Integrative role of plant mitochondria facing oxidative stress: The case of ozone

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This paper is published in memory of Dr Claude Lance, former professor at the University Pierre et Marie Curie Paris 6

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Abstract

Ozone is a secondary air pollutant, which causes oxidative stress in plants by producing reactive oxygen species (ROS) starting by an external attack of leaf apoplast. ROS have a dual role, acting as signaling molecules, regulating different physiological processes and response to stress, but also inducing oxidative damage. The production of ROS in plant cells is compartmented and regulated by scavengers and specific enzyme pathways. Chronic doses of ozone are known to trigger an important increase of the respiratory process while decreasing photosynthesis. Mitochondria, which normally operate with usual levels of intracellular ROS, would have to play a prominent role to cope with an enhanced ozone-derived ROS production. It is thus needed to compile the available literature on the effects of ozone on mitochondria to precise their strategy facing oxidative stress. An overview of the mitochondrial fate in three steps is proposed, i) starting with the initial responses of the mitochondria for alleviating the overproduction of ROS by the enhancement of existing antioxidant metabolism and adjustments of the electron transport chain, ii) followed by the setting up of detoxifying processes through exchanges between mitochondria and the cell, and iii) ending by an accelerated senescence initiated by mitochondrial membrane permeability and leading to programmed cell death.

Keywords: Ozone, respiration, mitochondria, oxidative stress, ROS, time-course response steps

Abbreviations – AOX: alternative oxidase, APX: ascorbate peroxidase, ASA: ascorbate, DHA: dehydroascorbate, DHAR: dehydroascorbate reductase, GABA: γ-Aminobutyric acid, GR: glutathione reductase, GSH: glutathione, HAF: Halliwell-Asada-Foyer cycle, MDHAR: monodehydroascorbate reductase, mETC: mitochondrial electron transport chain, O_3: ozone, OAA: oxaloacetate, OG: 2-oxoglutarate, OPPP: oxidative pentose phosphate pathway, PCD: programmed cell death, PEP: phosphoenolpyruvate, PEPcase: phosphoenolpyruvate carboxylase, TCA: tricarboxylic acid, ROS: reactive oxygen species, SA: salicylic acid, SDH: succinate dehydrogenase, UCP: uncoupling protein.

1. Introduction

In photosynthetically active cells, both photosynthesis and respiration produce energy in the form of ATP via photophosphorylation and oxidative phosphorylation, respectively in chloroplasts and mitochondria. Both organelles share the provision of energy whose the balance is tightly regulated under stress conditions especially (Gardestrom and Igamberdiev, 2016). Tropospheric ozone (O_3) is a secondary air pollutant, mainly produced by the photochemical dissociation of nitrogen oxides in the presence of organic compounds issued from car exhaust and industrial fuel combustion. O_3 concentrations increased fourfold during the last century reaching yearly average values of around 50 to 60 ppb but with peak values commonly observed between 100 and 250 ppb (Renaut et al., 2009). These concentrations have harmful impact on crops and forests (Wittig et al., 2009; Ainsworth et al., 2012; Matyssek et al., 2012; Jolivet et al., 2016; Cailleret et al., 2018; Emberson et al., 2018; Franz et al., 2018; Grulke and Heath, 2019). Plants exposed to moderate O_3 concentrations during prolonged time (chronic O_3 exposure) commonly exhibit a reduction in photosynthesis and photorespiration and an increase in respiration (Reich, 1983; Dizengremel, 2001; Bohler et al., 2007; Jolivet et al., 2016). The cellular changes induced by chronic O_3 injury were associated to an accelerated senescence in cell leaves (Pell et al., 1997; Pleijel et al., 1997; Miller et al., 1999; Gielen et al., 2007; Heath, 2008; Yendrek et al., 2017; Emberson et al., 2018; Vollenweider et al., 2019), the chloroplasts being early degraded while mitochondria remain intact until rather late (Sutinen et al., 1990; Holopainen et al., 1996). This delayed response of mitochondria thus confers a prominent role to this organelle in the mechanism of cell response to O_3 injury.

