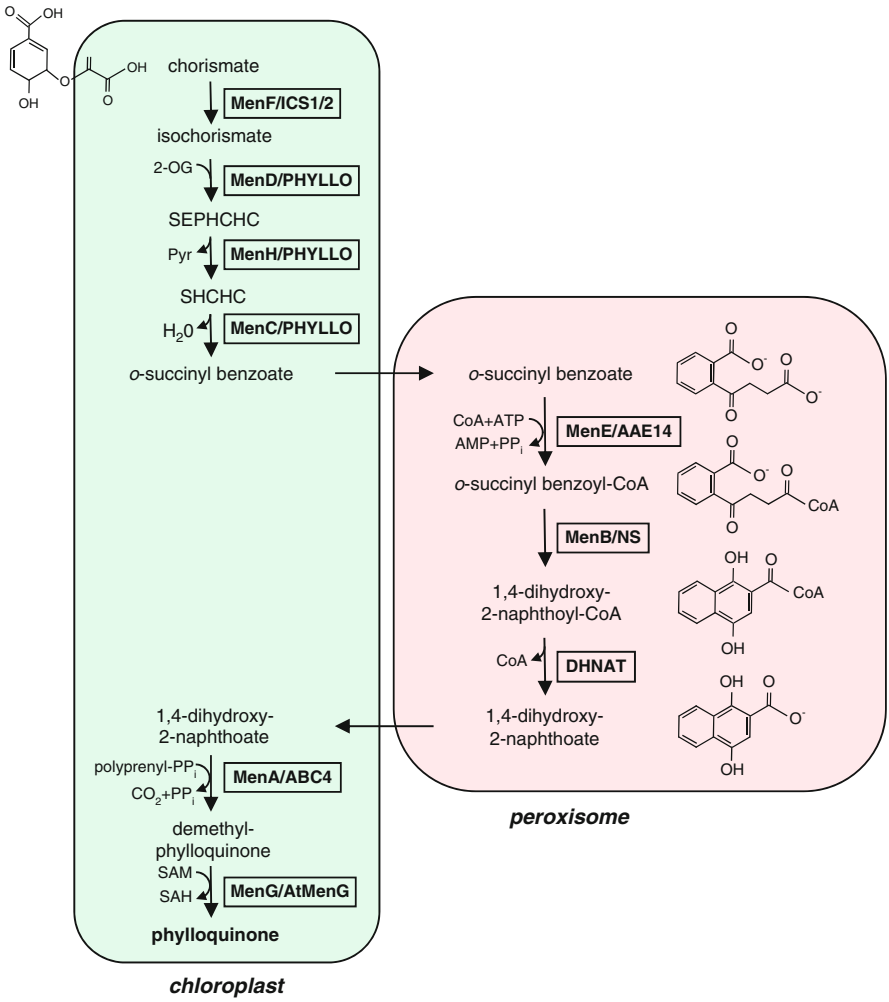

Fig. 12.2 Compartmentalisation of phylloquinone biosynthesis between chloroplasts and peroxisomes in higher plants. SEPHCHC, 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylic acid; SHCHC, (1*R*,6*R*)-2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid; 2-OG, 2-oxoglutarate. With permission modified after Babujee et al. (2010)

(Fitzpatrick et al. 2012), it is important to identify the complete set of enzymes involved in biosynthetic pathways, analyze their subcellular localization and study the pathway regulation and vitamin homeostasis.

In photosynthetic organisms phylloquinone is synthesized de novo in nine consecutive reactions from chorismate, which is produced from shikimate via the shikimate pathway (Fig. 12.2). The first four enzymes of phylloquinone biosynthesis, which convert chorismate to *o*-succinylbenzoate, are nuclear-encoded in

higher plants and localised in chloroplasts. The enzymes are referred to as Men (menaquinone) enzymes (MenF, D, H, and C) due to their functional similarity with menaquinone biosynthesis enzymes. Chorismate is first isomerized to isochorismate by isochorismate synthase, which is encoded by two paralogous genes in *Arabidopsis*. A multifunctional protein referred to as PHYLLO, which comprises three different enzymatic activities (MenD, H, and C), catalyzes sequential steps of the addition of 2-oxoglutarate to isochorismate and the elimination and aromatization to yield *o*-succinyl benzoate (Gross et al. 2006).

The next three biosynthetic steps comprise (i) activation of *o*-succinyl benzoate to the corresponding CoA ester, (ii) naphthoate ring formation by naphthoate synthase (MenB/NS), and (iii) thiolytic release of CoA by a thioesterase. Enzyme kinetic data, primary sequence and subcellular localization data for these three enzymes were long missing (see below). The last two pathway enzymes attach the phytol chain to the naphthoate ring catalyzed by 1,4-dihydroxy-2-naphthoic acid phytyltransferase (MenA/ABC4, Shimada et al. 2005). Finally, MenG methylates the precursor at position 3 (Lohmann et al. 2006). Both enzymes are also plastid-localised, as shown by in vivo subcellular targeting analysis (Shimada et al. 2005; Lohmann et al. 2006).

Phylloquinone biosynthesis was long thought to be exclusively compartmentalised in plastids in higher plants. Based on localisation of the first four and the last two out of nine pathway enzymes in chloroplasts (see above), it was more or less assumed that also the central three enzymatic steps (MenE/AAE14, MenB/NS, and DHNAT) were catalyzed by chloroplastic enzymes. This view was based on several lines of evidence and indications, including gene clustering in cyanobacteria, algae, and higher plants and experimental subcellular localisation data. For instance, several *Men* genes are plastid-encoded in red algae and, consequently, their gene products are plastid-localized (Gross et al. 2006, 2008). However, recent research data have shown that three central enzymes (MenE/AAE14, MenB/NS, and DHNAT) are peroxisomal.

12.5 Peroxisome Localization of MenB/NS

Naphthoate synthase (MenB/NS) catalyses the essential formation of the second aromatic ring in phylloquinone biosynthesis, i.e., the conversion of *o*-succinylbenzoyl-coenzyme A to 1,4-dihydroxy-2-naphthoyl-CoA (DHNA-CoA) (Fig. 12.2). The protein is orthologous to cyanobacterial MenB and encoded by a single gene in *Arabidopsis* and most higher plants (Johnson et al. 2001; Gross et al. 2008). First evidence for peroxisome localization of MenB/NS (At1g60550) has been obtained by proteome studies of leaf peroxisomes. When leaf peroxisomes were isolated from both spinach and *Arabidopsis* leaves and the proteins were separated by 2-dimensional electrophoresis, one of the novel proteins was identified as MenB/NS by mass spectrometry (Reumann et al. 2007; Babujee et al. 2010). Peroxisome targeting was further supported by the presence of a predicted canonical

PTS2 nonapeptide (RLx_5HL in *Arabidopsis*), and this targeting signal was found to be conserved among putative orthologs of higher plants (Reumann et al. 2007; Babujee et al. 2010). Peroxisome targeting was validated by in vivo subcellular targeting analysis using C-terminal reporter fusions (MenB/NS-EYFP), and the yellow fluorescent organelles were shown to coincide with peroxisomes labelled with cyan fluorescent protein (CFP) or red fluorescent protein (DsRed) (Babujee et al. 2010). Site-directed mutagenesis of the PTS2 nonapeptide (RLx_5HL to RLx_5VL) abolished peroxisome targeting and conclusively identified the PTS2 (Babujee et al. 2010). The absence of any predictions or indications for plastid targeting by neither in vivo subcellular targeting analysis nor proteomics allowed the conclusion that higher plant MenB/NS is exclusively targeted to peroxisomes. Functional data deduced from kinetic constants of recombinant *Arabidopsis* MenB/NS or complementation studies remain to be reported.

12.6 Peroxisome Targeting of MenE/AAE14

The peroxisomal localisation of MenB/NS raised the questions whether additional enzymes, primarily those catalyzing the preceding and the succeeding reactions of phylloquinone biosynthesis, were also located in peroxisomes. Kim et al. (2008) showed that acyl-CoA activating enzyme isoform 14 (AAE14) catalyses the reaction upstream of that of MenB, the activation of *o*-succinyl benzoate (OSB) to *o*-succinyl benzoyl-CoA (Fig. 12.2). Three *aae14* mutant alleles, identified by reverse genetics, were found to be seedling lethal. *Arabidopsis* AAE14 complemented the phenotype of an *Escherichia coli* mutant disrupted in the *menE* gene that encodes the bacterial OSB-CoA ligase and restored menaquinone biosynthesis (Kim et al. 2008).

MenE/AAE14 lacks a predicted transit peptide but had been reported to be plastid-targeted based on in vivo subcellular targeting data (Kim et al. 2008). However, chloroplast targeting of AAE14 is questionable because, according to the *Arabidopsis* Subcelllular Database (SUBA II, Heazlewood et al. 2007), AAE14 has not been identified in any of the numerous high-sensitivity proteomic analyses of chloroplasts. The characterization of a large variety of non-canonical PTS1 tripeptides in plant peroxisomal proteins led to the recognition that AAE14 also carries the non-canonical PTS1 tripeptide, SSL>, which had been previously validated as a functional PTS1 tripeptide for the C-terminal decapeptide of another PTS1 protein (Reumann et al. 2007). Indeed, the N-terminal reporter protein fusion (EYFP-AAE14) targeted peroxisomes, and the C-terminal decapeptide was demonstrated to represent a functional PTS1 domain sufficient to direct a reporter protein to peroxisomes (Babujee et al. 2010). Hence, MenE/AAE14 is unquestionably a peroxisomal enzyme in *Arabidopsis* and most other higher plants but might be dually targeted also to plastids (Fig. 12.2).

12.7 Peroxisome Targeting of DHNAT1/2

The thioesterase acting downstream of MenB/NS, DHNA-CoA thioesterase (DHNAT), has first been characterised biochemically and cloned from *Synechocystis* (Widhalm et al. 2009). By application of a comparative genomic approach, orthologous genes of unknown function were identified in most species of cyanobacteria that clustered with phylloquinone biosynthetic genes. The encoded proteins displayed homology with Hotdog domain-containing CoA thioesterases of certain soil bacteria involved in the catabolism of 4-hydroxybenzoyl-CoA (Widhalm et al. 2009). The recombinant *Synechocystis* ortholog produced DHNA with strict substrate specificity, and the corresponding knockout cells displayed the typical photosensitivity to high light associated with phylloquinone deficiency in cyanobacteria (Widhalm et al. 2009). However, *Synechocystis* DHNAT lacked closely related homologues in higher plants.

Similar to NS, first hints on the identity of higher plant DHNA-CoA thioesterase were obtained by proteomics. In total four small thioesterases of unknown function belonging to a seven-member family in *Arabidopsis* (Reumann et al. 2004, 2007) were identified by proteome analysis of leaf peroxisomes, and some of them had been validated as peroxisomal by in vivo subcellular targeting analysis (Reumann et al. 2009). Indeed, two of these small thioesterases were identified to function as DHNAT. Widhalm et al. (2012) applied a genomic approach to identify the gene(s) encoding higher plant DHNAT. Twelve *Arabidopsis* homologs encoding putative thioesterases were tested for their ability to functionally complement the phenotype of a *Synechocystis* mutant lacking DHNAT activity and to synthesize phylloquinone and grow photoautotrophically. Two *Arabidopsis* genes encoded functional DHNA-CoA thioesterases (DHNAT1/ST1, At1g48320, AKL>; DHNAT2/ST2, At5g48950, SKL>; Widhalm et al. 2012). The deduced plant proteins display low percentages of identity with cyanobacterial DHNA-CoA thioesterases, and do not even share the same catalytic motif. GFP fusion experiments demonstrated that the *Arabidopsis* proteins are targeted to peroxisomes (DHNAT1/ST1, Reumann et al. 2009; DHNAT2/ST2; Widhalm et al. 2012). Moreover, DHNA-CoA thioesterase activity was determined in isolated *Arabidopsis* leaf peroxisomes (Widhalm et al. 2012). In vitro assays with various aromatic and aliphatic acyl-CoA thioester substrates showed that the recombinant *Arabidopsis* enzymes preferred DHNA-CoA. In vivo evidence that the *Arabidopsis* enzymes were involved in phylloquinone biosynthesis was established by a reverse genetic approach. *Arabidopsis* T-DNA knock-down lines deficient in functional DHNAT protein/mRNA were isolated and displayed reduced DHNA-CoA thioesterase activity and phylloquinone content (Widhalm et al. 2012).

12.8 Evolution of Phylloquinone Biosynthesis Compartmentalization in Peroxisomes

Evolution is generally driven by increased fitness and proliferation. What might have been the advantage to compartmentalize three reactions of phylloquinone biosynthesis in peroxisomes in higher plants? MenE/AAE14 and MenB/NS are orthologous to *Synechocystis* enzymes of phylloquinone biosynthesis and have been inherited from the cyanobacterial endosymbiont that evolved into plastids. MenE/AAE14 orthologs are found in all photosynthetic eukaryotes, encoded either in the plastid genome (red algae) or in the nuclear genome (diatoms, chlorophytes and higher plants). The data suggest that the MenE/AAE14 gene was transferred from the cyanobacterial endosymbiont to the nuclear genome shortly after divergence of red algae. Similar to MenE/AAE14, MenB/NS orthologs are also found in all photosynthetic eukaryotes, either plastid- or nuclear-encoded, and were transferred to the nucleus together with MenE/AAE14 shortly after divergence of red algae. By contrast, higher plant DHNAT is neither of cyanobacterial nor of endosymbiotic origin. The cyanobacterial DHNAT gene appears having been lost at a rather early stage, possibly during gene transfer to the nucleus. The cyanobacterial enzyme has been replaced after divergence of chlorophytes by a bacterial thioesterase, most likely by lateral gene transfer, and this protein evolved to DHNAT in higher plants (Widhalm et al. 2012). In the transition phase, a pre-existing peroxisomal thioesterase of overlapping substrate activities might have taken over catalysis of the thiolytic cleavage of 1,4-dihydroxy-2-naphthoyl-CoA.

Detailed phylogenetic analyses of MenE/AAE14 and MenB/NS combined with PTS1 and PTS2 prediction analysis made possible by the web server PredPlantPTS1 (Lingner et al. 2011; Reumann et al. 2012, ppp.gobics.de) and the first plant-specific PTS2 protein prediction algorithms (T. Lingner and S. Reumann, unpubl. data) are expected to advance our understanding of the evolution of partial phylloquinone compartmentalization in higher plants.

12.9 Pathway Integration into Signaling Molecule Synthesis Networks

The pathway of phylloquinone biosynthesis reveals several branching points that lead to the synthesis of important signalling molecules such as SA (2-hydroxybenzoic acid) and derivatives of SA and BA. SA is an important regulator of plant resistance to biotrophic and hemi-biotrophic pathogens and functions as an endogenous signal molecule in systemic acquired resistance and thermogenesis (Raskin et al. 1987; Vlot et al. 2009). Volatile derivatives, such as methyl benzoate and methyl salicylate, are involved in plant-insect and plant-plant interactions serving as attractants for pollinators and seed dispersers in floral and fruit scents, respectively (Dudareva and Pichersky 2008). Moreover, BA and its

derivatives are important biosynthetic building blocks in primary metabolites (e.g. aromatic cytokinins), SA and compounds with chemotherapeutic activities like the anticancer agent taxol (Walker and Croteau 2000; Kliebenstein et al. 2007; Qualley and Dudareva 2008).

Salicylate can be synthesized from intermediates of phylloquinone biosynthesis by several pathways. The simplest and most direct is a 1-step reaction from isochorismate (Fig. 12.3, Wildermuth et al. 2001). Additionally, two so-called phenylalanine (Phe)-dependent pathways of SA biosynthesis branch from chorismate, first converting the intermediate to Phe via prephenate (Fig. 12.3). Subsequently, L-Phe ammonia-lyase, the first enzyme of the general phenylpropanoid pathway, produces *trans* cinnamic acid, which can be modified by cinnamate-4-hydroxylase to *p*-coumaric acid, the monomer used for the formation of flavonoids and lignins. Alternatively, *trans* cinnamic acid can be converted to BA and subsequently to SA by shortening of the side chain by two carbons. The two possible pathways are the so-called CoA-dependent, β-oxidative pathway, which is located in peroxisomes, and the CoA-independent, non-β-oxidative pathway (for review see Vlot et al. 2009).

In the CoA-dependent, β-oxidative pathway, *trans* cinnamic acid is first activated to cinnamoyl-CoA by the peroxisomal acyl-CoA activating enzyme cinnamate: CoA ligase (CNL) or initially referred to as putative benzoate: CoA ligase (BZO1, Kliebenstein et al. 2007; Klempien et al. 2012; Colquhoun et al. 2012; Lee et al. 2012; Gaid et al. 2012). This enzyme has an important function in channeling metabolic flux from the general phenylpropanoid pathway into benzenoid metabolism (Gaid et al. 2012). The CoA-dependent β-oxidative route has been shown to be active in several plant species. CNLs have been isolated and functionally characterized from *Arabidopsis*, *Petunia hybrida* and *Hypericum* (Kliebenstein et al. 2007; Klempien et al. 2012; Colquhoun et al. 2012; Lee et al. 2012). In *Hypericum calycinum* cell cultures, *CNL* is induced in response to elicitor treatment and is a key enzyme for the production of the BA-derived xanthone phytoalexin, hyperxanthone E (Gaid et al. 2012).

Subsequent intermediates of cinnamic acid β-oxidation are 3-hydroxy-3-phenylpropanoyl-CoA and 3-oxo-3-phenylpropanoyl-CoA (Fig. 12.3). In *P. hybrida*, the β-oxidation enzyme 3-ketoacyl-CoA thiolase was shown to cleave 3-oxo-3-phenylpropionyl-CoA to benzoyl-CoA using a gene silencing approach (Van Moerkercke et al. 2009). The CoA moiety of benzoyl-CoA is predicted being cleaved to release free BA by one of the seven peroxisomal small thioesterases carrying predicted PTS1s (Reumann et al. 2004; Lingner et al. 2011) but the exact isoform remains elusive. The peroxisomal *Arabidopsis* enzyme CHY1 (beta-hydroxyisobutyryl-CoA hydrolase), which is involved in valine catabolism, exhibits strong affinity for cinnamoyl-CoA and might play an additional, yet unknown, role in BA biosynthesis (Zolman et al. 2001; Ibdah and Pichersky 2009).

The CoA-independent, non-β-oxidative pathway leads via the intermediate benzaldehyde to the production of benzoate. The molecular basis of several enzymes and reaction mechanisms remains largely unknown and will not be reviewed here.

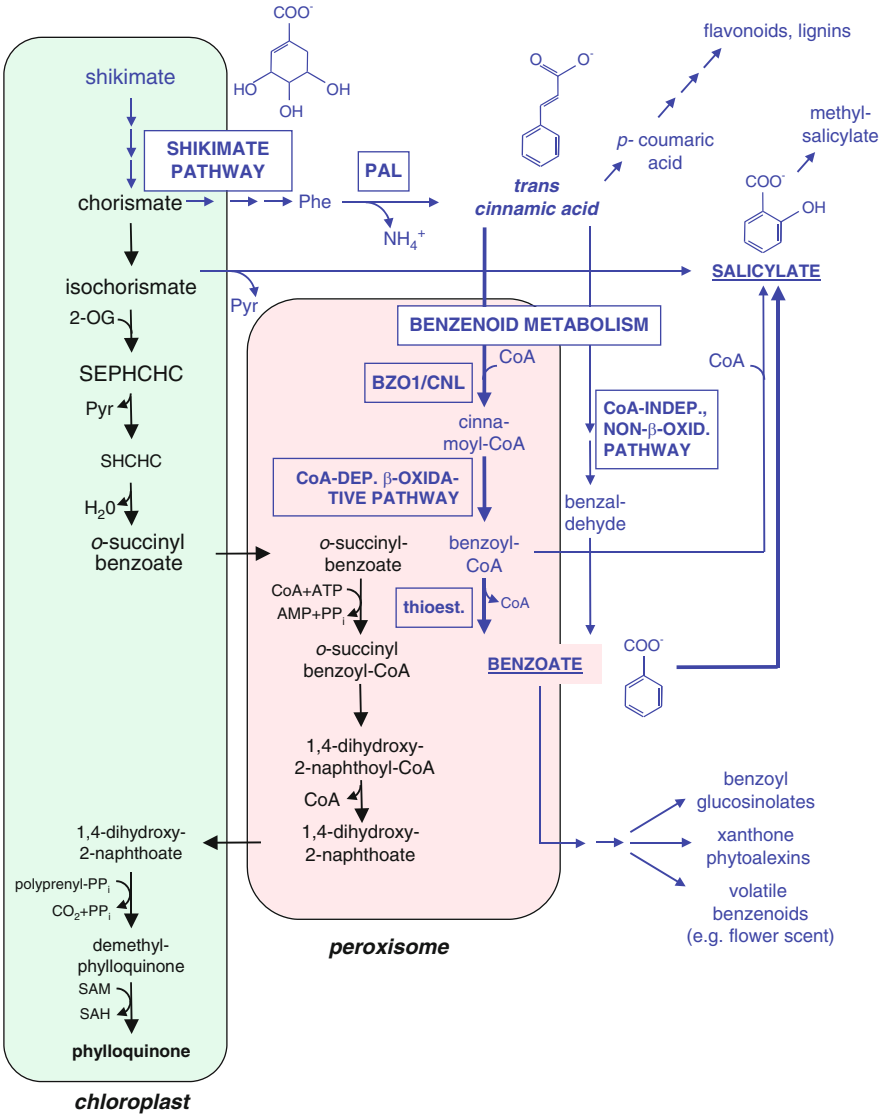

Fig. 12.3 Integration of phylloquinone biosynthesis into branching pathways for the synthesis of the signalling molecules. Biosynthetic pathways of signalling molecules such as SA associated with phylloquinone biosynthesis are added in *blue*. Two alternative Phe-dependent pathways lead to the synthesis of BA as SA precursor, namely the (generally dominating) peroxisomal CoA-dependent, β-oxidative pathway (*left*) and the CoA-independent, non-β-oxidative pathway (*right*). PAL, L-Phe ammonia-lyase

12.10 Future Perspectives

Nearly each year, novel peroxisomal enzymes are functionally characterized at the molecular level and novel metabolic functions of plant peroxisomes are uncovered. Plant peroxisomes thereby emerge as an important cell compartment for the interconnected networks of phylloquinone, benzoic and salicylic acid biosynthesis. Numerous mechanisms to regulate intermediate flux and the fine-tuned inducible production of secondary metabolites, including signalling molecules, await their characterization at the molecular level. The unexpected complexity and plasticity of plant peroxisomal functions appears to exceed that of animal and fungi peroxisomes. While plant peroxisome research, particularly that related to organelle biogenesis, used to benefit tremendously from the knowledge gained in baker's yeast and animal model systems, Arabidopsis now emerges as a valuable model organism to study unexpected functions of peroxisomes such as biosynthetic capabilities for secondary metabolites and adaptive mechanisms conferring resistance to abiotic and biotic stresses. Inducible (and hence difficult to describe de novo) and/or rudimentary stress adaptation functions, homologous or analogous to those described in plants, might also exist in fungi and animal peroxisomes and could be uncovered by advanced bioinformatic approaches followed by wet-lab validations and in-depth characterizations at the molecular level. It is time to pull down the walls of kingdom-specific organelle research and to jointly explore, collaborate and enjoy this exciting era of peroxisome research.

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Chapter 13
Role of Peroxisomes as a Source of Reactive Oxygen Species (ROS) Signaling Molecules

Luisa M. Sandalio, María Rodríguez-Serrano, María C. Romero-Puertas, and Luis A. del Río

Contents

Abstract Peroxisomes are very dynamic and metabolically active organelles and are a very important source of reactive oxygen species (ROS), H_2O_2, $O_2^{\cdot -}$ and $\cdot OH$, which are mainly produced in different metabolic pathways, including fatty acid β-oxidation, photorespiration, nucleic acid and polyamine catabolism, ureide metabolism, etc. ROS were originally associated to oxygen toxicity; however, these reactive species also play a central role in the signaling network regulating essential processes in the cell. Peroxisomes have the capacity to rapidly produce and scavenge H_2O_2 and $O_2^{\cdot -}$ which allows to regulate dynamic changes in ROS levels. This fact and the plasticity of these organelles, which allows adjusting

L.M. Sandalio (✉) • M. Rodríguez-Serrano • M.C. Romero-Puertas • L.A. del Río
Departmento de Bioquímica, Biología Celular y Molecular de Plantas,
Estación Experimental del Zaidín, CSIC, Apartado 419, E-18080 Granada, Spain
e-mail: luisamaria.sandalio@eez.csic.es

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their metabolism depending on different developmental and environmental cues, makes these organelles play a central role in cellular signal transduction. The use of catalase and glycolate oxidase loss-of-function mutants has allowed to study the consequences of changes in the levels of endogenous H_2O_2 in peroxisomes and has improved our knowledge of the transcriptomic profile of genes regulated by peroxisomal ROS. It is now known that peroxisomal ROS participate in more complex signaling networks involving calcium, hormones, and redox homeostasis which finally determine the response of plants to their environment.

Keywords Antioxidants • Peroxisomes • Reactive oxygen species • ROS • Signaling • Stress

Abbreviations

6PGDH	6-P-gluconate dehydrogenase
ACX	Acyl CoA oxidase
ALL	Allantoin
AO	Amine oxidase
APX	Ascorbate peroxidase
ASC	Reduced ascorbate
CAT	Catalase
CFP	Cyan fluorescent protein
DAR	Dehydroascorbate reductase
DHA	Dehydroascorbate
ESR	Electron spin resonance
G6PDH	Glucose-6-P-dehydrogenase
GFP	Green fluorescent protein
GOX	Glycolate oxidase
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSNO	*S*-nitrosoglutathione
GSSG	Oxidized glutathione
GST	Glutathione *S*-transferase
HAOX	2-hydroxy acid oxidase
IAA	Indole acetic acid
ICDH	Isocitrate dehydrogenase
JA	Jasmonic acid
MDAR	Monodehydroascorbate reductase
NDK	Nucleoside diphosphate kinase
$ONOO^-$	Peroxynitrite
PA	Polyamines
PEX	Peroxins

PMP	Peroxisomal membrane polypeptide
POX	Peroxidases
PPAR	Peroxisome proliferator-activated receptor
Prx	Peroxiredoxin
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SA	Salicylic acid
SO	Sulfite oxidase
SOD	Superoxide dismutase
SOX	Sarcosine oxidase
TPX	Thioredoxin-dependent peroxidase
TRX	Thioredoxin
UA	Uric acid
UO	Urate oxidase or uricase
XDH	Xanthine dehydrogenase
XOD	Xanthine oxidase
YFP	Yelow fluorescent protein

13.1 Introduction

Peroxisomes are ubiquitous organelles in eukaryotic cells bounded by a single membrane which do not contain DNA. De Duve and Baudhuin (1966) identified biochemically these organelles and proposed the functional term "peroxisomes" based on the presence of several H_2O_2-containg oxidases and catalase. Plant peroxisomes contain a granular matrix and can present crystalline or amorphous inclusions composed by catalase (Fig. 13.1A, B). Initially, peroxisomes were considered as cell garbage depots, where by the action of catalase the H_2O_2 produced by different oxidases present in these organelles was removed. However, now it is well known that peroxisomes are very dynamic and metabolically active organelles which participate in different cellular processes involved in development, morphogenesis and cell response to stress (del Río et al. 2006), being the detoxification of H_2O_2 and fatty acid β-oxidation perhaps the most conserved functions in all organisms from yeasts to humans (Hu et al. 2012). However, in recent years transcriptomic and proteomics approaches have revealed that these organelles are much more complex and new functions have been discovered (Reumann et al. 2009; Hu et al. 2012).

An important characteristic of peroxisomes is their oxidative metabolism. They contain an important number of oxidases which produce H_2O_2, and different sources of superoxide radicals have been also demonstrated in these organelles (del Río et al. 2002, 2006). Peroxisomes also contain a complex battery of antioxidant defences involved in the regulation of H_2O_2 and superoxide radical accumulation and avoiding their toxicity (del Río et al. 2002, 2006). Under stress conditions imposed by different abiotic factors such as xenobiotics, heavy metals, ozone or nutrient imbalances, alterations of H_2O_2 production and its scavenging can take

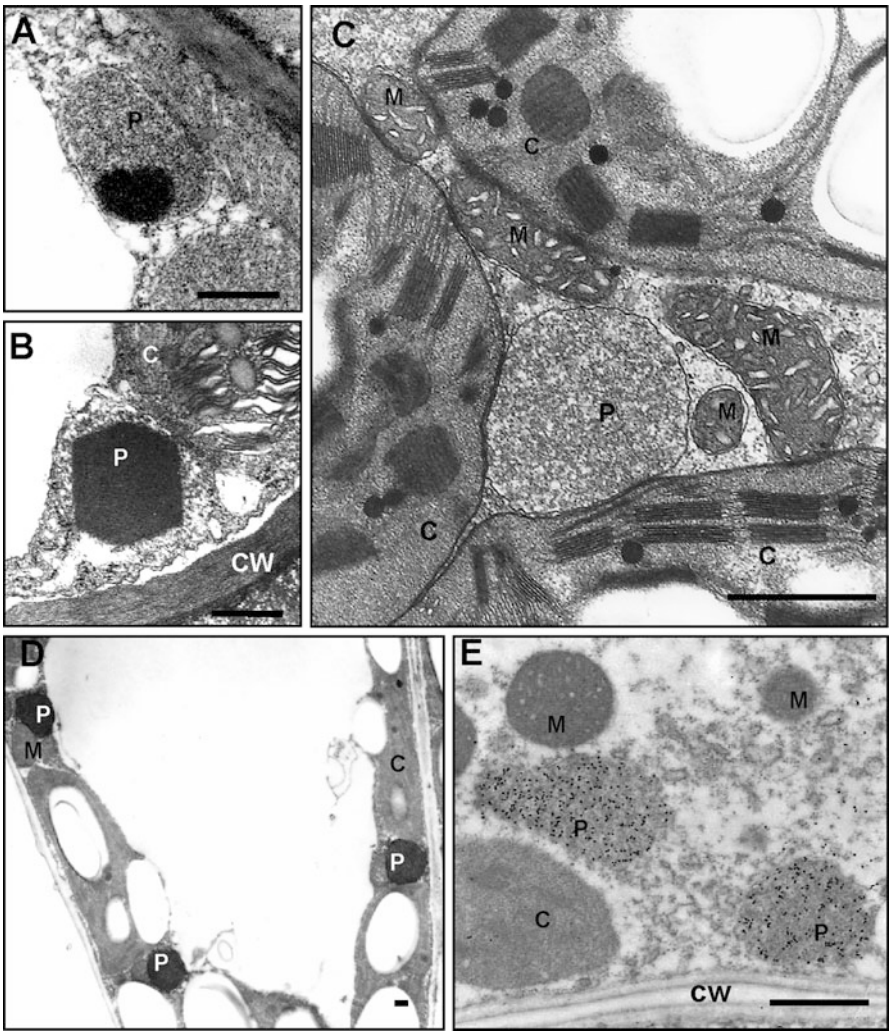

Fig. 13.1 Electron micrography showing the ultrastructure of peroxisomes from different plant leaves and the presence of catalase as the major component of peroxisomes. (**A**) Olive leaves showing an amorphous core inside the peroxisome. (**B**) Pepper leaves showing a peroxisome containing a cristal core. (**C**) Pea leaf showing close contact between chloroplasts, mitochondria and a peroxisome. (**D**) Cytochemical staining of catalase with 3,3′-diaminobenzidine (DAB) in mesophyll cells from pea leaves. (**E**) Immunolocalization of CAT in peroxisomes from pea leaves, using an antibody against pumpkin catalase. Immunogold particles are localized in peroxisomes. *C* chloroplast, *CW* cell wall, *M* mitochondrion, *P* peroxisome. Magnifcation bar = 1 μm

place, bringing about severe oxidative damages (del Río et al. 2002). However, H_2O_2 and other reactive oxygen species (ROS) can act as signaling molecules and are involved in the activation of different developmental and stress response mechanisms (Vanderauwera et al. 2009; Mittler et al. 2011). This review will

be focused on the production of ROS in peroxisomes and its scavenging by antioxidative enzymes and the role of peroxisomal ROS as signaling molecules that can trigger cell responses to biotic and abiotic stress conditions.

