ROS Generation in Peroxisomes and its Role in Cell Signaling

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In plant cells, as in most eukaryotic organisms, peroxisomes are probably the major sites of intracellular H_2O_2 production, as a result of their essentially oxidative type of metabolism. In recent years, it has become increasingly clear that peroxisomes carry out essential functions in eukaryotic cells. The generation of the important messenger molecule hydrogen peroxide (H_2O_2) by animal and plant peroxisomes and the presence of catalase in these organelles has been known for many years, but the generation of superoxide radicals (O_2^-) and the occurrence of the metalloenzyme superoxide dismutase was reported for the first time in peroxisomes from plant origin. Further research showed the presence in plant peroxisomes of a complex battery of antioxidant systems apart from catalase. The evidence available of reactive oxygen species (ROS) production in peroxisomes is presented, and the different antioxidant systems characterized in these organelles and their possible functions are described. Peroxisomes appear to have a ROS-mediated role in abiotic stress situations induced by the heavy metal cadmium (Cd) and the xenobiotic 2,4-D, and also in the oxidative reactions of leaf senescence. The toxicity of Cd and 2,4-D has an effect on the ROS metabolism and speed of movement (dynamics) of peroxisomes. The regulation of ROS production in peroxisomes can take place by post-translational modifications of those proteins involved in their production and/or scavenging. In recent years, different studies have been carried out on the proteome of ROS metabolism in peroxisomes. Diverse evidence obtained indicates that peroxisomes are an important cellular source of different signaling molecules, including ROS, involved in distinct processes of high physiological importance, and might play an important role in the maintenance of cellular redox homeostasis.

Introduction

Peroxisomes are ubiquitous subcellular organelles bounded by a single membrane and devoid of DNA, with an essentially oxidative type of metabolism, and are probably the major sites of intracellular H_2O_2 production. A general characteristic of peroxisomes is that they contain as basic enzymatic constituents catalase and hydrogen peroxide (H_2O_2)-producing flavin oxidases, and occur in almost all eukaryotic cells (Fahimi and Sies 1987, Baker and Graham 2002, del Río 2013). Fatty acid β-oxidation is a general feature of virtually all types of peroxisomes, and in recent years it has become increasingly clear that peroxisomes are involved in a range of important cellular functions in almost all eukaryotic cells (Terlecky and Titorenko 2009, Hu et al. 2012, Waterham and Wanders 2012, del Río 2013). The use of transcriptomic and proteomic approaches has identified new functions for peroxisomes (Reumann et al. 2009, Hu et al. 2012). **Table 1** shows some of the main functions that have been described so far for peroxisomes in plant cells.

Plant peroxisomes usually contain a granular matrix but can have crystalline or amorphous inclusions composed of catalase (del Río et al. 2002). Electron micrographs of plant peroxisomes from three different origins, namely olive (leaf and fruit), pea leaf and pepper (leaf and fruit), are presented in **Fig. 1**. The peroxisome of plant cells is a highly dynamic compartment that is dependent upon the actin cytoskeleton, not microtubules, for its subcellular distribution and movements (Mano et al. 2002, Mathur et al. 2002, Oikawa et al. 2015). Three important characteristic properties of peroxisomes are their oxidative type of metabolism, their capacity for sharing metabolic pathways with other cell compartments and their metabolic plasticity, because their enzymatic content can vary depending on the organism, cell/tissue type and environmental conditions (Fahimi and Sies 1987, Baker and Graham 2002, Titorenko and Terlecky 2011, del Río 2013). The presence of regulatory proteins in peroxisomes, such as heat shock proteins, kinases and phosphatases, has been reported (Hayashi and Nishimura 2003, Reumann et al. 2009, Hu et al. 2012). In plants, the cellular population of peroxisomes can proliferate during senescence

Table 1 Main functions reported for peroxisomes in plant cells

| Function	References
β-oxidation of fatty acids	Baker and Graham (2002); Nyathi and Baker (2006)
Photorespiration	Tolbert (1997)
Glyoxylate cycle	Pracharoenwattana and Smith (2008)
Metabolism of ureides	Schubert (1986); Baker and Graham (2002)
Purine catabolism	Corpas et al. (1997)
Sulfur metabolism	Corpas et al. (2009)
Polyamine catabolism	Moschou et al. (2008); Reumann et al. (2009)
Metabolism of reactive oxygen and nitrogen species (ROS and RNS)	del Río et al. (2002, 2006); del Río (2011)
Metabolism of reactive sulfur species (RSS)	Corpas and Barroso (2015)
Biosynthesis of phytohormones (auxin, jasmonic acid, salicylic acid)	Hu et al. (2012); del Río (2013)
Photomorphogenesis	Kaur et al. (2013)
Leaf senescence	del Río et al. (1998)
Plant defense against fungal infection	Koh et al. (2005)
Protection against herbivores	Shabab (2013)
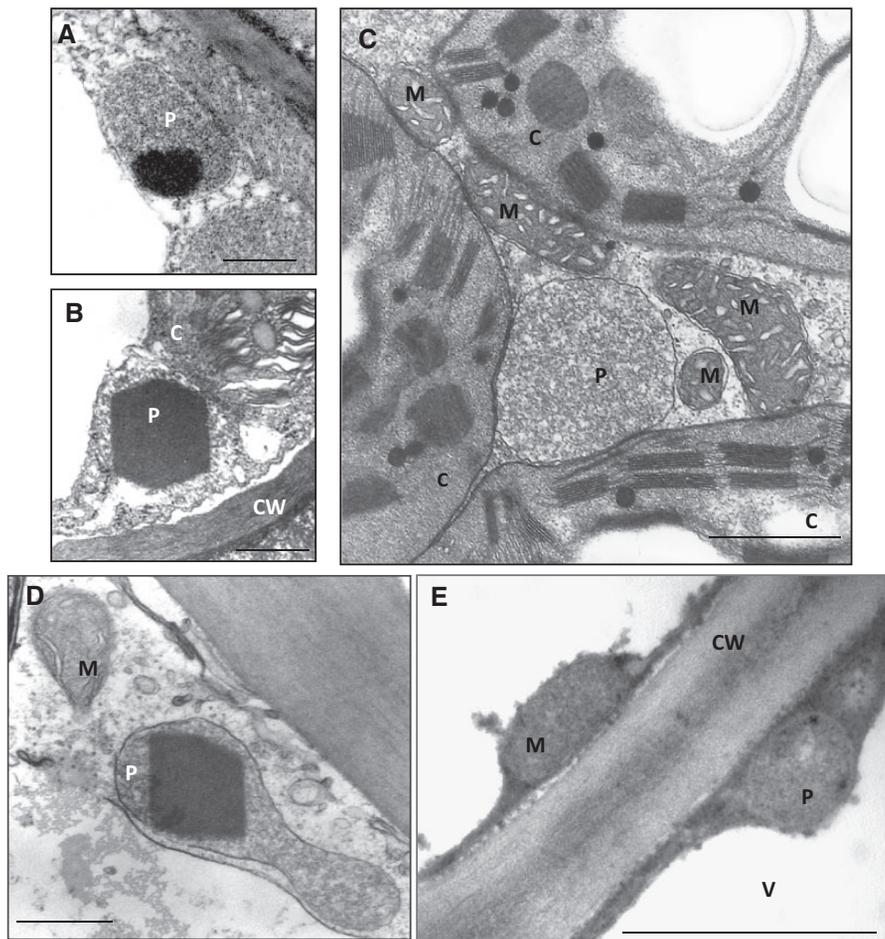
Fig. 1 Electron micrographs of peroxisomes from different plant origins. (A) Peroxisome from olive tree leaf (*Olea europaea* L.) showing an amorphous core inside the peroxisome. (B) Peroxisome from pepper leaf (*Capsicum annuum* L.) with a crystalline inclusion. (C) Peroxisome from pea leaf (*Pisum sativum* L.) showing a granular matrix and in close contact with chloroplasts and mitochondria. (D) Peroxisome from ripe pepper fruit (*Capsicum annuum* L.). (E) Peroxisome from mature olive fruit (*O. europaea* L.). C, chloroplast. CW, cell wall. M, mitochondrion. P, peroxisome. V, vacuole. Scale bar = 1 μm. Reproduced from Sandalio et al. (2013), Mateos et al (2003) and López-Huertas and del Río (2014).