Although occurring in normal conditions, the generation of reactive oxygen species (ROS) at the level of the mitochondrial electron transport chain (mETC) is strongly increased under chronic O_3 which induces oxidative stress (Baier et al., 2005). However, the increase in the respiratory process raises many questions even though it is usually associated to repair, detoxification and synthesis of defense compounds (Dizengremel, 2001; Ainsworth et al. 2012). Respiration not only provides energy to plant cells in the form of ATP and NAD(P)H but also contributes to the production of carbon skeletons and the maintenance of redox balancing. These three functions co-exist, could overlap and thus need to be coordinated to regulate plant respiration (O'Leary et al., 2019). A range of papers describe different aspects of the implication of the mitochondria in the O_3-induced increase of respiration, but a coordinated synthesis of the results is lacking. This review deepens the role of mitochondria in the response to O_3, highlighting its ability to catabolize carbon intermediates, balance reducing power and induce antioxidant systems. The time course of mitochondrial events under O_3 exposure, from a successful scavenging of ROS to the initiation of senescence, will be proposed. The integration of the mitochondrial behavior in a coordinated cellular response to O_3 will be discussed.

2. Ozone-increased carbohydrate breakdown in the cytosol to feed mitochondria

The increase in respiration observed in plants submitted to chronic O_3 exposure is associated to an increased activity of a set of enzymes belonging to the glycolytic pathway and the oxidative pentose phosphate pathway (OPPP)(Dizengremel et al., 1994; Sehmer et al. 1998; Dizengremel et al., 2009; Dghim et al., 2012). This increase provides additional reducing power in the form of NADH and/or NADPH, which are useful for detoxification mechanisms (Fig 1.) (Dizengremel, 2001; Dizengremel et al., 2008). It must also be emphasized that several metabolites branch off from glycolysis or/and OPPP (Plaxton and Podesta, 2006) to initiate biosynthetic pathways (Fig 1.). Thus, phosphoenolpyruvate (PEP), end-product of the glycolysis, has a central role under O_3 stress (Dizengremel et al., 2012), and can bind to erythrose-4-phosphate from OPPP, allowing an increased functioning of the phenylpropanoid pathway essential to the production of defense compounds. Furthermore, the carboxylation of PEP into oxaloacetate (OAA) via phosphoenolpyruvate carboxylase (PEPcase) could in part mitigate the loss of CO_2 fixation by Rubisco under O_3 (Fontaine et al., 1999; Renaut et al., 2009). Furthermore, an increase of pyruvate kinase activity, providing more pyruvate to the tricarboxylic acid (TCA) cycle, was also observed under O_3 exposure (Dizengremel et al., 1994, 2009). Another role of PEP will be however emphasized here in the context of an increased mitochondrial respiration under O_3 stress. OAA produced from PEPcase can be transported into the mitochondrion or produce malate in the cytosol (Fig 1.). Malate can thus either produce pyruvate through the O_3-increased activity of the cytosolic NADP-malic enzyme (Dghim et al., 2012; Chen et al., 2019), providing additional NADPH for detoxification purposes, or be transported into the mitochondrion. It cannot be excluded that malate could thus be decarboxylated to pyruvate within the mitochondrion via NAD(P)-malic enzyme (Møller and Rasmusson, 1998; Dizengremel et al., 2012). An increasing offer in OAA, malate and/or pyruvate, would then replenish the TCA cycle (Dizengremel et al., 2012).

3. Deciphering the role of mitochondria under ozone stress

During the first thirty years following the observation of damaging effects of O_3 on plants (Haagen-Smit et al., 1952), few studies were devoted to the effect of O_3 specifically on plant mitochondria (Dizengremel and Citerne, 1988). The concentration of O_3 used was often acute (1 ppm delivered for several hours) and the resulting effect was mainly an inhibition of the oxidative phosphorylation (Lee, 1967). More recent works using chronic O_3 exposure explored the responses of the different mitochondrial pathways contributing to the oxidative degradation of the organic acids.

3.1. A noncyclic anabolic function of TCA to enhance antioxidant defense

The TCA cycle operates in the matrix of mitochondria by fully decarboxylating the organic acids; however, a noncyclic functioning of TCA can also occur (Tcherkez et al., 2009; Sweetlove et al., 2010) allowing the leak of some organic acids (citrate, isocitrate, oxoglutarate) towards amino acid

