Hydrogen Peroxide and Nitric Oxide Generation in Plant Cells: Overview and Queries

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Abstract Hydrogen peroxide (H_2O_2) and nitric oxide (NO) are two key molecules representative of two families of related compounds designated as reactive oxygen and nitrogen species (ROS and RNS, respectively). Our present knowledge about where, when, and how these molecules are produced in a specific plant tissue either under physiological or stress conditions and how they interact support the relevant crosstalk between these molecules which in many cases are autoregulated throughout posttranslational modifications. Thus, either *S*-nitrosation or nitration of different enzymes of the ROS metabolism including superoxide-generating NADPH oxidase (NOX) or antioxidant enzymes such as catalase and superoxide dismutase (SOD) and components of the ascorbate-glutathione cycle may take place under diverse situations. However, H_2O_2 and NO may react among them giving rise to a more powerful toxic species, the hydroxyl radical ($^{\cdot}OH$), which may react with most biomolecules (nucleic acids, proteins, and lipids), leading to irreversible damages within cells. This chapter will provide a comprehensive and easy overview about H_2O_2 and NO production, on how these molecules are generated within different cell compartments, and about their metabolic interaction. A proposed model on how such interaction between H_2O_2 and NO may influence the organelles' signaling

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network under normal physiological and stress conditions and/or developmental metabolic shifts is discussed.

Keywords Chloroplast · Hydrogen peroxide · Hydroxyl radical · Mitochondrion · Nitric oxide · Peroxisome · Reactive oxygen species · Reactive nitrogen species · Signaling · *S*-nitrosoglutathione · *S*-nitrosylation

Abbreviations

GSNO	*S*-nitrosoglutathione
GSH	Reduced glutathione
H_2O_2	Hydrogen peroxide
NR	Nitrate reductase
NO	Nitric oxide
$ONOO^-$	Peroxynitrite
PTM	Posttranslational modification
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SNOs	*S*-nitrosothiols

1 Introduction

Hydrogen peroxide (H_2O_2) and nitric oxide (NO) are two key molecules representative of two families of related compounds designated as reactive oxygen and nitrogen species (ROS and RNS, respectively). Both families of molecules participate in a myriad of plant processes (del Río 2015; Lindermayr 2017; Corpas and Barroso 2018a), and their relevance in plant cells is well recognized because they are involved in dual roles under both physiological events (seed and pollen germination, plant development and growth, stomatal movement, leaf senescence, and fruit ripening, among others) and in the mechanism of response against biotic and abiotic stresses. This dual face of both molecules implies their role in signaling processes during the initial phases of the plant response to diverse situations and as potential responsible of cellular damages when these molecules are overproduced without control. Figure 1 summarizes some of the plant processes where both H_2O_2 and NO participate at different level. Our present knowledge about where, when, and how these molecules are produced in a specific plant tissue either under physiological or stress conditions and how they interact supports the relevant interrelationship between these molecules which in many cases are autoregulated throughout post-translational modifications (PTMs). As a matter of fact, these families of molecules cannot be studied as separated areas because they are interconnected at metabolic level where many of the enzymes involved in their corresponding metabolisms are autoregulated by those PTMs. Thus, either *S*-nitrosation or nitration of different enzymes of the ROS metabolism including superoxide-generating NADPH oxidase (NOX) or antioxidant enzymes such as catalase and superoxide dismutase (SOD)