13.2 Peroxisomes Are an Important Source of Reactive Oxygen Species

The term reactive oxygen species (ROS) refers to species derived from the reduction of oxygen, including the free radicals superoxide ($O_2^{\cdot -}$), hydroxyl (·OH), alkoxyl (RO·) and peroxyl (ROO·), but also some non-radical compounds such as hydrogen peroxide (H_2O_2), the excited oxygen species singlet oxygen ($^1O_2$), ozone (O_3), hypochlorous acid (HOCl), peroxynitrite ($ONOO^-$), etc (Halliwell and Gutteridge 2007). These species are continuously produced as by-products of aerobic metabolism in different metabolic pathways and their accumulation is regulated by a complex system of antioxidative defences. The reactivity of each ROS can vary, being ·OH the stronger oxidising species which has a very short half-life and can react with all types of biomolecules, mainly membrane fatty acids, DNA and proteins, giving rise to severe disturbances in cell metabolism (Halliwell and Gutteridge 2007).

ROS production take place in many compartments of plant cells, including chloroplasts, mitochondria, plasma membrane, apoplasts and nuclei. Although mitochondria and chloroplasts were considered the most prominent sources of ROS due to the electron transport chain present in these organelles, in the last years peroxisomes have gained protagonism as one of the main contributors for ROS production in cells.

13.2.1 Sources of Superoxide Radicals

The production of superoxide radicals in peroxisomes was first demonstrated in peroxisomes from watermelon cotyledons (Sandalio et al. 1988) and in pea leaf peroxisomes (del Río et al. 1989). Two different sources of $O_2^{\cdot -}$ were demonstrated which were associated to the peroxisomal matrix and membranes, respectively. Superoxide radical production in the peroxisomal matrix was detected by both biochemical methods and electron spin resonance (ESR) spectroscopy in peroxisomes purified from pea leaves and watermelon cotyledons, and was due to the enzyme xanthine oxidase (XOD) (Sandalio et al. 1988; del Río et al. 1989). The presence of XOD in peroxisomes from pea leaves has been confirmed by immunogold electron microscopy (Corpas et al. 2008), although in other plant species it has been described to be present in the cytosol (Werner and Witte 2011). Xanthine oxidase is a xanthine oxidoreductase which catalyses the oxidation of xanthine or hypoxanthine to uric acid which is further used by urate oxidase or uricase (UO) giving rise to allantoin (Werner and Witte 2011). Both enzymes (XOD and UO) are

key enzymes in the catabolism of nucleic acids (Werner and Witte 2011). Xanthine oxidoreductases are present in two forms differing in their electron aceptor, being NAD^+ the preferred electron acceptor (xanthine dehydrogenase form, XDH), but in absence of NAD^+ the enzyme catalyzes the reduction of O_2 to $O_2^{\cdot -}$ (xanthine oxidase form, XOD). However, in *Arabidopsis* plants XDH can also produce $O_2^{\cdot -}$ (Hesberg et al. 2004). XOD and XDH are interconverted by proteolysis (Corpas et al. 2008) and by regulating the sulfuration state of the molybdenum cofactor in the protein (Werner and Witte 2011). Urate oxidase is also localized in peroxisomes and can produce $O_2^{\cdot -}$ (Sandalio et al. 1988).

Another source of $O_2^{\cdot -}$ in peroxisomes is associated to an electron transport chain in the peroxisomal membrane (Sandalio et al. 1988; del Río et al. 1989; del Río and Donaldson 1995). This electron transport chain appears to be similar to that reported by Fang et al. (1987) which was composed by NADH:ferrycianide reductase and a cytochrome *b*. Three integral peroxisomal membrane polypeptides (PMPs) were characterized as responsible for $O_2^{\cdot -}$ production in pea leaf peroxisomal membranes, having molecular masses of 18, 29, and 32 kDa. The PMP18 is the main source of $O_2^{\cdot -}$, requires NADH and was proposed to be a cyt *b* (López-Huertas et al. 1999). The PMP32 is a flavoprotein which uses NADH and can transfer electrons to cytochrome *c* or oxygen and, on the basis of its immunoreactivity and biochemical properties could be the monodehydroascorbate reductase (MDAR) (López-Huertas et al. 1999). The PMP29 uses NADPH as electron donor and can transfer electrons to cytochrome *c* and O_2, and has been proposed to be related to the peroxisomal NADPH:cytochrome P-450 reductase (López-Huertas et al. 1999). This electron transport chain could participate in the regeneration of NAD^+ necessary for different metabolic pathways in peroxisomes, and the production of $O_2^{\cdot -}$ could be a consequence of the normal function of this electron-transport chain (del Río et al. 1990; del Río and Donaldson 1995; López-Huertas et al. 1999; Donaldson 2002).

Plant sulfite oxidase (SO) participates in sulfite detoxification and the assimilatory reduction of sulfate and can react with O_2 producing $O_2^{\cdot -}$ (Byrne et al. 2009). Immunogold electron microscopy and transient expression of SO-GFP have demonstrated the presence of this protein in plant peroxisomes (Nowak et al. 2004; Byrne et al. 2009). The function of this enzyme is not very well known and it could be to protect peroxisomal enzymes, like catalase, from inactivation by sulfite.

Superoxide accumulation in peroxisomes can be imaged *in vivo* by confocal lasser microscopy using fluorescent probes such as dihydroethidium (Rodriguez-Serrano et al. 2009; Fig. 13.2 panel A).

13.2.2 Sources of Hydrogen Peroxide

One of the main sources of H_2O_2 in peroxisomes is the photorespiratory reaction of glycolate oxidase (GOX). Photorespiration is considered as a protective mechanism of plants to prevent photoinhibition under conditions of low CO_2 availability

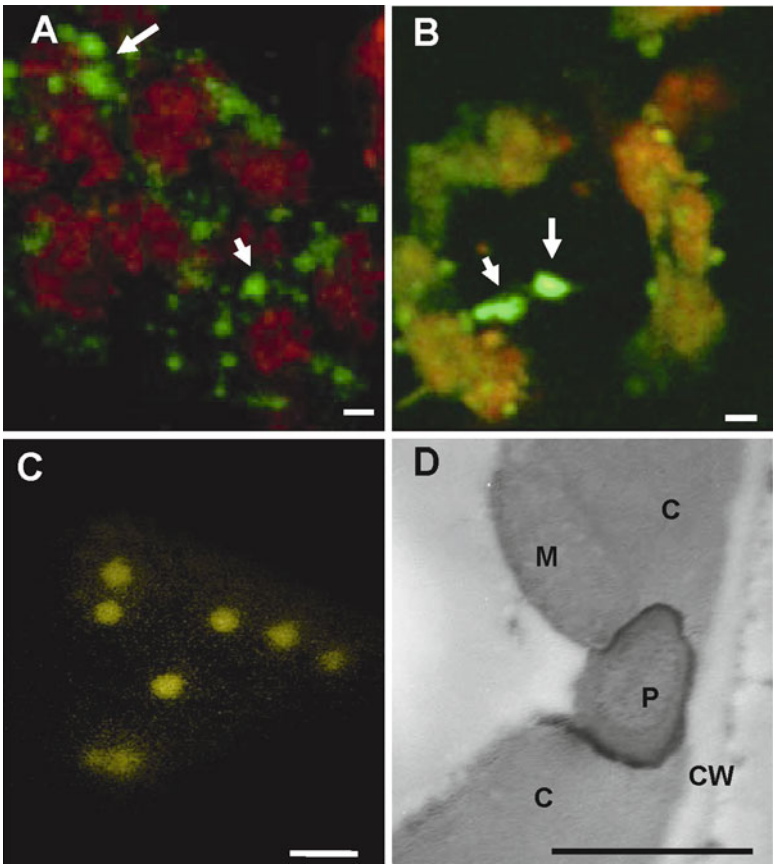

Fig. 13.2 Imaging of ROS production in peroxisomes. (**A**) $O_2^{\cdot -}$ production induced by $CdCl_2$ (50 μM) in pea leaves, detected by confocal laser microscopy using dihydroethidium. (**B**) H_2O_2 accumulation induced by $CdCl_2$ (50 μM) in pea leaves, by confocal laser microscopy using 2′,7′-dichlorofluorescein diacetate. (**C**) Detection by fluorescence microscopy of H_2O_2 induced by the herbicide 2,4-D (23 mM) in tobacco leaves transiently expressing SKL-HyperAs. (**D**) Cytochemical detection of H_2O_2 with $CeCl_3$ in leaf cells from pea plants treated with $CdCl_2$. Image shows a gradient of cerium perhydroxide precipitates, being maximum at the peroxisomal membrane in contact with mitochondria and chloroplasts. *C* chloroplast, *CW* cell wall, *M* mitochondrion, *P* peroxisome. *Arrows* in panels A and B show peroxisomes. Bars = 2 μm

and high irradiation (Yamaguchi and Nishimura 2000; Reumann 2002). The rate of H_2O_2 production in peroxisomes during this process is twice higher than in chloroplasts and even 50-fold higher than in mitochondria (Foyer and Noctor 2003). Photorespiration is a light-dependent process which results in the uptake of O_2 and the release of CO_2, and is compartmentalized in chloroplasts, peroxisomes and mitochondria (Foyer et al. 2010). The close metabolic relationship between these organelles is visible at ultrastructural level by the physical contact between

them (Fig. 13.1C). Basically, during photorespiration glycolate from chloroplasts enters into peroxisomes where it is oxidized to glyoxylate by the glycolate oxidase with production of H_2O_2 (Foyer et al. 2010). The genome of *Arabidopsis thaliana* reveals the existence of five genes of glycolate oxidase: *GOX1* and *GOX*2 are very similar and are the main photorespiratory enzymes; *GOX3* is more abundant in non-photosynthetic tissues; and the more divergent genes, *HAOX1* and *HAOX2,* whose function is not well known and could be involved in the metabolism of 2-hydroxy acids (Reumann 2002).

Fatty acid β-oxidation is another important source of H_2O_2 in peroxisomes. This metabolic pathway is more predominant in peroxisomes of germinating seeds where stored lipids are used to feed the new seedling, although fatty acid β-oxidation is also involved in the metabolism of the phytohormones jasmonic acid and auxin (Baker et al. 2006; see Chaps. 14 and 16). The β-oxidation starts with the acyl-CoA oxidase (ACX) reaction which catalyses the oxidation of acyl-CoA to *trans*-2-enoyl-CoA with production of H_2O_2 (Baker et al. 2006; Kaur et al. 2009). Fatty acid β-oxidation pathway is needed for plant growth and development and is induced in senescence where degraded lipids can be used as a source of carbon (Castillo and León 2008; Yang and Ohlrogge 2009). In contrast to animals and yeasts, in plants the regulation at transcriptional and posttranscriptional level of those genes involved in β-oxidation is not well known. In mammal cells β-oxidation genes, including ACX, are regulated by peroxisome proliferators, mainly polyunsaturated fatty acids and xenobiotics such as clofibrate, which activates nuclear transcription factors called peroxisome proliferator-activated receptors (PPARs) (Reddy and Hashimoto 2001). β-oxidation in plants is also regulated by peroxisome proliferators such as herbicides (Romero-Puertas et al. 2004a; McCarthy-Suárez et al. 2011) and clofibrate (Palma et al. 1991; Nila et al. 2006), and the induction of β-oxidation is accompanied by changes in the number and size of peroxisomes (Palma et al. 1991; Nila et al. 2006; Castillo et al. 2008). Although in plants PPARs have not been identified so far, the expression of α-PPAR from *Xenopus laevis* in tobacco plants gave rise to a similar response to that observed in mammalian cells which demonstrates the existence of a heterologous system in plants (Nila et al. 2006).

Both the spontaneous and the enzymatic dismutation of $O_2^{\cdot -}$, catalyzed by SOD, is another source of H_2O_2. Sarcosine oxidase (SOX) has been recently incorporated to the list of peroxisomal H_2O_2-producing proteins in plants. SOX is a FAD-dependent oxidase that catalyzes the oxidation of sarcosine, some *N*-methyl amino acids and l-pipecolate, producing formaldehyde, glycine and H_2O_2 (Goyer et al. 2004). The catabolism of polyamines (PA) is another source of H_2O_2 in peroxisomes. Diamine oxidases and polyamine oxidases have been localized in peroxisomes from *Arabidopsis thaliana* (Kamada-Nobusada et al. 2008) and rice plants (Osno et al. 2012), which suggests a role for peroxisomes in the catabolism of polyamines.

The production of H_2O_2 at subcellular level can be visualized by using fluorescent probes such as 2′,7′-dichlorofluorescein diacetate and confocal microscopy (Fig. 13.2 panel B; see Rodriguez-Serrano et al. 2009) by expressing transient

or constitutively specific H_2O_2 biosensors, like HyperAs, targeted to peroxisomes (Fig. 13.2C; see Costa et al. 2010) or by a cytochemical approach using $CeCl_3$ (Fig. 13.2 panel D; see Romero-Puertas et al. 2004b).

13.3 Antioxidant Defences in Peroxisomes

Reactive oxygen species are continuously being produced during the aerobic metabolism of cells and can have a signaling role in the regulation of important cellular processes such as development or cell responses to biotic and abiotic stresses. However, the accumulation of ROS is dangerous to aerobic organisms because they can promote oxidative damages to lipids, proteins and DNA which can compromise the cell viability (Halliwell and Gutteridge 2007). It is for this reason why the steady-state levels of these reactive molecules are regulated by a complex set of ROS-scavenging systems. The subtle control of ROS production and ROS scavenging will enable these reactive species to act as signaling molecules or damaging molecules. Figure 13.3 summarizes the different metabolic pathways involved in ROS production in peroxisomes and the antioxidative defenses which have been described to be present in these organelles.

13.3.1 Catalase

Catalase (CAT) is one of the most abundant proteins in plant peroxisomes, comprising 10–25 % of total peroxisomal protein (Tolbert 1980) and is widely used as a peroxisomal marker (Fig. 13.1D, E). CAT catalyzes the degradation of H_2O_2 to H_2O and O_2, but also can reduce H_2O_2 to H_2O using different electron donors, thus showing peroxidatic activity (Fig. 13.1D). CAT shows a weak affinity for its substrate, H_2O_2, and, therefore, it needs high H_2O_2 concentrations to work efficiently (Mhamdi et al. 2012), but plant catalase is activated by Ca^{+2} and calmodulin (Yang and Poovaiah 2002; Costa et al. 2010). Three genes encoding CAT have been identified in tobacco, *Arabidopsis*, pumpkin or maize (Mhamdi et al. 2012). In *Arabidopsis*, *CAT2* expression is associated to the photorespiration pathway, *CAT1* expression is associated to fatty acid β-oxidation and *CAT3* is linked to senescence processes (Mhamdi et al. 2012).

13.3.2 Superoxide Dismutases

Superoxide dismutases constitute a very important antioxidative defence carrying out the dismutation of $O_2^{\cdot -}$ to H_2O_2 and O_2, and prevent oxidative damages to other enzymes, such CAT. SODs are present in most cell compartments,

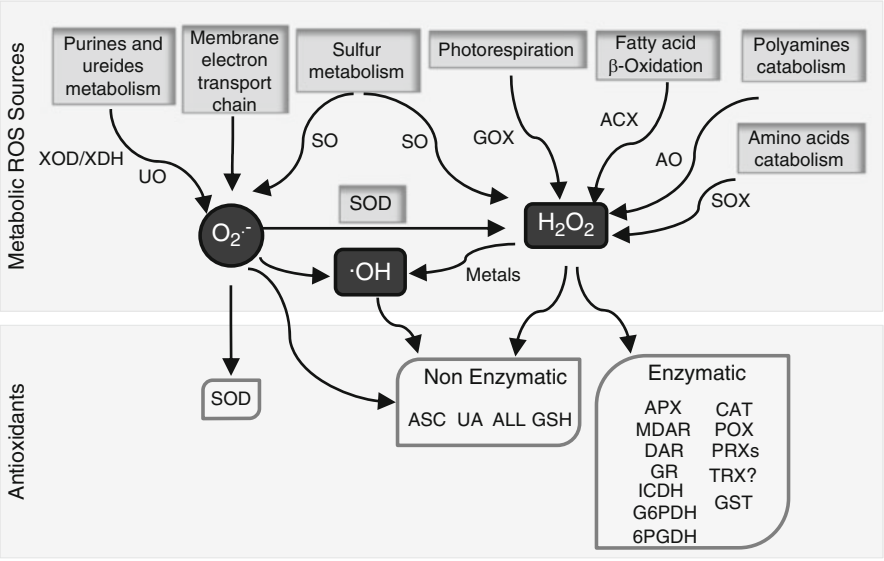

Fig. 13.3 Hypothetical model of ROS production and scavenging in plant peroxisomes. *ACX* acyl CoA oxidase, *ASC* reduced ascorbate, *ALL* allantoin, *AO* amine oxidase, *APX* ascorbate peroxidase, *CAT* catalase, *DAR* dehydroascorbate reductase, *GOX* glycolate oxidase, *GR* glutathione reductase, *G6PDH* glucose-6-P-dehydrogenase, *GSH* reduced glutathione, *GST* glutathione *S*-transferase, *ICDH* isocitrate dehydrogenase, *MDAR* monodehydroascorbate reductase, *6PGDH* 6-P-gluconate dehydrogenase, *POX* peroxidases, *PRXs* peroxiredoxins, *SO* sulfite oxidase, *SOX* sarcosine oxidase, *SOD* superoxide dismutase, *TRX* thioredoxin, *UA* uric acid, *UO* urate oxidase or uricase, *XOD* xanthine oxidase, *XDH* xanthine dehydrogenase

including chloroplasts, mitochondria, nuclei, peroxisomes, cytoplasm and apoplasts (Alscher et al. 2002). Essentially, there are three SOD families differing in the metal present in its prosthetic group: Fe-SODs, Cu,Zn-SODs and Mn-SODs. The presence of SOD in peroxisomes was first demonstrated in pea leaf peroxisomes by immunocytochemistry and biochemical analysis of purified peroxisomes, where a Mn-SOD was detected (del Río et al. 1983; Sandalio et al. 1987). Further studies in peroxisomes from watermelon (glyoxysomes) demonstrated the presence of a Mn-SOD in the peroxisomal membrane and a CuZ-SOD in the matrix (Sandalio and del Río 1988; Sandalio et al. 1997; Rodríguez-Serrano et al. 2007). The occurrence of SOD in peroxisomes was extended to other plant species and even human and animal cells (Keller et al. 1991; del Río and Donaldson 1995; del Río et al. 2002) and yeast (Petrova et al. 2009). However, the gene encoding the Mn-SOD from pea leaf peroxisomes, in contrast to the mitochondrial enzyme, has not been identified so far. Differential splicing or post-translational modifications of Mn-SOD could explain its dual localization in mitochondria and peroxisomes (del Río et al. 2003). CuZn-SOD was further on associated to *Arabidopsis* peroxisomes, being this protein encoded by *CSD3* (Kliebenstein et al. 1998).

13.3.3 Ascorbate-Glutathione Cycle

In addition to CAT, peroxisomes have the components involved in the ascorbate-glutathione cycle (ASC-GSH), also called Foyer-Halliwell-Asada cycle, which is an efficient system to decompose H_2O_2 (Foyer and Noctor 2011; del Río 2011). This cycle is present in chloroplasts, cytoplasm, and mitochondria (Foyer and Noctor 2011) and was demonstrated to be present also in peroxisomes from pea leaves by Jimenez et al. (1997). Later on, the occurrence of the ASC-GSH cycle was also reported in peroxisomes of tomato plants (Mittova et al. 2004). This cycle is composed by four enzymes, ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DAR), and glutathione reductase (GR), and needs ascorbate (ASC), dehydroascorbate (DHA), reduced glutathione (GSH) and oxidized glutathione (GSSG) (Halliwell and Gutteridge 2007; del Río 2011). The distribution of the different enzymes has been studied in pea leaf peroxisomes, and APX was located in the cytosolic side of peroxisomal membrane (Jiménez et al. 1997), MDAR was located in both membrane and matrix (López-Huertas et al. 1999; Leterrier et al. 2005; Lisenbee et al. 2005), and GR was only observed in the matrix (Jiménez et al. 1997; Romero-Puertas et al. 2006). The MDAR and APX activities associated to the peroxisomal membranes could participate in the regeneration of NAD^+ needed for peroxisomal metabolism (Fang et al. 1987; del Río and Donaldson 1995; Lisenbee et al. 2005) but also could regulate the H_2O_2 produced by the spontaneous dismutation of $O_2^{\cdot -}$ generated in the NAD(P)H-dependent electron transport chain of the peroxisomal membrane (López-Huertas et al. 1999).

Peroxisomal MDAR requires NADH and has been associated to the 32-KDa PMP in castor bean peroxisomes (Bowditch and Donaldson 1990) and pea leaf peroxisomes (López-Huertas et al. 1999) and it has been characterized at molecular and functional level in pea leaves (MDAR1) (Leterrier et al. 2005).

13.3.4 Glutathione Peroxidase, Glutathione S-Transferase and Peroxiredoxins

The presence of glutathione peroxidase in peroxisomes has been demonstrated in yeast where *GPX1* encodes a glutathione peroxidase which also has an atypical 2-Cys peroxiredoxin activity (Ohdate and Inoue 2012). In *Candida boidini* a glutathione peroxidase was also found in peroxisomes (CbPMP20) (Horiguchi et al. 2001). In the *Arabidopsis* genome two PMP20 homologues, *AtTPX1* and *AtTPX2*, have been demonstrated and the protein encoded by *AtTPX2* has thioredoxin-dependent peroxidase activity in vitro (Verdoucq et al. 1999). AtTPX2 has been considered as a type II peroxiredoxin A and apparently is located in peroxisomes from eukaryote cells (Verdoucq et al. 1999; Dietz 2003). Peroxiredoxins (Prxs) are thioredoxin-dependent peroxidases that catalyze the reduction of H_2O_2, organic

hydroperoxides and peroxinitrite to water, alcohols and nitrite, respectively, but can also sense redox state, and transmit redox information to other partners (Dietz 2003). Prxs have been demonstrated to be present in many cell compartments and also have been detected in peroxisomes from mammalian cells (PrxV) (Seo et al. 2000) and *Hansenula polymorpha* (PMP20) (Aksam et al. 2008). The presence of a peroxiredoxin in pea leaf peroxisomes has also been described although neither the protein has been characterized nor its gene identified yet (del Río et al. 2006). The presence of Prxs in peroxisomes could increase the antioxidant capacity of these organelles, although some other regulatory function for these proteins cannot be ruled out. In addition, three families of glutathione *S*-transferases GSTT1, GSTT2, and GSTT3 have been identified in peroxisomes where they could participate in removing toxic hydroperoxides due to their glutathione peroxidase activity (Dixon et al. 2009).

13.3.5 NADPH-Generating Dehydrogenases

Peroxisomal metabolism consume NADPH in different pathways, such as the ASC-GSH cycle, the fatty acid β-oxidation, or the jasmonic acid biosynthesis. To keep functional those enzymes plant peroxisomes contain at least three different sources of NADPH, the enzyme isocitrate dehydrogenase (ICDH), and two oxidative enzymes of the pentose phosphate pathway, including glucose-6-P-dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGDH) (Corpas et al. 1999; del Río et al. 2002). More recently, proteomics studies in *Arabidopsis* have corroborated the presence of ICDH and 6PGDH in peroxisomes (Fukao et al. 2003; Reumann et al. 2009). On the other hand, *de novo* biosynthesis of NADPH can also take place in peroxisomes by phosphorylation of NADH catalysed by a NADH kinase 3 recently localized in peroxisomes (Waller et al. 2010).

13.4 Post-Translational Regulation of ROS Production in Peroxisomes

Post-translational modifications have an important role in the regulation of catalytic activity of proteins, their stability, interactions between proteins, and subcellular localization (Van Bentem et al. 2006). The regulation of ROS metabolism by post-translational modification such as oxidation, phosphorylation or *S*-nitrosylation has not been explored in-depth so far. Due to the high production of ROS in peroxisomes, mainly under certain adverse conditions, peroxisomal proteins should be prone to oxidation by carbonylation of C groups. Romero-Puertas et al. (2002) have demonstrated that oxidative stress induced by cadmium produces the oxidation of peroxisomal proteins, being CAT, glutathione reductase and Mn-SOD some of

the carbonylated proteins identified. These proteins were more efficiently degraded by the peroxisomal proteases induced by the cadmium treatment (Romero-Puertas et al. 2002; McCarthy et al. 2001). Malate synthase, isocitrate lyase, malate dehydrogenase and CAT have been found to be carbonylated in peroxisomes isolated from castorbean endosperm (Nguyen and Donaldson 2005). However, the nature of the proteases involved in degrading oxidatively modified proteins in peroxisomes and the genes encoding these proteins are not well known. The *Arabidopsis* genome analysis and proteomic studies have disclosed at least nine proteases predicted to be in peroxisomes and some of them have been demonstrated to be involved in the import and processing of proteins in these organelles and in peroxisomes biogenesis (Helm et al. 2007; Lingard and Bartel 2009). Less information is available on proteases involved in the regulation of protein turnover inside peroxisomes, although the presence of a serine protease and different endoproteases has been demonstrated in purified peroxisomes from pea leaves, being CAT, GOX and G6PDH the target of these proteases (Distefano et al. 1997, 1999). Peroxisomal endoproteases have also being suggested to be involved in the conversion of xanthine dehydrogenase into the superoxide-generating xanthine oxidase in these organelles (Distefano et al. 1999). However, the proteolytic activity inside peroxisomes is a theme of debate and there are some results suggesting that damaged or obsolete proteins should be transported outside the organelles for degradation with the assistance of peroxins PEX4, PEX6 and PEX22 (Lingard et al. 2009).

Nitric oxide (NO) and other RNS can oxidize, nitrate or nitrosylate proteins. *S*-nitrosylation refers to the binding of a NO group to a cysteine residue and it plays a significant role in NO-mediated signaling (see Chap. 15). Six peroxisomal proteins have been recently identified as putative targets of *S*-nitrosylation and they are involved in photorespiration, β-oxidation, and ROS detoxification (Ortega-Galisteo et al. 2012). The activity of three of these proteins (CAT, GOX and malate dehydrogenase) was inhibited by NO donors and the *S*-nitrosylation level of CAT and GOX changed in plants treated with Cd and the herbicide 2,4-D, suggesting that this posttranslational alteration could be involved in the regulation of H_2O_2 accumulation under abiotic stress and also could regulate in peroxisomes the flux of metabolites between different metabolic pathways (Ortega-Galisteo et al. 2012).

Phosphorylation/dephosphorylation is one of the most common mechanisms used by the cell to regulate the activity or functionality of different proteins. The phosphoproteome of leaf peroxisomes has been studied and CAT and GOX have been identified as targets of this post-translational modification (Pazmiño 2009). Analysis of the activity of these proteins in the presence of kinases and phosphatases inhibitors demonstrated a fine regulation of both enzymes by coordinated phosphorylation/dephosphorylation (Pazmiño 2009). The phosphoproteome analysis of pea leaves peroxisomes and proteomic studies in wheat embryos have shown that Mn-SOD is also phosphorylated, although there is no information on the effect of this modification on Mn-SOD activity (Pazmiño 2009; Irar et al. 2010). However in *Listeria* Mn-SOD was down-regulated by phosphorylation (Archanbaud et al. 2006). Xanthine oxidase has also been found to be phosphorylated in animal tissues, althougt its effect on XOD activity has not been studied (Kayyali et al. 2001). The

presence of kinases (Fukao et al. 2003; Reumann et al. 2009; Dammann et al. 2009; Coca and San Segundo 2010) and phosphatases (Matre et al. 2009) in peroxisomes has been reported in recent years, although their specific targets and function have not been established yet. In *Arabidopsis* plants, the interaction between CAT and the nucleoside diphosphate kinase (NDK-1) has been studied, and *Arabidopsis* lines overexpressing NDK-1 showed higher tolerance to oxidative stress imposed by paraquat and H_2O_2 (Fukamatsu et al. 2003).

All these results suggest that in response to metabolic or environmental changes the accumulation of H_2O_2 and $O_2^{\cdot -}$ in plant peroxisomes can be regulated by post-translational modification of those proteins involved in their production and/or scavenging, which allows a fine level of regulation of ROS function in the cell and a fast response to the environment.

13.5 Peroxisomes as Producers of ROS Signaling Molecules

Reactive oxygen species were originally associated to oxygen toxicity derived from aerobic metabolism. Different studies in plants demonstrated that ROS over-accumulation is responsible for the toxicity of several stress factors such as high light intensity, ozone, heavy metals, xenobiotics, low and high temperatures, mechanical wounding or pathogen infection (Dat et al. 2000; Sandalio et al. 2012; Mullineaux et al. 2006). Plant peroxisomes are one of the main cellular sources of ROS and different studies have demonstrated their participation in the oxidative stress induced by xenobiotics like clofibrate or 2,4-dichlorophenoxyacetic acid (Palma et al. 1991; Nila et al. 2006; Romero-Puertas et al. 2004a; McCarthy-Suárez et al. 2011), heavy metals (Romero-Puertas et al. 1999), salinity (del Río et al. 2002; Mittova et al. 2004), ozone (Pellinen et al. 1999) or senescence (del Río et al. 1998; Rosenwasser et al. 2011). However, during the last decade it has been demonstrated that ROS play a central role in the complex signaling network which regulates essential processes in the cell including stress response (Vanderauwera et al. 2009; Mittler et al. 2011).

Most sources of ROS are associated to metabolic pathways, such as photorespiration, ureide metabolism or fatty acid β-oxidation, in addition to electron transport chains. For this reason, disturbances in any of those processes would give rise to changes in the accumulation of ROS, and this situation can be perceived by the cell as an alarm, and so triggering a cascade of events to promote defence responses. The accumulation of ROS in a specific cell compartment can be in itself needed to trigger a specific response (Mittler et al. 2011).

Taken into account the diverse and characteristic metabolism of peroxisomes, these organelles can be considered as an important source of signaling molecules. Peroxisomes have the capacity to rapidly produce and scavenge H_2O_2 and $O_2^{\cdot -}$ thanks to the important battery of antioxidants present in these organelles. Another advantage of peroxisomes as a source of signaling molecules is their metabolic plasticity which allows metabolic adjustments depending on developmental and

environmental cues (del Río et al. 2002, 2006), as well as the ability to rapidly change their motility and population number in response to the plant environmental conditions (Palma et al. 1991; López-Huertas et al. 2000; Castillo et al. 2008; Rodríguez-Serrano et al. 2009; Hu et al. 2012).

Catalase loss-of-function mutants have been an excellent tool to study the consequences of increased levels of endogenous H_2O_2 in peroxisomes. The use of catalase-deficient plants from *Nicotiana tabacum* and *Arabidopsis thaliana* has allowed to study the transcriptional response associated to H_2O_2 and has led to identify genes responsive to elevated levels of photorespiratory H_2O_2 in leaves (Takahashi et al. 1997; Vandenabeele et al. 2004; Vanderauwera et al. 2005). The comparison of different microarray data sets that profiled the *Arabidopsis* transcriptome during elevated photorespiratory H_2O_2 has revealed that a total of 783 transcripts modify their expression in response to elevated levels of photorespiratory H_2O_2 and the majority of them were associated with stress responses, being the greatest overlap observed with heat and osmotic stress (Foyer et al. 2010; Inzé et al. 2012). The subcellular localization of those hydrogen peroxide-induced proteins has been carried out, being most of them associated to nucleus and cytosol (Inzé et al. 2012).

