Plant peroxisomes: A nitro-oxidative cocktail

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ABSTRACT

Although peroxisomes are very simple organelles, research on different species has provided us with an understanding of their importance in terms of cell viability. In addition to the significant role played by plant peroxisomes in the metabolism of reactive oxygen species (ROS), data gathered over the last two decades show that these organelles are an endogenous source of nitric oxide (NO) and related molecules called reactive nitrogen species (RNS). Molecules such as NO and H2O2 act as retrograde signals among the different cellular compartments, thus facilitating integral cellular adaptation to physiological and environmental changes. However, under nitro-oxidative conditions, part of this network can be overloaded, possibly leading to cellular damage and even cell death. This review aims to update our knowledge of the ROS/RNS metabolism, whose important role in plant peroxisomes is still underestimated. However, this pioneering approach, in which key elements such as β-oxidation, superoxide dismutase (SOD) and NO have been mainly described in relation to plant peroxisomes, could also be used to explore peroxisomes from other organisms.

1. Introduction

In morphological terms, peroxisomes are very simple organelles composed of a dense matrix surrounded by a single membrane. Antioxidant catalase and H2O2-producing flavin oxidases are essential enzymatic components of these organelles. However, it is surprising to note that their biochemical composition can change depending on the organism, organ type, development stage and environment involved [25,44,53,88,97,112,113].

In higher plants, peroxisomes possess extraordinary metabolic plasticity because they house a wide range of pathways such as fatty acid β-oxidation, glyoxylate cycles, photorespiration, purine catabolism, plant hormone biosynthesis (indole-3-acetic acid and jasmonic acid) and polyamine catabolism [20,54,63,85,88,108]. Many of these pathways involve other subcellular compartments, suggesting that peroxisomes must have a pertinent retrograde signaling among the different subcellular compartments which must integrate their functions under optimal physiological conditions or trigger appropriate responses in unforeseen adverse situations [55].