and under different stress conditions produced by xenobiotics, ozone, cadmium (Cd), H_2O_2, salinity (del Río et al. 1998, Romero-Puertas et al. 1999, del Río et al. 2003b, Oksanen et al. 2003, Nila et al. 2006, Castillo et al. 2008, Mitsuya et al. 2010) and light (Desai and Hu 2008). The peroxisome proliferator-activated receptor (PPAR), the transcription factor involved in peroxisomal proliferation and induction of peroxisomal fatty acid β-oxidation in animal tissues, was demonstrated to be functional in transgenic tobacco plants, and its expression led to changes in general lipid metabolism, and induced the proliferation of peroxisomes, as reported in animal tissues (Nila et al. 2006).

In the last three decades, different experimental evidence has indicated the existence of cellular functions for peroxisomes related to reactive oxygen species (ROS) and reactive nitrogen species (RNS) (del Río et al. 2002, del Río et al. 2006, Corpas et al. 2009, del Río 2011, Fransen et al. 2012, Sandalio and Foyer 2015), and a function for peroxisomes as key centers of the cellular signaling apparatus has been postulated (del Río 2013). The diverse key physiological functions that have been demonstrated for peroxisomes from different origins (Table 1) strongly indicate the interest of peroxisomes as a cellular source of different signaling molecules, including ROS. However, studies carried out in animal peroxisomes have indicated that, contrary to what was a widely held belief, peroxisomes are inefficient at degrading cytoplasmic H_2O_2, and peroxisomal catalase should no longer be considered as an important detoxification site of cytosolic H_2O_2 (Fritz et al. 2007).

This review will analyze the generation of ROS in peroxisomes and its regulation, the different antioxidant systems present in these organelles, and the proteomics of ROS metabolism, in the context of a function for peroxisomes as a source of ROS signaling molecules that can participate in plant cell metabolism under both physiological and stress conditions.

Production of Reactive Oxygen Species in Peroxisomes

The generation of different ROS, including H_2O_2, $O_2 \cdot^-$ and singlet oxygen ($^1O_2$), has been reported in peroxisomes.

Hydrogen peroxide (H_2O_2)

The main metabolic processes responsible for the generation of H_2O_2 in different types of peroxisomes are, in decreasing order, the photorespiratory glycolate oxidase (GOX) reaction (in green tissues), the main enzyme of fatty acid β-oxidation, acyl-CoA oxidase, the enzymatic reaction of flavin oxidases and the spontaneous or enzymatic dismutation of O_2^- radicals (Baker and Graham 2002, del Río et al. 2002, Foyer et al. 2009, del Río 2013). Peroxisomes contain numerous H_2O_2-producing flavin oxidases depending on the organism and tissue origin, and they can include GOX, xanthine oxidase (XOD), sulfite oxidase, sarcosine oxidase and enzymes of polyamine catabolism (diamine oxidase and polyamine oxidase), whose presence has been identified in peroxisomes, among others (Corpas et al. 2009, del Río 2013, Sandalio and Romero-Puertas 2015).

The rate of H_2O_2 production by the peroxisomal GOX is about 2- and 50-fold higher than that reported for chloroplasts and mitochondria, respectively (Foyer et al. 2009). The generation of H_2O_2 in peroxisomes can be imaged in vivo by confocal microscopy, using fluorescent probes such as 2′,7′-dichlorofluorescein diacetate (Sandalio et al. 2008), or by expressing transient or constitutively specific H_2O_2 biosensors, such as HyPer, targeted to peroxisomes (Costa et al. 2010). Additionally, H_2O_2 can also be detected by cytochemistry using $CdCl_3$ and visualization by electron microscopy (Romero-Puertas et al. 2004a).

Superoxide radicals (O_2^-)

It has been demonstrated that peroxisomes, like mitochondria and chloroplasts, also produce superoxide radicals (O_2^-) as a consequence of their normal metabolism. In peroxisomes from pea leaves and watermelon cotyledons, using biochemical and electron spin resonance spectroscopy (ESR) methods, the existence of, at least, two sites of O_2^- generation was found: one in the organelle matrix, in which the generating system was identified as XOD, and another site in the peroxisomal membranes dependent on NAD(P)H (Sandalio et al. 1988, del Río et al. 1989, del Río and Donaldson 1995, del Río et al. 2002). XOD catalyzes the oxidation of xanthine and hypoxanthine to uric acid and is a well-known producer of superoxide radicals (Halliwell and Gutteridge 2015). Xanthine oxidoreductases (XORs) are present in two forms, depending on their electron acceptor. Experiments involving incubation of peroxisomal matrices from pea leaves with microbial XOR showed that peroxisomal endoproteases (EPs) could carry out the irreversible conversion of xanthine dehydrogenase (XDH) into the superoxide-generating XOD (Distefano et al. 1999, Palma et al. 2002). Urate oxidase is also present in the matrix of peroxisomes and it was also found to be an O_2^- producer but weaker than XOD, judging by the fainter O_2^- ESR signals obtained (Sandalio et al. 1988). The peroxisomal XOD from pea leaves has been characterized, and the presence of this enzyme in plant peroxisomes has been confirmed by immunogold electron microscopy (Corpas et al. 2008).