The deficiency of CAT under conditions where photorespiration is very active produces severe disturbances in the redox status, triggering the induction of pathogen–associated processes, such as SA accumulation or pathogenesis-related proteins (PRs) (Takahashi et al. 1997), and the day-length determine the production of lesions by a process which is dependent on SA (Queval et al. 2007; Chaouch et al. 2010). *Arabidopsis* plants over-expressing ectopically GOX in chloroplasts showed similar effects with accumulation of H_2O_2 in these organelles and development of lesions (Fahnenstich et al. 2008). Transcriptomic studies using *Arabidopsis* mutants deficient in GR1 (*gr1*) and CAT2 (*cat2*) have identified some similarities in gene expression profile which was in both cases dependent on growth day length (Mhamdi et al. 2010). The analysis of double mutants *cat2-gr1* showed that GR1-dependent glutathione status regulates the accumulation of H_2O_2 and some processes associated to it, such as lesion formation, SA accumulation, induction of PRs genes, and signaling mediated by JA pathways (Mhamdi et al. 2010). *Arabidopsis* and tobacco double mutants deficient in CAT and APX were more tolerant to stress than single mutants (Rizhsky et al. 2002; Vanderauwera et al. 2011). In the *Arabidopsis* double mutants lacking *APX1* and *CAT2* a specific acclimation response was triggered involving the activation of DNA repair, cell cycle regulation and antiprogrammed cell death mechanisms (Vanderauwera et al. 2011). These experimental approaches have demonstrated a close interaction between H_2O_2 and phytohormone-dependent signaling involving ET, JA, IAA and SA and suggest that redox homeostasis, in particular the rate GSH/GSSG, could modulate this relationship (Queval et al. 2007; Tognetti et al. 2012; Mhamdi et al. 2010).

A transcriptomic study carried out in T-DNA-*Arabidopsis* mutants defective in acyl-CoA oxidase (*ACX1*) has identified genes directly regulated by ACX-dependent H_2O_2 categorized in the following processes: phosphorylation, stress

responses, oxidative stress response, metabolism, defence response, transcription factors, hormone response and signal transduction (Romero-Puertas et al., unpublished results). Comparative analysis of these results with other microarray profiles showed that 90 % of these genes were also affected by other conditions causing oxidative stress (Romero-Puertas et al., unpublished results).

The use of *Arabidopsis* mutants deficient in the peroxisomal enzyme GOX has allowed studying the contribution of H_2O_2 from each GOX isoform to the regulation of cell response to the infection by *Pseudomonas*. Hydrogen peroxide generated specifically by *HAOX2* and *GOX3* activates components of the SA signal transduction cascade and also seems to regulate JA and ET pathways, while *GOX1* and *GOX2* only play a secondary or indirect role on defence responses (Rojas et al. 2012). H_2O_2 generated by GOX could represent a secondary oxidative burst after 24 h of inoculation of *Nicotiana* and *Arabidopsis* plants, triggering a defence response different from that regulated by NADPH oxidases (Rojas et al. 2012). The increase of GOX and glyoxylate aminotranferase has also been associated to the hypersensitive response in *Cucumis melo* line P1 infected with the oomycete *P. cubensis* (Taler et al. 2004).

The balance between ROS production and scavenging is crucial in the regulation of cell response to infection. Recently, Valenzuela-Soto et al. (2011) have observed that transgenic tobacco plants over-expressing a peroxisome proliferator-activated receptor gene from *Xenopus laevis* (*xPPARα*) show higher susceptibility to virulent *Pseudomonas syringae* and a partial loss of resistance to avirulent *Pseudomonas syringae* pathogens as result of a generalized reduction in H_2O_2 and SA levels and an increase in the expression of ET and JA biosynthesis genes (Valenzuela-Soto et al. 2011). Under these conditions, the hypersensitive-response, oxidative burst and systemic-acquired resistance apparently were not affected in the transgenic plants (Valenzuela-Soto et al. 2011). These results suggest that peroxisome proliferation could lead to increased susceptibility to bacterial pathogens in tobacco by altering the redox balance of the plant and the expression pattern of key genes of defense signaling pathways (Valenzuela-Soto et al. 2011). On the other hand, it has been reported that the proliferation of peroxisomes in *Arabidopsis* plants over-expressing *PEX11e* did not improve the tolerance to salt stress (Mitsuya et al. 2011).

The existence of peroxisomal ROS receptors to decode endogenous ROS signals has not been established so far and the role of more complex networks involving calcium, protein phosphorylation or protein *S*-nitrosylation/nitration has to be investigated in-depth. Peroxisomes can store Ca^{2+} (see Chap. 7), which can contribute to regulate H_2O_2 accumulation in these organelles. Costa et al. (2010) have observed that the induction of Ca^{2+} in the cytosol is followed by an increase of Ca^{2+} in peroxisomes which, in turn, give rise to a reduction of H_2O_2 by a Ca^{2+}-dependent activation of catalase. Rodríguez-Serrano et al. (2009) demonstrated the existence of Ca^{2+}-dependent changes in the dynamics of peroxisomes in *Arabidopsis* plants under abiotic stress conditions. Peroxisomes are also a source of NO (see Chap. 15) and also participate in SA, IAA and JA biosynthesis (see Chaps. 14 and 16) which suggests the possibility of cross-talk between ROS and NO, and ROS and the hormones SA, JA and IAA. The integration of environmental stress-related signals

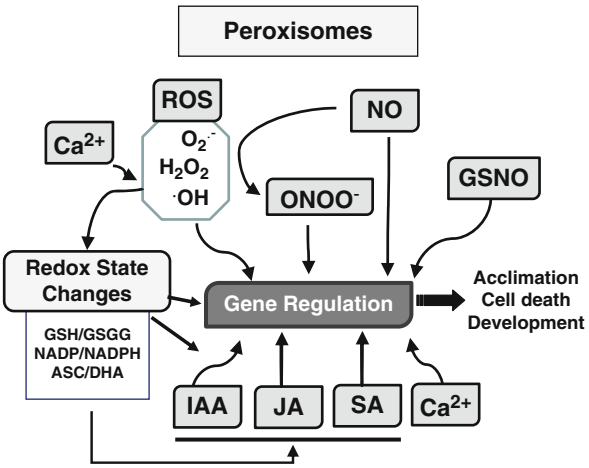

Fig. 13.4 Cross-talk between ROS and different signaling molecules produced in peroxisomes. The interplay between, ROS, RNS (NO, $ONOO^-$ and GSNO), and redox homeostasis, determined by the redox state of glutathione, ascorbate and pyridine nucleotides, calcium concentration and hormone balance, control the peroxisomal signaling network involved in the regulation of acclimation, cell death induction or plant development. *GSNOS*-nitrosoglutathione, *IAA* indole acetic acid, *JA* jasmonic acid, *$ONOO^-$* peroxynitrite, *SA* salicylic acid

by cross-talk between ROS and auxin regulatory networks is an important emerging mechanism to understand the modulation of response to cell stress (Tognetti et al. 2012). Unravelling the different connexions between these networks would be necessary to know the different signaling pathways and the role of peroxisomes in regulating cellular response. Figure 13.4 shows a model of cross-talk between ROS and different signals that could be involved in the peroxisomal ROS-dependent transcription regulation of development and stress response in plants.

13.6 Peroxisomes as Sensors of ROS/Redox Changes

A characteristic property of peroxisomes is their plasticity because they can undergo changes in their size, morphology, metabolism and population depending on the developmental stage or environmental conditions (del Río et al. 2002, 2006; Hu et al. 2012). The population of peroxisomes can increase by dividing pre-existing peroxisomes and this process is associated with cell division and growth, but in plants it can also take place under stress conditions induced by ozone, light, xenobiotics, salinity or metals in a process referred to as peroxisome proliferation (del Río et al. 2002; Oksanen et al. 2003; Kaur et al. 2009; Mitsuya et al. 2011).

One of the challenges in peroxisome research is to dissect signaling pathways governing the regulation of the peroxisomal population under different

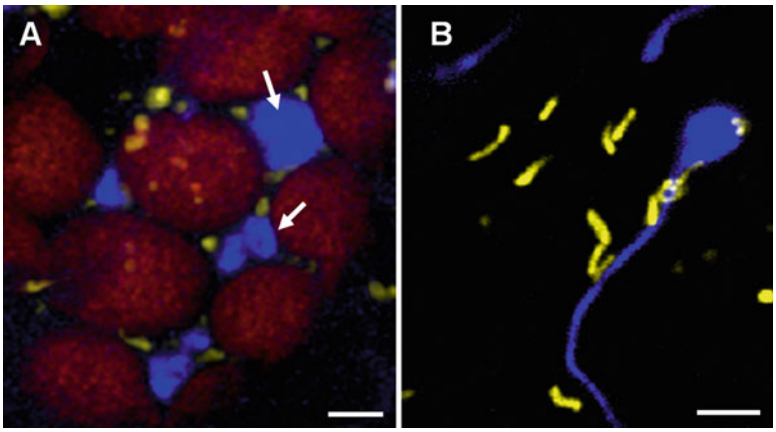

Fig. 13.5 Imaging of peroxisomes, mitochondria and chloroplasts of *Arabidopsis* leaves by confocal laser microscopy. Peroxisomes and mitochondria were imaged in *Arabidopsis lines* expressing CFP in peroxisomes (*blue*) and YFP in mitochondria (*yellow*), and chloroplasts were detected by chlorophyll fluorescence (*red*). (**A**) Mesophyll cells showing close contact between chloroplasts, mitochondria and peroxisomes. (**B**) Coordinated formation of peroxules and matrixules induced by Cd treatment in epidermal cells. Bars = 5 μm

environmental and metabolic conditions. López-Huertas et al. (2000) demonstrated that the induction of peroxisome biogenesis genes is regulated by H_2O_2 in both plant and animal cells and also takes place in response to wounding and infection with an avirulent pathogen (López-Huertas et al. 2000), suggesting that proliferation of peroxisomes could be a protective mechanism to cope with oxidative stress. However, *Arabidopsis* and tobacco lines showing constitutive proliferation of peroxisomes are not more resistant to infection or salt treatment (Mitsuya et al. 2011; Valenzuela-Soto et al. 2011). More recently, Sinclair et al. (2009) have observed that exogenous ROS sources induce the formation of peroxules, which are protuberances of peroxisomes produced previous to elongation, fission and division of these organelles. These changes are observed after few minutes treatment with exogenous ROS sources, and $\cdot$OH appears to be the main ROS involved in this process (Sinclair et al. 2009). Apparently, peroxules formation is a common feature in plants in response to different toxic conditions inducing oxidative stress (Rodríguez-Serrano et al., unpublished results; Fig. 13.5). In *Arabidopsis* plants under stress by cadmium, the dynamics of peroxisomes, in terms of speed of movement, is regulated by ROS and is also dependent on Ca^{+2} ions (Rodríguez-Serrano et al. 2009). These results indicate that peroxisomes could act as cellular sensors of ROS/redox changes by triggering a fast and probably very specific response against environmental cues.

By using *Arabidopsis* lines expressing CFP in peroxisomes and YFP in mitochondria, it has been found that in response to abiotic stress imposed by Cd, peroxisome proliferation takes place in a coordinated way with mitochondria

proliferation (Rodríguez-Serrano et al., unpublished results). ROS produced by environmental cues could induce peroxisome proliferation which, in its turn, could activate the cellular signaling network involving Ca^{+2}, changes in redox homeostasis, phosphorylation/dephosphorylation of proteins and hormones biosynthesis, although the mechanisms of sensing endogenous or external environment changes and the specific role of ROS in these processes are still unknown.

13.7 Conclusions

The presence of different sources of ROS in peroxisomes associated to important metabolic pathways, and the complex battery of antioxidants present in these organelles demonstrate an important role of peroxisomes in the cellular oxidative metabolism. Peroxisomes can act as stress sources, when over-accumulation of ROS takes place, but they can also participate as sensors of oxidative stress induced by different stimuli, and as effectors of the cell response. In addition to ROS, peroxisomes also have a role in hormone biosynthesis and production of reactive nitrogen species (RNS) which can participate in the network involved in the regulation of gene transcription dependent on peroxisomal ROS. On the other hand, the ROS-dependent regulation of peroxisome proliferation in a coordinated way with mitochondria proliferation confers to these organelles a central role in the modulation of cell responses to environmental changes. Further research is necessary to elucidate the molecular mechanisms of perception by peroxisomes of different signals and how they regulate their metabolism, morphology and proliferation, as well as the role of ROS and post-translational modifications of peroxisomal proteins in these processes. The coordination of peroxisomal and mitochondrial proliferation, the cross-talk between both organelles and the signaling events governing this interplay is another exciting field in cell biology that has to be studied in-depth in order to better understand the regulation of plant cell development and the cell response to environmental changes.

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Chapter 14
Peroxisomes as a Source of Auxin Signaling Molecules

Gretchen M. Spiess and Bethany K. Zolman

Contents

Abstract Peroxisomes house many metabolic processes that allow organisms to safely sequester reactions with potentially damaging byproducts. Peroxisomes also produce signaling molecules; in plants, these include the hormones indole-3-acetic acid (IAA) and jasmonic acid (JA). Indole-3-butyric acid (IBA) is a chain-elongated form of the active auxin IAA and is a key tool for horticulturists and plant breeders for inducing rooting in plant cultures and callus. IBA is both made from and converted to IAA, providing a mechanism to maintain optimal IAA levels. Based

G.M. Spiess • B.K. Zolman (✉)
Department of Biology, University of Missouri – St. Louis, St. Louis, USA
e-mail: zolmanb@umsl.edu

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on genetic analysis and studies of IBA metabolism, IBA conversion to IAA occurs in peroxisomes, and the timing and activity of peroxisomal import and metabolism thereby contribute to the IAA pool in a plant. Four enzymes have been hypothesized to act specifically in peroxisomal IBA conversion to IAA. Loss of these enzymes results in decreased IAA levels, a reduction in auxin-induced gene expression, and strong disruptions in cell elongation resulting in developmental abnormalities. Additional activity by known fatty acid β-oxidation enzymes also may contribute to IBA β-oxidation via direct activity or indirect effects. This review will discuss the peroxisomal enzymes that have been implicated in auxin homeostasis and the importance of IBA-derived IAA in plant growth and development.

Keywords Indole-3-butyric acid • IBA • Indole-3-acetic acid • IAA • Auxin • Peroxisome

Abbreviations

2,4-D	2,4-dichloroacetic acid
2,4-DB	2,4-dichlorobutyric acid
ACAD	Acyl-CoA dehydrogenase
APH	Aminoglycoside phosphotransferase
Col	Columbia-0
ECH	Enoyl-CoA hydratase
ETF	Electron transfer flavoprotein
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IBR	IBA-response
JA	Jasmonic acid
NAA	1-napthaleneacetic acid
PAA	Phenylacetic acid
PED	Peroxisome defective
PEX	Peroxin
SDR	Short-chain dehydrogenase/reductase
Ws	Wassilewskija

14.1 IAA Is an Essential Plant Hormone with Tightly Regulated Levels

Auxins are an important class of plant hormones, identified over a century ago and now recognized as key regulators of plant development. Auxins regulate cell division and cell elongation (Perrot-Rechenmann 2010) to influence numerous physiological responses, including embryogenesis, stem elongation and apical

dominance, root architecture, vascular development, expansion of cotyledons and flowers, apical hook maintenance, and differential growth leading to tropic changes (Davies 2004). Indole-3-acetic acid (IAA) is the principal auxin, although related compounds have been identified in plants and several synthetic compounds produce auxin-like responses following application. IAA regulates expression of thousands of genes (Paponov et al. 2008; Simon and Petrasek 2011). Additional non-transcriptional responses, particularly based on changes in membrane biology, regulate auxin responses faster than transcriptional changes allow (Perrot-Rechenmann 2010; Simon and Petrasek 2011).

Mutants with altered auxin levels have dramatic phenotypes, indicating that a specific level of IAA is optimal for proper growth and development. For example, mutants in auxin biosynthesis genes have reduced IAA levels, resulting in significant phenotypic defects. The *yuc1yuc4* mutant has defects in floral development, making the plant sterile (Cheng et al. 2006), and *yucca* quadruple mutants lack normal meristems (Cheng et al. 2007). Similarly, mutants with high auxin levels have developmental changes. The *yuc6-1D* dominant mutant and a *CYP79B2* overexpression line with increased IAA have increased hypocotyl length and long, narrow leaves (Kim et al. 2011).

IAA homeostasis is maintained by biosynthesis, degradation, sequestration, and transport. Modifications of the IAA structure alter its ability to be transported and/or activate signaling. In many cases, these modifications are reversible, allowing sequestration but a quick influx to the active pool when required (Woodward and Bartel 2005). In this review, we will focus on indole-3-butryic acid (IBA). The indole backbone is identical between IBA and IAA, but the acetyl side chain in IAA is elongated in IBA (Fig. 14.1a). Many connections between peroxisomes and IBA metabolism indicate that peroxisomes produce IAA signaling molecules from IBA, which are necessary to maximize plant growth during development and changing environmental conditions.

14.2 The History of IBA

IBA has been used as an exogenous auxin in agriculture and horticulture for many years. IBA is highly efficient at inducing roots on new plants and cuttings (Fig. 14.1b–d). Although all natural and synthetic auxins induce rooting, researchers have chosen IBA based on the efficacy of the response. At the concentrations that IAA is useful for inducing secondary root formation, IAA significantly inhibits root elongation (de Klerk et al. 1999; Zolman et al. 2000). Alternatively, at concentrations in which IBA induces secondary root formation, the inhibition of root elongation is not as strong. As shown in Fig. 14.1b, when concentrations are used that inhibit root growth equally, IBA induces more roots than IAA. The synthetic auxin 1-napthaleneacetic acid (NAA) shows similar efficacy.

Most early IBA research focused on determining the optimal concentrations and application timing for individual species. IBA is widely used in regeneration

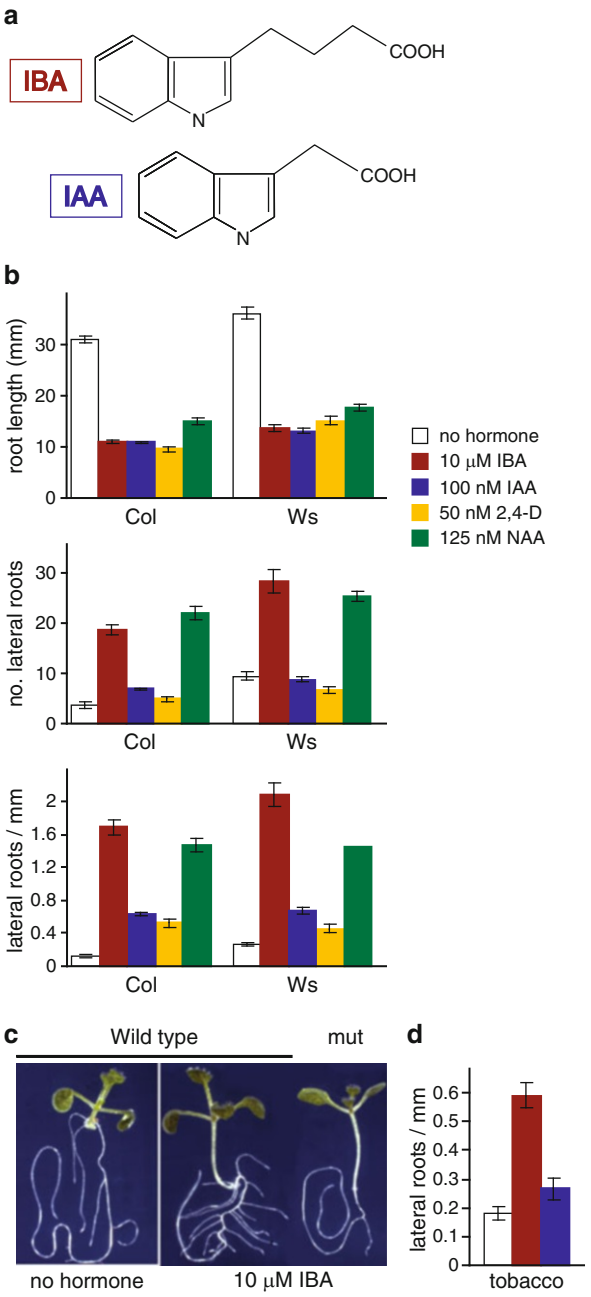

Fig. 14.1 Auxin influences on rooting. (**a**) Structures of indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA). (**b**) Root development following application of the endogenous auxins IAA and IBA or the synthetic auxins 2,4-D and NAA. *Arabidopsis* wild-type seedlings from the Columbia-0 (Col) or Wassilewskija (Ws) accessions were grown at 22 °C for 4 days on media without hormone, then transferred to the indicated conditions and grown for 5 days before root length and lateral root number were assayed. Bars represent means ± standard error, $n \geq 9$. (**c**) Col wild-type seedlings grown on no hormone or IBA. For comparison, the *pxa1-1* mutant (Zolman et al. 2001) is shown as a representative IBA-response mutant, with a long primary root and reduced root induction. (**d**) *Nicotiana benthamiana* seedlings were examined as described in part B, except measurements were done after 16 days

of cuttings or following plant transformations. For example, IBA was used for regeneration of *Jatropha curcas* cotyledon segment into an adult plant (Khemkladngoen et al. 2011), micropropagation of olive trees (Padilla et al. 2009), and *in vitro* rooting of sugarcane (Shahid et al. 2011). Improved rooting in cultures has enhanced production of compounds with commercial or medicinal value. For instance, IBA stimulated root production in cultures of the medicinal plant *Hypercicum perforatum*, increasing yields of antifungals for purification (Tocci et al. 2012).

Using a variety of methods, IBA has been identified as an endogenous compound in many species (Bartel et al. 2001; Ludwig-Müller 1993), including apple (van der Krieken et al. 1992), *Arabidopsis* (Ludwig-Müller 1993; Strader et al. 2010; Tognetti et al. 2010), maize (Barkawi et al. 2008; Epstein et al. 1989), mung bean (Xi et al. 2009), nasturtium (Ludwig-Müller and Cohen 2002), oilseed rape (Fan et al. 2011), pea (Nordström et al. 1991; Schneider et al. 1985), pear (Baraldi et al. 1993), and seaweed (Gupta et al. 2011). The presence of IBA in diverse plants suggests an important role for the compound in plant growth and development. However, identification of IBA as an endogenous plant compound was recently questioned (Novák et al. 2012) and accumulation differences have been noted between plant tissues and accessions (for example, (Epstein et al. 1989; Nordström et al. 1991; Schneider et al. 1985)). In studies showing accumulation, IBA typically was at a reduced level compared to IAA (Ludwig-Müller 1993).

Epstein and Lavee (1984) reported that labeled IBA was converted to IAA in grapevine and olive cuttings, followed by similar studies in other species using both labeled and internal measurements (Bartel et al. 2001; Liu et al. 2012; Ludwig-Müller 1993; Růžička et al. 2010). In a recent *Arabidopsis* study by Strader et al. (2010), incubation with labeled IBA resulted in IAA formation within 1 h. Mutants defective in auxin signaling responded normally, indicating that the IBA conversion is independent of signal transduction. The importance of IBA to root initiation connects to the conversion to IAA. In apple cuttings, radiolabeled IBA application elevates levels of both IBA and IAA; when concentrations of exogenous IBA and IAA were applied that generated equivalent levels of free internal IAA, IBA induced a higher number of lateral roots (van der Krieken et al. 1992). A study in pear connected a defect in root initiation with decreased IBA to IAA conversion (Baraldi et al. 1993). The increased rooting ability of IBA could be due to its stability or slow metabolism, providing a longer source of IAA for the plant.

In maize, application of labeled IAA led to IBA formation, with tissue and accession specific characteristics for the reaction (Ludwig-Müller and Epstein 1991). Similar responses were noted in *Arabidopsis* seedlings (Ludwig-Müller and Epstein 1994), although this conversion has not been noted in all systems (for instance, apples (van der Krieken et al. 1992)). This conversion suggested that IBA could act as a storage form of IAA, being produced if too much IAA was present and contributing to the auxin pool when needed.

To better understand the role of IBA in plants, either as an endogenous compound or an applied auxin, more recent research has focused on the molecular mechanism of IBA metabolism. These studies have led to a proposed role for peroxisomes in producing auxin signaling molecules.

14.3 IBA Metabolism Occurs in Peroxisomes

14.3.1 Two Carbon Units are Metabolized in Peroxisomes

IBA metabolism was suggested to be a peroxisomal β-oxidation pathway based on early testing of elongated molecules for auxin responses. The synthetic equivalent of IBA is 2,4-dichlorophenoxybutyric acid (2,4-DB); this compound is a chain-elongated form of the synthetic auxin dichlorophenoxyacetic acid (2,4-D), analogous to the relationship between IBA and IAA. Wain and Wightman (1954) tested a series of elongated 2,4-D molecules for three auxin responses: wheat cylinder elongation, pea curvature, and tomato leaf epinasty. In addition to activity by 2,4-D (acetic acid side chain), 2,4-DB (butyric side chain) and 2,4-dichlorocaprioic acid (caproic side chain) also showed activity (Wain and Wightman 1954). Alternatively, molecules with propionic, valeric, or heptanoic side chains did not induce responses (Wain and Wightman 1954).

Elongated IAA chains were studied similarly (Fawcett et al. 1960). IBA and IAA derivatives were effective auxins in wheat cylinder and pea elongation assays, particularly if the carbon chain was increased by two, four, or six carbons (Fawcett et al. 1960). Extracts were tested by paper chromatography and shown to shorten derivatives in two carbon steps (Fawcett et al. 1960). These similarities to fatty acid metabolism suggested that a β-oxidation mechanism was being employed in auxin production. In plants, peroxisomes are the site of fatty acid β-oxidation (Graham 2008).

Exogenous 2,4-D application often is used as an herbicide, based on the toxicity of such molecules in high doses. Recent work has shown that peroxisomes respond to 2,4-D, contributing to senescence induction. 2,4-D exposure increases activity of several peroxisomal enzymes, resulting in increased ROS; this accumulation leads to changes in gene expression, organelle appearance, and cellular organization (McCarthy-Suárez et al. 2011b; Pazmiño et al. 2011; Romero-Puertas et al. 2004), resulting in organ-specific disruptions of growth (McCarthy-Suárez et al. 2011a; Pazmiño et al. 2011).

14.3.2 IBA-Response Mutant Screens

To understand IBA roles in plants and the molecular mechanism of IBA activity, genetic screens have been employed. Screens have taken advantage of growth inhibition at high IBA or 2,4-DB concentrations, looking for mutagenized plants with normal growth (Hayashi et al. 1998; Strader et al. 2011; Zolman et al. 2000). For instance, wild-type plants grown on IBA have a short primary root with many lateral roots, whereas mutants retain long primary roots with few lateral organs (Fig. 14.1c). Similarly, high IBA concentrations inhibit wild-type hypocotyl elongation. Hypocotyl resistant mutants were identified with an elongated hypocotyl

compared to wild type. These growth differences could be due to decreased IBA uptake or transport, reduced metabolism, or signaling disruptions. The caveat in these screens was that responses to the target auxin (IAA or 2,4-D) remained normal to avoid mutants with general defects in auxin transport or signaling.

Three broad mutant categories have been identified with altered IBA or 2,4-DB responses. Mutants in the first group also show general peroxisomal defects, including developmental or growth delays and sucrose-dependent seedling establishment indicative of reduced fatty acid β-oxidation. Identification of such mutants in an IBA context provided a strong suggestion that IBA was metabolized in peroxisomes.

The second category describes mutants with defects in genes encoding β-oxidation like enzymes. Peroxisomes import all proteins post-translationally from the cytoplasm using one of two peroxisomal targeting signals (Hu et al. 2012); each of these enzymes have a peroxisomal targeting signal. Unlike the first group, these mutants showed IBA-specific phenotypes with normal peroxisomal function. Therefore, these were hypothesized to play roles in peroxisomal IBA metabolism.

A third category includes proteins involved in IBA transport. These proteins affect IBA movement throughout the plant. A recent review describes these proteins (Strader and Bartel 2011); this review will focus on IBA metabolism within a cell.

Mutants identified in these screens have been named *ibr* (*IB*A *r*esponse) or *ped* (*pe*roxisome *d*efective) mutants or renamed based on their protein/enzyme function. The current mutant collection, described below, indicates that most IBA activity is disrupted based on issues with metabolism or transport, with no established contribution from signaling. Several mutants have revealed connections to peroxisomes, indicating that peroxisomes play a key role in producing auxin molecules.

14.3.3 A Model for IBA β-Oxidation

A model has been proposed for peroxisomal IBA conversion to IAA using a β-oxidation like mechanism (Fig. 14.2). In this model, IBA would be imported into the peroxisome, followed by addition of a CoA side chain necessary for metabolism. The proposed IBA intermediates are based on the general mechanism of fatty acid β-oxidation. Cleavage of the CoA moiety would release active IAA, which would be exported from the peroxisome to act in signal transduction.

14.4 The Peroxisomal Conversion of IBA to IAA

14.4.1 Peroxins

Many plant peroxins (PEX proteins) acting in peroxisomal biogenesis or matrix protein import were found based on *Arabidopsis* forward genetic screens (Hu et al. 2012), both facilitating the identification of plants peroxins and cementing

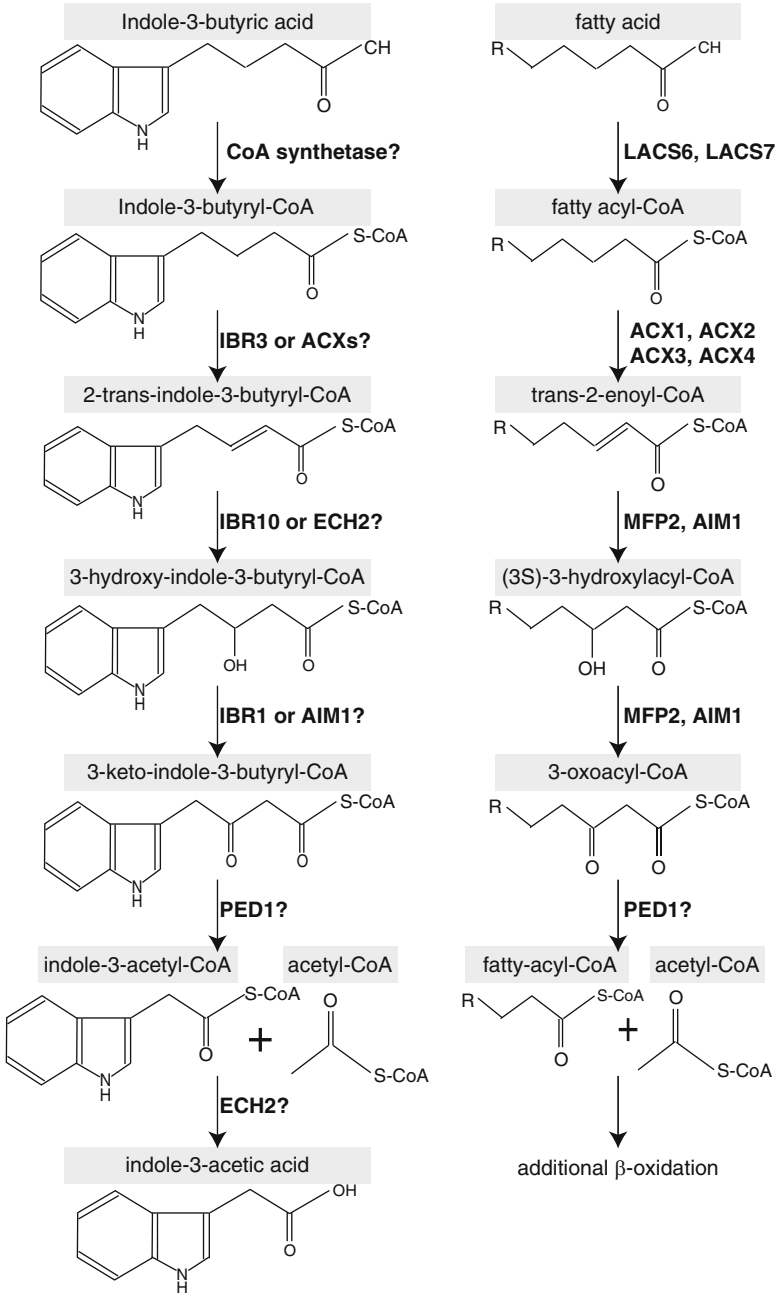

Fig. 14.2 Predicted mechanism for IBA conversion to IAA. Proposed enzymes are indicated to the *right* of the *arrow*. Fatty acid β-oxidation is shown for comparison (R = 2–18 carbon chain)

the connection between auxin metabolism and peroxisomes. Mutants disrupting the peroxisome matrix protein receptors PEX5 (Zolman et al. 2000) and PEX7 (Ramón and Bartel 2010) and the membrane associated proteins PEX4 (Zolman et al. 2005), PEX6 (Zolman and Bartel 2004), and PEX14 (Hayashi et al. 2000; Monroe-Augustus et al. 2011) were identified in IBA- or 2,4-DB resistance screens. Peroxin mutants identified by reverse genetics had similar phenotypes (Hu et al. 2012). The *pex6-1* mutant had decreased conversion of labeled IBA to IAA (Strader et al. 2010), further indicating that the conversion of IBA to IAA is peroxisomal. Disruptions in peroxins that reduce peroxisomal import would reduce the import of IBA β-oxidation enzymes, leading to decreased IAA production and, therefore, IBA-resistant growth.