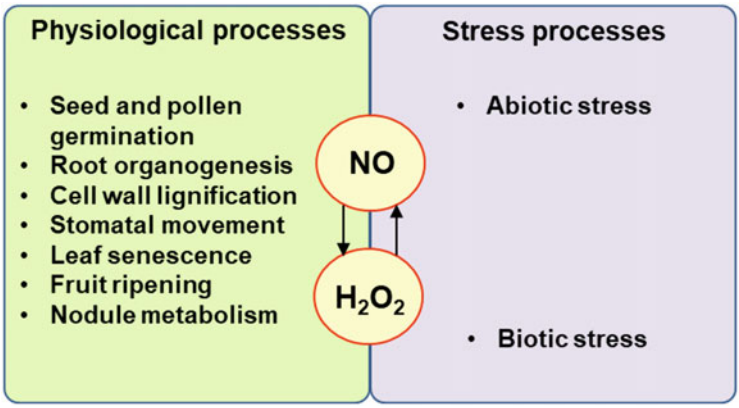

Fig. 1 Nitric oxide (NO) and hydrogen peroxide (H_2O_2) in plant cells participate in complex processes both under physiological conditions (seed and pollen germination, plant development and growth, stomatal movement, leaf senescence, fruit ripening, and others) and in the mechanism of response against biotic and abiotic stresses. They play their roles independently or interacting one with another under two perspectives, as signaling molecules in the initial phases of their respective response and as potential responsible of cellular damages when these molecules are overproduced without control

and components of the ascorbate-glutathione cycle may take place under diverse situations (Yun et al. 2011; Begara-Morales et al. 2015; Chaki et al. 2015).

Both reactive species (H_2O_2 and NO) are generated in multiple cell loci (Foyer and Noctor 2003; Corpas et al. 2015; Gupta et al. 2018a), but their respective diffusion rates within the cell spaces allow them to move from one organelle to another. Accordingly, the potential interaction between H_2O_2 and NO may have repercussions not only in situ where they are produced but also several dozens of micrometers away, thus exporting their effect to other cell compartments.

Consequently, the main goal of this chapter is to provide a wide and comprehensive overview of the metabolism of these two molecules indicating how they are produced and how they are interrelated in the plant metabolism. A prospective of the cell scenario that can be found under physiological and stress conditions will be also depicted.

2 Generation and Scavenging of H_2O_2 in Plant Cells

Hydrogen peroxide (H_2O_2) is one of the main reactive oxygen species (ROS) basically generated in living beings as a secondary metabolite of the aerobic metabolism. Early studies in cell organelles found out initially this molecule as the principal ROS directly generated in chloroplasts, mitochondria, and peroxisomes. Thus, in chloroplast, the first conclusive reports on the production of H_2O_2 were given by Mehler, who discovered that these organelles, besides producing oxygen

by the Hill reaction, also consume it through the so-called Mehler reaction (Mehler 1951). Years later, it was proved that this species was not directly generated in chloroplasts, but through the dismutation of superoxide radicals ($O_2^{\cdot-}$) spontaneously or achieved by the enzymatic system superoxide dismutase (SOD; EC 1.15.1.1) (Asada et al. 1974). Indeed, it was demonstrated that superoxide radicals are firstly produced, by the autoxidation of reduced ferredoxin at the photosystem PSI and the plastoquinone level in photosystem PSII, and then dismutated into H_2O_2 by the action of either chloroplastic Fe-SOD or CuZn-SODs (Asada 2006; Corpas et al. 2015). Diverse aspects complementing this scheme have been recently reviewed (Smirnoff and Arnaud 2018).

Once H_2O_2 is produced, it is decomposed basically by both stromal and thylakoidal ascorbate peroxidase (sAPX and tAPX, respectively; EC 1.11.1.11) (Yoshimura et al. 1999; Shigeoka et al. 2002; Maruta et al. 2016) which could work in cooperation with the other enzymes of the ascorbate-glutathione cycle, with consumption of reduced ascorbate and NADPH provided by the Calvin-Benson cycle. All these actors which play a role in the chloroplast scenario (PSI and PSII with their respective electron acceptors, ferredoxin, SOD, APX, and the ascorbate-glutathione cycle) are integrated within the water-water cycle which years ago postulated Professor Asada's works (Asada 1999, 2006; Corpas et al. 2015; Mano et al. 2016) and was accepted worldwide. Peroxiredoxins (Prxs) and thioredoxins (Trxs) are also systems with coordinated functions among them involved in the hydrogen peroxide scavenging in chloroplasts (Puerto-Galán et al. 2013). They can also interact with PSI through the ferredoxin site, thus sharing some connection points with the water-water cycle (Asada 2006; Nikkanen and Rintamäki 2014).

Globally, all these partners which participate in the H_2O_2 metabolism within chloroplasts are key points to modulate the concentration of this ROS for signaling purposes and confer to this organelle a relevant role in the signal transduction network within the plant cell (Smirnoff and Arnaud 2018) which will be dependent on the lighting conditions.