synthesis (Fig. 1A, O'Leary et al., 2011). This anaplerotic pathway would be solicited under chronic O_3 exposure (Dizengremel et al., 2012). Citrate is exported from the mitochondrion to the cytosol to produce 2-oxoglutarate (OG) via cytosolic isocitrate dehydrogenase (IDH), a reaction that provides NADPH and is increased under O_3 stress (Dghim et al., 2012). Mitochondrial OG can also be directly exported after being produced from isocitrate by the mitochondrial NAD(P)-IDH. OG may thus be integrated into the glutamine synthetase 2 / glutamate oxoglutarate aminotransferase or into the glutamine synthetase 1 (GS1) / aspartate aminotransferase cycles to give glutamate in the chloroplast or cytosol, respectively (Fig 1A). The activity of GS1 was shown to increase in plants fumigated with O_3 (Galant et al., 2012). This could allow to cope with a diminished NH_4^+ remobilisation due to O_3-decreased photorespiratory activity (Bagard et al., 2008). Interestingly, a higher supply of cytosolic glutamate could also support *de novo* glutathione (GSH) synthesis by γ-glutamylcysteine synthetase and glutathione synthetase and therefore contributes to cell ROS detoxification. In animal cells, a specific OG transporter has been described in the mitochondrial inner membrane (Lash, 2006). This transporter carries out OG to the cytosol in exchange for cytosolic GSH (Fig. 1A). The presence of such a transporter still needs to be investigated in plants in which GSH biosynthesis only occurs in cytosol and chloroplast. Such transporters would therefore insure mitochondrial import of cytosolic GSH during stress. The enhancement of noncyclic TCA and anaplerotic pathway under O_3 would thus feed cytosolic antioxidant synthesis in carbon skeletons and simultaneously import antioxidants in mitochondria.

O_3 enters the leaf through stomata and rapidly generate various ROS (Kangasjarvi et al., 1995) that cause the peroxidation of membrane lipids (Ranieri et al., 1996; Loreto and Velikova, 2001). In this respect, the leak of compounds out of TCA cycle may result from inactivation of some downstream enzymes of the cycle by the lipid peroxidation products, as 4-hydroxy-2-nonenal, which has been shown to specifically inhibit oxoglutarate dehydrogenase and pyruvate dehydrogenase complexes by about 80% (Millar and Leaver, 2000). To maintain the rate of respiration, part of the TCA cycle can be by-passed, as do the γ-aminobutyrate shunt (GABA shunt) for succinate synthesis in bypassing oxoglutarate dehydrogenase and succinyl-coA-synthetase (Fig. 1B, Rhoads et al., 2006; Dizengremel et al., 2012). GABA synthesis is catalyzed by glutamate dehydrogenase and glutamic acid decarboxylase, which both increased in O_3-treated rice leaves (Cho et al., 2008). Increased concentrations of GABA were generally observed in response to stress, conferring on the GABA shunt an adaptive mechanism (Michaeli and Fromm, 2015). Recently, Che-Othman et al. (2019) concluded that increase in GABA shunt activity in wheat leaves overcome the inhibition of the TCA cycle key enzymes, providing an alternative carbon source to support the stress-dependent increase in mitochondrial respiration. In response to O_3, GABA could also act as a transmembrane signal

indicating changes in TCA cycle activity from cell to cell (Ramesh et al., 2015; Gilliham and Tyerman, 2016).

The exchange of metabolites between chloroplast and mitochondria allows balancing the cellular redox within plant cell (Hoefnagel et al., 1998; Gardestrom and Igamberdiev, 2016; O'Leary et al., 2019). This is mediated via a system, often called malate valve, which couples a malate/OAA redox shuttle based on mitochondrial NAD-malate dehydrogenase to a chloroplastic NADP-malate dehydrogenase (Hoefnagel et al., 1998; Scheibe, 2004). When the NADPH/NADP ratio is high, malate is transported from the chloroplast into the mitochondria where it is transformed in OAA, resulting in the transfer of chloroplast NADPH in excess to mitochondrial NADH (Fig 1.)(Scheibe, 2004, Yoshida et al., 2007). This system allows to minimize chloroplast endogenous ROS production due to an overly reduced photosynthetic reaction system and a lack of NADP (Finnegan et al., 2004). This could happen under O_3 stress when a transient stromal over-reduction due to damaged Calvin cycle occurs before any impairment of the light reactions (Dizengremel, 2001; Dizengremel et al., 2009). In addition, O_3 is well known to induce stomatal closure (Wittig et al., 2007; Hoshika et al., 2015) that would also drive a NADPH accumulation in chloroplast by reducing CO_2 levels. Malate valve contributes to import reducing power in mitochondria and therefore to detoxification capacity in supporting mitochondrial antioxidant regeneration and to minimize chloroplastic ETC imbalance resulting in ROS generation.