14.4.2 Peroxisomal Import

IBA is imported into peroxisomes by the ABC-family transporter ABCD1. This protein was connected to IBA metabolism via identification of the *pxa1* (Zolman et al. 2001) and *ped3* (Hayashi et al. 2002) mutants in IBA and 2,4-DB resistance screens. A third set of *abcd1* mutants were identified as *comatose (cts)*; these strong alleles were named for their forever dormant phenotypes (Footitt et al. 2007). These studies and others have revealed a role for ABCD1 in peroxisomal substrate import (Linka and Esser 2012), including IBA or an IBA precursor. The reduced conversion of labeled IBA to IAA in *pxa1-1* supports a role for ABCD1 in IBA import (Strader et al. 2010).

14.4.3 Four Peroxisomal Enzymes Specifically Connected to IBA Responses

Four matrix proteins have been genetically connected to IBA metabolism. *ech2*, *ibr1*, *ibr3*, and *ibr10* were identified as IBA-response mutants (Goepfert et al. 2006; Strader et al. 2011; Wiszniewski et al. 2009; Zolman et al. 2000, 2007, 2008). All four genes are expressed throughout the plant during development (Goepfert et al. 2006; Zolman et al. 2008). The predicted protein domains are similar to mitochondrial or peroxisomal β-oxidation related domains (Figs. 14.3, 14.4, 14.5 and 14.6) and each enzyme has a peroxisomal targeting signal. Each protein has homologs in a range of plant species, including both monocots and dicots, with apparent duplications occurring in the past. Although not as tightly related, proteins with homology and similar domain structures also can be identified in the moss *Physcomitrella patens,* the fern *Selaginella moellindorffii* and the algae *Chlamydomonas reihardtii* and *Volvox carteri*.

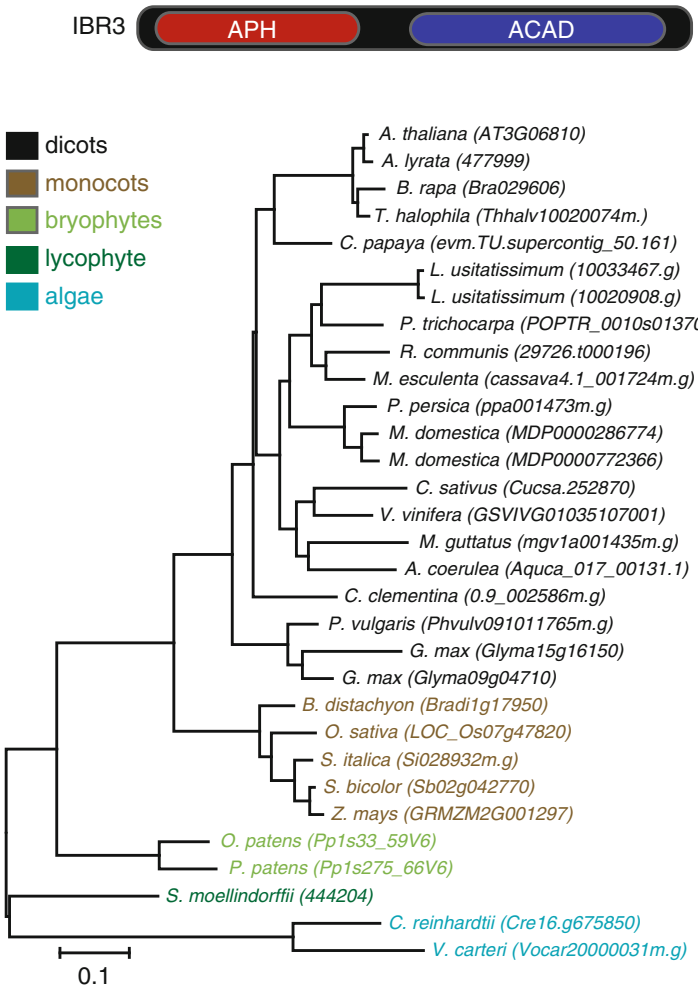

Fig. 14.3 IBR3. IBR3 domain structure includes an N-terminal ACAD10_aa like domain with predicted APH activity and a C-terminal ACAD domain proposed to act in IBA metabolism. Phylogenetic analysis of Arabidopsis At3g06810. Sequences were obtained using a BLASTP search of the phytozome (Goodstein et al. 2012) and NCBI databases for similar sequences. ClustalW protein alignments were performed using MEGA 5.05 (Tamura et al. 2011). Maximum-likelihood trees were constructed using MEGA 5.05. Species included are dicots {*Aquilegia caerulea* (Colorado Blue Columbine), *Arabidopsis lyrata*, *Arabidopsis thaliana*, *Brassica rapa* (turnip), *Capsella rubella*, *Carica papaya* (papaya), *Citrus clementina* (Clementine), *Citrus sinensis* (orange), *Cucumis sativus* (cucumber), *Eucalyptus grandis* (Rose gum), *Glycine max* (soybean), *Linum usitatissimum* (flax), *Malus domestica* (apple), *Manihot esculenta* (Cassava), *Medicago truncatula* (Barrel Clover), *Mumulus guttatus* (Monkey flower), *Populus trichocarpa* (Poplar), *Phaseolus vulgaris* (Green bean), *Prunus persica* (peach), *Ricinus communis* (castor oil), *Thellungiella halophila* (Salt tolerant relative of *A. thaliana*), and *Vitis vinifera* (Grape Vine)}; monocots {*Brachypodium distachyon* (purple false brome), *Oryza sativa* (Rice), *Setaria italic* (foxtail Millet), *Sorghum bicolor* (Sorghum), and *Zea mays* (corn)}; a lycophyte {*Selaginella moellendorfii* (fern)}; a bryophyte {*Physcomitrella patens* (moss)}; and alga {*Chlamydomonas reinhardtii* (green alga) and *Volvox carteri* (Chlorophyte alga)}. The number at the bottom indicates the number of amino acid substitutions per site

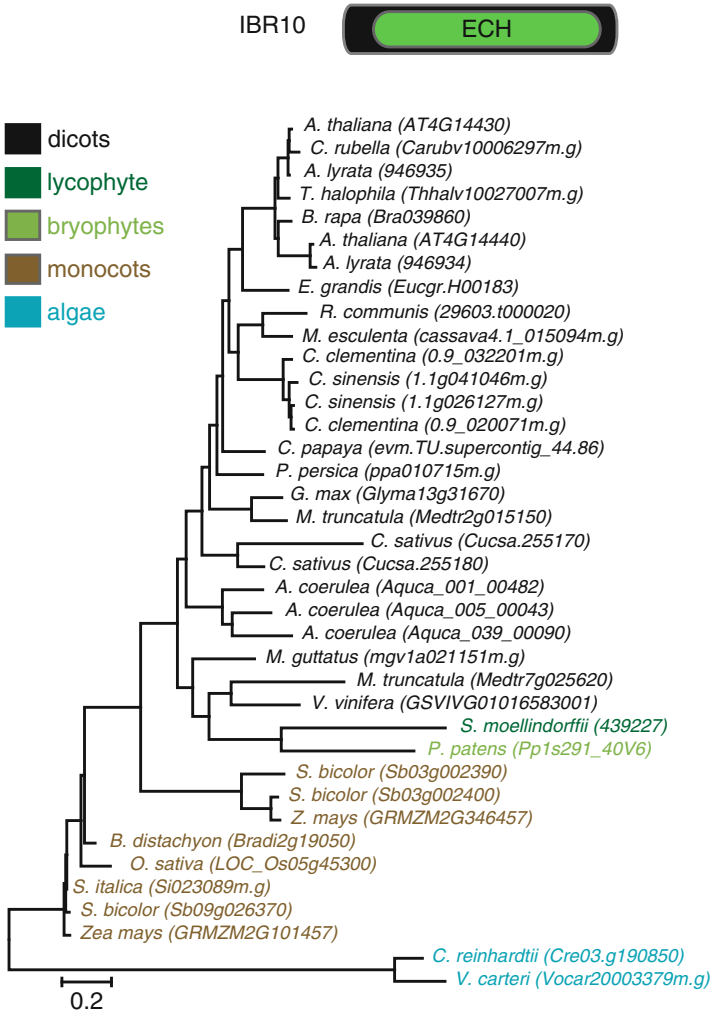

Fig. 14.4 IBR10. IBR10 domain structure includes a large crotonase-like/enoyl CoA hydratase (ECH) domain. Phylogenetic analysis of Arabidopsis At4g14430, generated as described in Fig. 14.3

ibr1, *ibr3*, and *ibr10* mutants have specific IBA-response phenotypes, with no disruptions in other hormone pathways or overall growth and development (Zolman et al. 2007, 2008). *ech2* acts similarly (Strader et al. 2011), with an additional phenotype of altered accumulation of certain fatty acid substrates (Goepfert et al. 2006). Based on the targeting signals and predicted enzymatic activities, these four enzymes have been hypothesized to act in the conversion of IBA to IAA. Supporting this hypothesis, an *ibr1 ibr3 ibr10* triple mutant has a defect converting labeled IBA to IAA (Strader et al. 2010) and an *ech2 ibr1 ibr3 ibr10* quadruple mutant has a

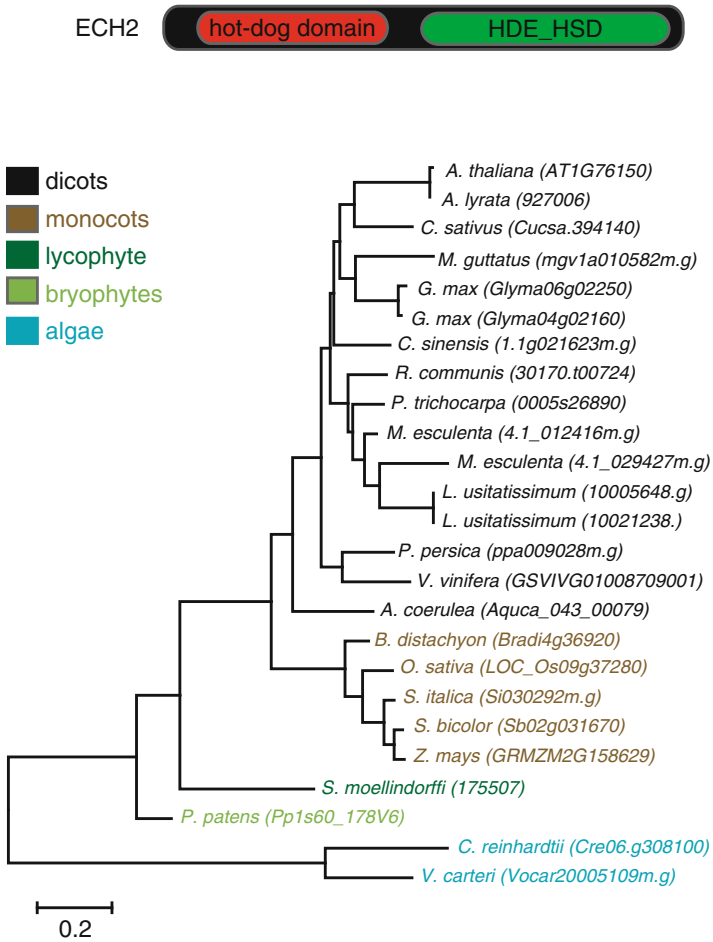

Fig. 14.5 ECH2. ECH2 domain structure has an N-terminal hotdog domain with a C-terminal HDE_HSD hydratase domain associated with enoyl-CoA *h*ydratase, *d*ehydrogenase, and *e*pimerase activities. Phylogenetic analysis of Arabidopsis At1g76150, generated as described in Fig. 14.3

20 % reduction in free IAA levels (Strader et al. 2011). Although these enzymes have been connected to IBA responses, future biochemical studies are required to confirm the specific roles suggested in IBA metabolism.

14.4.3.1 IBR3 (*Arabidopsis* At3g06810)

IBR3 contains two domains: an acyl-CoA dehydrogenase domain (ACAD) and an aminoglycoside phosphotransferase domain (APH; Fig. 14.3). Similar proteins are

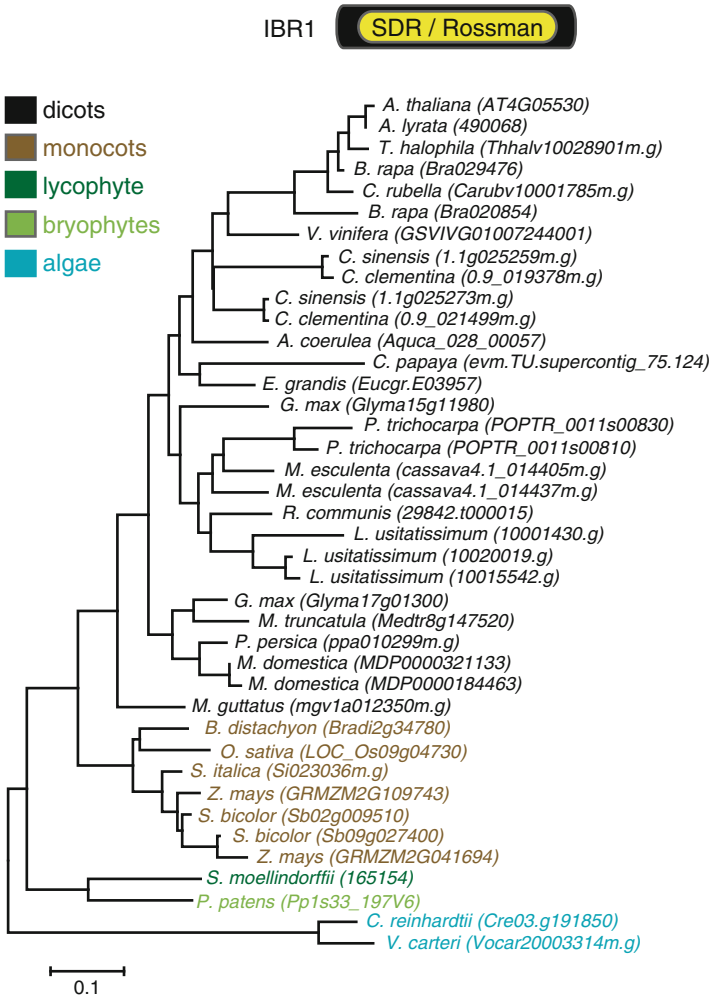

Fig. 14.6 IBR1. IBR1 domain structure has a short-chain dehydrogenase/reductase (*SDR*) domain with a Rossman fold for cofactor binding. Phylogenetic analysis of Arabidopsis At4g05530, generated as described in Fig. 14.3

found in many organisms, primarily plants and mammals (Zolman et al. 2007). Many bacterial proteins have each domain, but not the combination of ACAD and APH domains.

The C terminal domain is an ACAD_FadE2 domain (Zolman et al. 2007). Many members of this family have published roles in β-oxidation and amino acid catabolism. This domain includes an FAD binding site and is typically found in proteins with an acyl CoA dehydrogenase/oxidoreductase activity. One question regarding IBR3 activity is in regards to its catalytic role: is it a dehydrogenase or an

oxidase? Similar domains are common in mitochondrial ACAD proteins and peroxisomal oxidase proteins; these enzymes have similar dehydrogenation activities, but differ based on the oxidative half-reaction associated with the reduction (Arent et al. 2008). In general, dehydrogenases are mitochondrial and use the electron transfer flavoprotein (ETF) to transfer electrons from FADH to the electron transport chain. A similar reaction is catalyzed by peroxisomal oxidases, but peroxisomal enzymes use molecular oxygen in place of ETF. This reaction generates an H_2O_2 byproduct, which is quickly metabolized by peroxisomal catalases. Although IBR3 is most similar to mitochondrial proteins with dehydrogenase activity, the targeting signal would indicate that it is localized to the peroxisome and therefore likely to have oxidase activity. IBR3 was predicted to convert IBA-CoA to the α,β-unsaturated thioester (Fig. 14.2).

The N-terminal domain is an ACAD10_aa like domain, which has an ATP binding site and contains a catalytic domain similar to phosphotransferases and Ser/Thr kinases (Zolman et al. 2007). Although this domain is named for two vertebrate ACAD family members, most family members do not contain this domain and the substrate is not known for ACAD10 or ACAD11. Small molecule phosphorylation by APH-domain proteins is common in substrate inactivation; the stereotypical examples are aminoglycoside antibiotics, in which phosphorylation reduces antibiotic efficacy leading to host antibiotic resistance (Azucena and Mobashery 2001). The importance of this domain in IBA metabolism remains to be determined.

Several *ibr3* mutant alleles have been identified, including T-DNA insertion alleles and point mutants (Zolman et al. 2007). Because many alleles have been identified and the mutants have a less pronounced phenotype than other IBA-response mutants (Strader et al. 2011; Zolman et al. 2008), this step may have some redundancy or be less important for flux through the pathway.

14.4.3.2 IBR10 (*Arabidopsis* At4g14430)

IBR10 is also known as ECI2, ECHIb, and PEC12, all names that reflect the major domain of the protein. The protein is in the crotonase-like/enoyl-CoA hydratase (ECH) superfamily (Fig. 14.4), which includes hydratases, dehydratases, and isomerases (Zolman et al. 2008). Although a range of substrates can be metabolized in this family, many have a CoA binding site, similar to the predicted IBA intermediate. Enoyl-CoA hydratase activity in fatty acid metabolism is performed by one of the two multifunctional proteins (see below).

In IBA metabolism, the IBR10 protein is predicted to produce the IBA hydroxylacyl-CoA thioester intermediate (Fig. 14.2). Unlike *ibr3*, only a single *ibr10* allele has been reported: *ibr10-1* has a 78 bp deletion, resulting in a 26 amino acid deletion from the middle of IBR10 (Zolman et al. 2008). Notably, the C-terminal active sites are normal. No insertions have been reported in this gene, which complicates the analysis of whether *ibr10-1* is a null allele or if the deletion allows some activity.

14.4.3.3 ECH2 (*Arabidopsis* At1g76150)

The *ech2-1* mutant was identified in a screen for hypocotyl resistance to IBA (Strader et al. 2011). This mutant allele has a Gly-to-Glu mutation in a conserved amino acid. The ECH2 protein was previously characterized in a reverse genetics study as an enoyl-CoA hydratase (Goepfert et al. 2006). In yeast complementation assays, ECH2 had bidirectional enoyl-CoA hydratase activity and plants with altered *ECH2* levels showed changes in the metabolism of unsaturated fatty acids (Goepfert et al. 2006).

This protein has a hotdog fold, which consists of a series of β-sheets that surround a central α-helix (Fig. 14.5; (Strader et al. 2011)). The hotdog domain at the N terminus is similar to *E. coli* FabA and continues as part of the HDE_HSD hydratase like hotdog fold of *h*ydratase-*d*ehydrogenase-*e*pimerase and *h*ydroxy*s*teroid *d*ehydrogenase proteins. Many proteins containing this fold have roles in fatty acid metabolism, which may indicate the origins of this protein. Notable activities that could be related to IBA metabolism include thioester hydrolysis, hydratase, dehydrogenase, epimerase, and short chain reductase. Among other roles, the PaaI hotdog domain-containing protein has been hypothesized to act as a thioesterase on phenylacetyl-CoA in *E. coli* phenylacetic acid (PAA) degradation (Dillon and Bateman 2004).

IBR10 and ECH2 contain similar domains, leading to a question of redundancy, especially given that each only has been identified once in mutant screens. However, *ECH2* expression did not rescue the *ibr10* phenotype and *IBR10* expression did not rescue *ech2*, suggesting the two play independent roles in IBA response (Strader et al. 2011). One possibility is that both enzymes act as hydratases at the same step (Fig. 14.2), but with tissue or condition specificity based on changes in expression. Another possibility is that ECH2, which has a hotdog domain that has also been associated with thioesterase activity, acts at a later step in IBA metabolism (Strader et al. 2011).

14.4.3.4 IBR1 (*Arabidopsis* At4g05530)

IBR1, also known as SDR4 or SDRA (Wiszniewski et al. 2009), is in the short-chain dehydrogenase/reductase (SDR) family with predicted oxidoreductase activity (Fig. 14.6). Characterized enzymatic reactions in this family include isomerization, epimerization, dehydration, and enoyl-CoA reduction. Enzymes in this family require a cofactor (NAD, NADP, or FAD) as an electron carrier (Zolman et al. 2008). The Rossman fold binds NAD(P)+, matching the predicted cofactor for IBR1 (Zolman et al. 2008). Substrates catalyzed by this family include lipids, carbohydrates, aromatic compounds, and amino acids.

This protein is in the dehydrogenase subcategory of the family and is proposed to catalyze the dehydrogenation of 3-hydroxy indole-3-butyryl-CoA (Fig. 14.2). Multiple *ibr1* alleles have been identified, including point mutations, T-DNA insertions, and large deletions (Wiszniewski et al. 2009; Zolman et al. 2008).

ibr1 mutants have a generally weaker phenotype than *ibr10* and *ech2* (Strader et al. 2011), suggesting other enzymes may be contributing to IBA metabolism at this step.

14.4.4 β-Oxidation Connections to IBA Metabolism?

The proposed β-oxidation enzymes described above were identified in forward genetics mutant screens as IBA-specific mutants. None have phenotypes indicative of a defect in β-oxidation of seed storage fatty acids (Strader et al. 2011; Zolman et al. 2007, 2008). However, other mutants were identified that had phenotypes indicative of IBA metabolism defects and fatty acid defects. In addition, labeled IBA conversion to IAA was disrupted in two fatty acid β-oxidation mutants, indicating that disruptions in fatty acid β-oxidation can affect IBA metabolism (Strader et al. 2010). However, the specific role of these β-oxidation enzymes remains unclear. The enzymes acting in fatty acid β-oxidation may directly act on IBA substrates, given that the predicted mechanism is similar. Alternatively, specific enzymes could act in each pathway, allowing substrate specific enzymes to regulate each pathway independently.

14.4.4.1 A Synthetase

β-oxidation of fatty acid substrates requires activation by addition of a CoA side chain (Fig. 14.2). Fatty acids are activated by the LACS6 and LACS7 synthetases, but *lacs6 lacs7* has normal IBA responses and the enzymes have no activity on IBA (Fulda et al. 2004). *AAE18* encodes a peroxisomal acyl-activating enzyme that is active on 2,4DB but has wild-type IBA responses (Wiszniewski et al. 2009). This mutant is a rare example in which 2,4-DB and IBA responses are not equal and leads to the question of how IBA is activated for metabolism. One hypothesis is that an AAE18 or LACS family member acts on IBA substrates (Wiszniewski et al. 2009). Because the PXA1 transporter appears to import CoA activated substrates (Linka and Esser 2012), a separate possibility is that IBA is imported into peroxisomes as IBA-CoA and that activation occurs in the cytosol.

14.4.4.2 Acyl-CoA Oxidases

The acyl-CoA oxidases (ACXs) also are implicated in fatty acid metabolism and IBA oxidation. Four *Arabidopsis* ACX proteins have been tested biochemically; all catalyze the initial oxidation of CoA-activated substrates with high substrate specificity (Fig. 14.2; (Arent et al. 2008)). For example, ACX4 specifically catalyzes short-chain acyl-CoAs with carbon chains of 4–8 carbons, whereas ACX3 prefers

medium-chain substrates (8–14 carbons). ACX2 is a very-long chain oxidase (substrates over 16 carbons) and ACX1 has strongest activity on long-chain substrates.

acx1 and *acx3* mutants were identified in forward genetics screens as IBA resistant (Adham et al. 2005) and additional reverse genetics studies identified these and *acx4* with IBA- or 2,4-DB-resistant phenotypes (Adham et al. 2005; Eastmond et al. 2000; Pinfield-Wells et al. 2005; Rylott et al. 2003). Higher-order mutants show enhanced IBA resistance, indicating that multiple ACX proteins might be involved in IBA metabolism (Adham et al. 2005; Khan et al. 2012). The most dramatic resistance is seen with *acx1acx3acx4*, a mutant which combines the most active and most highly expressed isozymes (Khan et al. 2012). This result could indicate that multiple ACX enzymes are acting in IBA β-oxidation (Adham et al. 2005). These phenotypic responses are supported by a reduced conversion of IBA to IAA in the *acx1 acx2* double mutant (Strader et al. 2010). However, the high substrate specificity would seemingly discount the idea that multiple ACX proteins act on IBA substrates.

The reaction catalyzed by the ACX proteins is the same step for which IBR3 has been suggested (Fig. 14.2). Combining *ibr3* and *acx* mutations enhance the IBA-resistant phenotypes (Zolman et al. 2007), suggesting that different pathways are disrupted. A question arises, therefore, about whether both sets of enzymes catalyze this reaction or if only one is necessary. A hypothesis has been suggested that the ACX enzymes are indirectly responsible for IBA metabolism and IBR3 plays the direct role in metabolism (Adham et al. 2005). In this indirect scenario, CoA substrates that build up in the *acx* mutants, particularly in higher-order lines, would cause a decrease in available CoA for IBA activation. This decrease would lead to reduced IBA conversion and IBA-resistant growth.

14.4.4.3 Multifunctional Enzymes

The middle steps of fatty acid β-oxidation are catalyzed by one of two multifunctional enzymes (Fig. 14.2; (Richmond and Bleecker 1999; Rylott et al. 2006)). These large, multi-domain proteins catalyze both the hydration and dehydrogenation steps of fatty acid β-oxidation. *aim1* mutants are resistant to 2,4-DB (Richmond and Bleecker 1999) and IBA (Zolman et al. 2000). These results perhaps indicate that the AIM1 protein can act on one or both of the middle steps of IBA metabolism. *mfp2* mutant alleles are not resistant to chain-elongated auxins (2,4-DB), consistent with a preference for longer-chain substrates (Rylott et al. 2006).

The combined activities of IBR10 and IBR1 equal the activities found in peroxisomal multifunctional proteins (Fig. 14.2; (Zolman et al. 2008)), suggesting that these two proteins could catalyze these reactions either with or instead of AIM1. Again, this could be a situation where multiple enzymes catalyze the same reactions in IBA β-oxidation. Overlapping AIM1 and IBR1 activity could explain the weaker phenotype seen in *ibr1*. However, a similar indirect mechanism could be envisioned, perhaps based on limitations in the NADP cofactor levels or changes in the organelle based on substrate accumulation.

14.4.4.4 PED1, a Thiolase

The *ped1* mutant was isolated as 2,4-DB resistant in the original β-oxidation mutant screen (Hayashi et al. 1998). In addition, the mutant showed a strong seedling growth defect that could be rescued by sucrose application, indicating a block in fatty acid metabolism that was bypassed if a carbon source was present (Hayashi et al. 1998). *ped1* is defective in a ketoacyl-CoA thiolase, charged with removing a two carbon unit (acetyl CoA) and adding a CoA to the chain-shortened substrate that will re-enter the β-oxidation cycle (Fig. 14.2). To date, no IBA-specific mutant has been identified to specifically act at this step but a thiolase disruption leads to defects in IBA conversion to IAA (Strader et al. 2010). Therefore, PED1 appears likely to be working not just in fatty acid β-oxidation but also on IBA.

14.5 The Roles of IBA in a Plant

IBA-response mutants show many phenotypes, indicating that IBA (or other substrates metabolized by these enzymes) are contributing to the auxin pool in important ways. In particular, a triple *ibr1 ibr3 ibr10* mutant and a quadruple *ech2 ibr1 ibr3 ibr10* mutant have revealed much about the importance of these peroxisomal enzymes (Strader et al. 2010, 2011; Zolman et al. 2008). In addition, studies with *pdr8*, which disrupts IBA transport, are used here as one example of responses to increased intracellular IBA (Strader and Bartel 2009). These efflux mutants lead to increased IBA levels, presumably increasing IAA as well; indeed, many of the *pdr8* mutant phenotypes are suppressed if combined with *pex5* or *pxa1* disrupting peroxisomal function.

IBA-response mutants have notable changes in root architecture, including reduced lateral root systems, under both inducible and normal growth conditions (Fig. 14.1c and d; (Strader et al. 2011; Zolman et al. 2000)). As described above, the most practical use of IBA is in regeneration of callus or induction of roots on cuttings. In IBA-response single mutants, defects in lateral root initiation can be rescued by other auxins, indicating that the defect in the pathway is reduced initiation, not an inability to make lateral roots (Zolman et al. 2000, 2007, 2008). In higher-order mutants, however, the defect could not be fully rescued, suggesting that these proteins are necessary for full root initiation in endogenous tissues (Strader et al. 2011). Exogenous IBA also is known to play roles in adventitious root initiation (King and Stimart 1998; Ludwig-Müller et al. 2005). Furthermore, the quadruple mutants have smaller root meristems, although organization is normal, with resulting decreases in root length (Strader et al. 2011). Root hair elongation also is affected by IBA. Shorter root hairs are seen in the *ibr* triple mutant with reduced IBA conversion; this phenotype is rescued by exogenous IAA (Strader et al. 2010). The IBA efflux mutant *pdr8* has longer root hairs, with enhanced responses following IAA application (Strader and Bartel 2009).

Mutant seedling phenotypes include altered cotyledon expansion: mutants disrupted in IBR enzymes have smaller cotyledons (Strader et al. 2010), whereas IBA efflux mutants have larger cotyledons (Strader and Bartel 2009). Vascular development in cotyledons and epidermal cell patterning are disrupted in *ech2 ibr1 ibr3 ibr10* (Strader et al. 2011). Further, maintenance of the apical hook is relaxed in these mutants, a phenotype consistent with other mutants defective in auxin responses (Strader et al. 2011). In growing plants, IBA induces hypocotyl (Strader et al. 2011) and stem (Yang and Davies 1999) elongation. Finally, the *comatose* mutant has reduced stamen elongation (Footitt et al. 2007). Because this disruption is restored by exogenous NAA, loss of peroxisomal IBA-derived IAA may be contributing to stamen elongation.

Molecular studies using these mutants revealed a connection between auxin levels, gene expression, and phenotypes. Auxin-induced reporter gene expression was reduced in mutants defective in *ECH2* and *IBR10*, both in root tips and apical hooks (Strader et al. 2011). The *ech2 ibr1 ibr3 ibr10* quadruple mutant has a 20 % reduction in free auxin in root tips, which limited auxin-induced gene expression (Strader et al. 2011). These results indicate important roles for IBA-derived IAA in plant growth and development.

Notably, this list of phenotypes does not completely overlap with the list of known IAA responses. IBA is one mechanism of many that regulate IAA levels, including conjugation of IAA to amino acids/proteins and sugars, de novo biosynthesis, degradation, and transport (Woodward and Bartel 2005). The IBA-response mutant phenotypes indicate that the auxin derived from IBA is not influencing all phenotypes, but that the temporal or spatial distribution of IBA-derived IAA from peroxisomes is leading to changes in the auxin pool that are physiologically important. Failure to generate these pools results in a reduction in responses that cannot be compensated by de novo auxin biosynthesis or other IAA input pathways. However, other responses may derive from other auxin input pathways. For instance, hypocotyl elongation appears to be more highly influenced by IAA-conjugate storage pools than IBA (Strader et al. 2010). Additionally, whereas many auxin biosynthetic mutants have embryogenesis and morphological phenotypes (see above), even the quadruple IBA-response mutants have normal morphology.

14.6 Open Questions

14.6.1 Is IBA an Endogenous Auxin? Are Longer Side-Chain Molecules in Plants?

Exogenous IBA has been used for many years in horticulture and agriculture. As described above, studies have reported IBA in a wide range of plants. However, Novak et al. (2012) recently reported that IBA was not detectable in *Arabidopsis*, *Populus*, or wheat. Further work characterizing auxin intermediates will be important to

reconcile the differences in these studies. Longer-chain substrates also could be converted to IAA and therefore may have auxin-like activity with IBA intermediates, as indicated by Fawcett et al. (1960). Regardless of the above question, IBA is an effective auxin and a detailed understanding of its activity will improve our ability to induce rooting in plants.

