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

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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

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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

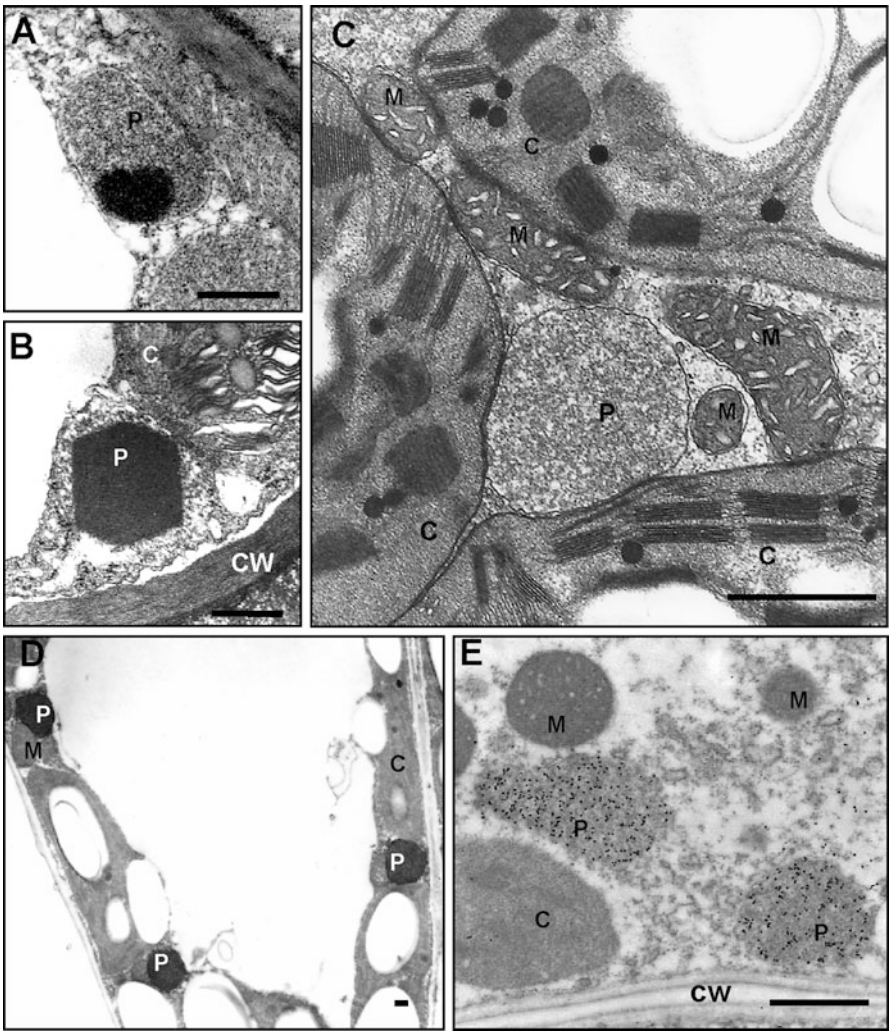

Fig. 13.1 Electron micrography showing the ultrastructure of peroxisomes from different plant leaves and the presence of catalase as the major component of peroxisomes. (**A**) Olive leaves showing an amorphous core inside the peroxisome. (**B**) Pepper leaves showing a peroxisome containing a cristal 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

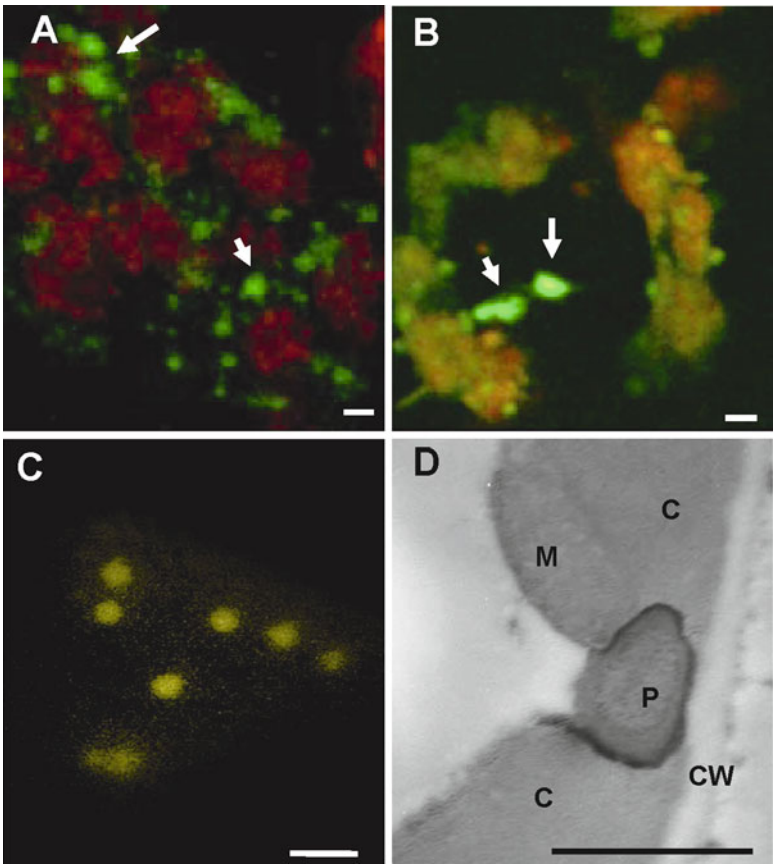

Fig. 13.2 Imaging of ROS production in peroxisomes. (**A**) $O_2^{\cdot -}$ production induced by $CdCl_2$ (50 μM) in pea leaves, detected by confocal laser microscopy using dihydroethidium. (**B**) H_2O_2 accumulation induced by $CdCl_2$ (50 μM) in pea leaves, by confocal laser microscopy using 2′,7′-dichlorofluorescein 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,