3.2. Role of the mitochondrial electron transport chain

The mETC is a major site of ATP synthesis, but is also a source of ROS (Fig. 1C) through complex I (NADH: ubiquinone oxidoreductase) and complex III (UQH_2: cytochrome c oxidoreductase; cytochrome bc1 complex) (Murphy, 2009). More recently, it was shown that complex II (SDH) can also produce O_2^- at the FAD-binding site through the monovalent electron reduction of O_2 (Jardim-Messeder et al., 2015; Huang et al, 2019). Endogenous ROS production by mETC can however be mitigated by the use of alternative pathways. The plant mETC possesses an alternative non-proton pumping pathway, by-passing complexes III and IV, and mediated by alternative oxidase (AOX) (Fig 1C)(Lance et al., 1985; Vanlerberghe and McIntosh, 1997; Millar et al., 2011). AOX might act to maintain a basal ubiquinone pool reduction state, preventing over-reduction of upstream electron-transport components and lowering ROS production (Purvis and Shewfelt, 1993; Rhoads et al., 2006 ; Mittler, 2002; Umbach et al., 2005; Saha et al., 2016). It has been stated that the participation of the alternative oxidase is maintained very low under standard conditions by repressing its gene expression, potentially because of its wasteful nature (Selinski et al. 2018). However, the activity of AOX can be enhanced by several parameters acting at different levels (transcriptional, translational, post-translational) in response to a large range of stressors. The AOX gene expression was shown to be increased by H_2O_2 itself (Vanlerberghe & McIntosh, 1997) or through specific transcription factors

(e.g. NAC17; Ng et al., 2013). Furthermore, differential expression of AOX isogenes can lead to the production of heterodimers having varying catalytic properties which potentially offer a fine-tune mechanism of AOX activity in response to stress. Under O_3 exposure, an activation of AOX at both transcriptional and translational levels was observed in poplar, tobacco and *Arabidopsis* (Jolivet et al., 1997; Dizengremel, 2001, Ederli et al., 2006; Tosti et al., 2006). In tobacco, the O_3 induced activation of gene expression of AOX1a was suggested to be NO-dependent, in cooperation with ethylene signaling, and correlated to an inhibition of cytochrome oxidase by NO (Ederli et al., 2006, Kumari et al., 2019). This is a good example of a disfunctioning of mETC triggering the expression of a nuclear gene encoding a mitochondrial protein (retrograde signaling system, Ng et al., 2014).

AOX activity can also be regulated by redox state. The reduced form of the AOX dimer is more active than the oxidized form, suggesting that AOX activity can be modulated depending on reducing power availability, but may be affected by excessive accumulation of ROS. Thioredoxins belonging to Trxo and Trxh families are able to render AOX operational by reducing disulfide bonds (Trxo family: Marti et al., 2009; Umekawa and Ito, 2019; Trxh family: Gelhaye et al., 2004). The redox state activation of AOX protein is also coupled to ketocarboxylic acids binding that enhance its activity: pyruvate and OG are very effective activators in lowering Km for UQ substrate (Umbach et al., 2002; Selinski et al., 2018). These two systems of AOX activation, redox state and metabolite binding, interact with each other to complete AOX activity regulation. The increased availability of pyruvate under O_3 (Dizengremel et al., 1994, 2009) could explain the enhancing participation of AOX to the mitochondrial electron flow; however this activation would require sufficient reducing power availability to maintain AOX in its reduced form and minimize endogenous ROS production by mETC. In addition, AOX, that has a lower O_2 affinity than cytochrome oxidase (Km of 1-2.5µm vs. 0.1-0.2µm), allows to maintain a low level of O_2 in mitochondria that therefore contributes to minimize ROS production (Gupta et al. 2015).