14.6.2 Why Is IBA Conversion to IAA a Peroxisomal Process?

IBA conversion to IAA in the peroxisome is one of several IAA inputs. Other steps in auxin metabolism occur in the cytosol, the chloroplast, and the ER (Woodward and Bartel 2005). The sequestration between organelles could be an additional mechanism to ensure that IAA levels are precisely maintained. In addition, or alternatively, methods of producing auxin signaling molecules could be based on endogenous pathways. *Arabidopsis* and other oilseed plants have well-established mechanisms to metabolize long-chain fatty acids for energy during early seedling development; IBA conversion to IAA could parallel this process. Because fatty acid and IBA enzymes overlap, peroxisomal localization of both pathways facilitates activity. Future studies need to examine the regulation of each pathway and how they might tie together, especially in terms of overlapping enzyme activity, parallel use of cofactors, and any differences between oilseeds and other plants.

14.6.3 The Missing Steps

The enzymatic activities of these proteins must be tested to confirm their position in peroxisomal IBA conversion. The function of ACX family members, AIM1, and PED1 must be evaluated to determine if the IBA-resistant nature of these mutants is based on a direct role in activity or an indirect connection between peroxisomal fatty acid β-oxidation and IBA metabolism. We do not yet know how IBA is activated by CoA addition. Although we know that conversion is peroxisomal, we do not know how IBA-derived IAA is exported to the cytosol. Finally, we do not know the triggers that initiate IAA production from IBA.

This pathway may be a target for engineering root systems; however, to manipulate this pathway, we must know more about the activity of each protein as well as the biochemical characteristics of the pathway, especially regarding redundancy. Studies of the regulation, distribution, and timing of this conversion await a more complete understanding of the enzymes involved.

14.6.4 IBA Homeostasis

Although the focus of this review is the conversion of IBA to IAA, understanding IBA metabolism will facilitate our understanding of this auxin source. Several IBA

biosynthesis studies have been done (Ludwig-Müller 1993, 2000). However, no genes or proteins have been identified. Maize results suggest IBA is made from IAA in a two-step process, with enhanced production if the cofactors acetyl-CoA and ATP are added (Ludwig-Müller et al. 1995). IBA was made specifically with IAA and not from 1-IAA, the synthetic auxins NAA or 2,4-D, or the endogenous compound PAA (Ludwig-Müller and Epstein 1994; Ludwig-Müller and Hilgenberg 1995). This reaction is different in light and dark and between tissues, with highest production in dark-grown roots (Ludwig-Müller and Hilgenberg 1995). *Arabidopsis* seedling IBA production peaked at 3–5 days but all tissues were able to convert IAA to IBA (Ludwig-Müller and Epstein 1994) and IBA synthase activity was found in a wide variety of plants (Ludwig-Müller and Hilgenberg 1995). IBA formation from IAA may be triggered when IAA levels are above optimal, relieving any stress caused by high IAA-induced responses. Because this conversion has not been fully elucidated, a second hypothesis is that IBA is an intermediate in one of the several IAA biosynthesis pathways (Strader et al. 2011).

In addition to IBA conversion to IAA, IBA conjugation also occurs rapidly, especially ester linkages to sugars; conjugation is a common mechanism for plant hormones to limit responses (Ludwig-Müller 1993, 2000). These IBA conjugates have been implicated in abiotic stress response (Tognetti et al. 2010), which is notable when connected with the idea that IBA synthesis also increases during drought stress (Ludwig-Müller et al. 1995) and that IBA has been connected to stomatal opening (Cousson 2010). The roles of IBA warrant future study in the context of stress biology.

14.7 Conclusions

Because IBA activity is based on its conversion to IAA, IBA is considered a proto-auxin. The presence of IBA in species ranging from seaweed to trees and the conservation of peroxisomal enzymes proposed to act in IBA responses suggests that IBA is an important contributor to auxin homeostasis. The significant phenotypes found in mutants with alterations in IBA metabolism and transport demonstrate the importance of these peroxisomal enzymes in auxin homeostasis and IBA-derived IAA in plant development. IBA biosynthesis from IAA may relieve IAA levels that are above optimal; IBA to IAA conversion could increase free (active) IAA when more auxin is needed by the plant following developmental cues or changing environmental conditions. Regardless of the recent question regarding the endogenous accumulation of IBA, IBA metabolism needs to be investigated due to its usage in agricultural and horticultural situations. The peroxisomal nature of these IBA-response enzymes indicates a role for peroxisomal metabolism of IBA (or a related substrate) feeding into the auxin cycle and playing a critical role in plant growth and development.

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Chapter 15
Peroxisomes as Cell Generators of Reactive Nitrogen Species (RNS) Signal Molecules

Francisco J. Corpas, Juan B. Barroso, José M. Palma, and Luis A. del Río

Contents

Abstract Nitric oxide is a gaseous free radical with a wide range of direct and indirect actions in plant cells. However, the enzymatic sources of NO and its subcellular localization in plants are still under debate. Among the different subcellular compartments where NO has been found to be produced, peroxisomes are the best characterized since in these organelles it has been demonstrated the presence of NO and it has been biochemically characterized a L-arginine-dependent nitric oxide synthase activity. This chapter summarizes the present knowledge of the NO metabolism and its derived reactive nitrogen species (RNS) in plant peroxisomes and how this gaseous free radical is involved in natural senescence, and is released to the cytosol under salinity stress conditions acting as a signal molecule.

F.J. Corpas (✉) • J.M. Palma • L.A. del Río
Departamento de Bioquímica, Biología Celular y Molecular de Plantas,
Estación Experimental del Zaidín, CSIC, Apartado 419, E-18080 Granada, Spain
e-mail: javier.corpas@eez.csic.es

J.B. Barroso
Grupo de Señalización Molecular y Sistemas Antioxidantes en Plantas, Unidad Asociada al CSIC, Área de Bioquímica y Biología Molecular, Universidad de Jaén, E-23071 Jaén, Spain

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15.1 Introduction

Nitric oxide (NO) is a free radical that has attracted the attention of many plant biologists during last decade, mainly due to the involvement of this molecule in many physiological and phyto-pathological processes. In higher plants, it has been demonstrated that NO has an important function in plant growth and development, including seed germination (Bethke et al. 2006), primary and lateral root growth (Lanteri et al. 2006), development of functional nodules (Baudouin et al. 2006; Leach et al. 2010), flowering (Zafra et al. 2010), pollen tube growth regulation (Prado et al. 2004; Šírová et al. 2011), fruit ripening, senescence (Leshem et al. 1998; Corpas et al. 2004a; Procházková and Wilhelmová, 2011), pathogen response (Mur et al. 2006; Yoshioka et al. 2011) and abiotic stress (Corpas et al. 2011), being also a key signaling molecule in different intracellular processes (Shapiro 2005; Neill et al. 2008). The main physiological processes of higher plants where NO is involved are summarized in Fig. 15.1.

Peroxisomes in higher plants are organelles with a diverse range of specific metabolic functions depending on the tissue localization, the plant developmental

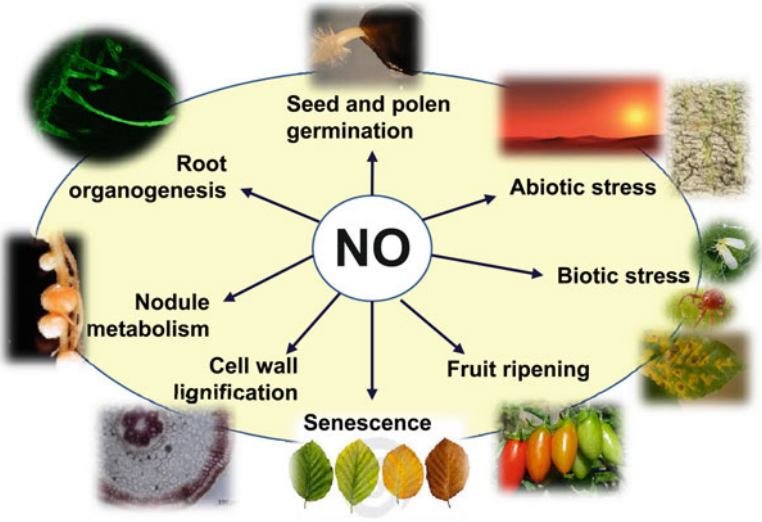

Fig. 15.1 Summary of the main functions of NO in different plant physiological and pathological processes

step and the environmental conditions. For this reason, these organelles have received different names, like glyoxysomes, leaf-peroxisomes, microbodies, gerontosomes, etc., although, at present, the preferred name is peroxisome (Pracharoenwattana and Smith 2008). Among the most significant metabolic functions of this cell compartment are photorespiration, fatty acid β-oxidation, glyoxylate cycle, and metabolism of reactive oxygen species (ROS), and the peroxisomal characteristic enzymes are catalase and H_2O_2-generating flavin oxidases, indicating a prominent oxidative metabolism (del Río et al. 1992, 2002; del Río 2011; Corpas et al. 2001; Hu et al. 2012). Nevertheless, the potential functions of these organelles are still growing since new proteins are continuously been reported what indicates the metabolic plasticity of peroxisomes in plant cells (Reumann et al. 2009). Additionally, since the discovery of a L-arginine-dependent nitric oxide synthase activity in peroxisomes, increasingly growing evidence are giving to these organelles an important role in the NO metabolism. In this work, an overview of the contribution of peroxisomes to the nitric oxide metabolism in plant cells under physiological and stress conditions is presented.

15.2 Metabolism of Nitric Oxide (NO) and Related Molecules in Higher Plants

Nitric oxide, whose more precise chemical name is nitrogen monoxide, is a free radical since this molecule has an unpaired electron in its π orbital. This unpaired electron in NO provides it with a high reactivity with different bio-molecules (proteins, nucleic acids, and lipids), affecting their biological functions. The term RNS designates a group of NO-derived molecules, such as peroxynitrite ($ONOO^-$), nitrogen dioxide (NO_2), *S*-nitrosothiols (SNOs), and *S*-nitrosoglutathione (GSNO) which can mediate modification of different bio-molecules, being the proteins the most studied.

Nitric oxide has an important interaction with sulfhydryl-containing molecules such as reduced glutathione (GSH) or proteins. This post-translational process is designated as *S*-nitrosylation and has become an important area of research trying to identify protein targets of this interaction. In recent years using different approaches a significant number of *S*-nitrosylated proteins have been identified (Lindermayr et al. 2005; Romero-Puertas et al. 2008; Abat and Deswal 2009; Palmieri et al. 2010). In rat liver, analysis by the biotin switch assay of mitochondrial extracts treated with *S*-nitrosoglutathione (GSNO) allowed identifying some peroxisomal enzymes which co-purified with the mitochondrial fractions such as catalase and malate dehydrogenase (Foster and Stamler 2004). A similar result was obtained in *Arabidopsis* mitochondrial fractions where catalase was also identified as a target of *S*-nitrosylation (Palmieri et al. 2010). More recently, a pharmacological approach using pea leaf peroxisomes treated with *S*-nitrosoglutathione (GSNO) also showed

that catalase and malate dehydrogenase, plus glycolate oxidase and hydroxypyruvate reductase are potential candidates of *S*-nitrosylation (Ortega-Galisteo et al. 2012). In this sense, an *in vitro* assay of purified tobacco catalase incubated with different NO donors (SNAP, GSNO or NOC-9) produced an inhibition of the enzyme activity between 70 % and 90 %, being in part restored if these NO donors were removed (Clark et al. 2000). This reversible inhibition suggests a process of *S*-nitrosylation.

Although it can be assumed the presence of GSNO in plant cells, to our knowledge the first demonstration of the presence of this molecule in higher plants was reported in pea leaves by an immunological approach combined with confocal laser scanning microscopy (Barroso et al. 2006). Later, using the same technique, the presence of GSNO was extended to olive and sunflower (Valderrama et al. 2007; Chaki et al. 2009). An important advance in the research on GSNO in plants has been its detection and quantification by chromatography-electrospray/mass spectrometry (LC-ES/MS) in different organs of pepper and *Arabidopsis* plants (Airaki et al. 2011; Leterrier et al. 2012).

The presence of GSNO in peroxisomes has been demonstrated by electron microscopy immunogold labeling in sunflower hypocotyls (Chaki 2007) and pea leaves (Ortega-Galisteo et al. 2012). GSNO is considered an important mobile reservoir of NO bioactivity (Durner and Klessig 1999; Noble et al. 1999; Stamler et al. 2001) and this molecule can mediate the signaling pathway through specific post-translational modifications of redox-sensitive proteins by a reaction of trans-nitrosylation from GSNO to Cys-proteins, leading to *S*-nitrosothiols.

Peroxynitrite is the product of the rapid reaction between superoxide radicals ($O_2^{\cdot -}$) and nitric oxide ($k = 1.9 \times 10^{10}\ M^{-1}\ s^{-1}$) (Kissner et al. 1997), being an important molecule that produces the oxidation and nitration of many biomolecules. However, peroxynitrite has a very short half-life and its action must be correlated with the site of generation of both superoxide and nitric oxide (Szabó et al. 2007). Peroxynitrite, direct or indirectly, is considered to be responsible of the nitration process, a post-translational modification that could affect protein function (Radi 2004). Tyrosine nitration is at present the most studied protein modifications by peroxinitrite although other amino acids such as cysteine, methionine and tryptophan can be also nitrated. In higher plants, tyrosine nitration is under intense study because it is usually associated with environmental stress processes, and the identification of nitrated proteins is increasingly growing (Chaki et al. 2009, 2011; Lozano-Juste et al. 2011). Among the potential targets of protein nitration there are also some peroxisomal enzymes such as catalase, glycolate oxidase and malate dehydrogenase (Lozano-Juste et al. 2011). However, the specific effect on their respective enzymatic activities needs to be determined.

Table 15.1 summarizes the set of peroxisomal proteins which have been shown to be affected by post-translational modifications mediated by NO, $ONOO^-$ and GSNO suggesting the existence of an active NO metabolism within peroxisomes.

Table 15.1 Summary of peroxisomal proteins susceptible of post-translational modifications mediated by reactive nitrogen species

Post-translational modification	Peroxisomal enzyme	Identification
Tyrosine nitration	Catalase Glycolate oxidase Malate dehydrogenase	Immunoreactivity with antibody against nitrotyrosines[a]
S-nitrosylation	Catalase Glycolate oxidase Malate dehydrogenase Hydroxypyruvate reductase	Preincubation with GSNO (NO donor) and biotin-switch[b]

[a]Lozano-Juste et al. 2011; [b]Ortega-Galisteo et al. 2012

15.3 Evidence of the Presence of L-Arginine-Dependent NOS Activity in Peroxisomes

In animal systems the majority of the NO produced is due to a nitric oxide synthase (NOS; EC 1.14.13.39) (Moncada et al. 1991; Alderton et al. 2001; Ghosh and Salerno 2003). This enzyme catalyses the conversion of L-arginine, oxygen and NADPH to NO, citrulline and $NADP^+$. There are three distinct isoforms of NOS designated as neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3). These isoforms are also classified on the basis of either their constitutive (eNOS and nNOS) or inducible (iNOS) expression, and their dependence (eNOS and nNOS) or independence (iNOS) on calcium (Alderton et al. 2001). However, in plant systems there are several potential sources of NO including enzymatic and non-enzymatic mechanisms (Wojtaszek 2000; del Río et al. 2004; del Río 2011; Hancock 2012). Although the specific enzymatic source of NO is still controversial, there are a significant number of evidence obtained using physiological and/or pharmacological approaches with inhibitors analogous to L-arginine, such as L-NAME (N^G-nitro-L-arginine methyl ester), L-NNA (N^G-nitro-L-arginine) or L-NMMA (L-N^G-monomethyl-arginine monoacetate) that support the existence of a L-arginine-dependent NOS activity in plants (Corpas et al. 2009b). A comparison of the biochemical properties of animal and plant NOS activities is shown in Table 15.2. Recently it has been reported the characterization of a nitric oxide synthase in *Ostreocccus tauri*, a unicellular species of marine green alga (Foresi et al. 2010). The full-length sequence of *O. tauri* NOS showed a similarity of 42 %, 43 %, and 34 % with respect to eNOS, iNOS, and nNOS, respectively. Moreover, the authors suggested that the active form of *O. tauri* NOS is a dimer with a subunit of 119 kDa which is close to the molecular mass of the animal NOS subunits (Foresi et al. 2010).

Table 15.2 Comparison between animal and plant NOS activities and their subcellular localization. In plants the L-arginine-dependent NOS activity was determined in extracts of olive leaves (Valderrama et al. 2007), pea leaves (Corpas et al. 2004a, 2006), and sunflower hypocotyls (Chaki et al. 2009) using the ozone chemiluminiscence assay (NOA). Reproduced from Corpas et al. 2009b. New Phytologist 184:9–14

Biochemical parameters	Mammal NOS	Plant NOS
Substrate	L-Arginine	L-Arginine
Cofactors requirement:		
NADPH	+	+
Ca^{2+}	+	+
Calmodulin	+	+
FAD, FMN, BH_4	+	+
Sensitivity to inhibitors of NOS activity (aminoguanidine, L-NNA, L-NMMA, L-NAME)	+	+
Isozymes	3	n.d.
Subcellular localization	Cytosol Mitochondria Peroxisomes	Chloroplasts Peroxisomes

n.d. not determined

The first biochemical characterization of a nitric oxide synthase activity in higher plants was accomplished in isolated peroxisomes (Barroso et al. 1999). Using peroxisomes purified from pea leaves, a NOS activity was determined by monitoring the conversion of L-[^{3}H]arginine into L-[^{3}H]citrulline (Barroso et al. 1999). With this method it was also possible to confirm that this activity was strictly dependent on L-arginine, NADPH, calcium, calmodulin, FAD, FMN and BH_4, the same cofactors necessary for the animal NOS (Table 15.2). Likewise, this peroxisomal activity was sensitive to characteristic inhibitors of the three NOS isoforms.

Due to the still existing controversy on the presence of this NOS activity in plants, mainly derived from the existence of different pathways which can metabolize the amino acid L-arginine including arginase (which catalyses the hydrolysis of L-arginine to L-ornithine and urea) and arginine decarboxylase (involved in the synthesis of polyamines) which mimicks a false NOS, an alternative method to determine this NOS activity in peroxisomes was set up. Consequently, the NOS activity was assayed by a spectrofluorometric method using the fluorescence probe DAF-2 DA, and also by ozone chemiluminescence (Corpas et al. 2004a, 2008). The ozone chemiluminescence method is based on the following reactions:

$NO + O_3 \rightarrow NO_2^* + O_2$ and $NO_2^* \rightarrow NO_2 + h\nu$, where NO_2^* denotes the NO_2 molecule in the excited state and $h\nu$ represents an emitted photon. Chemiluminescence resulting from these reactions is detected by a photomultiplier tube (Corpas et al. 2008). Figure 15.3a shows the biochemical characterization of NOS activity in peroxisomes purified from pea leaves. Again this NOS activity required the same cofactors obtained by the previous assay where the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline was monitored (arginine-citrulline assay).

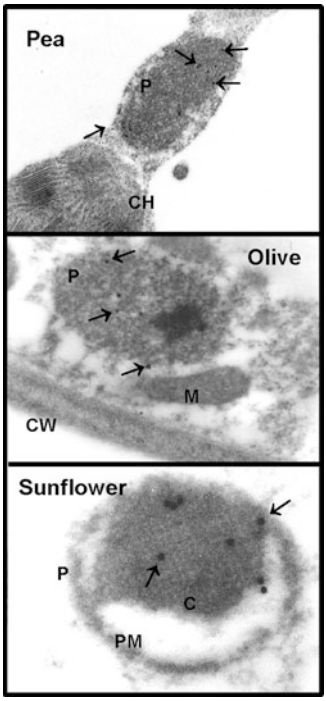

Fig. 15.2 Immunogold electron microscopy localization of NOS in peroxisomes from three plant species: pea leaves (×10,000), olive leaves (×16,000) and sunflower hypocotyls (×63,000). *C* peroxisomal core, *CH* chloroplast, *CW* cell wall, *M* mitochondrion, *P* peroxisome, *PM* peroxisomal membrane. Arrows indicate 15 nm gold particles. Reproduced from Corpas et al. 2004b. Studium Press, pp 111–129

Using an antibody against murine iNOS, it was demonstrated by western blotting the presence in peroxisomes from pea leaves of an immunoreactive polypeptide of about 130 kDa. The NOS activity was immunolocalized in these peroxisomes by electron microscopy (Barroso et al. 1999), and its peroxisomal presence was ratified by confocal laser scanning microscopy which showed that the NOS activity colocalized with catalase, a characteristic peroxisomal marker (Corpas et al. 2004a). Later the immunolocalization of this protein in pea leaf peroxisomes was also corroborated in other plant species using the same antibody. Figure 15.2 shows representative electron micrographs of thin sections of pea leaf, olive leaf and sunflower hypocotyl where the presence of immuno-reactivity in peroxisomes was detected (Corpas et al. 2004b).

Therefore, on the basis of the different experimental approaches reported so far, there are clear and conclusive evidence which support the existence of an L-arginine-dependent NOS activity in plant peroxisomes (Corpas et al. 2001, 2009b; del Río 2011).

In the case of animal peroxisomes, the occurrence of an iNOS in peroxisomes of rat hepatocytes was also reported (Stolz et al. 2002) being later demonstrated that this peroxisomal iNOS was the monomeric form of the dimeric iNOS found in the cytosol (Loughran et al. 2005).

15.4 Detection of Endogenous NO in Plant Peroxisomes

Although the presence of NO in peroxisomes as a consequence of the existence of nitric oxide synthase activity could be clearly deduced, an unavoidable task to corroborate it was the detection of this free radical within the organelle. Two different but complementary approaches have been used, including: 1. Electron paramagnetic resonance (EPR) spectroscopy with the spin trap Fe(II)-dithiocarbamate [$Fe(MGD)_2$] that reacts with NO forming a stable NO-$Fe(MGD)_2$ complex; and 2. The subcellular co-localization of NO by confocal laser scanning microscopy (CLSM) in *Arabidopsis* plants expressing GFP through the addition of a peroxisomal targeting signal 1 (PTS1) (Mano et al. 2002). Figure 15.3b shows a representative EPR spectrum of the NO-$Fe(MGD)_2$ spin adduct produced in isolated peroxisomes from pea leaves with the typical triplet signal of this spin adduct ($g = 2.05$ and $a_N = 12.8$ G). As positive control of NO production, commercial neuronal NOS was used under the standard assay conditions (Corpas et al. 2004a). In addition, Fig. 15.3 (panels c–e) shows the *in vivo* CLSM visualization of NO in root peroxisomes from transgenic *Arabidopsis* plants expressing the green fluorescence protein (GFP) through the addition of a peroxisomal targeting signal 1 (PTS1). Peroxisomes appeared as green spherical spots in all root tip cells (Fig. 15.3c). Using the fluorescent probe diaminorhodamine-4 M acetoxymethyl ester (DAR-4 M AM) it was possible to detect NO as an intense red fluorescence in spots with a similar pattern to that of GFP-PTS1 (Fig. 15.3d). Figure 15.3e shows a merged image indicating a complete overlap of the two punctuate patterns, what corroborates the presence of NO in *Arabidopsis* root peroxisomes (Corpas et al. 2009a). Therefore, the use of these two complementary technical approaches, EPR spectroscopy and CLSM colocalization in transgenic *Arabidopsis* plants expressing GFP in peroxisomes, allows concluding that NO is present in plant peroxisomes.

15.5 Function of Peroxisomal NO During Natural Senescence

Natural senescence of plants is a complex process controlled by multiple developmental and environmental signals (Buchanan-Wollaston 1997; del Río et al. 1998). Pioneer works on nitric oxide showed that the application of exogenous NO to senescent pea plants reduced the generation of ethylene which is a phyto-hormone involved in the regulation of senescence (Leshem and Haramaty 1996). Moreover, exogenous NO can retard the flower and plant senescence as well as fruit ripening (Leshem et al. 1998; Leshem 2000). Similarly, it has been reported that exogenous NO counteracts the senescence of rice (*Oryza sativa*) leaves induced by abscisic acid (Hung and Kao 2003). Although there is little information on the molecular function of NO in these processes, there are different reports suggesting that NO and other RNS are involved in plant senescence (Procházková and Wilhelmová, 2011).

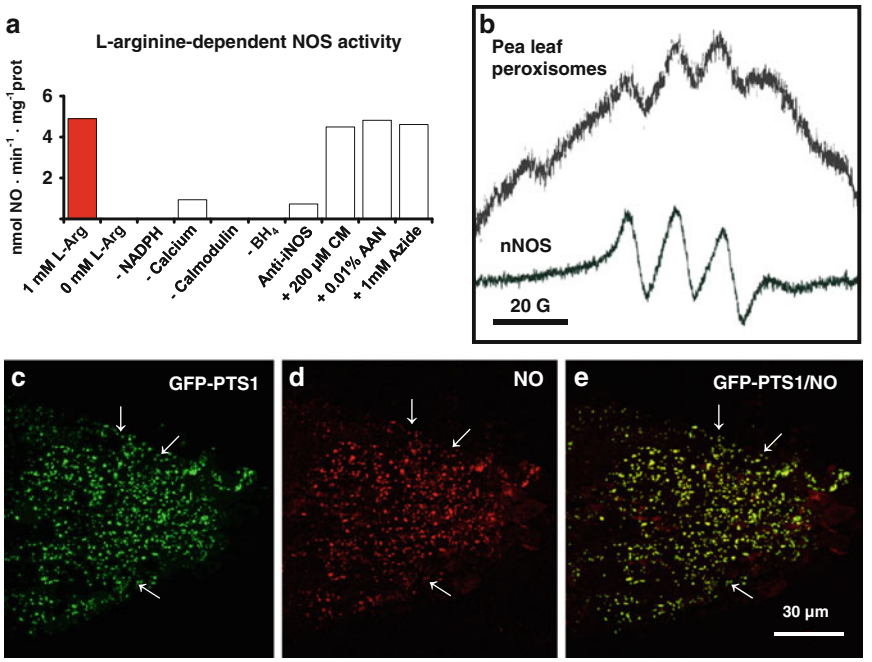

Fig. 15.3 Determination of nitric oxide synthase activity and nitric oxide in plant peroxisomes. (**a**). Biochemical characterization of L-arginine-dependent NOS activity in peroxisomes isolated from pea leaves by the ozone chemiluminiscence method. Reaction mixtures containing peroxisomes that were incubated in the absence and presence of L-arginine (1 mM), NADPH (1 mM), EGTA (0.5 mM), cofactors (10 μM FAD, 10 μM FMN and 10 μM BH_4), antibody against animal iNOS (Anti-iNOS), carboxymethoxylamine (CM) and aminoacetonitrile (AAN), two inhibitors of the P protein of the glycine decarboxylase, or azide (an inhibitor of nitrate reductase). Reproduced from Corpas et al. (2009b) New Phytologist. (**b**). EPR spectra of the NO-spin adduct of the $Fe(MGD)_2$ complex obtained with purified pea leaf peroxisomes and commercial neuronal nitric oxide synthase (nNOS). Reproduced from Corpas et al. (2004a) Plant Physiol 136:2722–2733, Copyright American Society of Plant Biologists. (**c**) Representative image illustrating the CLSM detection of peroxisomes in root tips of *Arabidopsis* seedlings expressing GFP through the addition of a peroxisomal targeting signal 1 (GFP-PTS1) where the fluorescence punctates (*green*), attributable to GFP-PTS1, indicate the localization of peroxisomes. (**d**) Fluorescence punctates (*red*) attributable to NO detected in the same root area. (**e**) Merged image of (**c**) and (**d**) showing colocalized fluorescence punctates (*yellow*). NO was detected with DAR-4M AM (excitation 543 nm; emission 575 nm) and peroxisomes with GFP (excitation 495 nm; emission 515 nm). Arrows indicate representative punctuate spots corresponding to NO and peroxisome localization. Reproduced with permission from Plant Physiol. (2009a) 151:2083–2094. Copyright American Society of Plant Biologists

Plant peroxisomes have a prominent oxidative metabolism and it has been reported that peroxisomal ROS metabolism is also involved in the process of senescence (del Río et al. 1998). Thus, during leaf senescence some peroxisomal enzymes, such as catalase and certain photorespiration enzymes, are down-regulated

(Strother 1988; Pastori and del Río 1997) while some enzymes of the glyoxylate cycle, like malate synthase and isocitrate lyase, are up-regulated (De Bellis et al. 1990; Pastori and del Río 1997). In this content, it has been reported that the peroxisomal L-arginine-dependent NOS activity was down regulated by 72 % in senescent pea leaves and it was accompanied by a reduction of NO content (Corpas et al. 2004a). These data imply that the peroxisomal metabolism, including nitric oxide, is involved in the process of plant senescence.

15.6 Peroxisomal NO Under Salinity Stress

In the previous sections, the presence in peroxisomes of a L-arginine-dependent NOS activity and the product of this reaction, nitric oxide, has been shown. Beyond this point, the question to be addressed is if the peroxisomal NO can be released into the cytosol and which is its physiological function at cellular level. To reach this objective, a strategy was designed which consisted in comparing the NO content in different types of *Arabidopsis* mutants expressing GFP-PTS1 that were exposed to salinity stress, since it has been shown that this abiotic condition induces both oxidative and nitrosative stress, characterized by a rise in the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), respectively (Valderrama et al. 2007). For these sets of experiments, it was selected the *Arabidopsis* mutant designated as *apm4/pex12* (Mano et al. 2006) which is defective in PTS1-dependent protein transport to peroxisomes and displayed lower NO content (Corpas et al. 2009a).