The other site of O_2^- production is the peroxisomal membrane, where a small electron transport chain appears to be involved. This is composed of a flavoprotein NADH:ferricyanide reductase of about 32 kDa and a Cyt b (Bowditch and Donaldson 1990). The integral peroxisomal membrane polypeptides (PMPs) of pea leaf peroxisomes were identified, and three of these membrane polypeptides, with molecular masses of 18, 29 and 32 kDa, have been characterized and demonstrated to be responsible for O_2^- generation (López-Huertas et al. 1999). The 18 kDa PMP was the main producer of superoxide radicals in the peroxisomal membrane and was identified as a Cyt b (López-Huertas et al. 1999). While the 18 and 32 kDa PMPs use NADH as the electron donor for O_2^- production, the 29 kDa PMP was dependent on NADPH, and was able to reduce Cyt c with NADPH as electron donor (López-Huertas et al. 1999, del Río et al. 2002). PMP32 very probably corresponds to the enzyme of the ascorbate–glutathione cycle,

monodehydroascorbate reductase (MDAR) (López-Huertas et al. 1999), whose activity was previously detected in pea leaf peroxisomal membranes (Jiménez et al. 1997). The third O_2^--generating polypeptide, PMP29, could be related to the peroxisomal NADPH:cytochrome P450 reductase (López-Huertas et al. 1999). Superoxide production by peroxisomal membranes may be an obligatory consequence of NADH re-oxidation by the peroxisomal electron transport chain, in order to regenerate NAD^+ to be re-utilized in peroxisomal metabolic processes (del Río et al. 1990, del Río et al. 1992, del Río and Donaldson 1995). The superoxide accumulation in peroxisomes can be imaged in vivo by confocal laser microscopy, using fluorescent probes such as dihydroethidium (Sandalio et al. 2008).

Singlet oxygen ($^1O_2$)

The non-radical ROS, singlet oxygen ($^1O_2$), is normally produced in chloroplasts through different photodynamic reactions (Asada 2006, Triantaphylidés and Havaux 2009), but a recent report has shown the production of singlet oxygen, independent of light, in peroxisomes, mitochondria and the nucleus of *Arabidopsis thaliana* non-photosynthetic tissue. In vivo imaging, using the singlet oxygen-specific probe singlet oxygen sensor green (SOSG) and confocal microscopy, showed the generation of $^1O_2$ in peroxisomes, mitochondria and the nucleus, and singlet oxygen accumulation was intensified in roots from *A. thaliana* plants subjected to various biotic and abiotic stresses in the dark (Mor et al. 2014). It has been suggested that the origin of $^1O_2$ could be the reaction of O_2^- with H_2O_2 via a Haber–Weiss mechanism (Mor et al. 2014).

Antioxidant Systems in Peroxisomes

The presence of catalase in peroxisomes has been known since the characterization of these organelles in mammalian tissues (De Duve and Baudhuin 1966) and today it is known that catalase, apart from its function in the control of H_2O_2 generated in peroxisomes, can also have a key role in the maintenance of cellular redox homeostasis (Mhamdi et al. 2012, Inzé et al. 2012, Sandalio and Romero-Puertas 2015). Three genes encoding catalase have been identified in *A. thaliana* and other plants. *CAT2* expression is associated with the photorespiration pathway, *CAT1* expression is associated with fatty acid β-oxidation, and *CAT3* is related to senescence processes (Mhamdi et al. 2010, Mhamdi et al. 2012). Nonetheless, besides catalase, a complex battery of antioxidative systems has been demonstrated in plant peroxisomes.

Superoxide dismutases

The family of metalloenzymes, superoxide dismutases (SODs), catalyze the dismutation of superoxide radicals into molecular oxygen and H_2O_2 (Fridovich 1995, Imlay 2011). SODs are mainly distributed in chloroplasts, cytoplasm, mitochondria, apoplasts and nuclei (Ogawa et al. 1996, Asada 2006, Rodríguez-Serrano et al. 2007), but the presence of SOD in peroxisomes was demonstrated for the first time in plant tissues. In protoplasts from pea leaves, by immunofluorescence and immunoelectron microscopy, using a polyclonal antibody against Mn-SOD purified from pea leaves, the metalloenzyme Mn-SOD was found to be localized in peroxisomes and was absent in chloroplasts (del Río et al. 1983). Later, the presence of Mn-SOD in peroxisomes was confirmed in organelles isolated from pea leaves by cell biology methods (Sandalio et al. 1987). Since then, the occurrence of different types of SODs in plant peroxisomes has been reported in at least 10 distinct plant species, and in five of these plants the presence of SOD in peroxisomes has been confirmed by immunogold electron microscopy (del Río et al. 2002, Mateos et al. 2003, Corpas et al. 2006, Rodríguez-Serrano et al. 2007). Results obtained concerning the presence of SOD in plant peroxisomes were extended years later to human, animal and yeast peroxisomes (see del Río et al. 2002, Petrova et al. 2009). Three SODs of peroxisomal origin have been purified and characterized, a CuZn-SOD and a Mn-SOD from watermelon cotyledons and an Mn-SOD from pea leaves (Bueno et al. 1995, Palma et al. 1998, Rodríguez-Serrano et al. 2007). However, the genes encoding the peroxisomal SODs have not been characterized yet. Mn-SOD, which is distributed in peroxisomes and mitochondria, could be produced from a single gene by alternative splicing or alternative targeting (see del Río et al. 2003a). This could explain the extremely similar molecular properties of the Mn-SODs from both oxidative organelles (Sevilla et al. 1982, Palma et al. 1998).