As in chloroplasts, the former reports in the 1960s on ROS in mitochondria demonstrated the generation of H_2O_2 in this cell compartment (Jensen 1966a, b), and still some years later, this issue was corroborated under different conditions (Boveris and Chance 1973). However, soon after the proposal of superoxide a radical as precursors of H_2O_2 in mitochondria was issued (Loschen et al. 1974). Later, a series of well-designed studies were developed that were focused on complexes I and III from the mitochondrial electron transport chain (ETC) as sources of $O_2^{\cdot-}$ (Boveris and Cadenas 1982; Turrens 1997; Raha and Robinson 2000; Murphy 2009; Huang et al. 2016). Further detection of Mn-SOD activity in these organelles (Weisiger and Fridovich 1973) supported this precursory of superoxide radicals as source of H_2O_2. A thorough review on this subject can be followed in Corpas et al. (2015).

In animals, it has been reported that the H_2O_2 formed at the mitochondrial ETC is scavenged by a selenium-dependent glutathione peroxidase (SeGPX), which uses reduced glutathione (GSH) provided by a glutathione reductase (GR) located at the matrix site (Ursini et al. 1995; Handy et al. 2009; Halliwell and Gutteridge 2015). In plants, a role of GPX in the mitochondrial H_2O_2 homeostasis has been also

referenced (Passaia et al. 2013), but the early report of all enzymatic components of the ascorbate-glutathione cycle (Jiménez et al. 1997) allowed proposing this pathway as the main H_2O_2 processing route in these cell loci (Corpas et al. 2015; Mittova et al. 2015). Besides, a thioredoxin-peroxiredoxin system has been also reported in the matrix which could remove H_2O_2 with the participation of a thioredoxin reductase that would utilize NADPH provided by a NADP-dependent isocitrate dehydrogenase as electron donor (Murphy 2009; Corpas et al. 2015; Sevilla et al. 2015). H_2O_2 can escape from the organelle and be pumped off to the cytosol where it can be either detoxified by diverse systems, including peroxisomes when it is released at high concentration, or driven to signaling processes (Foyer and Noctor 2003; Smirnoff and Arnaud 2018).

Regarding peroxisomes, this descriptive name was proposed in Professor de Duve's laboratories in which it was found that the "microbodies" reported in the mid-1950s (Rhodin 1954) displayed a very active H_2O_2 metabolism (de Duve and Baudhuin 1966). Peroxisomes are organelles with a highly oxidative metabolism whose main characteristics are the presence of flavin oxidases responsible for the H_2O_2 production and catalase (CAT; EC 1.11.1.6), the principal enzyme which removes H_2O_2 in the eukaryotic cell. Enzymes such as acyl-CoA oxidase, xanthine oxidase, urate oxidase, glycolate oxidase (mainly in plants), diamine oxidase, and polyamine oxidase, among others, have been reported in peroxisomes, all of them acting as sources of hydrogen peroxide (Corpas et al. 2015, 2019; Smirnoff and Arnaud 2018). Besides, the relevant presence in peroxisomes of superoxide dismutase (SOD) activity, either as Mn-SOD, Fe-SOD, or CuZn-SOD, can be also considered as a significant generator of H_2O_2 at the cell level (del Río 2011; Palma et al. 2015; del Río et al. 2018).

As indicated above, peroxisomes bear the H_2O_2-scavenging catalase as its archetypical enzyme. In fact, this protein is considered as the typical marker for peroxisomes in biochemistry and cell biology research. However, this plant organelle also contains the four enzymes of the ascorbate-glutathione cycle (Jiménez et al. 1997; del Río 2011) which finely tune the concentration of H_2O_2 within this compartment. Thus, the peroxisomal APX has been well studied in many species and under different situations (Corpas et al. 1994, 2015; Yamaguchi et al. 1995; Bunkelmann and Trelease 1996; Corpas and Trelease 1998; Narendra et al. 2006; Palma et al. 2006).

Some other cell sources of H_2O_2 have been reported in the cell, including the plasma membrane and the apoplast and the endoplasmic reticulum (Smirnoff and Arnaud 2018), but because the main organelles involved in the metabolism of this ROS (Gupta et al. 2018a) and its relationship with NO are better documented in chloroplasts, mitochondria, and peroxisomes, we will focus our attention in the potential signaling networks where these three cell organelles may be integrated.