The localization and production of NO in roots of *Arabidopsis* plants expressing GFP-PTS1 under salinity stress (100 mM NaCl) is presented in Fig. 15.4 (panels a–f). As it is shown, root peroxisomes are subcellular compartments where NO is produced mainly in control (0 mM NaCl) plants (Fig. 15.4a, c), as both punctuate patterns are seen to overlap in the merged image (Fig. 15.4e). Under salinity conditions, the pattern of peroxisomes did not appreciably change compared with control (0 mM NaCl) plants; however, NO production increased significantly under salinity conditions (Fig. 15.4d). The distribution of NO fluorescence detected in both peroxisomes and cytosol also varied considerably (Fig. 15.4d, f). This suggests that under salinity stress NO is released from the peroxisomes into the cytosol. To corroborate this hypothesis, the effect of salinity stress was studied in *apm4/pex12* mutants (Fig. 15.4g–l) which are defective in the PTS1-dependent protein transport machinery to peroxisomes. In Fig. 15.4g, h, the pattern of peroxisomes appears as green spots in the roots of control and salt-stressed plants, with a slight increase in the number of peroxisomes being observed under salinity stress. Figure 15.4i shows the location of NO (red color) in the same root area of Fig. 15.4g (0 mM NaCl) where NO was almost totally absent. Under salinity stress (Fig. 15.4j), NO slightly increased. Figures 15.4k, l, show the merged images of control and stressed roots, respectively, where it can be clearly observed that NO is present in

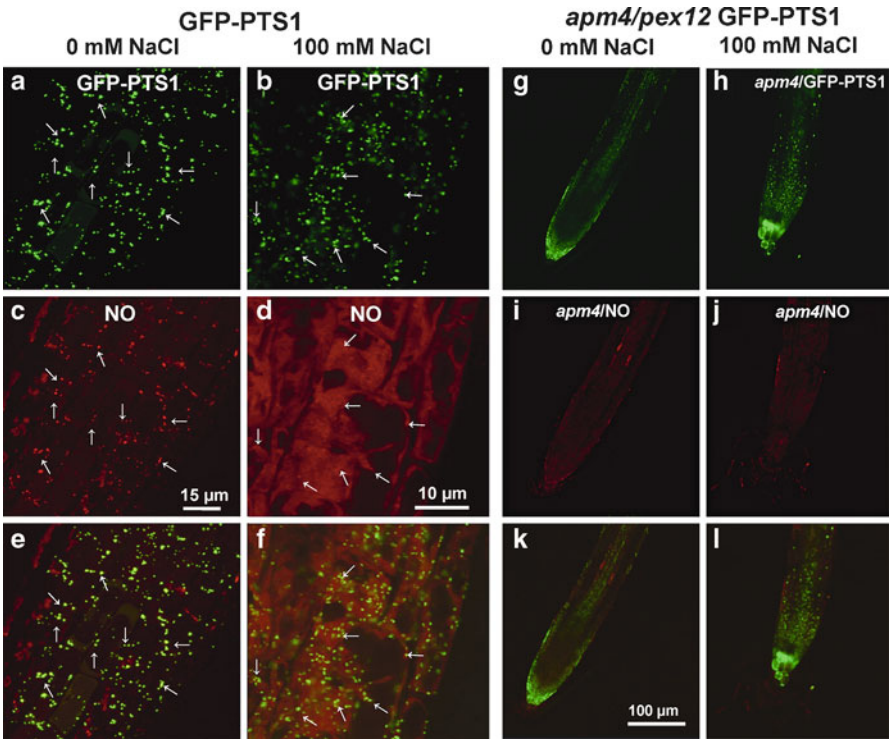

Fig. 15.4 *In vivo* NO detection by confocal laser scanning microscopy (CLSM) in root tips of wild type (**a**–**f**) and *apm4/pex12* mutants of *Arabidopsis* seedlings exposed to 100 mM NaCl. In both *Arabidopsis* plants the expression of GFP-PTS1 was detected as green spots, whereas red labelling corresponded to NO detection. (**a** and **b**), Fluorescence punctuates attributable to GFP-PTS1, indicating the localization of peroxisomes in control (0 mM NaCl) and salt-treated plants, respectively. (**c** and **d**), Fluorescence punctuates attributable to NO detection in control (0 mM NaCl) and salt-treated plants, respectively. (**e**), Merged image of (**a**) and (**c**) showing colocalized fluorescence punctates (*yellow*). (**f**), Merged image of (**b**) and (**d**) showing colocalized fluorescence punctates (*yellow*). (**g** and **h**), Fluorescence punctuates attributable to GFP-PTS1, indicating the location of peroxisomes in control (0 mM NaCl) and salt-treated *apm4/pex12* mutants, respectively. (**i** and **j**), Fluorescence punctuates attributable to NO detection in the same root area of (**g**) and (**h**), respectively. (**k**), Merged image of G and I showing colocalized fluorescence punctuates (*yellow*). (**l**), Merged image of (**h**) and (**j**) showing colocalized fluorescence punctuates (*yellow*). NO was detected with DAR-4M AM (excitation 543 nm; emission 575 nm) and peroxisomes with GFP (excitation 495 nm; emission 515 nm). Arrows indicate representative punctuate spots corresponding to NO and peroxisome localization. Reproduced with permission from Plant Physiol. (2009a) 151:2083–2094. Copyright American Society of Plant Biologists

cytosol, indicating that the peroxisomal protein responsible for NO generation was not imported into the peroxisomes due to the malfunction of the protein targeting mechanism. These data suggest that in *Arabidopsis* roots, peroxisomes are the main source of NO under normal and stress conditions.

15.7 Conclusions

Reactive nitrogen species (RNS) constitute a NO-derived family of molecules produced in different cell compartments, under physiological and stress conditions, where peroxisomes are important components. The emerging roles of some of these molecules in signal transduction pathways of cell communication have attracted the attention of many scientists interested in their physiological implications. Thus, one of the challenges in research of plant peroxisome biology is to demonstrate that RNS messenger molecules, such as NO, generated inside peroxisomes can diffuse out of these organelles to exert their effects in other cell loci. Considering the actual knowledge available, Fig. 15.5 summarizes schematically the main components involved in the metabolism of nitric oxide in plant peroxisomes. The NO generated from L-arginine by a NO synthase activity can react with different molecules of peroxisomal metabolism, such as superoxide radicals ($O_2^{\cdot -}$), to generate peroxynitrite ($ONOO^-$) which can mediate a process of protein tyrosine nitration in some peroxisomal proteins, including catalase, glycolate oxidase and malate dehydrogenase (Lozano-Juste et al. 2011). Alternatively, NO can interact

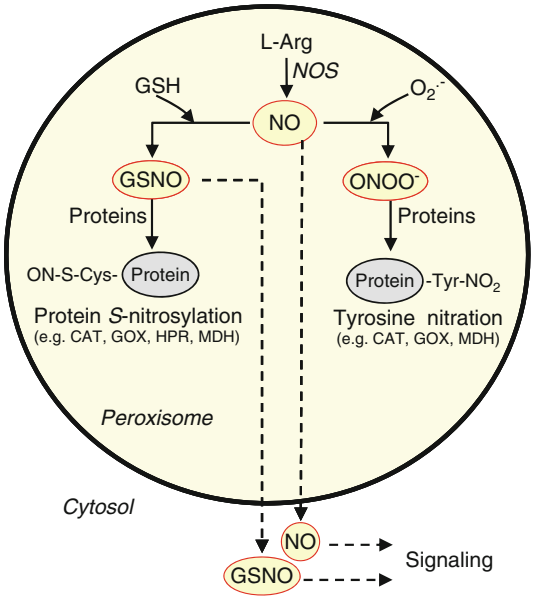

Fig. 15.5 Schematic model of nitric oxide metabolism in plant peroxisomes. L-Arginine-dependent nitric oxide synthase (NOS) generates NO which can react with reduced glutathione (GSH) in the presence of O_2 to form *S*-nitrosoglutathione (GSNO). This metabolite can interact with SH-containing proteins by a process of *S*-nitrosylation affecting their function. On the other hand, NO can react with superoxide radicals ($O_2^{\cdot -}$) to generate peroxynitrate ($ONOO^-$) which can mediate a process of tyrosine nitration of proteins. Alternatively, either NO or GSNO can be released to the cytosol to participate in signaling cascades. *CAT* catalase, *GOX* glycolate oxidase, *MDH* malate dehydrogenase, *HPR* hydroxypyruvate reductase

with reduced glutathione to produce *S*-nitrosoglutathione which can carry out the *S*-nitrosylation of some peroxisomal enzymes like malate dehydrogenase, catalase, glycolate oxidase and hydroxypyruvate reductase (Clark et al. 2000; Ortega-Galisteo et al. 2012). On the other hand, both NO and GSNO could be released to the cytosol, being this process specially significant under stress conditions, such as salinity, where NO is overproduced (Corpas et al. 2009a) and initiate a signaling cascade or interact with other biomolecules causing post-translational modifications. Future research is needed to discover the identity of the peroxisomal enzyme(s) responsible for NO generation from L-arginine as well as more specific peroxisomal targets susceptible of post-translational modifications.

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Chapter 16
Role of Plant Peroxisomes in the Production of Jasmonic Acid-Based Signals

José León

Contents

Abstract Jasmonates are a family of oxylipins derived from linolenic acid that control plant responses to biotic and abiotic stress factors and also regulate plant growth and development. Jasmonic acid (JA) is synthesized through the octadecanoid pathway that involves the translocation of lipid intermediates from the chloroplast membranes to the cytoplasm and later on into peroxisomes. The peroxisomal steps of the pathway involve the reduction of *cis*-(+)-12-oxophytodienoic acid (12-OPDA) and dinor-OPDA, which are the final products of the choroplastic phase of the biosynthetic pathway acting on 18:3 and 16:3 fatty acids, respectively. Further shortening of the carbon side-chain by successive rounds of β-oxidation reactions are required to complete JA biosynthesis. After peroxisomal reactions are completed, (+)-7-*iso*-JA is synthesized and then transported to the cytoplasm where is conjugated to the amino acid isoleucine to form the bioactive form of the hormone (+)-7-*iso*-JA-Ile (JA-Ile). Further regulatory activity of JA-Ile triggering gene activation in the jasmonate-dependent signaling cascades is exerted

J. León (✉)
Instituto de Biología Molecular y Celular de Plantas, CSIC – Universidad Politécnica de Valencia, Valencia, Spain
e-mail: jleon@ibmcp.upv.es

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through a process mediated by the perception via the E3 ubiquitin ligase COI1 and further ligand-activated interaction with the family of JAZ repressor proteins. Upon interaction, JAZ are ubiquitinated and degraded by the proteasome, thus releasing transcription factors such as MYC2 from repression and allowing the activation of JA-responsive genes.

Keywords Oxylipins • Chloroplast • Peroxisome • Jasmonates • Wounding • Reproductive development

16.1 Introduction

Peroxisomes have been traditionally linked to the metabolism of hydrogen peroxide and the oxidation of fatty acids in diverse biological systems ranging from mammals and plants to yeasts. Peroxisomes are highly dynamic organelles that are continuously being formed and degraded, and also undergo transitions between different types with varied enzyme endowment and alternative metabolic functions (Michels et al. 2005; Kaur et al. 2009; Rodríguez-Serrano et al. 2009). Moreover, the number of peroxisomes per cell is also changeable and responds to environmental and endogenous cues (Kaur et al. 2009; del Río 2011; Islinger et al. 2012). Plant peroxisomes have been extensively characterized as the site for β-oxidation-mediated degradation of fatty acids, the photorespiratory glycolate pathway and the glyoxylate cycle. However, increasing work during the last decades allowed the characterization of new biological functions exerted by plant peroxisomes. Among them, peroxisomes have been characterized as factories for the production of signaling molecules (Masters 1996; Corpas et al. 2001; Nyathi and Baker 2006). Peroxisomal β-oxidation-mediated shortening of large precursors yield molecules with hormonal activity such as jasmonates (JA) (Gfeller et al. 2010) or the auxin indole-3-acetic acid (IAA) (Normanly 2010; see also Chap. 14 of this volume). Besides, peroxisomes are involved in the production and metabolism of reactive oxygen and nitrogen species (Corpas et al. 2001; del Río 2011; see also Chaps. 13 and 15 of this volume) with demonstrated signaling potential in plant responses to abiotic and biotic stress (Molassiotis and Fotopoulos 2011). Peroxisomes participate in the regulation of many developmental processes of plants through the synthesis of hormones and redox-related signaling molecules (see Chap. 11 of this volume) including photomorphogenesis (Desai and Hu 2008), fertilization (Boisson-Dernier et al. 2008), seed production (Footitt et al. 2007), embryogenesis (Sparkes et al. 2003), germination and early postgerminative growth (Footitt et al. 2006; Rylott et al. 2006), inflorescence formation (Richmond and Bleecker 1999) and leaf senescence (del Río et al. 1998). Moreover, substantial evidence for a role of leaf peroxisomes in defense against pathogens and herbivores has been also reported (Imazaki et al. 2010; see Chaps. 17 and 18 of this volume). Many of the above mentioned developmental and defense-related responses are indeed regulated by jasmonates (JAs), which are synthesized through the so-called

octadecanoid pathway. This biosynthetic pathway is a complex process involving different subcellular organelles such as plastids and peroxisomes. Upon completion of JA biosynthesis inside peroxisomes, this molecule is extensively metabolized in the cytoplasm to a wide array of JA-derived metabolites some of which will be active in controlling gene expression and some others will be either inactivated forms or storage forms for further mobilization.

16.2 Early Steps of JA Biosynthesis Occur in Chloroplasts

JAs are oxylipins derived from polyunsaturated fatty acids, mainly 18C linoleic acid and linolenic acid and 16C hexadecatrienoic acid. The jasmonate family of phytohormones comprises several chemicals sharing a cyclopentenone motif with two aliphatic side chains similar to that present in animal prostaglandins (Fig. 16.1). The pathway of JA biosynthesis starts at the chloroplast membranes where phospholipases catalyze the hydrolysis of unsaturated fatty acids from membrane galactolipids. Figure 16.2 summarizes the multiorganelle-based pathway of JA biosynthesis. In *Arabidopsis*, two enzymes with *sn-1*-specific galactolipase activity DEFECTIVE IN ANTHER DEHISCENCE 1 (DAD1) and DONGLE (DGL) have been reported to be localized in plastids and to be essential for basal JA biosynthesis (Ishiguro et al. 2001; Hyun et al. 2008). However, they seem to be dispensable for wound- and pathogen-induced JA biosynthesis, where phospholipase PLA-Iγ1 plays an important role (Ellinger et al. 2010). Moreover, it has also been recently reported that the patatin-like phospholipase pPLAIIa

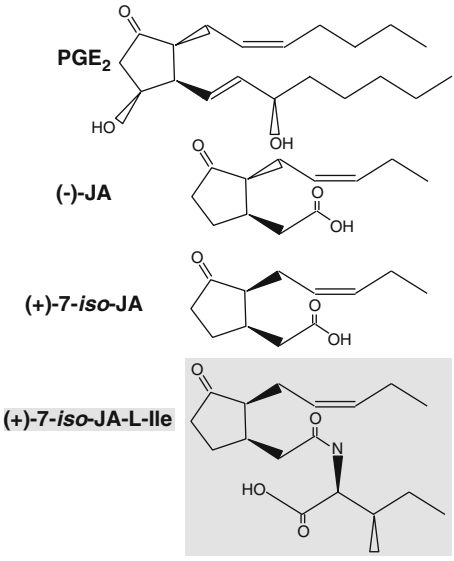

Fig. 16.1 Chemical structures of prostaglandin PGE2 and jasmonates (*JA*s) sharing the cyclopentenone ring with variable side aliphatic chains. The plant active JA form jasmonoyl-isoleucine is highlighted in *gray*

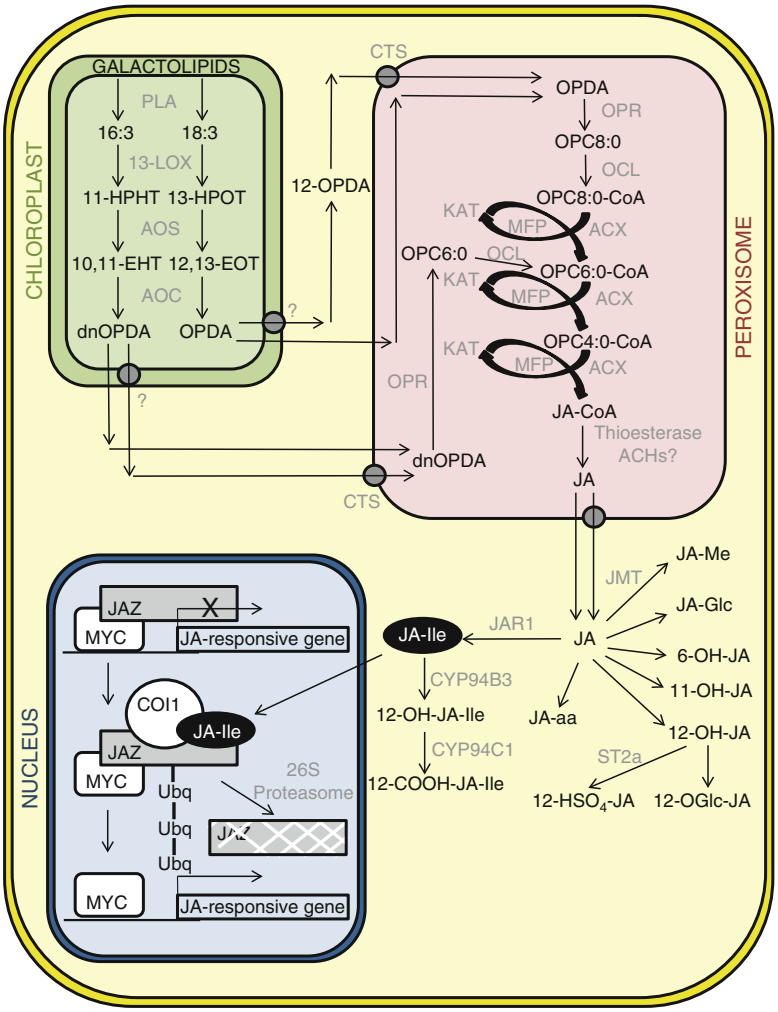

Fig. 16.2 Multiorganelle-based biosynthetic pathway of JA in plants. JA is synthesized in chloroplasts and peroxisomes, metabolized in the cytoplasm and perceived in the nucleus as JA-Ile, where it modulates gene expression through ubiquitin (*Ubq*)- and proteasomal degradation of JAZ repressors of transcription factors of the MYC family. Enzymes catalyzing the different biosynthetic or catabolic steps are marked in *gray*

negatively regulates JA production (Yang et al. 2012), and it may be involved in removing oxidated fatty acid from membranes. Unsaturated fatty acids released from galactolipids should incorporate molecular oxygen into the C-13 position in reactions catalyzed by dioxygenases of the 13-lipoxygenases (13-LOX) family (Feussner and Wasternack 2002) before further transformations in the chloroplast to occur. LOX families of different plants usually comprise quite a large number of

members. In *Arabidopsis*, only a subset of four members corresponds to 13-LOX (Bannenberg et al. 2009). From them, only AtLOX2 has been confirmed to be responsible for JA biosynthesis in wound-activated responses (Bell et al. 1995). Nevertheless, the product of 13-LOX action on unsaturated fatty acids is the 13-hydroperoxide of the corresponding fatty acids, which are substrates for enzymes of a subfamily of cytochrome P450s called allene oxide synthases (AOS). In model systems such as *Arabidopsis* and tomato, one and two genes coding for AOS have been identified (Laudert et al. 1996; Howe et al. 2000), and the enzyme is targeted to chloroplast and associated to plastid membranes (Farmaki et al. 2007). The last reaction occurring inside chloroplasts involved cyclation of allene oxide to *cis*-(+)-12-oxophytodienoic acid (OPDA) or dinor-OPDA when coming from 18:3 or 16:3 fatty acids, respectively (Stumpe and Feussner 2006), a process catalyzed by different enzymes of the allene oxide cyclase family (Schaller et al. 2008). OPDA has biological activity as demonstrated by inducing JA-independent gene expression (Dave and Graham 2012).

The chloroplastic phase of jasmonate biosynthesis concludes with OPDA or dinor-OPDA synthesis, which have already the enantiomeric structure of jasmonates but require of further hydrocarbon shortening. Plant cells use the β-oxidation machinery to shorten the lateral hydrocarbon chain of the cyclopentenone core structure (Fig. 16.2). This biochemical process occurs mainly in peroxisomes (Baker et al. 2006). Because OPDA and dinor-OPDA were synthesized in the chloroplasts, transport from chloroplasts to peroxisomes is required. It is still not completely clear how OPDAs are exported from the chloroplast to the cytoplasm. Since OPDA is itself a molecule with signaling potential (Dave and Graham 2012), it is likely that the control of its movement between subcellular compartments would be highly regulated. To complete JA biosynthesis it is anyway necessary a further transport from cytoplasm into peroxisomes. Both passive and active transport across peroxisomal membrane have been reported. Active transport is mediated by the ATP-binding cassette transporter COMATOSE (CTS)/PXA1/PED3 that is required to import precursors into peroxisome and to complete JA biosynthesis (Theodoulou et al. 2005; see Chap. 9). Because *ped3* mutants were not completely depleted of JAs either another redundant transporter or passive movement across membrane should be also operative. The physicochemical properties of OPDA make it sufficiently lipophilic to cross membranes passively through ion trapping mechanism in peroxisomes but with lower efficiency than the ATP-driven CTS-mediated active transport (Theodoulou et al. 2005). Because OPDAs are synthesized in chloroplasts and transformed in peroxisomes, there must be OPDAs pools in all three subcellular compartments. Different subcellular localizations of OPDAs may be related to different functions as JA precursors or as direct JA-independent regulators. Regarding this, OPDA or an OPDA-related compound seems to be essential for proper embryo development in tomato (Goetz et al. 2012), for inhibition of seed germination coordinately with ABA (Dave et al. 2011) and for COI1-mediated induction of *PHO1;H10* gene, involved in the transfer of phosphate to the xylem in the root (Ribot et al. 2008), all functions for which JAs are ineffective.

16.3 Peroxisomal β-Oxidation Completes JA Biosynthesis

As shown in Fig. 16.2, once OPDAs have been transported into peroxisomes and before undergoing β-oxidation-mediated chain shortening, substrates are reduced by a small family of flavin-dependent 12-oxophytodienoate reductases (OPR). OPR families comprise different number of members in different plants ranging from three members in tomato to ten in rice (Li et al. 2009). However, the relevant (9*S*,13*S*)-enantiomer of OPDA is substrate only for OPR3 from *Arabidopsis* and tomato and its ortholog in other plants such as OsOPR7 from rice (Breithaupt et al. 2009), and thus the only ones involved in JA biosynthesis. OPR3 acting on (9*S*,13*S*)-12-oxophytodienoic acid or dinor-OPDA leads to 3-oxo-2-(2′-[Z]-pentenyl)-cyclopentane-1-octanoic acid (OPC-8) or 3-oxo-2-(2′-[Z]-pentenyl)-cyclopentane-1-hexanoic acid (OPC-6), respectively. OPC-8 and OPC-6 have to be converted to the corresponding acyl-CoA derivatives in reactions catalyzed by acyl-CoA synthetases before being substrates for three or two rounds of β-oxidation, respectively. Acyl-CoA synthetases are encoded by gene families that comprise a varied number of components depending on the plant species. In *Arabidopsis*, acyl-CoA synthetases belong to the acyl-activating enzymes, which comprises more than 60 genes and 17 of them are presumably peroxisome-targeted proteins (Reumann et al. 2004). Based on co-expression analysis with OPR3, an OPC-8:0 CoA ligase 1 (OPCL1) protein was demonstrated to be involved in JA biosynthesis in *Arabidopsis* (Koo et al. 2006). To complete the biosynthesis of JA, the side chains of the above mentioned precursors must be shortened by β-oxidation in six and four carbons, respectively.

Each round of β-oxidation involves the sequential function of three enzymes, acyl-CoA oxidases (ACX), multifunctional proteins (MFP) and 3-ketoacyl-CoA thiolases (KAT) and removes two C units from the aliphatic chains. In *Arabidopsis*, there are six ACXs with different substrate specificities for short, medium and long chain fatty acyl-CoA (Schilmiller et al. 2007). Besides, there are two MFPs (AIM1 and MFP2; Richmond and Bleecker 1999; Rylott et al. 2006) and three KATs (KAT1, KAT2, KAT5; Castillo et al. 2004), respectively, with still undefined substrate specificity. The biochemical characterization of *Arabidopsis* peroxisomal enzymes allowed to define that ACX1, ACX5 and KAT2 are involved in jasmonate biosynthesis (Castillo et al. 2004; Afitlhile et al. 2005; Li et al. 2005; Schilmiller et al. 2007), and it still remains undefined whether AIM1, MFP2 or both participate in that process. These MFP proteins have at least two enzyme activities namely 2E-enoyl-CoA hydratase and 3S-hydroxyacyl-CoA hydratase (Richmond and Bleecker 1999; Rylott et al. 2006). In addition to these enzyme activities, the multifunctional proteins may have also delta 3, delta 2-enoyl-CoA isomerase activity (Engeland and Kindl 1991).

After the three rounds of β-oxidation are completed, the jasmonoyl-CoA produced by KAT activity must be hydrolyzed by a still unidentified thiosterase to release (+)-7-*iso*-JA. There are two acyl-CoA thioesterases AtACH1 and AtACH2

in *Arabidopsis* with peroxisome targeting sequences (Tilton et al. 2000) but their involvement in releasing (+)-7-*iso*-JA has not been demonstrated so far for any of them.

16.4 Further Metabolism of JA Renders Both Active and Inactive Forms

Accumulated evidences support that the active form of JAs is actually the amino acid conjugate (+)-7-*iso*-Jasmonoyl-L-isoleucine (JA-Ile) (Fonseca et al. 2009). JA-Ile is perceived by a receptor complex comprising an F-box protein called Coronatine Insensitive 1 (COI1) in Arabidopsis (Yan et al. 2009), which has been extensively documented as essential for jasmonate-triggered responses (Xu et al. 2002), a JAZ protein repressor (Thines et al. 2007; Chini et al. 2007) and the cofactor inositol pentakisphosphate (Sheard et al. 2010). The JA perception system functions in such a way that binding of the bioactive hormone to the COI1 receptor induces ubiquitin-directed and proteasomal-mediated degradation of JAZ proteins. Because JAZs act as repressors of major JA-related transcription factors such as MYC2, their degradation would release MYC2 from repression and thus allowing the expression of JA-regulated genes (Fig. 16.2).

Conjugates of JA with amino acids, such as Ile, Leu, Val, Ala, Tyr, Trp and Phe have been detected in different plants, being JA-Ile the most abundant one (Piotrowska and Bajguz 2011). The Mg-ATP-dependent enzyme catalyzing the activation of JAs by conjugating it to Ile was called JAR1 in *Arabidopsis* (Staswick and Tyriaki 2004) and belongs to the acyladenylate-forming firefly luciferase superfamily. Two JAR1 homologues, JAR4 and JAR6, which seems to be not specific for JA-Ile but involved in the synthesis of several JA conjugates to Val and Leu, have been identified and characterized in *Nicotiana attenuata* (Wang et al. 2007).

The biosynthesis of JA-Ile is not only limited by the availability of precursor molecules of the chloroplastic and peroxisomal phases of the octadecanoid pathway but also by competition for free JA with several metabolic reactions including glycosylation, hydroxylation, methyl esterification and sulfonation (Fig. 16.2). For instance, it has been reported that over-expression of JA carboxyl methyltransferases, involved in converting JA into MeJA, led to reduced accumulation of JA-Ile and decreased biological impact exerted by the bioactive hormone (Stitz et al. 2011). Neither MeJA nor JA are bioactive in promoting the interaction between COI1 and JAZ as JA-Ile does. Moreover, JA-Ile can be also deactivated by other metabolic reactions (Koo and Howe 2012). The esterification with sugars such as glucose or gentiobiose reduces their activity. Because those sugar-conjugated JAs are soluble in water and they can release free JA upon β-glucosidase-based hydrolysis, they are likely storage forms of JAs. However, this is not the only way to inactivate jasmonates. It has been described that hydroxylation events at different carbons of

the JA molecule might render JAs with altered activity including (−)-12-hydroxyJA, (−)-9,10-dihydroJA, (−)-11,12-dihydroJA, or cucurbic acid. Not all of them are inactive, instead some others such as (−)-12-hydroxyJA, also called tuberonic acid, has been described to be extraordinarily effective in inducing tuberization in potato (Yoshihara et al. 1989). Finally, it has been also proposed that another way to inactivate JAs is through sulfonation. The formation of sulfate conjugates of JAs have been reported in several plants (Achenbach et al. 1994). In *Arabidopsis*, it seems to be catalyzed by the AtST2a sulfotransferase, which has strict specificity for (−)-11-and (−)-12-hydroxyjasmonic acid as substrates (Gidda et al. 2003). It is noteworthy that only (−) derivatives have been identified, suggesting that metabolism of JAs occurs basically from the (−) instead of (+) stereoisomer. Moreover, also inactivation of the amino acid conjugates of JAs has been described. Two wound-inducible cytochromes P450, CYP94C1 and CYP94B3, are involved in JA-Ile oxidation and inactivation (Heitz et al. 2012).

16.5 JAs Regulate Development and Defense

During the last two decades, an increasing body of evidence accumulated pointing to JAs as important regulators of multiple aspects of plant development and responses to stress. JAs have been reported to control many processes throughout the whole life cycle of the plant, ranging from seed germination to reproductive development. It is now known that JAs regulate seed germination in tight coordination with ABA, gibberellins and ethylene, although it remains controversial the way they perform this regulatory function (Linkies and Leubner-Metzger 2012). JAs also regulate vegetative development in roots (Staswick et al. 1992) and promote secondary growth in shoots by stimulating cell division (Sehr et al. 2010), but, at the same time, is involved in regulating cell death and leaf senescence (Reinbothe et al. 2009). Also leaf movements seem to be regulated by JAs (Nakamura et al. 2011). The control of reproductive development by JAs is, by far, the best documented developmental roles of these phytohormones. Flower maturation is essentially controlled through functional interactions between auxins and JAs (Reeves et al. 2012), thus ensuring a timely stamen maturation and elongation (Song et al. 2011) and anther dehiscence (Wilson et al. 2011), finally leading to the proper deposit of pollen on receptive stigma and further pollen germination. However, despite their roles in regulating development, JAs have been well characterized as regulators of a wide array of biotic and abiotic stress factors. Their involvement in regulating wound activated responses is likely the best studied role of JAs, being involved in both local signal production at wounding sites as well as transmission and perception of those signals systemically (León et al. 2001; Koo and Howe 2009). JAs also participate in regulating responses of different plants to drought and salt stress in tight association with ABA (Seo et al. 2011; Ismail et al. 2012), as well as in mediating responses to heat stress in cooperation with salicylates (Li et al. 2010). Besides responses to abiotic stresses, JAs are one of the hormonal components participating in the complex activation of defense responses to pathogens. In this

context, it has been particularly well documented the antagonistic interactions between JAs and salicylates in the activation of defense against biotrophic pathogens (Thaler et al. 2012) and the positive role of JAs in the activation of defense responses to necrotrophic pathogens (Glazebrook 2005).

16.6 Peroxisomes, JAs and Responses to Wounding

Plants have primary pre-existing defensive barriers that help them to remain isolated from adverse environmental effects. However, these physical barriers are often damaged by the action of either unfavorable weather conditions, such as strong winds or extreme temperatures, or by the chewing activity of herbivores. Interestingly, once plant is damaged there is no possibility to mobilize specialized cells to the wound site like happens in healing responses in mammals. Plants have evolved to make every cell competent for activating defense against wounding through a double level mechanism. The first level acts through repairing the damaged tissue and blocking further access of negative agents to inside the plant, and the second level requires the activation of defense mechanisms directed to prevent further damage or the onslaught of secondary biotic stress factors (León et al. 2001). Wound activated responses occur in the time frame of minutes to few hours after damage and include the generation or release of local signals, their perception both locally and systemically, and the downstream signaling leading to activated expression of specific genes. Whereas repairing and healing are local responses, the activation of defense must occur both locally and systemically. Local and systemic wound-activated defenses are based on the production of different signals depending on the plant and the herbivore, but often including small mobile peptides such as systemin, oligosaccharides released from plant cell wall and hormones such as JA, ethylene and ABA (León et al. 2001). Despite the central role exerted by JA in regulating many wound activated responses, it has been characterized both JA-dependent and JA-independent pathways for the activation of gene expression (Titarenko et al. 1997). In *Arabidopsis*, both pathways seem to be oppositely regulated by common elements including reversible protein phosphorylation (Rojo et al. 1998) and calcium/calmodulin-regulated events (León et al. 1998).

After the early responses occurring immediately after wounding and involving local regulation of gene expression, a second wave of wound-triggered signals are required for further local and systemic responses. Oxylipins, and markedly JAs among them, are key molecules regulating those secondary responses. The role of plant peroxisomes in the last steps of JAs biosynthesis by hosting β-oxidation machinery action on fatty acid derivatives has been extensively documented in the response of plants to wounding or mechanical damage (Li et al. 2005). The integrity of peroxisomes appears to be essential for a fully functional β-oxidation of JA precursors independently of the dynamics of biogenesis, proliferation and autophagic degradation of the organelles. Similarly to what happen in mammals, plant cells have the capacity to change the number of peroxisomes per cell either in response to stress factors or after treatment with synthetic peroxisome

proliferators (del Río 2011). Plants, despite having components of the pathway required to trigger peroxisome proliferation such as PEX11 or the dynamin-related DRP3A proteins, do not have homologues of the Peroxisome Proliferator-Activated Receptors (PPARs), which function indeed as endogenous metabolic sensors of peroxisome proliferators (Nila et al. 2006). In *Arabidopsis*, clofibrate-induced peroxisome proliferation is accompanied by the COI1-mediated activation of wound- and JA-activated signaling, and wounding activated the expression of peroxisome biogenesis genes (Castillo et al. 2008). However, although there is a significant cross-regulation of clofibrate- and wound activated signaling, both processes remained uncoupled in *Arabidopsis*. Moreover, JA that is a positive regulator of wound-activated defense is indeed a negative regulator of peroxisome proliferation in *Arabidopsis* (Castillo et al. 2008).