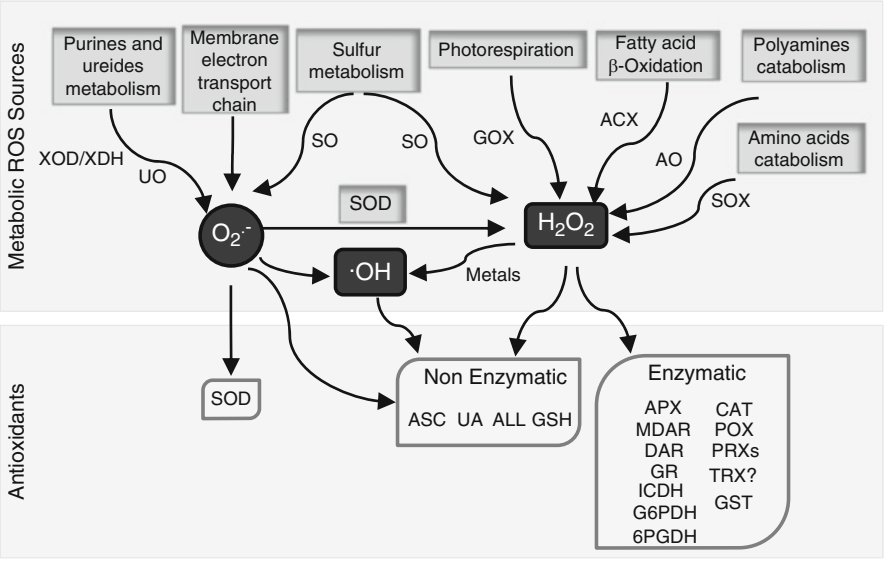

Fig. 13.3 Hypothetical model of ROS production and scavenging in plant peroxisomes. *ACX* acyl CoA oxidase, *ASC* reduced ascorbate, *ALL* allantoin, *AO* amine oxidase, *APX* ascorbate peroxidase, *CAT* catalase, *DAR* dehydroascorbate reductase, *GOX* glycolate oxidase, *GR* glutathione reductase, *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

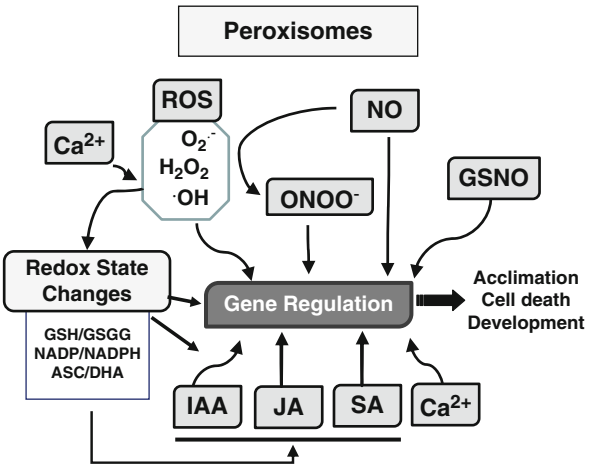

Fig. 13.4 Cross-talk between ROS and different signaling molecules produced in peroxisomes. The interplay between, ROS, RNS (NO, $ONOO^-$ and GSNO), and redox homeostasis, determined by the redox state of glutathione, ascorbate and pyridine nucleotides, calcium concentration and hormone balance, control 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

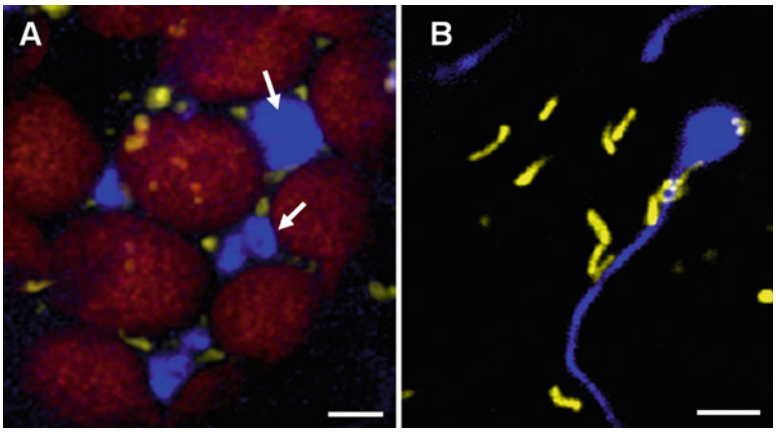

Fig. 13.5 Imaging of peroxisomes, mitochondria and chloroplasts of *Arabidopsis* leaves by confocal laser microscopy. Peroxisomes and mitochondria were imaged in *Arabidopsis lines* expressing CFP in peroxisomes (*blue*) and YFP in mitochondria (*yellow*), and chloroplasts were detected by chlorophyll 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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