Ascorbate–glutathione cycle (Foyer–Halliwell–Asada cycle)

The ascorbate–glutathione cycle, also known as the Foyer–Halliwell–Asada pathway after the names of the three major contributors, is a reaction sequence considered to be a crucial mechanism for H_2O_2 metabolism in both animals and plants (Asada 2006, Foyer and Noctor 2011). This cycle that occurs in chloroplasts, cytoplasm and mitochondria (Foyer and Noctor 2011) has also been demonstrated in peroxisomes (Jiménez et al. 1997). The four enzymes of the cycle, ascorbate peroxidase (APX), MDAR, dehydroascorbate reductase (DAR) and glutathione reductase (GR), are present in peroxisomes purified from pea leaves (Jiménez et al. 1997) and tomato leaves and roots (Mittova et al. 2004, Kuźniak and Skłodowska 2005). APX is perhaps the best characterized enzyme of this cycle at the molecular and functional level (Ishikawa et al. 1998). The peroxisomal GR of pea leaves has been purified and characterized (Romero-Puertas et al. 2006). The presence of reduced ascorbate and glutathione and their oxidized forms in purified peroxisomes was demonstrated by HPLC analysis (Jiménez et al. 1997). In *A. thaliana* the relative concentration of total glutathione and ascorbate in different cell organelles, including peroxisomes, was also quantified by immunogold cytochemistry based on antiglutathione and antiascorbate antisera and electron microscopy (Zechmann et al. 2008, Fernández-García et al. 2009, Koffler et al. 2015).

The intraperoxisomal distribution of the ascorbate–glutathione cycle was studied in pea leaves, and DAR and GR were found in the soluble fraction of peroxisomes, whereas APX activity was bound to the cytosolic side of the peroxisomal

membranes (Jiménez et al. 1997). MDAR was localized in both the membrane and matrix of pea peroxisomes (López-Huertas et al. 1999, Leterrier et al. 2005), and in pea and *A. thaliana* the peroxisomal MDAR was found to contain a peroxisomal targeting signal (PTS) type 1 (Leterrier et al. 2005, Lisenbee et al. 2005). The presence of APX and MDAR in the leaf peroxisomal membranes could serve to reoxidize endogenous NADH to maintain a constant supply of NAD^+ for peroxisomal metabolism (del Río and Donaldson 1995). Nevertheless, APX and MDAR could also have a modulating role of H_2O_2 leaking from peroxisomes, as well as the H_2O_2 that is being continuously formed by dismutation of the O_2^- generated in the NAD(P)H-dependent electron transport system of the peroxisomal membrane (López-Huertas et al. 1999, del Río et al. 2002, del Río et al. 2006). This membrane scavenging of H_2O_2 could also prevent a cytoplasmic accumulation of this metabolite, particularly under certain plant stress situations, when the level of H_2O_2 produced in peroxisomes can be substantially increased (del Río al. 1996).

Glutathione peroxidase and glutathione S-transferase

The presence of another peroxidase activity, glutathione peroxidase, has been reported in leaf peroxisomes of tomato plants (Kuźniak and Skłodowska 2005). In the yeast *Candida boidini*, a glutathione peroxidase was found in peroxisomes (Horiguchi et al. 2001), and in *Saccharomyces cerevisiae* the presence of glutathione peroxidase has been demonstrated in peroxisomes, and *GPX1* encoded a glutathione peroxidase which also had an atypical 2-Cys peroxiredoxin activity (Ohdate and Inoue 2012). Moreover, three families of glutathione *S*-transferases, GSTT1, GSTT2 and GSTT3, have been detected in peroxisomes where they could have a role in the removal of toxic hydroperoxides due to their glutathione peroxidase activity (Dixon et al. 2009).

NADPH-generating dehydrogenases

NADPH is an important component in cell redox homeostasis, and its regeneration is essential for reductive biosynthesis and detoxification pathways. NADPH has a relevant role in the protection against oxidative stress mainly due to its involvement in the ascorbate–glutathione cycle and the water–water cycle (Asada 2006, Foyer and Noctor 2011). This evidence has supported the notion of NADP-dependent dehydrogenases as antioxidative enzymes which can be included in the group of catalase, SOD, APX and GR/peroxidase (Corpas et al. 1999). In isolated plant peroxisomes, the presence of three NADP-dehydrogenases was demonstrated, including glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH) and isocitrate dehydrogenase (ICDH) (Corpas et al. 1998, Corpas et al. 1999, Valderrama et al. 2006). The localization of NADP-ICDH in *A. thaliana* peroxisomes has been demonstrated by immunocytochemistry, and this dehydrogenase has been postulated to be involved in stomatal movement, suggesting that H_2O_2, NADPH and nitric oxide (NO) generated in peroxisomes are necessary for stomatal movements (Leterrier et al. 2016). In addition, de novo

biosynthesis of NADP can also take place in peroxisomes by phosphorylation of NAD, catalyzed by NAD kinase3 (Waller et al. 2010).

The occurrence in peroxisomes of several dehydrogenases implies that these organelles have the capacity to reduce NADP to NADPH for its reutilization in their metabolism. In peroxisomal membranes NADPH is necessary for the function of the NADPH:cytochrome P450 reductase (Baker and Graham 2002) and the O_2^--generating polypeptide, PMP29 (López-Huertas et al. 1999). NADPH has been reported to protect catalase from oxidative damage (Kirkman et al. 1999). NADPH is also required for the GR activity of the ascorbate–glutathione cycle which recycles reduced glutathione (GSH) from its oxidized form (GSSG) (Foyer and Noctor 2011), and for the reduction of double bonds of unsaturated fatty acids by 2,4-dienoyl-CoA reductase (Reumann et al 2007). The peroxisomal NO-producing activity, nitric oxide synthase-like, also requires NADPH for its activity (Corpas et al. 2004).

Peroxiredoxins

Peroxiredoxins are a family of thiol-specific antioxidant enzymes, also known as thioredoxin-dependent peroxidases, which are present in bacteria, yeasts, plants and mammals (Dietz 2003). Peroxiredoxins have been localized in yeast and animal peroxisomes (Walbrecq et al. 2015). In plants, the presence of a protein immunorelated to peroxiredoxins with a molecular mass of about 50 kDa was localized in the matrix of pea leaf peroxisomes (Corpas, Pedrajas, Palma, Valderrama, Rodríguez-Ruiz, Chaki, del Río and Barroso, unpublished results) and its expression was found to be differentially modulated under oxidative stress conditions, suggesting that this peroxiredoxin could be involved in the mechanism of regulation of H_2O_2 and/or peroxynitrite.