In some cases, these metabolic interactions may involve dynamic and reversible functional and morphological adaptations between peroxisomes and other organelles such as chloroplasts and oil bodies during physiological processes such as photorespiration [19,51,84] and seed germination [35] which enable metabolic exchanges between the organelles to be optimized [63]. So far, over 300 plant peroxisomal proteins have been identified [69,93]; however, innovative approaches using proteomic and bioinformatic technologies, in which key components with hitherto unknown functions [3,47,60,62,71,88,99] have been described in relation to plant peroxisomes, could also be used to explore peroxisomes from other organisms.
metabolism with complex regulatory antioxidative machinery. The data indicate that plant peroxisomes have a nitro-oxidative behavior has been described during the natural senescence of leaves [16]. The antioxidant enzyme superoxide dismutase (SOD), which catalyzes the reduction of superoxide radicals (O$_2^-$) into H$_2$O$_2$ and O$_2$, is also present in peroxisomes. SOD, specifically Mn-SOD, was first described in plant peroxisomes in 1983 [43], whose presence was strongly questioned until CuZn-SOD was described in animal peroxisomes many years later [61]. Since then, an increasing number of reports have confirmed the presence of SOD in peroxisomes (see Table 2), which is now universally accepted as a key antioxidant enzyme in all types of peroxisomes. However, it is worth mentioning two important characteristics of the peroxisomal SOD. While CuZn-SOD has been exclusively found in the matrix of animal peroxisomes, the number, type and localization (matrix and/or membrane) of SOD in plant peroxisomes can change radically (HO$_2$), alkoxyl radicals (RO·), peroxyl radicals (ROO·) and non-radical O$_2$ derivatives such as hydrogen peroxide (H$_2$O$_2$). Superoxide radicals and hydrogen peroxide are two of the most studied ROS in plants. ROS are generated under physiological conditions and plants containing a complex battery of antioxidant defence systems to regulate their production [4]. Interestingly, peroxisomes are important sites for the generation of these ROS, mainly via photorespiration and fatty acid β-oxidation pathways [25,32,40,42,83]. Thus, peroxisomes as cell compartments, with high rates of H$_2$O$_2$ production, play an important role in defence mechanisms and ROS-mediated cross-talk between cell compartments. The principal ROS regulatory enzymatic systems in plant peroxisomes include catalase, all ascorbate-glutathione cycle components as well as superoxide dismutase (SOD). Interestingly, some of these antioxidant components are located in both the peroxisomal matrix and membrane. The peroxisomal β-oxidation pathway, which generates significant amounts of H$_2$O$_2$, was first described in the germinating castor bean [18] and was later found in rat liver [66]. In germinating seeds, triglycerides are mobilized as a source of energy during the non-autotrophic stage prior to photosynthesis. Thus, fatty acids enter peroxisomes via ATP-binding cassette (ABC) transporters of subfamily D. They are then oxidized to fatty acyl-CoA in peroxisomes and shortened by two carbons in each β-oxidation cycle. Finally, acetyl-CoA is converted to four-carbon molecules by the glyoxylate cycle, which then undergo gluconeogenesis in the mitochondrion and cytosol to provide energy for seedling development [14,57,78]. However, H$_2$O$_2$, which acts as a priming factor involving specific changes at the proteome, transcriptome and hormonal levels, is not simply a byproduct of peroxisomal β-oxidation [6,83,144]. Another important source of intracellular H$_2$O$_2$ is the peroxisomal glycolate oxidase (GOX), which is involved in photosynthesis [48]. In addition to the importance of this pathway as a major determinant of C$_3$ crop biomass production, photosynthetic H$_2$O$_2$ appears to participate in pathogen defense [16]. Catalase (CAT) is one of the most representative peroxisomal antioxidant enzymes. While only one catalase isoform is encoded by a single gene in animal cells, catalase is present in the form of multiple isoforms encoded by a small gene family in plant cells [46,49,57]; consequently, the number and expression of various CAT isoforms change during plant development and under different environmental conditions. Given the oxidative metabolism of peroxisomes, various studies have evaluated the oxidative stability of plant catalase activity in the presence of high concentrations of H$_2$O$_2$ (up to 100 mM). However, its oxidation at multiple sites does not affect catalase activity [2]. On the other hand, there is evidence to show that NO-donors, such as S-nitrosglutathione (GSNO) and DETA NONOate, as well as nitrating agents, such as peroxyxinitrite (ONO'O), cause the down-regulation of catalase activity ([15,17,87]). This inhibition of catalase activity could reflect a reduced capacity to remove H$_2$O$_2$ and consequently an increase in the nitro-oxidative metabolism [21,22]. This behavior has been described during the natural senescence of leaves and pepper fruit ripening, when both NO and catalase activity decline [15,24]. In this context, catalase can be regarded as a key enzyme involved in increasing cell longevity [36]. The antioxidant enzyme superoxide dismutase (SOD), which catalyzes the disproportionation of O$_2^-$ into H$_2$O$_2$ and O$_2$, is also present in peroxisomes. SOD, specifically Mn-SOD, was first described in plant peroxisomes in 1983 [43], whose presence was strongly questioned until CuZn-SOD was described in animal peroxisomes many years later [61]. Since then, an increasing number of reports have confirmed the presence of SOD in peroxisomes (see Table 2), which is now universally accepted as a key antioxidant enzyme in all types of peroxisomes. 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Table 2. Superoxide dismutases (SODs) localized in plant and animal peroxisomes.

| Source                | SOD Isozyme     | Reference |
|-----------------------|-----------------|-----------|
| **Plants**            |                 |           |
| Pea                   | Mn-SOD          | [43,90]   |
| Watermelon            | Cu/Zn-SOD, Mn-SOD | [100,104] |
| Carnation             | Fe-SOD, Mn-SOD  | [39]      |
| Castor bean           | Mn-SOD          | [41]      |
| Sunflower             | Cu/Zn-SOD       | [33]      |
| Cucumber              | Cu/Zn-SOD, Mn-SOD | [33]    |
| Cotton                | Cu/Zn-SOD       | [33]      |
| Tomato                | SOD             | [79]      |
| Olive                 | Cu/Zn-SOD       | [28,120]  |
| Pepper                | Mn-SOD          | [77]      |
| Rice                  | Fe-SOD, Cu/Zn-SOD | [91]     |
| **Animals**           |                 |           |
| Humans                |                 |           |
| Hepatoma cells        | Cu/Zn-SOD       | [61]      |
| Fibroblast            | Cu/Zn-SOD       | [61]      |
| **Rat**               |                 |           |
| Liver                 | Cu/Zn-SOD       | [37]      |
| Brain                 | Cu/Zn-SOD       | [80]      |
| Fish liver            | Cu/Zn-SOD       | [86]      |
| Molluscs digestive gland | Cu/Zn-SOD     | [86]      |
| Crustaceans digestive gland | Cu/Zn-SOD | [86]      |