Alternative pathways of mETC also include the shunt of complexes I and II, implying one of the four alternative non-proton pumping NAD(P)H dehydrogenases located either on the outer (NDex) or inner (NDin) surface of the inner mitochondrial membrane (Fig. 1C). The NDin might largely participate for the reoxidation of matrix NADH under conditions where its concentration is increased (Møller, 2001), which could be the case under O_3 stress as mentioned above. Matrix NAD(P)H can be produced via the NAD(P)-IDH and NAD-malic enzyme or imported through the malate/OAA shuttle. The activity of the NAD(P)-malic enzyme is increased under O_3 (Dizengremel et al., 2009; Dghim et al., 2012. NDin is thus probably competing with NAD(P)H-dependent ROS-detoxifying enzymes for matrix NAD(P)H (Møller, 2001). Although rather inactive in normal conditions, the Ca^{2+}-dependent NDex linked to the outer face of the inner mitochondrial membrane could be activated under stress by an increase in Ca^{2+} cytosolic concentration, as it has been previously shown under O_3 (Heath and Castillo,

1988; Clayton et al., 1999). In addition, an increased expression of the NDex was demonstrated to accompany increased AOX expression under several stress treatments (Rhoads et al., 2006). Under oxidative stress, alternative dehydrogenases could help decrease ROS production linked to a high reduction level of mETC, by keeping the cytosolic NAD(P) pool relatively oxidized (Rhoads et al., 2006). In addition, uncoupling proteins (UCP) cross the inner mitochondrial membrane (Vercesi et al., 2006) and mildly participate to the modulation of the transmembrane potential (Fig 1C)(Rhoads et al., 2006). UCP is also able to decrease ROS formation in reintegrating proton and thus bypassing respiratory control by ATP synthase (Rhoads et al., 2006). While AOX can be inactivated by a strong oxidative stress (Winger et al., 2005), UCP is able to keep operating when ROS level is much higher (Considine et al., 2003; Rhoads et al., 2006). The role of the NADH dehydrogenases and UCP under O_3-driven oxidative stress must however be confirmed, especially in regard to the role of AOX.

Beside electron transport, mETC also hosts the final step of ascorbate (ASA) biosynthesis that converts L-galactono-1,4-lactone to ASA and is catalysed by L-galactono-1,4-lactone dehydrogenase (Fig 1C)(Ôba et al., 1995; Bartoli et al., 2000). This enzyme is located on the inner mitochondrial membrane where it is physically associated to complex I and regulated by electron transport. Furthermore, oxidized cytochrome c is essential to the functioning of L-GLDH in acting as a specific electron acceptor (Fig 1C)(Bartoli et al., 2000). As an example, L-GLDH activity was decreased by 40% in Arabidopsis knockdown mutants under-expressing cytochrome c (Welchen et al. 2016). The question remains as the relative contribution of ASA synthesis to respiratory electron flux compared to that driven by TCA cycle activity and whether this contribution is modulated in O_3 stress conditions. In any case, produced ASA can thereafter be used by cytosolic and mitochondrial antioxidant systems to minimize ROS accumulation. Therefore, the coupling of L-GLDH and mETC activities via cytochrome c could contribute to couple respiration and antioxidant defense to cope with oxidative stress.

4. Antioxidant systems present in mitochondria and known effects of ozone

Several antioxidant mechanisms, mainly present in the soluble parts of the mitochondria (i.e. intermembrane space and matrix), can contribute to mitigate the increased ROS production during O_3 exposure. A matrix-localized Mn-superoxide dismutase was reported in literature, coping with endogenous O_2^- production by mETC, and involved in the protection against ROS produced by O_3 attack (Fig 1C)(Alscher et al., 2002). To manage superoxide dismutase products, the occurrence of a Halliwell-Asada-Foyer cycle (HAF, Fig. 1D) has been reported in cytosol, chloroplasts, peroxisomes as well as in mitochondria (Jiménez et al., 1997; Noctor and Foyer, 1998; Foyer and Noctor, 2011) even though the presence of dehydroascorbate reductase (DHAR) in mitochondria remains questionable (Møller, 2001; Noctor et al., 2018). Several ascorbate peroxidases (APX) have been identified in plant mitochondria, one intermembrane APX bound to the inner mitochondrial membrane

(De Leonardis et al., 2000) and two others located in the matrix, either soluble or bound to the inner membrane (Mittova et al., 2004). As mentioned above, ASA is delivered to the mitochondrial intermembrane space where it can be oxidized by the intermembrane APX. Dehydroascorbate (DHA) can then be transported into the matrix via a GLUT-like transporter of the glucose transporter family (Chew et al., 2003; Szarka et al., 2013). In addition, if the lack of DHAR in the matrix were confirmed, DHA will have to be reduced in ASA by the glutaredoxin or directly by GSH (Fig 1D)(Ehrhart and Zeevalk, 2003). Recent work also demonstrated the presence of mitochondrial ascorbate transporter-MAT, a member of the SLC25 family, to directly transport ASA through the mitochondrial inner membrane (Scalera et al., 2018).