In recent years, the antioxidant systems of peroxisomes from two different fruits have been characterized, including pepper (Mateos et al. 2003) and olive fruits (López-Huertas and del Río 2014). The metabolism of antioxidants was studied in peroxisomes from pepper fruits during the ripening process, and proteomic analysis revealed no changes between the antioxidant metabolism of immature (green) and ripe (red) fruits (Palma et al. 2015).

Effect of Abiotic Stress

It is known that when plants are subjected to different abiotic and biotic stresses a rapid accumulation of ROS (mainly H_2O_2) and other important signaling molecules such as RNS takes place (Baxter et al. 2014, Sandalio and Romero-Puertas 2015, Corpas 2015, del Río 2015).

Under normal physiological conditions, the production by peroxisomes of ROS is adequately regulated by various antioxidative enzymes present in peroxisomes. However, ROS production is also an important component of the signaling network that plants use, as signal transducers, for their development and for responding to environmental challenges (Foyer and Noctor 2005, Considine et al. 2015, Sandalio and Foyer 2015). In

peroxisomes from plants subjected to different abiotic stress situations (mainly salinity, Cd and xenobiotics), the effect of stress on ROS metabolism and organelle dynamics has been investigated.

The effect of NaCl salinity on the metabolism of ROS was studied in peroxisomes purified from leaves of two cultivars of pea plants with different sensitivity to NaCl (NaCl sensitive and NaCl tolerant). In both cultivars, salinity caused a decrease in catalase activity and an enhancement in the photorespiratory GOX activity (Corpas et al. 1993a). In peroxisomes of leaves and roots from salt-tolerant tomato plants, there was an up-regulation of the antioxidative systems in response to salt-induced oxidative stress (Mittova et al. 2004). In *A. thaliana* plants, salt stress induced the expression of three peroxisome-associated genes, namely thiolase (*PED1*), *PEX10* and *PEX1*, and required components of the ethylene, jasmonate and ABA signaling pathways (Charlton et al. 2005). In the extreme halophyte *Salicornia brachiata*, the peroxisomal APX gene (*SbpAPX*) imparts abiotic stress endurance and confers salt stress tolerance in transgenic peanut (*Arachis hypogaea*) (Singh et al. 2014).

The specific importance of ascorbate and glutathione in different cell compartments during salt stress was investigated in *A. thaliana* Col-0 and mutants deficient in ascorbate and glutathione, by immunogold electron microscopy with antibodies to total glutathione and ascorbate. The results obtained showed that the accumulation of total glutathione in peroxisomes and chloroplasts seemed to be an important defense strategy of wild-type plants against salt stress (Koffler et al. 2015).

Leaf peroxisomes are also involved in the toxicity produced in pea plants by heavy metals, such as Cd and copper (Cu) (del Río et al. 2003b, del Río et al. 2006). In peroxisomes from Cu-tolerant plants, higher activities of the antioxidative enzymes Mn-SOD and catalase were found compared with Cu-sensitive plants (Palma et al. 1987). This suggests that Cu-tolerant plants could have evolved protection mechanisms against the peroxisomal generation of O_2^{-}-dependent toxic species. In leaf peroxisomes from pea plants treated with Cd, an overproduction of O_2^{-} and H_2O_2 and the oxidative modification of some endogenous proteins was observed, which run parallel to a slight increase of the peroxisomal population of pea leaves (Romero-Puertas et al. 1999, Romero-Puertas et al. 2002). In *A. thaliana* seedlings exposed to Cd, the accumulation of O_2^{-} in peroxisomes was corroborated (Corpas and Barroso 2014). By using *A. thaliana* lines expressing cyan fluorescent protein (CFP) in peroxisomes and yellow fluorescent protein (YFP) in mitochondria, it has been found that Cd stress induces peroxisome proliferation which takes place in a co-ordinated way with mitochondrial proliferation (Sandalio et al., 2013).

Peroxisomes responded to Cd toxicity by increasing the activity of antioxidative enzymes involved in the Foyer–Halliwell–Asada cycle as well as the NADP-dehydrogenases located in these organelles. Cd also increased the activity of the endogenous proteases and glyoxylate cycle enzymes, malate synthase and isocitrate lyase. The enhancement of the activity of these two enzymes suggests that Cd induces senescence symptoms in peroxisomes and, probably, metabolic changes in leaf

peroxisomes with participation of the peroxisomal proteases in all these Cd-induced metabolic changes (McCarthy et al. 2001, Palma et al. 2002).

In a study carried out with an *A. thaliana* mutant expressing the green fluorescent protein (GFP)–SKL peptide targeted to peroxisomes, it was found that Cd increased the cellular speed of movement, or motility, of peroxisomes which was dependent on endogenous ROS and Ca^{2+} (Rodríguez-Serrano et al. 2009). This increase in peroxisome motility could be a cellular mechanism of protection against the Cd-imposed oxidative stress, although a function for this speed increase in peroxisome-derived signaling processes or import of new proteins cannot be discarded (Rodríguez-Serrano et al. 2009, Sandalio and Romero-Puertas 2015).

In leaf peroxisomes from pea plants subjected to stress conditions by xenobiotics, such as the hypolipidemic drug clofibrate (ethyl-α-p-chlorophenoxyisobutyrate) and the auxin herbicide 2,4-D, an oxidative stress mechanism mediated by ROS was demonstrated to be involved (Palma et al. 1991, del Río et al. 2003b, del Río et al. 2006). Clofibrate also induced a proliferation of the peroxisomal population in leaves of pea, tobacco and *A. thaliana* plants (Palma et al. 1991, Nila et al. 2006, Castillo et al. 2008), a similar effect to that first described in rodents by Reddy et al. (1982). The role of peroxisomes in the oxidative injury induced by the herbicide 2,4-D in leaves of pea plants was investigated. 2,4-D did not induce the proliferation of pea leaf peroxisomes but produced senescence-like morphological changes in these organelles, including a slight increase in size and a lower electron density of their matrix (McCarthy 2004, McCarthy et al. 2011). These changes resembled those occurring in peroxisomes during leaf senescence (del Río et al. 1998). Other senescence symptoms induced by 2,4-D in leaf peroxisomes were the decrease of protein content and GOX and hydroxypyruvate reductase activities, and the increase of endopeptidase, XOD, isocitrate lyase and acyl-CoA oxidase activities as well as the 3-ketoacyl-CoA thiolase and thiol-protease protein contents (McCarthy 2004, McCarthy et al. 2011). The results indicated that peroxisomes might contribute to 2,4-D toxicity in pea leaves by overproducing cell-damaging ROS, the main sources being fatty acid β-oxidation and XOD, and also by participating actively in 2,4-D-induced leaf senescence (Romero-Puertas et al. 2004b, McCarthy et al. 2011). In pea leaves sprayed with the herbicide 2,4-D, transcriptome analysis showed an induction of transcripts of the peroxisomal enzyme MDAR (*MDAR 1*) (Leterrier et al. 2005).