16.7 Other Lipid-Derived Signals

As previously characterized in animals, plants are able to produce a wide array of oxylipins. Similarly to animals transforming arachidonic acid to prostaglandins (PGs) through the cyclooxigenase pathway, plants transform linolenic acid into JAs. However, PGs and JAs are not the only final products of fatty acid metabolism with signaling potential. Animals and plants produce other structurally related oxylipins called isoprostanes and dinor-isoprostanes, respectively, which seem to be formed by non-enzymatic free radical-catalyzed mechanisms. Plant dinor-isoprostanes, also called phytoprostanes, are structurally similar to JAs and play varied regulatory activities including adaptive responses to oxidative stress, inhibition of cell cycle progression, and modulation of the action of transcription factors and redox regulators (Mueller et al. 2008). Most of these activities are independent of JA perception and signaling and seems to be mediated through the action of transcription factors of the TGA family (Mueller et al. 2008). However, some phytoprostanes also share some biological activities with JAs including inhibition of root growth and induction of phytoalexin biosynthesis (Thoma et al. 2003), which are mediated by the JA receptor COI1.

OPDA and dn-OPDA can be esterified to the monogalactosyl diacylglycerol or digalactosyl diacylglycerol leading to the formation of galactolipids called arabidopsides in *Arabidopsis thaliana* and some other relative species. Accumulation of arabidopsides has been observed in responses to wounding and avirulent bacterial pathogens and are fully dependent on COI1 and JAR1 functions (Vu et al. 2012).

16.8 Conclusions

The increasing evidences supporting extensive regulatory roles of JAs in controlling a wide array of plant physiological processes and the crucial involvement of peroxisomes in their biosynthesis point to this organelle as an important signaling

factory in plants. On the other hand, the highly dynamic nature of peroxisomes, which are able to change in number and potential function, makes them well suited for being involved in the activation of rapid responses to either environmental or endogenous cues. Although the information on the role of JAs and peroxisomes in plant physiology as well as in their molecular and cellular functions has progressively increased during the last decade, several still obscure topics are pending to be clarified. A better knowledge of the functional interactions between JAs and other peroxisome-derived signals, mainly auxins and reactive oxygen and nitrogen species (ROS and RNS), will help to understand how plants use peroxisomes to exert a precise and simultaneously complex control of plant growth.

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Chapter 17
Role of Plant Peroxisomes in Protection Against Herbivores

Mohammed Shabab

Contents

Abstract Peroxisomes are subcellular organelles of vital importance. They are ubiquitous, have a single membrane and execute numerous metabolic reactions in plants. Plant peroxisomes are multifaceted and have diverse functions including, but not limited to, photomorphogenesis, lipid metabolism, photorespiration, nitrogen metabolism, detoxification and plant biotic interactions. Plants have evolved a variety of defence barriers against herbivory. These barriers are unique and loaded with various metabolites. Peroxisomes play an important role in cells, maintaining the compartmentation of certain specific reactions. They serve as a first line of

M. Shabab (✉)
Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Hans-Knöll Straβe 8, D-07745 Jena, Germany

Chemistry Unit, USDA-ARS-CMAVE, 1700 SW 23rd Drive, Gainesville, FL 32608, USA
e-mail: mohammed.shabab@ars.usda.gov

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defence, as peroxisomes generate primary signals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). Both ROS and RNS sense the invasion by herbivores and dramatically reshape the plant transcriptomes, proteomes, and metabolomes, so indicating the importance of signals generated by peroxisomes. Peroxisomes also store a plethora of important enzymes, which have a key role in producing defence molecules. Some of the main enzymes in the biosynthesis of isoprenoids are present in peroxisomes. These enzymes generate plant volatiles, which have numerous functions and important roles in plant-herbivore communication.

Although disputed, the enzyme myrosinase has also been reported to be present in peroxisomes, and myrosinases are well known for their role in the mustard bomb, a powerful defence against herbivores. This chapter focuses on the diverse roles of peroxisomes in the generation of direct and indirect defenses against herbivores.

Keywords Herbivores • Peroxisomes • ROS • RNS • NOS • Myrosinase • Mevalonate pathways

Abbreviations

GSNO	*S*-Nitrosoglutathione
GSNOR	S-nitrosoglutathione reductase
GSSG	Glutathione disulfide
IDP	Isopentenyl diphosphate
DMADP	Dimethylallyl diphosphate
IDI	Isopentenyl diphosphate isomerase
JAZ3	Jasmonate ZIM-domain 3
MAPK	Mitogen activated protein kinase
MEP	2-C-methyl-D-erythritol 4-phosphate
MVA	Mevalonic acid
MVD	Mevalonate 5-diphosphate decarboxylase
MVK	Mevalonate kinase
NADPH	Nicotinamide adenine dinucleotide phosphate
PMK	5-phosphomevalonate kinase
NO	Nitric oxide
NOS	Nitric oxide synthase
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SIPK	Salicylic acid-induced protein kinase
TGG	Beta-thioglucosidase glucohydrolase
VIGS	Virus induced gene silencing
WIPK	Wound-induced protein kinase
YFP	Yellow fluorescent protein

17.1 What Are Plant Herbivore Interactions?

In order to survive in highly dynamic and challenging environments, plants have evolved extraordinary skills. These skills are for fighting against biotic and abiotic threats. Plants use various defense strategies to effectively combat attacks by herbivores. These mechanisms include pre-existing physical and chemical barriers, as well as inducible defense responses that become activated upon insect herbivory (Mauricio et al. 1997). One of the important plant chemical defenses is the ability to synthesize indigestible compounds and to produce allelochemicals. Allelochemicals exhibit a high degree of diversity in terms of structure and targets. The production of these important allelochemicals is tightly regulated and orchestrated by various factors such as phytohormones. The compartment system inside plant cells helps plants by means of the toxic effects of allelochemicals and many inducible signals, and also serves as armory against pathogens and herbivores. One of these key compartments are peroxisomes, where many important oxidative events take place.

17.2 Peroxisomes

Peroxisomes are single membrane bound cellular organelles, present in almost all eukaryotic cells, which have an essentially oxidative metabolism (del Río et al. 2002; Kaur et al. 2009). Peroxisomes contains a variety of enzymes that primarily function together to detoxify the toxins present in the cell and most notably hydrogen peroxide, which is the most common byproduct of cellular metabolism in living systems. These organelles contain enzymes that convert the hydrogen peroxide to water, rendering the potentially toxic substance safe for release back into the cell. Peroxisomes differ from mitochondria and chloroplasts in many ways. Most notably, they are surrounded by only a single membrane, and they do not contain DNA (del Río et al. 2002; Nyathi and Baker 2006). The roles of peroxisomes in plant biotic and abiotic defences are becoming clearer (del Río et al. 2006; Reumann et al. 2007; Palma et al. 2009; Corpas et al. 2010). Peroxisomes contain important enzymes and generate diverse metabolites that have a relevant role in the direct and indirect plant defence against herbivores. This chapter analyzes the general signaling pathways originated in these organelles and the early signaling events induced by herbivory.

17.3 General Signaling Pathways

17.3.1 Reactive Oxygen Species

Reactive oxygen species (ROS) are produced during normal cellular metabolism and are also induced by changes in the environmental conditions (Vranova et al. 2002;

Apel and Hirt 2004; Halliwell and Gutteridge 2007). The primary ROS formed in the cell is the superoxide free radical (O_2^-) which can initiate a cascade of reactions that result in the formation of a variety of ROS depending on the cell type or cellular compartment (Apel and Hirt 2004). H_2O_2 formed due to the enzymatic and spontaneous dismutation of superoxide, is a key signaling molecule in both plants and animals (see Chap. 13). These reactive molecules are generated in various organelles like mitochondria, peroxisomes and plasma membranes (Corpas et al. 2001). In peroxisomes, the production of H_2O_2 has been known for a long time, and there is abundant information on the biochemical pathways of H_2O_2 generation (del Río et al. 2002, 2006; Nyathi and Baker 2006; Kaur et al. 2009; see Chap. 13).

Different reports have shown the importance of ROS in herbivory-related responses. Feeding of *Helicoverpa zae* on soybean plants leads to considerably elevated ROS levels (Wu and baldwin 2010). Another sample of increased ROS generation was in *Medicago truncatula,* where the mechanical wounding was not sufficient to increase the amount of ROS but herbivory contribution was essential for that (Leitner et al. 2005). In another interesting study, Maffei et al. detected an increase in the ROS levels in lima bean plants after *S. littoralis* attack (Maffei et al. 2006).

NADPH oxidase is a plasma membrane enzyme system known to be involved in the generation of ROS (Apel and Hirt 2004). Inhibition of NADPH oxidase activity in tomato plants results in decreased levels of defense-related genes, such as protease inhibitors, and a polyphenol oxidase (Orozco-Cárdenas et al. 2001). Diamine oxidase is another key enzyme involved in ROS production in plants. H_2O_2 may also be generated and function belowground since the expression of a diamine oxidase gene was induced in *Arabidopsis* roots after inoculation with root herbivore nematodes (Møller et al. 1998). These examples show that ROS play an important role in plant-herbivore interactions. Nevertheless, more studies are required to identify in plants those key regulators in the ROS generation pathways of peroxisomes in response to herbivores.

17.3.2 Reactive Nitrogen Species

Reactive nitrogen species (RNS) are various nitric oxide-derived compounds, including nitric oxide (NO), higher oxides of nitrogen, *S*-nitrosothiols, dinitrosyl iron complexes, etc. (Halliwell and Gutteridge 2007; Lamattina and Polacco 2007). In plants, there is increasing evidence of a role of nitric oxide (NO) as an endogenous plant growth regulator as well as a signal molecule in the transduction pathways leading to the induction of defense responses (Delledonne et al. 1998; Lamattina and Polacco 2007; Corpas et al. 2010; see Chap. 15).

The enzyme nitric oxide synthase (NOS) catalyzes the oxygen- and NADPH-dependent oxidation of L-Arginine to NO and citrulline in a complex reaction requiring different cofactors (Alderton et al. 2001). The subcellular localization of NOS in plants was first described in leaves of pea plants (Barroso et al. 1999;

Corpas et al. 2004). *S*-Nitrosoglutathione (GSNO) is an endogenous *S*-nitrosothiol that serves as a source of bioavailable NO, and the enzyme *S*-nitrosoglutathione reductase (GSNOR) catalyzes the NADH-dependent reduction of GSNO to GSSG and NH_3 (Barroso et al. 2006). Recently, by a variety of techniques it has been shown that this enzyme is present in peroxisomes (Ortega-Galisteo et al. 2012). Using a virus-induced gene silencing system (VIGS), the activity of GSNOR in a wild tobacco species, *Nicotiana attenuata*, was knocked down and the function of GSNOR in defence against the herbivore insect *Manduca sexta* was examined (Wunche et al. 2011). Silencing GSNOR in *Nicotiana attenuata* decreased the herbivory-induced accumulation of jasmonic acid (JA) and ethylene, two important phytohormones regulating plant defence levels, without compromising the activity of two mitogen-activated protein kinases (MAPKs), salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK) (Wunche et al. 2011). Also a decreased activity of trypsin proteinase inhibitors (TPIs) was detected in GSNOR-silenced plants after simulated *M. sexta* feeding and bioassays indicated that GSNOR-silenced plants have elevated susceptibility to *M. sexta* attack. Furthermore, silenced GSNOR plants have altered methyl jasmonate induced responses. For example, methyl jasmonate induced accumulation of defence-related secondary metabolites (TPI, caffeoyl putrescine, and diterpene glycosides) is reduced in amount. But the silencing has no effect on the transcriptional regulation of JAZ3 (jasmonate ZIM-domain 3) and TD (threonine deaminase), indicating that GSNOR mediates certain but not all jasmonate-inducible responses. These works clearly evidence the importance of GSNOR in plant resistance to herbivory and jasmonate signaling, and proves the involvement of NO in plant–herbivore interactions.

17.4 Early Signaling Events Induced by Herbivory

17.4.1 Specialized Enzymes Localized in Peroxisomes: Myrosinase

Certain plants species are known to evolve a novel defense system known as myrosinase-glucosinolate. These plants belongs to families of *Brassicaceae*, *Capparidaceae*, and *Tropaeolaceae* which includes: white mustard (*Sinapis alba*), cress (*Lepidium sativum*), wasabi (*Wasabia japonica*), daikon (*Raphanus sativus*) and the well-known model system *Arabidopsis thaliana*. The myrosinase-glucosinolate system serves as a major chemical defense mechanism against insects, bacteria and fungi (Bones and Rossiter 1996; Raybould and Moyes 2001). Glucosinolates (β-thioglucoside-N-hydroxysulfates) are amino acid-derived secondary metabolites that can be cleaved by the enzyme myrosinase (β-thioglucoside glucohydrolase; EC 3.2.1.147) (Bones and Rossiter 1996; Wittstock and Halkier 2002) (Fig. 17.1).

Glucosinolates and myrosinase are compartmentalized or present in separate plant cells like myrosin cells and S-cells (Koroleva et al. 2000; Husebye et al. 2002;

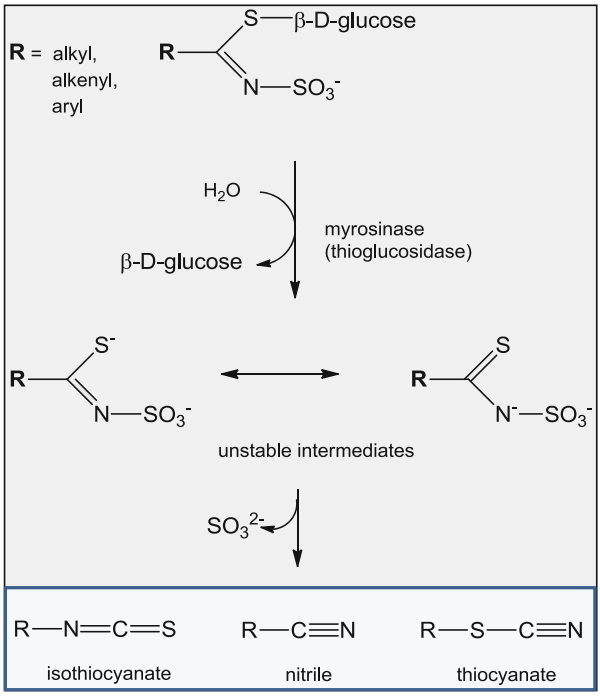

Fig. 17.1 Mechanism of glucosinolate hydrolysis by myrosinase. Myrosinase cleaves off the glucose from a glucosinolate. The remaining aglycone rearranges to an isothiocyanate, thiocyanate or a nitrile depending on physiological conditions such as pH and cofactors. The reactive products serve as defense for the plant. At neutral pH, the primary product is isothiocyanate. Under acidic conditions and in the presence of ferrous ions or certain proteins the formation of nitriles is favored instead

Thangstad et al. 2004), thus protected from self-damage to their own cells. In contrast to the glucosinolates, which are distributed in many plant tissues, myrosinase is localized in scattered cells only. Myrosinase is a hydrolyzing enzyme, which belongs to the Glycoside Hydrolase Family 1 in terms of enzyme classification. Recently, by using proteomics and gel free shot gun approaches, many myrosinases were found to be present in peroxisomes (Reumann et al. 2007). Herbivore attack, particularly by chewing insects, causes tissue damage, thereby both the enzyme and the glucosinolate substrate come into contact: Unstable aglycones are then released, and they spontaneously can rearrange into various active compounds, mainly nitriles and isothiocyanates, which tend to deter generalist herbivores.

Nitriles and isothiocyanates are toxic to the larvae of the black vine weevil, *Otiorhynchus sulcatus* (Borek et al. 1997). In a study showing that larvae of *Trichoplusia ni*, a lepidopteran generalist, avoided *Arabidopsis thaliana* ecotypes that produced isothiocyanates upon glucosinolate hydrolysis and, instead, fed on ecotypes that produced nitriles, the biological activity of isothiocyanates was

again clearly displayed (Lambrix et al. 2001). Interestingly, certain parasitoids use glucosinolates that are released by feeding herbivores to detect their host (Mithöfer and Boland 2012; Barth and Jander 2006). In such cases, the glucosinolates have a dual function for the infested plant in direct as well as indirect defense. The glucosinolate profiles of cruciferous plants are very diverse. Secondary modifications of amino acid side chains produce >140 known glucosinolate structures (Fahey et al. 2001). In *Arabidopsis*, there are at least 37 different glucosinolates, with side chains derived from methionine, tryptophan, phenylalanine and isoleucine (Reichelt et al. 2002).

17.4.2 Cellular Compartmentalization of Myrosinase

The localization of myrosinase in the cell is disputed. In earlier reports it was established that myrosinases were largely stored as myrosin grains in the vacuoles of particular idioblasts, called myrosin cells, which were situated near the phloem, at least in *Arabidopsis* (Bones and Rossiter 1996; Thangstad et al. 2004). Now we know that myrosinase is found in guard cells as well. But very recently it was demonstrated the presence of myrosinase in S-cells (the cells near the phloem that also contain the glucosinolates in Arabidopsis stems and leaves) (Koroleva and Cramer, unpublished results). It's certainly possible that the subcellular localization in these different cell types is similar, in either peroxisomes or vacuoles, but it may not be the same in these diverse cell types. The work on the S-cells is probably the most persuasive for myrosinase cellular localization, and we know that these cells are supposed to be near dead on maturity, just serving as repositories for glucosinolates. Here the myrosinase is suggested to be in the ER or in "derived vesicles". Recently, by using proteomics and gel free shot gun approaches, myrosinase was identified in peroxisomes (Reumann et al. 2007). Nevertheless, although the localization of myrosinase inside the cell is disputed, it is clear that intracellular compartmentalization plays an important role is maintaining plant defence against herbivores. Further work is needed to conclusively prove the localization of myrosinase in peroxisomes.

17.5 Mevalonate Pathway: Generation of Terpenoids and Terpenes

Terpenoids constitute the largest classes of natural products in nature. In plants, these chemicals perform many essential functions including photosynthesis, reproduction and adaptation to the environmental challenges as well as being involved in plant defences (Gershenzon and Dudareva 2007). Terpenoids contribute to both direct and indirect defenses. They are an extremely diverse group of carbon-

based compounds, all of which derived from five-carbon isoprene units and are ubiquitously distributed (Mithöfer and Boland 2012). Most are polycyclic structures which differ from one another in both functional groups and in basic carbon skeletons (Gershenson and Kreis 1999). All isoprenoids are synthesized via the condensation of the five carbon universal isoprenoid precursors, isopentenyl diphosphate (IDPIDP) and its allylic isomer dimethyl allyl diphosphate (DMADP) (Sapir-Mir et al. 2008). Two separate IDP-forming pathways exist in plants. The 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway that provides the precursors for the synthesis of monoterpenes, carotenoids, apocarotenoids and the side chain of chlorophylls, tocopherols and prenylquinones and the mevalonic acid (MVA) pathway that provides precursors for the formation of triterpenes, sesquiterpenes, phytosterols, ubiquinone, vitamin D and primary metabolites important for cell integrity (Rodríguez-Concepción and Boronat 2002; Bouvier et al. 2005). It has been shown that MVA pathways is subcellularly distributed in various cellular organelles like endoplasmic reticulum (ER), cytosol and peroxisomes. Three of the important enzymes of the MVA pathways are present in peroxisomes (Sapir-Mir et al. 2008; Simkin et al. 2011). These enzymes are 5-phosphomevalonate kinase (PMK) and mevalonate 5-diphosphate decarboxylase (MVD) and isopentenyl diphosphate isomerase (IDI). 5-phosphomevalonate kinase (PMK; EC 2.7.4.2) adds the phosphate group on mevalonate phosphate, generating mevalonate diphosphate, the decarboxylation of mevalonate diphosphate by mevalonate 5-diphosphate decarboxylase (MVD; EC 4.1.1.33) which yields IDP. IDP undergoes a reversible isomerization step catalyzed by the IDP isomerase (IDI; EC 5.3.3.2) which leads to the generation of DMADP, thus allowing the biogenesis of terpenoid compounds via IDP/DMADP condensations (Dewick 2002; Clastre et al. 2011). Figure 17.2 shows a picture of the MVA pathway and the related farnesyl diphosphate synthase (FPS) leading to the formation of C15 farnesyl diphosphate, which is the precursor of sterols and sesquiterpenes. The basic units of generation of terpenes are isopentenyl 5-diphosphate (IDP).

The MEP pathways and MVA pathways are interconnected with each other; there is a constant flow of different intermediate and precursors at least in lima bean (*Phaseolus lunatus*) (Bartram et al. 2006). The pathways are allocated dynamically rather than strictly in functioning (Jux et al. 2001). So the precise localization of terpenoids precursors cannot be decided, that's the reason why we are mentioning all the important volatiles generated by both pathways.

Several important defence compounds are generated by terpenoids. These include essential oils, volatiles and modified terpenes. Many of the volatile essential oils, e.g. citronella, limonene, menthol, camphor and pinene, are known to have anti herbivore properties. However, the key players in terpenoid volatiles are represented by mono-, sesqui-, and homoterpenoids, which all significantly contribute to any blend of plant-derived volatiles (Mithöfer and Boland 2012). In terms of indirect defenses, attracting parasitoids or parasites as well as repelling herbivores is very likely mediated by either the recognition of single volatile compounds or a specific volatile blend by an insect's particular olfactory system (Mumm et al. 2008). These interactions between terpenoids and insect sensory receptors have been suggested

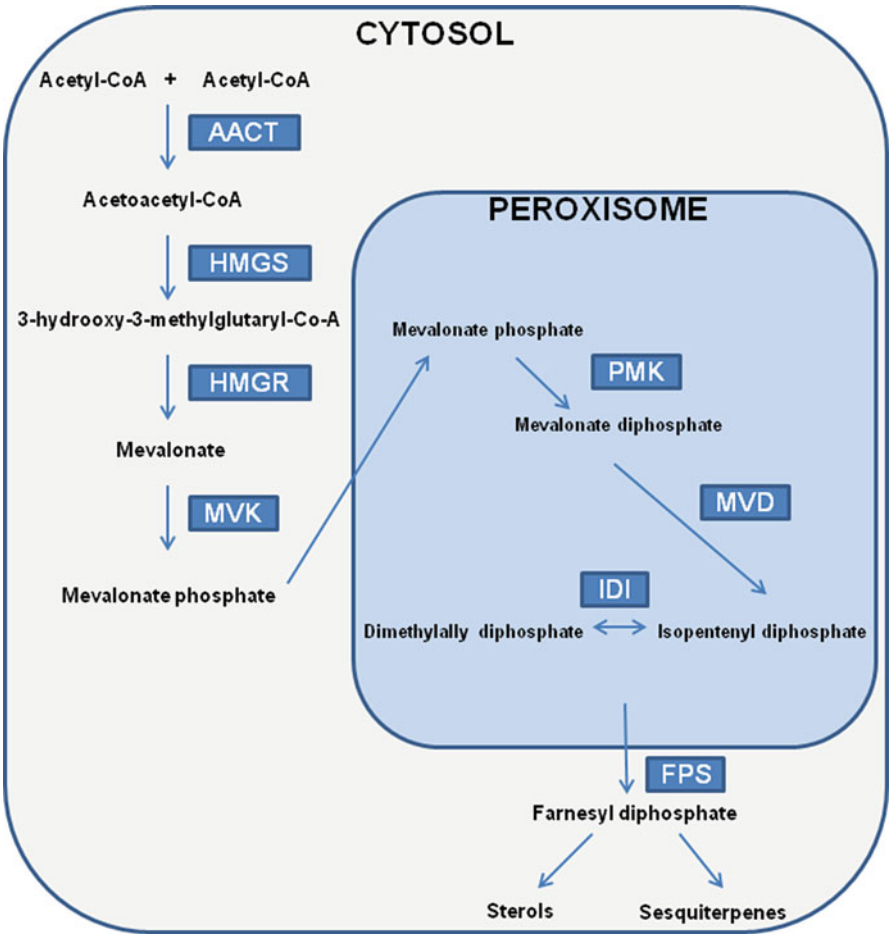

Fig. 17.2 Biosynthesis of terpenoids along the Mevalonate pathway leading to sesquiterpenes and sterols. according to Simkin et al. (2011). Transient transformations of *C. roseus* cells with yellow fluorescent protein (YFP)-fused constructs reveal that PMK and MVD are localised to the peroxisomes, while MVK was cytosolic

by Gershenzon and Croteau (1991). There are examples showing that individual volatile terpenoids attract the natural enemies of the herbivores. The predatory mite *P. persimilis*, for instance, was attracted by linalool, (*E*)-β-ocimene, and (3*E*)-4,8-dimethyl-1,3,7-nonatriene when exposed to individual chemicals in an olfactometer. (*E*)-α-bergamotene increased *Manduca sexta* egg predation rates on *Nicotiana attenuata* by a generalist predator, *Geocoris pallens* (Kessler and Baldwin 2001).

The best notable example is a sesquiterpene, (*E*)-β-farnesene which is a common aphid alarm pheromone that is released by attacked aphids and causes other aphids in the vicinity to stop feeding and move away. The same compound is

also present in some plant volatile mixtures. The function of (*E*)-β-farnesene in indirect defense has been demonstrated by expressing terpene synthases in transgenic plants of *Arabidopsis thaliana*. Overexpression of maize *TPS10*, which catalyzes the formation of (*E*)-β-farnesene, (*E*)-α-bergamotene and other herbivory-induced sesquiterpenes in *Arabidopsis*, resulted in plants emitting high quantities of corresponding volatiles, which were absent in the wild-type rosette leaves. In an olfactometer assay using the transgenic *Arabidopsis* plants as odor sources, females of the parasitoid *Cotesia marginiventris* learned to exploit the TPS10 sesquiterpenes to locate their lepidopteron hosts (Schnee et al. 2002; Unsicker et al. 2009).

(E)-β-caryophyllene is another example of sesquiterpene, a major component of rice volatiles, and is the product of the sesquiterpene synthase OsTPS3 isolated from rice (Cheng et al. 2007). Overexpression of OsTPS3 in rice resulted in transgenic plants that emit high quantities of (E)-β-caryophyllene, but only in response to treatment with methyl jasmonate. Behavioral assays with transgenic and wild-type plants both treated with methyl jasmonate showed that the parasitoid *A. nilaparvatae* is more attracted to the transgenic plants emitting higher levels of (E)-β-caryophyllene, indicating a possible role of OsTPS3 in the indirect defense of rice (Cheng et al. 2007). Overexpression of the herbivore-induced (E)-β–caryophyllene synthase TPS23 in *Arabidopsis* showed that the volatile (E)-β-caryophyllene product can also be associated with host presence by *C. marginiventris* (Köllner et al. 2008). A field experiment with maize varieties producing different amounts of (E)-β-caryophyllene was able to demonstrate the specificity and efficiency of this defense against larvae of the beetle *Diabrotica virgifera virgifera* in an agricultural setting (Rasmann et al. 2005). (E)-β-caryophyllene emitted from roots was a herbivore-induced underground signal that strongly attracts entomopathogenic nematodes, which are the natural enemies of the herbivores (Rasmann et al. 2005). Induction of (E)-β-caryophyllene after herbivore damage was also not only observed in several genotypes of maize, but also observed in six teosinte species. This indicates that this defense signal occurs in many grasses related to maize (Köllner et al. 2008).

(3*S*)-(*E*)-nerolidol is an acyclic sesquiterpene alcohol, which has a woody aroma reminiscent of fresh bark. The attraction of the predatory mite *Phytoseiulus persimilis* to (*3S*)-(*E*)-nerolidol has been well demonstrated (Dicke et al. 1990). Linalool is terpene alcohol, which has the dual function of repelling the herbivores as well as attracting the natural enemies of herbivores. Transgenic plants generated by FaNES1-linalool synthase form strawberry in *Arabidopsis* emitting volatiles terpenoids that are naturally not present in *Arabidopsis*, in the dual-choose assay for *M. Persicae* aphids between wild type plants and transgenic plants, aphids preferred leaves derived from wild type plants. (Aharoni et al. 2003). Diterpenoids, are widely distributed in plants as latex and resins, and can be quite toxic to herbivores. Certain diterpenes are known to make toxins against grazing herbivores like grayanotoxin present in leaves of Rhododendron. In cotton (*Gossypium* spp.), gossypol and related sesquiterpene aldehydes, all derived from the precursor (+)-δ-cadinene, provide both constitutive and inducible protection against pests.

In general, the exact mechanisms by which terpenoids directly act on insect pests are not known; processes such as the alkylation of nucleophiles, inhibition

of ATP-synthase, interference with insects' molting regulation, or the disturbance of the nervous system are very likely (Langenheim 1994). Recent progress in the study of insect olfaction has revealed that the heteromeric insect olfactory receptor complex forms a cation nonselective ion channel directly gated by odor or pheromone ligands independent of known olfactory receptor G-protein signaling pathways. Also there are pharmacological evidences of inhibition of acetylcholine esterase by α-pinene, limonene or eugenol (Maffei et al. 2011).

17.6 Conclusions

Plant responses as defence systems are very diverse and effective. To organize these defences, plants have to work in an organized and systemic manner. Compartmentalization and induction can solve this issue. Thus peroxisomes store important enzymes and metabolites and serve as an armory of defence weapons in plant cells. The reviewed literature shows that there is an increasing body of evidence proving that plant peroxisomes are indeed critical for defence against herbivores. Peroxisomal enzymes can generate various chemicals, which help plants to survive in hostile environments. Further studies are needed to end the confusion related to the controverted localization of myrosinase in peroxisomes and get deeper insights into the detailed mechanism of action of this enzyme.

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Chapter 18
Function of Peroxisomes in Plant-Pathogen Interactions

Yasuyuki Kubo

Contents

Abstract Peroxisomes are ubiquitous organelles of eukaryotic cells that accomplish a variety of biochemical functions, including β-oxidation of fatty acids, glyoxylate cycle, etc. Many reports have been accumulating that indicate peroxisome related metabolic functions are essential for pathogenic development of plant pathogenic fungi. They include peroxisome biogenesis proteins, peroxins and preferential destruction of peroxisomes, pexophagy. Gene disrupted mutants of anthracnose disease pathogen *Colletotrichum orbiculare* or rice blast pathogen *Magnaporthe* oryzae defective in peroxins or pexophagy showed deficiency in pathogenesis. Woronin body, a peroxisome related cellular organelle that is related to endurance of fungal cells against environmental damage has essential roles in pathogenesis of *M. oryzae*. Also, peroxisome related metabolisms such as β-oxidation and glyoxylate cycle are essential for pathogenesis in several plant pathogenic fungi. In addition, secondary metabolisms including polyketide melanin

Y. Kubo (✉)
Laboratory of Plant Pathology, Graduate School of Life and Environmental Sciences, Kyoto, 606-8522 Japan
e-mail: y_kubo@kpu.ac.jp

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biosynthesis of *C. orbiculare* and *M. oryzae,* and host selective toxins produced by necrotrophic pathogen *Alternaria alternata* have pivotal roles in fungal pathogenesis. Every such factor was listed and their functions for pathogenesis were demonstrated (Table 18.1 and Fig. 18.1).

Keywords Fungal pathogenesis • Appressoria • Lipolysis • β-oxidation • Glyoxylate cycle • Melanin biosynthesis • Pexophagy • Woronin body

18.1 Peroxisomes and Fungal Pathogenesis

Peroxisomes are single-membrane intracellular organelles present in most eukaryotic cells, which compartmentalize a variety of enzymes involved in diverse metabolic processes. They include β-oxidation involved in breakdown of fatty acids, glyoxylate cycle, a variation of the tricarboxylic acid cycle, cholesterol metabolisms, amino acids antibiotics, reactive oxygen species, and methanol assimilation (Brown and Baker 2008). In filamentous fungi, peroxisomal functions are revealed to play diverse roles in sexual reproduction in *Podospora anserin*a (Bonnet et al. 2006), secondary metabolisms of penicillin in *Penicillium chrysogenum* (Kiel et al. 2005), host selective toxin in *Altertnaria alternata* (Imazaki et al. 2010), asexual reproduction in *P. chrysogenum* (Kiel et al. 2004), Woronin body formation in *Neurospora crassa*, *M. oryzae* and *C. orbiculare* (Syn. *C. lagenarium*) (Soundararajan et al. 2004; Liu et al. 2008; Kubo 2012), and glucose metabolisms in *Cryptococcus neoformans* (Idnurm et al. 2007).