3 Generation of NO in Plant Cells

Such as it has been mentioned, NO could be considered the most relevant component of a family of related molecules designated as RNS. However, one of the key points in the metabolism of NO in plant cells is the identification and subcellular localization of the endogenous NO sources. Plants can generate NO by nonenzymatic and enzymatic mechanisms, but the contribution of each NO source to a specific physiological process is still unclear (Astier et al. 2018; Corpas and Palma 2018).

Figure 2 shows a simple model of the main recognized NO sources in higher plants. It is known that the nonenzymatic reduction of nitrite (NO_2^-) can lead to the formation of NO, and this reaction is favored at acidic pH. Thus, NO_2^- can also be chemically reduced by ascorbic acid at pH 3–6 to yield NO. This reaction could occur at micro-localized pH conditions in barley aleurone layers, in the chloroplast, and in apoplastic space where ascorbic acid is known to be present (Stöhr et al. 2001; Stöhr and Stremlau 2006). Another nonenzymatic mechanism proposed for NO formation is the light-mediated reduction of NO_2^- by carotenoids (Bethke et al. 2004).

Related with the enzymatic source of NO, there are two main candidates in higher plant, nitrate reductase (NR) and L-arginine-dependent nitric oxide synthase (NOS)-like activity (Corpas and Barroso 2017; Astier et al. 2018). NR is a molybdoenzyme that reduces nitrate (NO_3^-) to nitrite (NO_2^-) using NADH as electron donor. Thus, it has been shown that purified maize NR can generate NO in vitro conditions using NADH (Yamasaki et al. 1999) and this NO production seems to be implicated in some physiological processes such as stomatal closure (Chen et al. 2016). However, there is little information on the direct involvement of NR-derived NO in plant stress situations. More recently, using the unicellular alga *Chlamydomonas reinhardtii* as model photosynthetic organism, it has been demonstrated that the interaction between the mitochondrial amidoxime reducing component (mARC) and NR can generate NO from NO_2^- where the ARC catalyzes the NO generation from NO_2^- using electrons from NR (Chamizo-Ampudia et al. 2016). On the other hand, the NOS-like activity in higher plants is characterized to have similar requirements (L-Arg, NADPH, FMN, FAD, calmodulin, and Ca^{2+}) to that of the mammalian NOSs (Barroso et al. 1999; Corpas et al. 2004). Nevertheless, in higher plants, no

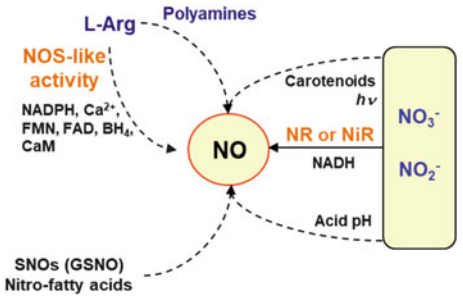

Fig. 2 Potential enzymatic and nonenzymatic sources of NO in higher plant cells. BH_4, tetrabiopterin; CaM, calmodulin; GSNO, *S*-nitrosoglutathione; L-Arg, arginine; NOS, nitric oxide synthase; NR, nitrate reductase; NiR, nitrite reductase; SNOs, nitrosothiols

ortholog genes have been found of any of the classic mammalian NO synthases (NOSs). In this sense, using also as a model the green alga *Ostreococcus tauri* it has been demonstrated the existence of an NOS-like protein (Foresi et al. 2010) whose occurrence has been extended to another 15 algal species (Jeandroz et al. 2016). On the other hand, there are evidences which correlated polyamines metabolism with NO generation (Tun et al. 2006; Wimalasekera et al. 2011; Agurla et al. 2018).

At subcellular level chloroplasts, mitochondria, and peroxisomes are the main organelles where NO generation has been mainly reported in higher plants. Although there is other potential place such as the apoplastic space, the available information is very limited (Stöhr and Ullrich 2002). The presence of NO into chloroplasts has been demonstrated by different experimental approaches such as electron spin resonance (ESR) and specific fluorescent probes (Jasid et al. 2006; Puntarulo et al. 2007; Galatro et al. 2013; Galatro and Puntarulo 2016), and the available data indicate that the main source of NO in this organelle is a NOS-like protein but not NR (Tewari et al. 2013; for more details see Chapter "Hydrogen Peroxide and Nitric Oxide Metabolism in Chloroplasts" of this book).