Depending on the plant species (see Table 2). Moreover, to our knowledge, none of the SOD proteins localized in peroxisomes contain a classic PTS1 or PTS2. In this context, peroxisomal Cu/Zn-SOD in mammals has been demonstrated to use a piggyback import mechanism, where physiological interaction with the copper chaperone of SOD (CCS) functions as a shuttle [51]. The ascorbate-glutathione (Asc-GSH) cycle constitutes a complementary system which enables plants to control H₂O₂ content. This cycle is composed of 4 enzymes: ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR), as well as antioxidants ascorbate and glutathione, and also requires NADPH as a reducing agent. Although the Asc-GSH cycle was initially found in cytosol and chloroplasts, these enzyme components have also been reported in plant peroxisomes, with APX being the first enzyme found to be present in peroxisomal membranes [13,26,117]. The other components were later identified and characterized in the peroxisomes of different plant species [59,77,81,102]. It is important to point out that the subcellular localization of the Asc-GSH cycle is peculiar to peroxisomes, as, while the APX enzyme is located on the outer surface of the membrane, the GR and DHAR enzymes are present in the matrix, and the MDAR enzyme is located in both the matrix and membrane [67,72]. Although the peroxisomal Asc-GSH cycle is involved in the mechanism of response to environmental stresses (for review, see [98]), it also appears to protect oil bodies close to peroxisomes from oxidative damage [45].

Nitric oxide (NO) is a free radical messenger belonging to a family of related molecules called reactive nitrogen species (RNS). These include molecules such as S-nitrosothiols (SNOs), S-nitrosoglutathione (GSNO), peroxynitrite (ONOO⁻) and nitro-fatty acids (NO₂⁻FA), which directly or indirectly perform a broad spectrum of regulatory functions. Peroxisomes from oxidative damage [45]. Later, it became possible to visualize peroxisomal NO production in vivo with the aid of specific, cell-permeable fluorescent probes to detect NO such as DAF-FM DA and DAR-4M AM. Confocal laser scanning microscopy (CLSM) was also used to analyze Arabidopsis thaliana transgenic plants expressing green or cyan fluorescent proteins by constructing peroxisomal targeting signal 1 (PTS1) [26]. Fig. 1 shows the visualization of NO and ONOO⁻ in the guard cells of transgenic Arabidopsis thaliana expressing CFP-PTS1. Another set of experimental data which evidence the RNS metabolism in plant peroxisomes is related to the identification of peroxisomal proteins which undergo RNS-mediated post-translational modifications, including nitration and S-nitrosylation and affect peroxisomal protein function (Table 3).

Additionally, genetic techniques using Arabidopsis knock out mutants have shown that peroxisomal NO is generated by an NOS-like protein. By using Arabidopsis pex12 and pex13 mutants, it has been possible to demonstrate the absence of peroxisomal NO generation, suggesting that an NOS-like protein is involved [27]. Moreover, analysis of peroxisomal NO content in Arabidopsis pex5 and pex7 knockout mutants subjected to RNA interference (RNAi) called pex5i and pex7i, respectively, enabled us to pinpoint the NOS-like protein responsible for NO generation in peroxisomes, which was imported via the PTS2 pathway [21,22]. Similar findings were also obtained with respect to the iNOS peroxisomal isoform present in rat hepatocytes, which is also imported through the PTS2 pathway [73], suggesting that both plants and animals contain a similar mechanism for importing the protein responsible for NO generation into peroxisomes.

3. Roles of peroxisomal ROS/RNS under physiological and stress conditions

The metabolic plasticity of plant peroxisomes is caused by their specific functions in various physiological processes and by their involvement in the mechanism of response to adverse environmental conditions.

3.1. Physiological conditions: seed and pollen germination, stomatal movement, senescence and fruit ripening

Peroxisomes are key elements in seed germination, as seedling growth in the dark requires the conversion of fatty acids to sugars through β-oxidation and gluconeogenesis [50,52,103]. During this process, large amounts of H₂O₂ are produced, thus promoting seed germination [114], which boosts the production of endogenous ethylene and leads to cell elongation in the root tip [56]. On the other hand, excess peroxisomal H₂O₂ appears to be regulated by the membrane-bound components of the ascorbate-glutathione (APX and MDAR) cycle in order to protect oil bodies against oxidative damage, which can inactivate the triacylglycerol lipase sugar-dependent1 and prevent the supply of carbon for seedling establishment [45]. Although seed germination is also stimulated by NO [9,64], the direct involvement of peroxisomal NO has not yet been confirmed. However, peroxisomal NO has been demonstrated to mediate auxin-induced lateral root organogenesis [105]. On the other hand, peroxisomal NO is directly involved in pollen germination and tube growth, and its target to the ovule [94–96].