Peroxisome also have pivotal role in lipolysis and secondary metabolisms starting from acetyl-CoA, essentially in *C. orbiculare* and *M. oryzae.* Turgor generation in appressorium, an infection structure of those species is derived from storage compounds in the spore, such as glycogen and lipids (de Jong et al. 1997; Wang et al. 2005). Lipid bodies are mobilized during appressorium formation and rapid lipolysis provides triacylglycerol and fatty acid (Schadeck et al. 1998; Weber et al. 2001; Wang et al. 2007; Fujihara et al. 2010). Production of acetyl-CoA through β-oxidation and glyoxylate cycle is critical for the pathogesis of plant pathogenic fungi, stimulating rapid lipolysis, providing source of an energy, turgor and the starter metabolite for secondary metabolic pathways, such as melanin biosynthesis and polyketide biosynthesis (Bhambra et al. 2006; Ramos-Pamplona and Naqvi 2006; Wang et al. 2007).

All of those metabolisms related to peroxisome function are essential for pathogenesis of plant pathogenic fungi and are listed in Table 18.1 and Fig. 18.1. Following are comprehensive descriptions of those essential factors for pathogenesis of plant pathogenic fungi.

Table 18.1 Pathogenesis related genes involved in peroxisome function in plant pathogenic fungi

Category	Protein	Gene	Fungus	Mutant phenotype				References
				Appressorial melanization	Fatty acid utilization	Host penetration	Invasive growth in host	
Peroxin	Peroxin 6	*PEX6*	*C. orbiculare*	±	−	−	+	Kimura et al. 2001
			M. oryzae	−	−	−	−	Ramos-Pamplona and Naqvi 2006; Wang et al. 2007
			A. alternata	/	−	−	−	Imazaki et al. 2010
	Peroxin 7	*PEX7*	*M. oryzae*	±	−	−	+	Goh et al. 2011
	Peroxin 13	*PEX13*	*C. orbiculare*	−	−	−	+	Fujihara et al. 2010
	Peroxin 22	*PEX22*	*C. orbiculare*	−	−	−	+	Kubo 2012
β oxidation	Carnitine acetyl-transferase	*PTH2*	*M. oryzae*	±	−	−	−	Ramos-Pamplona and Naqvi 2006; Bhambra et al. 2006
		CRC1	*M. oryzae*	±	−	−	−	Yang et al. 2012
	Mutifunctional beta-oxidation protein	*MFE1*	*C. orbiculare*	±	−	−	+	Asakura et al. 2012
		MFP1	*M. oryzae*	±	−	−	+	Wang et al. 2007
		MFE2	*U. maydis*	/	−	±	±	Klose and Kronstad 2006
Glyoxylate cycle	Isocitrate lyase	*ICL1*	*C.orbiculare*	+	−	−	+	Asakura et al. 2006
		ICL1	*M. oryzae*	+	−	±	±	Wang et al. 2003
		ICL1	*Leptosphaeria maculans*	/	−	±	±	Idnurm and Howlett 2002
	Malate synthase	*MLS1*	*Stagonospora nodorum*	/	−	−	/	Solomon et al., 2004
	Alanine:glyoxylate aminotransferase	*AGT1*	*M. oryzae*	+	+	−	/	Bhadauria et al. 2012

(continued)

Table 18.1 (continued)

Category	Protein	Gene	Fungus	Mutant phenotype				References
				Appressorial melanization	Fatty acid utilization	Host penetration	Invasive growth in host	
Pexophagy	Sterol glucosyltrans-ferase	*ATG26*	*C. orbiculare*	+	+	−	+	Asakura et al. 2009
Woronin body	Woronin body structural protein	*HEX1*	*M. oryzae*	+	+	±	±	Soundararajan et al. 2004
			C. orbiculare	+	+	+	+	Kubo 2012
	Peroxin 22	*PEX22*	*C. orbiculare*	−	−	−	+	Kubo 2012
	Membrane flavoprotein	*TmpL*	*A. brassicicola*	/	+	−	−	Kim et al. 2009
Melanin biosynthesis	Polyketide synthase	*PKS1*	*C. orbiculare*	−	+	−	+	Kubo et al. 1991, Takano et al. 1995
			M. oryzae	−	+	−	+	Chumley and Valent 1990
	Scytalone dehydratase	*SCD1*	*C.orbiculare*	−	+	−	+	Kubo et al. 1996
			M.oryzae	−	+	−	+	Chumley and Valent 1990
	Trihydroxynaphthalene resuctase	*THR1*	*C. orbiculare*	−	+	−	+	Perpetua et al. 1996
			M. oryzae	−	+	−	+	Chumley and Valent 1990
Secondary metabolisms	Carboxyl-activating enzyme	*AKT-1*	*A. alternata*	/	+	−	−	Tanaka and Tsuge 2000
	Estelate lipase family	*AKT-2*	*A. alternata*	/	+	−	−	Tanaka and Tsuge 2000
	Hydratse-somerase family	*AKT-3*	*A. alternata*	/	+	−	−	Tanaka and Tsuge 2000

+ possible; – impossible; / not applied

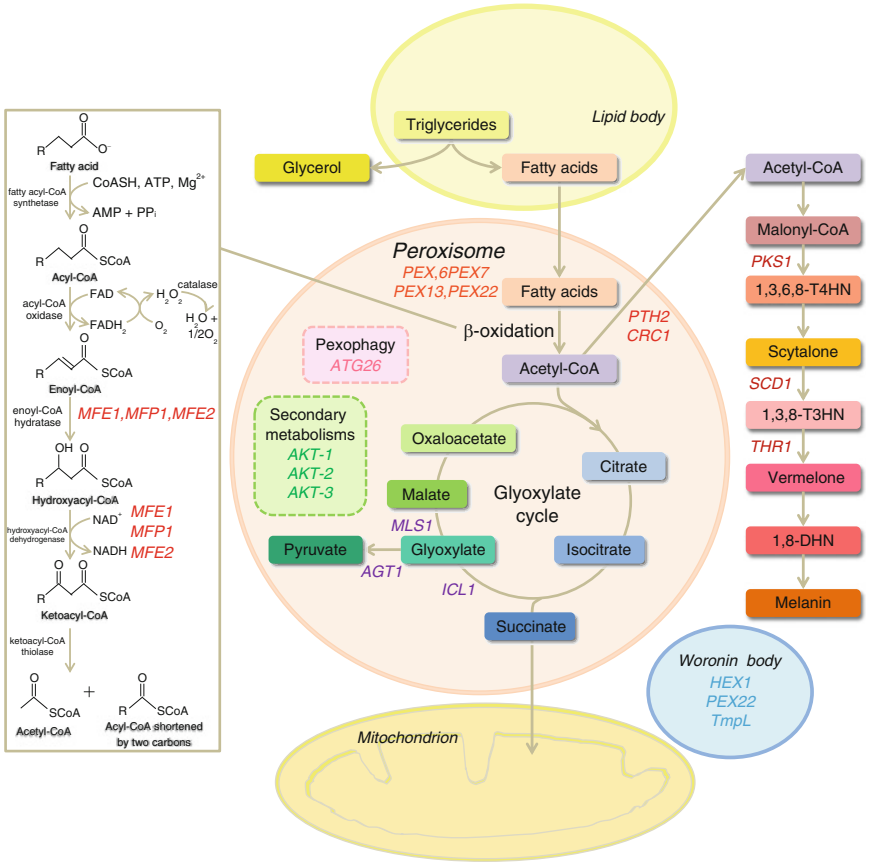

Fig. 18.1 Peroxisome related metabolic functions involved in pathogenesis of plant pathogenic fungi. Gene names, organisms, and defective mutant phenotypes were listed in Table 18.1

18.2 Peroxins

Peroxisome biogenesis proteins are collectively called peroxins and 32 PEX genes have been implicated in peroxisome biogenesis and their gene products (Wanders and Waterman 2004; Heiland and Erdmann 2005; Kiel et al. 2006). Out of them, four peroxins Pex6, Pex7 Pex13 and Pex22 are recognized as essential factors of fungal pathogenesis in *C. orbiculare* (Kimura et al. 2001; Fujihara et al. 2010; Kubo 2012), *M. oryzae* (Ramos-Pamplona and Naqvi 2006; Wang et al. 2007; Goh et al. 2011) and *A. alternata* (Imazaki et al. 2010). The process of peroxisomal matrix protein import is represented into four steps: (1) binding of receptors to matrix proteins; (2) docking of receptor complex to the peroxisome membrane proteins; (3) translocation of receptor complex into the peroxisome matrix; and (4) recycling of the receptors (Brown and Baker 2008). Peroxisomal matrix proteins

usually possess either a peroxisomal targeting signal PTS1 tripeptide sequence at the C-terminus, or a PTS2, a nonapeptide sequence at the N-terminus, which are recognized by the PTS1 receptor Pex5, and the PTS2 receptor Pex7, respectively (Titorenko and Rachubinski 2001).

18.2.1 Colletotrichum orbiculare (Syn. *C. lagenarium*)

C. orbiculare is an anthracnose pathogen of Cucurbitaceous plants and makes hemibiotrophic infection through melanized appressoria (Kubo et al. 1982; Kubo and Furusawa 1991; Kubo and Tanaka 2010; Kubo 2012). The first example of peroxisome biogenesis genes in relation to fungal pathogenicity was presented in *C. orbiculare PEX6* (Kimura et al. 2001) and followed by *PEX13* (Fujihara et al. 2010). The peroxin genes, *PEX6* and *PEX13* were identified by screening random insertional mutants for deficiency in pathogenesis and fatty acid utilization, respectively. *PEX6* codes for AAA-peroxin that heterodimerizes with AAA-peroxin Pex1p and along with *PEX22* participates in the recycling of peroxisomal signal receptor Pex5 from the peroxisomal membrane to the cytosol (Titorenko and Rachubinski 2000). *PEX13* gene codes for a docking protein for the Pex5 and Pex7 proteins receptor complex that functions for the import of PTS proteins into peroxisomes (Erdmann and Blobel 1996; Gould et al. 1996).

The two peroxin genes disruptants showed similar phonotype. They are unable to utilize fatty acids as a carbon source. And, PTS1 or PTS2 green fluorescent protein (GFP) fusions are not imported into peroxisome, thus import machinery for peroxisomal matrix proteins is impaired. In *C. orbiculare,* appressorium melanization is essential for host invasion (Kubo and Furusawa 1991; Kubo et al. 1991; Takano et al. 1995; Kubo et al. 1996; Perpetua et al. 1996) and the melanin is synthesized through the polyketide pathway, in which 1,3,6,8-tetrahydroxynaphthalene (T4HN) is synthesized by polyketide synthase from malonyl-CoA derived from acetyl-CoA (Fujii et al. 2000). Appressoria of both mutants showed defectiveness in melanization, due to the inability to produce acetyl-CoA for the polyketide melanin biosynthesis starter metabolite through the defect of β-oxidation of fatty acids. Scytalone, a melanin precursor application restored melanization of *pex13* and polyketide synthase deficient *pks1* mutant, but did not restore the penetration ability of *pex13* mutant appressoria unlike those of *pks1* mutant, indicating that the lack of cell wall melanin is not the only factor causing impaired penetration. Turgor pressure is the physical force required for direct plant penetration (Howard et al. 1991), and is necessary for accumulation of intracellular osmolites glycerol and the melanized thick cell wall (Fujihara et al. 2010). The concentration of intracellular glycerol is lower in *copex13* mutant appressoria than those of the wild-type. Other phenotype of *pex13* mutant includes: (1) the pronounced enlargement of lipid droplets in appressoria; (2) lipid bodies persistency in appressoria at a developmental stage when these were largely depleted in the wild-type appressoria; and (3) thinner and non-multiple layers differentiated appressorial cell walls (Fujihara

et al. 2010). Acetyl-CoA derived from peroxisomal β-oxidation of fatty acids is a substrate for the glyoxylate cycle and gluconeogenic pathway, which contribute to the synthesis of cell wall polymers such as chitin and glucan (Thines et al. 2000; Wang et al. 2003; Ramos-Pamplona and Naqvi 2006). Therefore, peroxin mutants are likely to display cell wall defects due to their inability to import enzymes of the glyoxylate cycle into peroxisomes (Bhambra et al. 2006). Conclusively, defects in melanization, cell wall synthesis, and turgor generation all contribute to the lack of host penetration ability shown by appressoria of the *C. orbiculare* peroxin mutants.

18.2.2 Magnaporthe oryzae

Rice blast pathogen *Magnaporthe oryzae* is a hemibiotrophic pathogen and makes similar infection process to *C. orbiculare via* melanized appressorium formation (Chumley and Valent 1990; Talbot 2003; Wilson and Talbot 2009). In this fungus, the role of two *PEX* genes *PEX6* and *PEX7* in pathogenesis was evaluated. *Pex6* mutant of *M. oryzae* showed essentially similar pathogenesis deficient phenotype to that of *C. orbiculare* (Ramos-Pamplona and Naqvi 2006; Wang et al. 2007). The mutant showed deficiency in fatty acid utilization, melanin biosynthesis and lipid body degradation, causing deficiency in appressorium function. However, different from *C. orbiculare*, wounded rice plant inoculated with *pex6* mutants did not develop rice blast symptoms or support fungal growth, indicating the loss of pathogenesis was not solely attributable to the lack of appressorium function. Therefore, peroxisome function is not essential for post penetrative invasive growth stage in *C. orbiculare* but necessary in *M. oryzae*. As in *C. orbiculare*, *M. oryzae PEX6* gene function is necessary for lipid body degradation in developing appressoria, demonstrating that peroxisomal β-oxidation could be involved in degradation of triacylglycerol through activity of triacylglycerol lipases.

Deficiency of the PEX7-mediated PTS2 import system during pathogenesis in *M. oryzae* caused loss of virulence on rice (Goh et al. 2011). The *pex7* mutant showed no disease symptoms on rice leaves. In connection with pathogenicity factors, *pex7* mutant showed delayed lipid translocation and degradation during appressorium maturation and defects in turgor generation and in plant penetration. Cell wall porosity was reported in previous research showing that maintenance of cell wall integrity and biosynthesis of cell wall components are important for pathogenic development of the fungus (Jeon et al. 2008; Choi et al. 2009). The *pex7* mutant showed defects in not only accumulation of osmolites but also in appressorial melanization and cell wall porosity. However, mycelial cell wall integrity did not seem to be affected in *pex7*. This defect in appressorial cell wall porosity does not seem to originate from changes in synthesis of major cell wall components like chitin or glucan but related to the production of minor cell wall components and/or arrangement of cell wall components required for cell wall pore density during appressorium development.

18.2.3 Alternaria alternata

The Japanese pear pathotype of *A. alternata* produces AK-toxin and causes black spot on susceptible Japanese pear cultivars (Nakashima et al. 1985). The gene cluster is involved in AK-toxin biosynthesis and includes four genes, *AKT1*, *AKT2*, *AKT3*, and *AKTR* (Tanaka and Tsuge 2000). *AKTR* encodes a transcription regulator of the Zn(II)2Cys6 family. *AKT1*, *AKT2*, and *AKT3* encode proteins with similarity to the carboxyl-activating enzymes, the estelase-lipase, and the hydratase-isomerase, respectively, and possess PTS1-like tripeptides SKI, SKL, and PKL, respectively at the C-terminal ends, suggesting that these enzymes are located in peroxisomes (Tanaka and Tsuge 2000). The *pex6* mutant of *A. alternata* shows deficiency in fatty acid utilization. The import of GFP-Akt1 into peroxisomes was impaired in the *pex6* mutant and the *pex6* mutant completely lost AK toxin production and pathogenicity on susceptible pear leaves (Imazaki et al. 2010). Thus, peroxisomes are essential for AK-toxin biosynthesis. Conidia of the *pex6* mutant retain normal morphogenesis of infection related morphogenesis and penetration ability assessed by onion epidermal cells entry. However, the ability of the *pex6* mutant to infect host cells was partially, but not completely, restored by the application of AK-toxin at the time of inoculation. Thus, peroxisome function is also necessary for initial plant infection and tissue colonization by this *A. alternata* pathotype.

18.3 β-Oxidation

β-oxidation is the principal metabolisms by which fatty acids are metabolized in fungal cells. The β-oxidation involves a set of four successive reactions catalyzed by four major enzymes in the process of fatty acid oxidation: acyl-CoA oxidase, 2-enoyl-CoA hydratase, 3-hydroxacyl-CoA dehydrogenenase, and 3-ketoacyl-CoA thiolase. Through this step, a two-carbon unit is divided from each fatty acid in the form of an acetyl-CoA unit, which is served into the glyoxylate cycle or degraded in the citric acid cycle. There are also several other enzymes, such as cis-enoyl-CoA isomerase, necessary for the degradation of unsaturated fatty acids. Multifunctional proteins (MFPs) comprise at least two of these enzymatic activities, 2-enoyl-CoA hydratase and 3-hydroxacyl-CoA dehydrogenenase, which are involved in the second and third steps of the fatty acid β-oxidation cycle. *M. oryzae mfp1* mutants were unable to use lipids or fatty acids as a sole carbon source and were attenuated in virulence (Wang et al. 2007). Although obvious peroxisomal targeting sequences were not predicted in Mfp1, the expression of an Mfp1-GFP gene fusion showed peroxisomes localization (Maggio-Hall and Keller 2004). The attenuated virulence shown by *mfp1* mutant was due to a defect in appressorium function that prevented the initial traversal of the host plant cuticle. Notably, turgor of the mutant was not significantly reduced, suggesting that glycerol is not likely to be derived from the degradation of fatty acids in *M. oryzae*. The virulence phenotype of *mfp1*

mutants provided evidence that peroxisomal β-oxidation is a critical component of appressorium function and is consistent with the idea that carnitine acetyl transferase (CAT) is necessary for *M. oryzae* pathogenesis (Bhambra et al. 2006; Ramos-Pamplona and Naqvi 2006; Yang et al. 2012).

Recently, MFP function was evaluated in *C. orbiculare* (Asakura et al. 2012). *C. orbiculare mfe1* disrupted mutants formed non-melanized appressoria with abundant lipid droplets, similar to those of *pex6* mutants, indicating that fatty acid β-oxidation in peroxisomes is critical for the appressorial melanization and lipolysis. Soraphen A, an inhibitor of acetyl-CoA carboxylase, inhibited appressorial lipolysis and melanization, suggesting that the conversion of acetyl-CoA, derived from fatty acid β-oxidation to malonyl-CoA is required for the lipolysis. Interestingly, defect in *PKS1*-dependent polyketide synthesis, an initial step in melanin biosynthesis, also affected appressorial lipolysis, indicating that melanin biosynthesis, as well as fatty acid β-oxidation, is involved in the regulation of lipolysis during development of infection structures.

In *Ustilago maydis*, a causal agent of smut disease of maize, the growth of the strains defective in *mfe2* was attenuated in utilization of fatty acids. The *MFE2* gene was not generally required for the production of filaments during mating in vitro, but not required for extensive proliferation of fungal filaments *in planta*, causing reduced virulence (Klose and Kronstad 2006; Kretschmer et al. 2012). Similarly, a defect in virulence was observed in developing ears upon infection of mature maize plants, causing delayed development of teliospores within mature tumor tissue. Notably, *MFE2* was required for filamentation in response to specific fatty acids. Oleic acid and linoleic acid induced highly branched filaments in wild-type cells, but the *mfe2* mutants formed filaments with short branches on oleic acid and did not respond morphologically to linoleic acid. Unlike wild-type strains, growth of the mutants was limited on both fatty acids. Thus, exogenous linoleic acid may have a specific signaling role either directly or after processing to form derivatives such as oxylipins. The *mfe2* mutation impaired symptom development during infection but did not completely abolish fungal growth within the plant. In general, biotrophic fungi evade plant defense mechanisms by mask or suppression of host responses (Schulze-Lefert and Panstruga 2003). A possibility was suggested that an enhanced defense response occurs upon infection by *mfe2* mutants due to the altered chemical environment caused by the metabolic defect in the pathogen.

The acetyl-CoA molecule is membrane-impermeable and its transport between intracellular compartments for energy production and biosynthesis functions is mediated through the formation of carnitine intermediates. CAT catalyzes the reversible reaction, which transfers the acetate moiety between coenzyme-A and carnitine (Ramsay and Naismith, 2003). *PTH2* (Sweigard et al. 1998; Bhambra et al. 2006; Ramos-Pamplona and Naqvi 2006) and *CRC1* (Yang et al. 2012), encoding CAT in *M. oryzae* were identified in a genetic screen for pathogenicity mutants. Characterization of these CAT deleted mutants revealed that those genes were indispensable for the appressorial function of host penetration. Furthermore, CAT mutants highlighted the significance of CAT to fungal pathogenicity. Those mutants are impaired in rice cuticle penetration with no observable difference

in appressorium turgor, and showed a slight reduction in melanin production, appressoria defectiveness and cell wall integrity, through defectiveness of acetyl CoA available to the glyoxylate cycle which supports glucan and chitin biosynthesis. Evidence to support such a role for CAT activity arises from the observation that addition of exogenous glucose or sucrose to appressoria of CAT mutants partially restores their ability to cause disease.

18.4 Glyoxylate Cycle

The glyoxylate cycle is widespread and well documented in archaea, bacteria, protists, plants, fungi and nematodes (Kondrashov et al. 2006). In addition to allowing the growth of bacteria on C2 compounds, the glyoxylate cycle is important for the growth of higher plant seedlings under most environmental conditions (Eastmond et al. 2000). Enzymes involved in the glyoxylate cycle are suggested to localize in peroxisomes in fungi, including *Yarrowia lipolytica* (Kionka and Kunau 1985; Valenciano et al. 1996; Titorenko et al. 1998), whereas *Saccharomyces cerevisiae* isocitrate lyase (ICL), a key component of the glyoxylate cycle, is solely cytosolic, even under growth conditions that induce peroxisome proliferation (Taylor et al. 1996). The significance of the glyoxylate cycle for fungal pathogenicity was first underscored by the requirement of isocitrate lyase for the virulence of the pathogenic yeast *Candida albicans* in mouse (Lorenz and Fink GR 2001). Important roles of ICL in virulence of fungal pathogens on plants were reported. In *Leptosphaeria maculans*, a causal agent of blackleg of crucifers, it was shown that the *ICL1* gene is expressed during its infection of *Brassica napu*s cotyledons and mutation of this locus causes attenuation in germination of spores, pathogenicity on cotyledons, and hyphal growth on canola. It was suggested that inability of the *icl* mutant to utilize carbon sources provided by the plant is the cause of the reduced pathogenicity (Idnurm and Howlett 2002).

Essential role of ICL in fungal pathogenesis was also reported in *M. oryzae*. During infection by *M. oryzae*, *ICL* gene was expressed in conidia, appressoria, and mycelia. Deletion of the *ICL1* gene caused a reduction in appressorium formation, conidiation and cuticle penetration, and an overall decrease in damage to leaves of rice and barley. Thus ICL is essential for full virulence, due to multiple attenuations in *M. oryzae* (Rauyaree et al. 2001; Wang et al. 2003).

Stagonospora nodorum is a necrotrophic pathogen producing leaf and glume blotch disease on wheat and other cereals. Initial steps of infection by the pathogen depend on the metabolism of stored lipids *via* the glyoxylate pathway to produce glucose that support fungal development (Solomon et al. 2004). During its interaction with wheat an increased expression of *MLS1* gene took place in pregerminated spores followed by a decrease in transcription after germination. Inconsistently, malate synthase activity was undetectable in pregerminated spores but increased to a significant level after germination. Spores of the *mls1* mutant inoculated onto wheat were unable to induce necrotic lesions, indicating that this gene is essential for

virulence on wheat. The requirement for malate synthase to cause infection depends on lipid metabolism as a key factor for the germination of *S. nodorum* (Solomon et al. 2004). This phenotype was not consistent with that of *M. oryzae icl* mutant. Whereas *S. nodorum* enters the leaf both through natural openings such as stomata and directly through the cuticle, *M. oryzae* infects through appressoria that makes use of massive amounts of glycerol. This may explain the different way of infection observed.

Functional role of ICL in *C. orbiculare* was demonstrated by Asakura et al. (2006). The *icl1* mutant of *C. orbiculare* failed to grow on acetate or fatty acids and the green fluorescent protein (GFP) gene fused *ICL1–GFP* expression product was detected in peroxisomes, conidia, appressoria and hyphae of the fungus. The *icl1* mutant was able to germinate and develop appressoria unlike *M. oryzae*, thus contribution of the glyoxylate cycle to infection-related morphogenesis seems to be uncommon among these pathogens. The *icl1* mutant was capable of degrading lipid bodies as well as the wild-type strain. However, conidia from the *icl1* mutant inoculated onto cucumber leaves and cotyledons formed a reduced number of lesions on leaves. In invasive growth experiments such as the inoculation of conidia into wounded sites, no defect was observed in the *icl1* mutant, while in penetration assays on cucumber cotyledons the mutant was unable to develop penetrating hyphae, indicating a requirement for *ICL* at this early stage of *C. orbiculare* infection. Furthermore, the *icl1* mutants partially restored invasion of the host plant by the addition of glucose or sucrose, or heat-treated cucumber plant. Gluconeogenesis mediated by the glyoxylate cycle is probably important for appressorium mediated host invasion by evading host defense responses.

In *M. oryzae* a gene encoding an alanine, glyoxylate aminotransferase (AGT) is required for pathogenicity (Bhadauria et al. 2012). The *agt1* mutant resulted in the failure of penetration from appressoria, showing attenuated pathogenesis to its hosts rice and barley. Analysis of green fluorescent protein expression using the transcriptional and translational fusion with the *AGT1* promoter and open reading frame, respectively, revealed that *AGT1* expressed constitutively in all grown cell types and during in planta colonization, and localized in peroxisomes. Notably, conidia produced by the *agt1* mutant were unable to form appressoria on artificial inductive surfaces. Supplementation with nicotinamide adenine dinucleotide (NAD^+)+pyruvate restored appressorium formation on an artificial inductive surface. Thus, it was shown that AGT1-dependent pyruvate formation by transferring an amino group of alanine to glyoxylate, an intermediate of the glyoxylate cycle is obligatory for lipid mobilization and utilization.

18.5 Pexophagy and Pathogenesis

Autophagy is a cellular self-eating process where proteins, cytoplasm, organelles are surrounded by an autophagic membrane and delivered to the lysosome for degradation. Autophagy usually occurs in all eukaryotic cells and has different purposes:

disposal of extra or damaged organelles, recycling of nutrients during starvation, restoration of cell contents and structures during development or apoptosis, and defense against pathogens (Mizushima and Klionsky 2007; Pollack et al. 2009). A type of selective autophagy known as pexophagy comprises the preferential destruction of peroxisomes (Farré and Subramani 2004). Pexophagy of yeast *Pichia pastoris* has been studied extensively through metabolic changes induced by *in vitro* carbon source alteration.

The role of peroxisome function and pexophagy in phytopathogenicity was first described in *C. orbiculare* (Bertoni 2009). By random insertional mutant screening of *C. orbiculare* for mutants defective in pathogenicity on cucumber leaves, *P. pastoris ATG26* homolog gene encoding a sterol glucosyltransferase that activates pexophagy was identified (Asakura et al. 2009; Takano et al. 2009). The mutant did not invade intact cucumber leaf epidermis cells, but penetrated the epidermis of heat treated immune compromised leaf cells or wounded leaf tissue. These data suggest that the melanized appressoria retains sufficient mechanical force for penetration, but that host defense provided resistance towards penetration by the *atg26* mutant appressoria, and that *ATG* gene is essentially indispensable in the initial infection stage of the pathogen. Using GFP fused with peroxisomal targeting signal to monitor peroxisome dynamics, it was shown that the *atg26* mutant revealed proper peroxisome biogenesis in conidia, but defective in peroxisome degradation in appressoria. The expression of truncated variants of the *ATG26* gene in the mutant revealed that peroxisome degradation depends on both the presence of the catalytic domain of the enzyme and the proper intracellular localization mediated by the non-catalytic N-terminal domains. Thus, the results of this work confirm that peroxisome degradation is required for the formation of functional appressoria suitable to penetrate the host epidermal cell wall (Asakura et al. 2009), and suggest that there could be a potential link between peroxisome homeostasis and infection-related morphogenesis. In contrast to *C. orbiculare*, deletion of *atg26* in *M. oryzae* did not result in reduced pathogenicity towards rice (Kershaw and Talbot 2009). The mutant was able to cause rice blast disease and the mutation did not affect conidial or appressorial autophagy. Role of pexophagy may be different depending on the infection strategy employed by pathogens.

18.6 Woronin Body and Pathogenesis

Woronin bodies are peroxin derived organelles unique in the filamentous ascomycetes and function to seal septal pores when hyphal cells were damaged (Trinci and Collinge 1974; Markham and Collinge 1987; Jedd and Chua 2000; Momany et al. 2002). The major Woronin body structural protein, Hex1p has peroxisome targeting signal-1 and self-assembles to form the crystalline core (Jedd and Chua 2000). It was indicated that Woronin body function is essential for pathogenesis in *M. oryzae* (Soundararajan et al. 2004). Although the *hex1* mutant of *M. oryzae* retains normal mycelial growth, conidiation, and mating response, appressoria showed

severe morphological defects and delayed host penetration. In addition, the mutant infection hyphae showed defect in survival in the host environment by increased cell death in response to nitrogen starvation. These anomalies caused the *hex1* strain to have attenuated pathogenesis. In *M. oryzae*, Woronin bodies localize at the tips of secondary infection hyphae that necrotrophically develop in host tissue. This was consistent with reports on Woronin body localization in other fungi showing localization in non septal regions, such as the tips of the germlings or at the cell periphery (Markham and Collinge 1987; Momany et al. 2002; Tey et al. 2005). Deletion of the *PEX6* peroxin gene encoding not only prevents peroxisome biogenesis but also lack Woronin bodies and renders *M. oryzae* completely non-pathogenic, highlighting the diverse functions fulfilled by the peroxisomal compartment in fungi and the significance of peroxisomal activities in appressorium function (Ramos-Pamplona and Naqvi 2006). In *C. orbiculare*, *pex6*, *pex13* and *pex22* mutant showed deficiency in pathogenesis, however *hex1* mutant retained full pathogenesis, indicating that Woronin body function in pathogenesis is different in these fungi (Kubo 2012).

Alternaria brassicicola is a necrotrophic plant pathogen that infects *Brassica* and *Arabidopsis* plants. TmpL encodes a predicted hybrid membrane protein containing an AMP-binding domain, six putative transmembrane domains, and FAD/NAD(P)-binding domain and is necessary for regulation of intracellular reactive oxygen species (ROS) levels and tolerance to external ROS (Kim et al. 2009). *A. brassicicola tmpL* mutants exhibited dramatically reduced invasive growth on host plants although it retained initial penetration. Localization and gene expression analyses in *A. brassicicola* indicated that TmpL is associated with the Woronin body, and expressed during conidiation and initial invasive growth stage *in planta*. Cytological analysis revealed sequential association between TmpL and peroxisomes and it was suggested that TmpL is targeted into peroxisomes followed by Woronin body biogenesis and maturation. The regulation of intracellular levels ROS is crucial for fungal virulence of many pathogenic fungi. *A. brassicicola tmpL* mutants exhibited attenuated conidiogenesis, accelerated aging, enhanced oxidative burst during conidiation, and hypersensitivity to oxidative stress compared to wild-type strains. Constitutive expression of the *A. brassicicola YAP1, a* redox-regulating transcription factor gene in *tmpL* mutants resulted in tolerance against oxidative stress and partially restored virulence on plants, confirming that dysfunction in oxidative stress homeostasis caused by *TmpL* mutation is the cause of the developmental and virulence defect. *A. brassicicola tmpL* mutants exhibited dramatically reduced invasive growth on host plants although it retained initial penetration (Kim et al. 2009).

18.7 Conclusion

The importance of peroxisome function in the infection process by plant pathogenic fungi highlights the dependence of foliar plant pathogens on lipid and fatty acid metabolisms for supporting initial growth and development into the leaf tissue. The absence of external nutrients during spore germination requires lipid mobilization,

peroxisomal fatty acid β-oxidation leading to generation of a pool of acetyl CoA, and glyoxylate cycle that constitute pivotal roles for fungal pathogens to enter its host. Also, for those fungi that infect through melanized and elaborate appressoria such as *Colletotrichum* and *Magnaporthe* species, the peroxisome related metabolisms are crucial for appressorium function endowed by melanization and turgor generation. Coordinate control of primary and secondary metabolisms including lipolysis and melanin biosynthesis could be key regulatory machinery for pathogenesis to those fungi (Asakura et al. 2012). Thus, peroxisomes has pivotal roles in the development of fungal pathogenesis as a source of signaling molecules.

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