In leaves of *A. thaliana*, 2,4-D also promoted oxidative stress, giving rise to post-translational changes of actin (oxidation and *S*-nitrosylation), causing disturbances in the actin cytoskeleton and thereby affecting the dynamics and metabolism of peroxisomes and mitochondria (Rodríguez-Serrano et al. 2014). 2,4-D altered the dynamics of the *A. thaliana* peroxisomes, slowing the speed and shortening the distances by which these organelles were displaced. Interestingly, Cd and 2,4-D have opposing effects on the dynamics of peroxisomes in leaves of *A. thaliana* plants, increasing and slowing their motility, respectively, in a process regulated by ROS (Rodríguez-Serrano et al. 2009, Rodríguez-Serrano et al. 2014).

The signaling pathways that control the peroxisome proliferation process under different environmental conditions are not well known (reviewed in del Río 2013). Two opposing hypotheses to explain the function of peroxisome proliferation in plants have been proposed. One is related to toxicity and postulates that peroxisome proliferation could form part of the mechanism of oxidative stress induced by some xenobiotics such as clofibrate (Reddy et al. 1982, Palma et al. 1991). On the other hand, an opposite and more beneficial hypothesis establishes that peroxisome proliferation, making use of these organelles' antioxidants, might be a mechanism of protection against oxidative stress situations. This hypothesis is based on the up-regulation of peroxisome biogenesis genes (PEX) by H_2O_2 observed in transformed A. thaliana plants (López-Huertas et al. 2000).

The biotechnological potential of plant peroxisomes has been highlighted by Kessel-Vigelius et al. (2013). The well-known functions of peroxisomes in abiotic stress response and pathogen defense have suggested that manipulating endogenous peroxisomal pathways, such as fatty acid β-oxidation, up-regulating the peroxisomal ROS-scavenging systems and increasing the peroxisomal population could be a good strategy to improve abiotic and biotic stress tolerance, circumventing oxidative stress (Kessel-Vigelius et al. 2013). However, the engineering of plant peroxisomes still requires an improvement in our knowledge of how peroxisomal metabolism is regulated and co-ordinated.

Effect of Senescence

Peroxisomes have been demonstrated to have a ROS-mediated function in the oxidative reactions characteristic of senescence. The senescence-induced changes in the reactive oxygen metabolism of peroxisomes are mainly characterized by the disappearance of catalase activity and an overproduction of $O_2^{\cdot-}$ and H_2O_2 accompanied by a strong decrease of APX and MDAR activities (del Río et al. 1998). Since $O_2^{\cdot-}$ radicals under physiological conditions quickly dismutate into H_2O_2 and O_2, the final result of senescence is a build-up in leaf peroxisomes of the more stable metabolite H_2O_2, which can diffuse into the cytoplasm mainly through channel proteins such as certain aquaporins (Bienert et al. 2007) or by other alternative mechanisms (Bienert et al. 2006, Fritz et al. 2007). This could represent a serious situation not only for peroxisomes but also for other cell organelles such as mitochondria, chloroplasts and nuclei, due to the possible formation of the strongly oxidizing hydroxyl radicals (·OH) by the metal-catalyzed reaction of H_2O_2 with $O_2^{\cdot-}$ (Halliwell and Gutteridge 2015). On the other hand, the endogenous proteases of peroxisomes are induced by senescence and they probably participate in the important metabolic changes that take place in these organelles as a result of senescence (McCarthy et al. 2001, Palma et al. 2002).

Regulation of ROS Generation in Peroxisomes

Experimental evidence suggests that the accumulation in peroxisomes of H_2O_2 and $O_2^{\cdot-}$, in response to metabolic or environmental changes, can be regulated by post-translational modifications of those proteins involved in their production and/or scavenging (Sandalio et al. 2013). Possible post-translational modifications of proteins in peroxisomes could involve oxidation, phosphorylation and S-nitrosylation, among others (Friso and van Wijk 2015). Many peroxisomal proteins have been identified as targets of post-translational modifications associated with ROS and RNS (Chaki et al. 2015, Sandalio and Romero-Puertas 2015). In plants under adverse environmental conditions there is an overproduction of ROS in peroxisomes that can oxidize proteins and produce the carbonylation of their C groups. In pea plants under Cd toxicity, oxidative stress is induced and several peroxisomal proteins are oxidized (carbonylated), including at least catalase, GR and Mn-SOD (Romero-Puertas et al. 2002). These oxidatively modified proteins are degraded by the peroxisomal proteases induced by Cd toxicity (McCarthy et al. 2001, Palma et al. 2002, Romero-Puertas et al. 2002). In peroxisomes from castor bean endosperm, malate synthase, isocitrate lyase, malate dehydrogenase and catalase have been found to be carbonylated by metal-catalyzed oxidation with loss of their enzymatic activities (Nguyen and Donaldson 2005).

The presence of endo- and exoproteolytic activity in plant peroxisomes was demonstrated for the first time in cell organelles purified from pea leaves (Corpas et al. 1993b, Distefano et al. 1997). One leucine aminopeptidase was identified in the soluble fraction of peroxisomes, and was characterized as a serine protease (Corpas et al. 1993b). The endoproteolytic activity of pea leaf peroxisomes was also characterized, and seven EP isoenzymes were found in peroxisomes from senescent pea leaves, and the serine-proteinase isoenzymes represent approximately 70% of the total EP activity of pea leaf peroxisomes (Distefano et al., 1997). The peroxisomal enzymes GOX, catalase and G6PDH were susceptible to proteolytic degradation by peroxisomal endoproteases, whereas peroxisomal Mn-SOD was not endoproteolytically degraded (Distefano et al. 1999). Peroxisomal endoproteases could carry out the irreversible conversion of XDH into the superoxide-generating XOD in these organelles (Distefano et al. 1999, Palma et al. 2002). In A. thaliana, by genome analysis and proteomic studies, at least nine proteases have been predicted in peroxisomes, and some of them are involved in the import and processing of proteins in these organelles and in peroxisome biogenesis (Lingard and Monroe-Augustus 2009, Quan et al. 2013). One of the proteases was identified by proteome analysis as a cysteine protease and is involved in β-oxidation, development and stress response (Quan et al. 2013).