In leaves, stomatal movement is highly regulated by external stimuli (light, CO₂ levels, water balance and pathogens) and also by internal molecules including hormones (ABA and salicylic acid) and ROS/RNS (H₂O₂ and NO) [10,82,111]. Fig. 1B shows the presence of...
chloroplasts. D and H, merged images for corresponding panels. C and G, chlorophyll autofluorescence (blue) attributable to the detection in the same guard cells of nitric oxide and peroxynitrite, respectively. A and E, seedlings expressing CFP-PTS1. A and B, fluorescence punctates (red) attributable to CFP-PTS1, indicating the localization of peroxisomes in guard cells. B and F, fluorescence punctates (green) attributable to the detection in the same guard cells of nitric oxide and peroxynitrite, respectively. C and G, chlorophyll autofluorescence (blue) attributable to the detection of chloroplasts. D and H, merged images for corresponding panels.

Peroxisomal NO and ROS are also involved in leaf senescence, which is characterized by programmed degeneration controlled by multiple developmental and environmental signals. This process is mainly characterized by a decline in photosynthesis, marked chlorophyll and protein loss, disintegration of organelle structures and a dramatic increase in lipid peroxidation where the imbalance between ROS production and antioxidative systems in the different subcellular compartments is significantly affected [12,89,92]. As the senescence of Arabidopsis leaves progresses, the isoforms Cat2 and Cat3 have been shown to decrease, which could be a signal for cells to promote senescence [122]. Similarly, during the natural senescence of pea leaves, the decrease in catalase activity is accompanied by a down-regulation of NO generation [24]. A similar observation has been made during pepper fruit ripening, which can be regarded as a process of senescence, where catalase activity is also down-regulated and is accompanied by increased nitration [15]. Furthermore, Arabidopsis mutants called pepu (peroxisome unusual positioning), characterized as containing aggregated peroxisomes, have also been found to contain high levels of oxidized proteins and inactive catalase. These aggregates are damaged peroxisomes which are selectively degraded by pexophagy at the senescence stage [107]. This suggests that H$_2$O$_2$ is a signal for pexophagy which contributes to the removal of damaged peroxisomes in the cell [118]. However, other data indicate that misfolded or aggregated peroxisomal matrix proteins may be cellular signals for pexophagy, where the protease LON2 is involved in protein quality control [119].

### Table 3

| NO-derived post-translational modification | Peroxisomal enzyme | Identification |
|------------------------------------------|--------------------|----------------|
| Tyrosine nitration                        | Catalase           | Immunoactive with antibody against nitrotyrosine$^c$ |
|                                          | Monodehydroascorbate reductase$^a$ |                |
|                                          | Glycolate oxidase   |                |
|                                          | Malate dehydrogenase, Hydroxypyruvate reductase$^a$ |                |
| S-nitrosylation                          | Catalase           | Preincubation with GSNO (NO donor) and biotin-switch$^b$ |
|                                          | Monodehydroascorbate reductase$^a$ |                |
|                                          | Glycolate oxidase   |                |
|                                          | Malate dehydrogenase, Hydroxypyruvate reductase |                |

$^a$ Lozano-Juste et al., 2011 [75]; Chaki et al., 2015 [15].
$^b$ Ortega-Galisteo et al., 2012 [87].
$^c$ Begara-Morales et al., 2015 [8].
$^d$ Corpas et al., 2013 [30].

NO (green color) in peroxisomes and the cytosol. Although it has been well established that NO induces stomatal closure as part of a mechanism of response to water deficit [82] and also restricts the entry of pathogenic microorganisms [1], the direct involvement of peroxisomal NO has not yet been confirmed. In this respect, it has been reported that Arabidopsis peroxisomal NADP-isocitrate dehydrogenase (ICDH) knock out mutants show a reduced stomatal aperture as compared to wild type plants [68]. The fact that NADPH generated by this enzyme is necessary for peroxisomal NO generation can be used as indirect evidence of the involvement of peroxisomal NO in stomatal movement. ROS have also been shown to be involved in stomatal movement. Thus, in maize plants grown under drought stress conditions, high accumulation rates of H$_2$O$_2$ in guard cells have been reported to result in stomatal closure [116]. This closely correlates with the regulation of Arabidopsis catalase 3, which, in turn, enables H$_2$O$_2$ to be modulated in stomatal guard cells [123].