Plant mitochondria are the major producers of ATP via oxidative phosphorylation with the O_2 being the terminal electron acceptor of the mitochondrial electron transport chain (ETC). So far the mitochondrial NO generation is through the reduction of NO_2^- where the electron donors could be different depending of the mitochondrial oxygen tension, principally under hypoxia/anoxia conditions because the NO_2^- reduction to ammonium is inhibited and, consequently, NO_2^- is accumulated allowing the NO generation. In this situation the electron donors to generate NO is through the action of cytochrome *c* oxidase and other ETC components, such as complexes III and IV, by using NO_2^- (Wulff et al. 2009; Blokhina and Fagerstedt 2010; Igamberdiev et al. 2014; Gupta et al. 2018b). There are also some evidences indicating that plant mitochondria also generate NO even under normoxic conditions through alternative oxidase (AOX) (Alber et al. 2017; for more details see Chapter "Metabolism and Interplay of Reactive Oxygen and Nitrogen Species in Plant Mitochondria" of this book).

Plant peroxisomes are organelles where the presence of NO generation has been also shown by different technical approaches including spin-trapping electron paramagnetic resonance (EPR) spectroscopy and fluorescence-specific probes (Corpas et al. 2004, 2009). In this case, the experimental biochemical data support that the NO is generated by an L-arginine-dependent NOS-like activity that requires the same cofactors of animal NOSs including NADPH, FAD, FMN, calmodulin, and calcium (Barroso et al. 1999; Corpas et al. 2004). Additionally, different reports have also provided evidences that plant peroxisomes contain other NO-derived molecules including *S*-nitrosoglutathione (GSNO) and peroxynitrite ($ONOO^-$) (Barroso et al. 2013; Corpas and Barroso 2014a). Furthermore, additional analyses demonstrate that plant peroxisomes have the capacity to generate NADPH (for a review see Corpas and Barroso 2018b) and contain both calmodulin (Chigri et al. 2012) and Ca^{2+} (Costa et al. 2010; Corpas and Barroso 2018a). All these

experimental data are in good agreement with the presence of a NOS protein in animal peroxisomes (Stolz et al. 2002; Loughran et al. 2005).

4 Interplay Among Cell Organelles by NO and H_2O_2 Signaling: Overview and Queries

Both H_2O_2 and NO are able to exert their respective roles both as signal molecules but also as damaging species by themselves, as already probed and thoroughly reported (see diverse chapters in this book). But they can also react between them, thus generating a more powerful ROS, hydroxyl radicals ($^{\cdot}OH$) according to the following reaction (Gray et al. 1972; Nappi and Vass 1998):

$$H_2O_2 + NO \rightarrow {}^{\cdot}OH + HNO_2$$

This could be a feasible mechanism to generate hydroxyl radicals in biological systems in the absence of transition metals as it occurs in the superoxide-mediated Fenton reaction, thus providing a new focus to address tissue-specific damage caused by this ROS (Nappi and Vass 1998). But also, the way on how many moles of each species is consumed by this reaction opens novel concerns on the ways to conceive signaling processes leaded by either H_2O_2 or NO. Thus, in Fig. 3, as an exemplifying model, the specific interaction of these species generated in plant peroxisomes with other cell compartments is depicted. Under normal physiological conditions, catalase (CAT) and ascorbate peroxidase (APX) control the level of internally generated H_2O_2 by either a battery of oxidases, superoxide dismutases, or spontaneous dismutation from superoxide radicals, as well as the H_2O_2 imported from other cell loci (Corpas et al. 2015). Due to the presence of peroxisomal NOS-like activity, the formation of NO usually takes place within the organelle. Under those circumstances the small H_2O_2 amount which might escape from the action of the tandem CAT/APX could react with NO, thus giving rise to certain levels of $^{\cdot}OH$, and this radical may exert its damaging effect not only in the own peroxisome but also in neighboring organelles/loci (Fig. 3a) (Corpas et al. 2015). However, this potential mechanism seems to be finely regulated since no episodes promoted through these events have been neither observed nor reported under normal physiological conditions. This appears to mirror what hypothetically could take place in peroxisomes (and other organelles) where the simultaneous presence of diverse ROS and RNS could lead to the formation of singlet oxygen ($^1O_2$), hydroxyl radicals ($^{\cdot}OH$), peroxynitrite ($ONOO^-$), and other highly reacting molecules if their formation were not tuned with precision (Corpas et al. 2017).