Concerning phosphorylation, the phosphoproteome of leaf peroxisomes has been studied, and catalase and GOX have been found as targets of this post-translation modification (Sandalio et al. 2013). The activity of these two enzymes was finely regulated by co-ordinated phosphorylation/dephosphorylation. The interaction between catalase and the nucleoside diphosphate kinase (NDK-1) has been studied in A. thaliana, and lines overexpressing NDK-1 had higher tolerance to oxidative stress (Fukumatsu et al. 2003). On the other hand, peroxisomes have been demonstrated to store Ca^{2+} which can contribute to

modulate the accumulation of H_2O_2 in peroxisomes by activating catalase (Costa et al. 2010).

Proteomic Studies of ROS Metabolism

The main functions of peroxisomes known so far were established on the basis of peroxisomes purified and analyzed by different classical cell biology and biochemical methods (Palma et al. 2009, del Río 2013). However, proteome analysis has made possible the detection of many new proteins in peroxisomes which provides important information about novel metabolic functions of these organelles and an understanding of their metabolic and regulatory networks. In recent years, a proteomic burst has taken place in peroxisome biology (Saleem et al. 2006; Arai et al. 2008, Palma et al. 2009, Reumann et al. 2009, Kaur and Hu 2011). Most proteome studies of plant peroxisomes have been carried out in A. thaliana, soybean, spinach and rice (Kaur and Hu 2011, Reumann 2011), and very recently in pepper fruits (Palma et al. 2015). Many new A. thaliana proteins have been established in recent years by peroxisome researchers, with major contributions from the Arabidopsis Peroxisome 2010 project (Reumann 2011, Hu et al. 2012, Quan et al. 2013).

Screening the A. thaliana genome has led to the identification of about 280 genes that encode proteins containing putative peroxisomal targeting signals PTS-1 (220) and PTS-2 (60) peptides (Hayashi and Nishimura 2003, Reumann et al. 2009). PTSs are located at either the C- (PTS-1) or the N-terminus (PTS-2). PTS-2 corresponds to polypeptides that are proteolytically cleaved upon their entrance into the organelle (Hu et al. 2012). In order to define the complete proteome of plant peroxisomes, the bioinformatic prediction of peroxisome-targeted proteins from plant genome sequences is a very important complementary approach (Kaur and Hu 2011, Reumann 2011). These in silico analyses show that although only a few dozen proteins have been functionally characterized as peroxisomal proteins, the total number of proteins predicted in the peroxisomal plant proteome may be up to 670 (Bussell et al. 2013). By fluorescence microscopy, the subcellular localization of >100 putative novel peroxisomal proteins identified from proteomics and in silico PTS searches of the A. thaliana genome was assayed, and the peroxisomal targeting for about 50 of them was confirmed (Reumann et al. 2009, Kaur and Hu 2011, Reumann et al. 2011).

Proteomic analysis has confirmed the presence of different peroxisomal enzymes of ROS metabolism which had been previously identified in these organelles by cell biology and biochemical methods, and/or immunocytochemistry. These included catalases, GOXs, acyl-CoA oxidase, NADP-dehydrogenases, hydroxypyruvate reductase, MDAR, DAR, APX, GR, GST, SOD and different proteases, among others (Palma et al. 2009, Reumann 2009, Kaur and Hu 2011, Reumann 2011, Quan et al. 2013). In a recent proteomic analysis of peroxisomes purified from pepper fruits, by combining 2-D and MALDI-TOF/TOF (matrix-assisted laser deionization-time of flight) mass spectrometry (MS), the identification of catalase and

two different SODs (Fe-SOD and Mn-SOD) was achieved (Palma et al. 2015).

The availability of new methods to obtain pure and intact peroxisomes with higher yields combined with more sensitive proteomics and MS technologies will allow identification of low-abundance and transient peroxisomal proteins and, therefore, increase our knowledge of the metabolic and regulatory networks in these important oxidative organelles.

Peroxisomes as Generators of ROS and Other Signaling Molecules

In the last two decades the idea that peroxisomes could be a cellular source of the signal molecules ROS and NO was proposed (del Río and Donaldson 1995, del Río et al. 1996, Corpas et al. 2001). The idea of a signaling function for plant peroxisomes was extended years later to other molecules which are also produced in peroxisomes and derived from β-oxidation, including jasmonic acid and its derivatives, salicylic acid and IAA, among others (Nyathi and Baker 2006; see del Río 2013).

Peroxisomes are probably the major sites of intracellular H_2O_2 production and are an important source of O_2^- radicals. A model of the role of peroxisomes in the generation of the ROS H_2O_2, O_2^- and $^1O_2$ is shown in **Fig. 2**. To prevent oxidative stress situations, peroxisomes have different enzymatic and non-enzymatic antioxidants which, under normal conditions, can scavenge the excess oxidants produced and so avoid the deleterious effects on plant cell biomolecules. However, it is now known that ROS play a key role in the complex signaling network that regulates essential cellular processes including stress response (Mittler et al. 2011, Considine et al. 2015). H_2O_2 is an important transduction signal in plant–pathogen interactions, response to wounding, stomatal closure, osmotic stress and excess light stress, where H_2O_2 leads to the induction of defense genes encoding different cellular protectants (Inzé et al. 2012).