### 3.2. Stress conditions: salinity and heavy metals (Cd and lead)

The involvement of the plant peroxisomal ROS metabolism in the mechanism of response to biotic and abiotic stress has been confirmed by numerous examples (see [20]). However, less information is available on the involvement of peroxisomal NO. In this context, several studies have provided sufficient evidence to indicate that, under certain environmental stress conditions such as salinity and heavy metals, the peroxisomal NO metabolism is significantly triggered, resulting in the generation of cellular nitro-oxidative stress (Table 4).

In Arabidopsis thaliana seedlings grown under salinity stress...
(100 mM NaCl), an increase in peroxisomal NO content has been reported. This also promotes its accumulation in the cytosol, thus contributing to the generation of ONOO⁻ and consequently to an increase in protein tyrosine nitration [29], which is regarded as a marker of nitrosative stress [27]. Similarly, in Arabidopsis seedlings exposed to cadmium (150 µM) stress, peroxisomal NO content was found to increase, which was accompanied by increased superoxide radical content and, accordingly, higher peroxynitrite production [21,22]. Likewise, Arabidopsis grown in the presence of 150 µM lead (Pb²⁺) also caused NO, O₂⁻ and ONOO⁻ overproduction. Furthermore, biochemical and gene expression analyses of peroxisomal enzymes, including the antioxidant catalase and two photorespiration enzymes glycolate oxidase (GOX) and hydroxypyruvate reductase (HPR), have shown that only the catalase was clearly a marker of nitrosative stress [27]. Similarly, in Arabidopsis seedlings exposed to cadmium (150 µM) stress, peroxisomal NO content was found to increase, which was accompanied by increased superoxide radical content and, accordingly, higher peroxynitrite production [21,22]. Likewise, Arabidopsis grown in the presence of 150 µM lead (Pb²⁺) also caused NO, O₂⁻ and ONOO⁻ overproduction. Furthermore, biochemical and gene expression analyses of peroxisomal enzymes, including the antioxidant catalase and two photorespiration enzymes glycolate oxidase (GOX) and hydroxypyruvate reductase (HPR), have shown that only the catalase was clearly a marker of nitrosative stress [27].

4. Conclusions

In plants, RNS and ROS are two families of related molecules generated in the cellular metabolism where peroxisomes are important organelles. Molecules, such as NO, GSNO and H₂O₂, which are endogenously produced by peroxisomes, can act as signal elements involved in different transduction pathways of cellular communication. Fig. 2 summarizes the principal elements involved in the endogenous peroxisomal metabolism of NO/H₂O₂, which can diffuse out of these organelles and affect other cell compartments. Nitric oxide generated from L-arginine by NOS-like activity interacts with superoxide radicals (O₂⁻) to generate peroxynitrite (ONOO⁻), which can mediate the protein tyrosine nitration of specific peroxisomal target proteins (Table 3). On the other hand, NO can react with reduced glutathione (GSH) to produce S-nitrosoglutathione (GSNO) which, through a process of transnitrosylation, affects other peroxisomal enzymes (Table 3). Although, little is known about the permeability of GSNO through cellular membranes [11,101], it is possible to suggest that NO, and perhaps GSNO, is released through the peroxisomal membrane to the cytosol and initiates a signaling cascade or interacts with other biomolecules; this causes post-translational modifications or is part of a mechanism of response to various types of stresses. Hydrogen peroxide is produced via different biochemical pathways, such as β-oxidation and photorespiration, in plant peroxisomes. Additionally, O₂⁻ generated by certain enzymes, such as xanthine oxidase (XOD, part of the purine metabolism) [32], can be dismutated to H₂O₂ by the

![Peroxisome Diagram](image-url)
enzyme superoxide dismutase (SOD). Although, peroxisomal H₂O₂ levels are controlled by catalase located in the matrix or by membrane-bound ascorbate peroxidase (APX), this does not discard the potential presence of H₂O₂-transporting aquaporins in the peroxisomal membrane [115]. Additionally, in this peroxisomal model, it is also important to mention the presence of other ascorbate-glutathione cycle components (MDAR, DHAR and GR) as well as a group of NADPH-regenerating enzymes glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH) and NADP-dependent isocitrate dehydrogenase (NADP-ICDH). This group of enzymes provides the NADPH necessary for both GR and L-arginine NOS-like activities.

In summary, peroxisomal studies have opened up exciting new avenues of investigation for the community of researchers working on cellular integration. Molecules such as NO and H₂O₂ act as retrograde avenues of investigation for the community of researchers working on the role of peroxisomes in the immune response and in various diseases. The importance of peroxisomes in health and disease, as well as the potential therapeutic applications, is highlighted in the recent review by F. J. Corpas et al. [136].

**Conflict of interest**

The authors declare that there are no conflicts of interest.

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