However, this tight equilibrium could be disturbed under certain conditions such as those promoted by any kind of stress (biotic and abiotic) as well as by metabolic changes triggered by shifts in the developmental stages (seed germination, fruit ripening, etc.) (Corpas and Barroso 2014a; Corpas et al. 2017). Under those

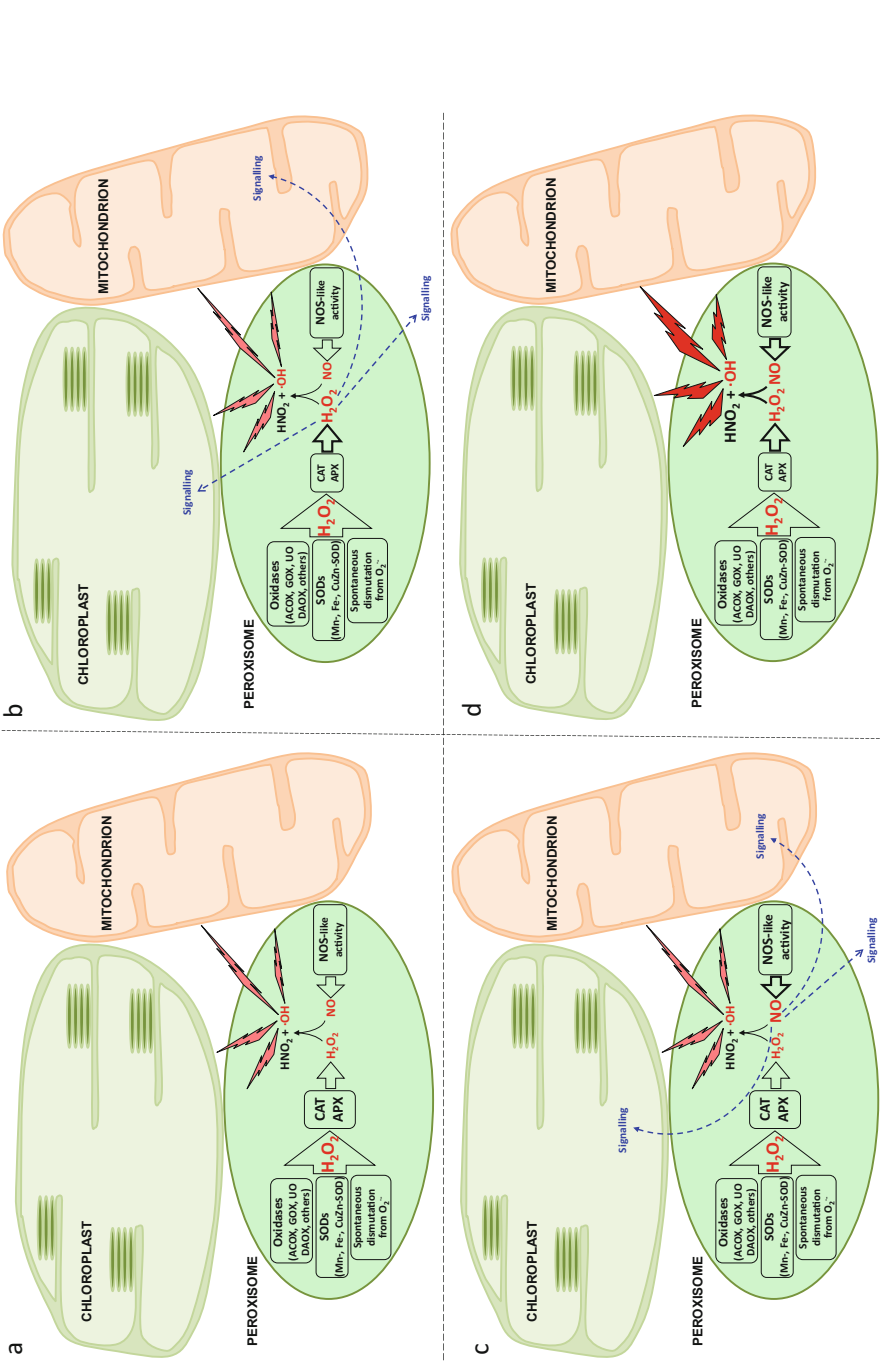

Fig. 3 Interaction of H_2O_2 and NO in peroxisomes from plant cells and their repercussions in the cell physiology. H_2O_2 can be generated by spontaneous dismutation and through the action of diverse enzymes. This species is basically removed by catalase and ascorbate peroxidase. NO is produced in the organelle

Fig. 3 (continued) by a NOS-like activity, and its reaction with H_2O_2 can give rise to hydroxyl radicals (˙OH) which is one of the most powerful damaging reactive oxygen species. H_2O_2 and NO can not only act as signaling molecules at the peroxisomal level but also interact with other cell organelles. (**a**) Normal physiological conditions in which H_2O_2 an NO reacts in a controlled form. (**b**) Situations where H_2O_2 is overproduced. In such cases this species may also act as signal molecule. (**c**) NO synthesis exceeds the H_2O_2 concentration with imbalanced stoichiometry being used for signaling purposes. (**d**) The overproduction of both, H_2O_2 and NO, leads to the generation of high ˙OH levels with strong damaging effects. ACOX, acyl-CoA oxidase; GOX, glycolate oxidase; UO, urate oxidase; DAOX, diamine oxidase; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; NOS, nitric oxide synthase

circumstances, the ability of CAT and APX to scavenge all the generated H_2O_2 might be limited by the inhibition and/or repression of both enzymes, and the concentration of H_2O_2 could overtake that of the NO formation. The generation of $^{\cdot}OH$ could be maintained, but the H_2O_2 excess could be driven to diverse signaling processes which involve cell organelles such as chloroplasts, mitochondria, and others (Fig. 3b). It may also happen that the NO synthesis within peroxisomes overcomes the H_2O_2 levels, so, besides the formation of $^{\cdot}OH$ and all its negative effects, NO could also participate in signaling events (Fig. 3c) through posttranslational modifications facilitated by *S*-nitrosation and nitration