An advantage of peroxisomes as a source of signaling molecules is their metabolic plasticity that permits metabolic adjustments depending on developmental and environmental situations (del Río et al. 2006), as well as the ability to change their motility and population in response to plant environmental conditions. Plant mutants deficient in catalase have been an excellent tool to study the consequences of an increase in the endogenous levels of H_2O_2 in peroxisomes. Microarrays studies in catalase loss-of-function A. thaliana mutants have demonstrated that a total of 783 transcripts modified their expression in response to high levels of photorespiratory H_2O_2, and the majority of transcripts were associated with abiotic stress responses (Foyer et al. 2009, Inzé et al. 2012). Most of those H_2O_2-induced proteins were localized in the nucleus and cytosol (Inzé et al. 2012). Different transcriptomic studies have also demonstrated a close interaction between peroxisomal H_2O_2, oxidative stress and phytohormone-dependent signaling involving ethylene, auxins, jasmonic acid, salicylic acid and ABA, suggesting that redox homeostasis (mainly the GSH/GSSG ratio), linked to NAD and NADP systems, might modulate this relationship (Yun et al.
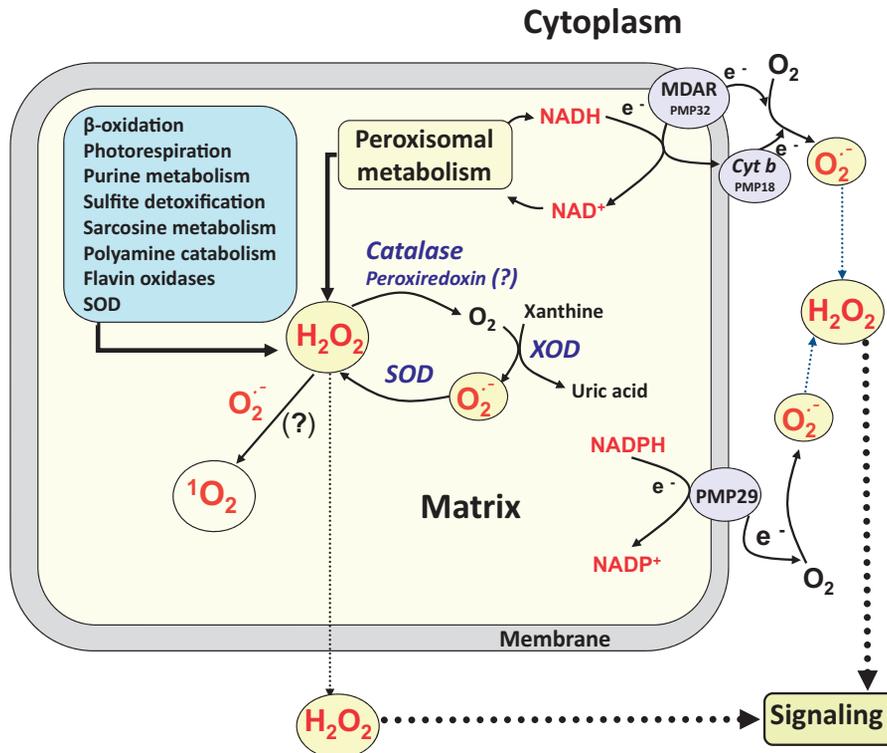
Fig. 2 Scheme of the different sources of the ROS, $O_2^{.-}$, H_2O_2 and $^1O_2$, in plant peroxisomes. PMP, peroxisomal membrane polypeptide. Cyt *b*, a *b*-type cytochrome (PMP18). MDAR, monodehydroascorbate reductase (PMP32). PMP29, peroxisomal membrane polypeptide of 29 kDa probably related to the NADPH:cytochrome P450 reductase. SOD, superoxide dismutase. XOD, xanthine oxidase. Broken arrows indicate signaling. Modified from del Río et al. (2006).

2012, Jones and Sies 2015, Sandalio and Romero-Puertas 2015, Wang et al. 2015). Moreover, the use of *A. thaliana* GOX loss-of-function mutants has permitted study of the contribution of H_2O_2 from each GOX isoform to the regulation of cell response to the plant infection by *Pseudomonas*. H_2O_2 generated by GOX could be a secondary oxidative burst response different from that regulated by NADPH oxidases (Rojas et al. 2012, Sandalio et al. 2013).

The role of peroxisomal complex networks involving calcium, protein phosphorylation or protein S-nitrosylation/nitration needs further investigation. Peroxisomes can store cellular Ca^{2+}, and it has been demonstrated that the complex Ca^{2+}/calmodulin can activate plant catalases (CAT3) and, therefore, regulate the H_2O_2 accumulation in these organelles (Costa et al. 2013). Peroxisomes are also a source of NO and other RNS signal molecules (Corpas et al. 2004, del Río 2011, Corpas and Barroso 2014, del Río et al. 2014). Furthermore, in plants, these organelles are the only site for β-oxidation of fatty acids, and several signaling molecules derived from β-oxidation, which are generated in peroxisomes, have been suggested as possible signaling molecules. These mainly include jasmonic acid and its derivatives (methyl jasmonate, Z-jasmonate and tuberonic acid), salicylic acid and IAA (Nyathi and Baker 2006; see del Río 2013). This suggests the possibility of cross-talk between ROS and RNS, and ROS and the hormones jasmonic acid, salicylic acid and IAA. Detailed knowledge of the interactions among these networks is necessary to know the different signaling pathways and the function of peroxisomes in the regulation of cellular responses (Sandalio et al. 2013).

Concluding Remarks

The existence of ROS metabolism in plant peroxisomes and the presence in these organelles of an important set of antioxidative enzymes emphasizes the importance of these organelles in cellular oxidative metabolism. Plant peroxisomes have a ROS- and RNS-mediated metabolic function in leaf senescence and certain types of abiotic stress, and peroxisomes can have a dual role in cells, as oxidative stress generators and as a source of ROS, RNS and other phytohormone-related molecules. These organelles could act as subcellular indicators or sensors of plant stress by releasing the signaling molecules $O_2^{.-}$ and H_2O_2, as well as other second messengers, into the cytoplasm and triggering specific changes in the expression of defense genes (stress signaling). ROS, NO and the hormone biosynthesis-derived signal molecules confer to peroxisomes a key role in the network regulating gene transcription in response to environmental signals, and also in the cross-talk between peroxisomes, chloroplasts and mitochondria. This signal molecule-producing function of plant peroxisomes is still more significant from a physiological viewpoint, considering that the cellular population of these organelles can proliferate in plants under different physiological and stress conditions, and their dynamics/motility

can be altered by some of those situations. Results obtained in recent years suggest that peroxisomes might play an important role in the maintenance of cellular redox homeostasis.

The ROS generation and metabolism in plant peroxisomes adds to the seminal research carried out by Professor Kozi Asada's group on production and scavenging of ROS in chloroplasts related to photosynthesis and photoinhibition, as well as to the environmental responses of photosynthesis (Asada 2006). Professor Asada proposed the existence of the water–water cycle in chloroplasts and contributed significantly to understanding the physiology of alternative electron flows in photosynthesis (Asada 1999). Apart from many other achievements, in his laboratory the presence of SOD in chloroplasts was demonstrated for the first time and its purification and characterization accomplished (Asada et al. 1973).

Very important milestones in ROS research in plants were established by Professor Kozi Asada. For this reason, it is our honor to contribute this review article on peroxisomes and ROS to the Special Issue of *Plant and Cell Physiology* dedicated to the memory of this distinguished Japanese scientist.

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