events (Corpas and Barroso 2014b; Corpas et al. 2017). Finally, the levels of both species could be considerably enhanced due to activation of the NOS-like activity and lowered CAT and APX activities, thus rendering an environment where the formation of $^{\cdot}OH$ is potentiated. In those conditions, the damaging processes would prevail leading to degradation and disorganization of cell components (Fig. 3d).

It should be kept in mind that all these events may also be triggered by the interactions between the H_2O_2 and NO generated in other organelles (chloroplasts, mitochondria, cytosol, etc.), thus building a complex ROS/RNS network where a considerable number of actors participate and that can be altered by many factors and situations. In the following chapters of this book, we will learn more precisely on how all this metabolic labyrinth is depicted according to the latest contributing knowledge in this field.

5 Conclusions

Hydrogen peroxide and NO are common metabolites in the cell which are generated in most organelles. Due to their relative moderate life span and diffusion rates, they are good candidates to exert independently a role in the signaling network either directly or indirectly. Nevertheless, both molecules can react in the cell loci where they are produced (chloroplasts, mitochondria, peroxisomes, and others), thus generating the more powerful reactive species hydroxyl radical ($^{\cdot}OH$), which can trigger deleterious effects for life. Under normal physiological stages, this condition is somehow balanced by the own cell metabolism, and the action of $^{\cdot}OH$ is controlled. Under pathological situations and/or unfavorable conditions, this balance could be broken down displacing the molecular stoichiometry of these species to favor non-coordinated signaling processes or, even worse, to enhance the production of $^{\cdot}OH$ promoting a cell-wide damage. The ROS/RNS homeostasis within each cell organelle would be fundamental to avoid the expansive wave of this eventuality inside of the cell. Understanding the intimate regulation of the interaction between H_2O_2 and NO will be useful to know how important physiological processes such as fruit ripening, which is regulated by NO (Corpas et al. 2018), occur.

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