The synthesis of GSH only occurs in cytosol and chloroplast (Rausch et al., 2007); however GSH presence has been reported in mitochondria suggesting a crossing of the inner mitochondrial membrane via a still unknown transporter in plants (Fig. 1A; Chen and Lash, 1998; Maughan and Foyer, 2006). The presence of gluthatione reductase (GR) was confirmed in mitochondria to allow GSH regeneration where its activity is NADPH dependent (Fig. 1D, Edwards et al., 1990; Jimenez et al., 1997). Under O_3, the synthesis of GSH is increased likely to maintain glutathione redox state (Dumont et al., 2014) and compensates ROS scavenging by glutathione. Similarly, the activities of APX, monodehydroascorbate reductase (MDHAR) and GR have been shown to increase under O_3 (Dusart et al., 2019; Gandin et al., 2019).

As previously mentioned, thioredoxins have been reported in plant mitochondria (Sweetlove and Foyer, 2004; Navrot et al., 2007; Vieira Dos Santos and Rey, 2008). Also, a type II peroxiredoxin (PrxII F), has been identified in plant mitochondria (Rhoads et al., 2006; Finkemeier et., 2005), where it could reduce mitochondrial H_2O_2 by using thioredoxin or GSH (Fig 1D). The latter are then regenerated by thioredoxin reductase or GR (Sweetlove et al., 2004; Rhoads et al., 2006). The type II peroxiredoxin/thioredoxin system could also reduce lipid peroxides (Rouhier et al., 2004) and could therefore contribute to maintain mitochondria membrane integrity under stress. A mitochondrial protein disulfide isomerase functioning together with thioredoxin and NADPH could also reduce disulfides resulting from protein oxidation (Sweetlove et al., 2002; Rhoads et al., 2006). In addition to the above-mentioned effect of thioredoxin on AOX, the association of all these processes would lower the content of ROS in the matrix in O_3 exposed plants.

5. Suggested steps of the response of mitochondria to ozone: from resistance to cell death

O_3 being a powerful oxidant, a plausible scenario of the successive mechanisms coping with the progressively rising intensity of the O_3-related stress is proposed below (Table 1). The sequential presentation is probably an idealized view of more intricate relationships between the responses to O_3. In addition, ROS, responsible for oxidative damages caused by O_3, are also known to play an

important role in triggering signaling pathways. Thus, there is a permanent fine-tuning of ROS accumulation to balance beneficial signaling and phytotoxic effect. The steps described below lie on results obtained from a series of O_3 experiments and, in a minor way, are complement in integrating probable events derived from general oxidative stress.

5.1. Mobilisation of pre-existing mechanisms to cope with ROS

The first impact of O_3 on photosynthesis, and especially on the biochemical components, leads to excessive NADPH accumulation within the chloroplast. A series of metabolic pathways, generally associated to carbohydrate breakdown as glycolysis and OPP, are also enhanced and result in even more NAD(P)H produced (Fig1, Table 1). This additional reducing power allows the existing detoxification processes to function at optimal level. An increase in APX activity has been described under O_3 and shown to be supported by enhanced ASA regeneration by NADH-dependent MDHAR (Gandin et al., 2019). In addition, the cytosolic carboxylating enzyme, PEPcase, is requested, either to cope with the decreased CO_2 fixation by Rubisco and/or to contribute to the anaplerotic pathway (Dizengremel et al., 2012). Several intermediates of catabolism are therefore favoured, either by pyruvate kinase (e.g. pyruvate) or/and by PEPcase (e.g. OAA and malate), and have impact on mitochondria functioning (Dghim et al., 2012). These organic acids will be transported into the mitochondria to feed TCA cycle and finally provide NAD(P)H to the mETC. Additional reducing power is also provided by the transfer of redox equivalents (e.g. through malate/OAA shuttle) either from chloroplasts, in which the redox pressure is transiently high, or from cytosol. However, increasing feeding of mitochondria with NAD(P)H threaten to over-reduce mETC and induce endogenous ROS production.

To maintain the mETC in a sufficiently oxidized state, ATP synthesis and respiratory control could be balanced by the UCP while the electron flow may use the activated AOX and some of the four NAD(P)H dehydrogenases located on both sides of the inner mitochondrial membrane (Table 1). Three NAD(P)H-DH are activated by Ca^{2+} which concentration is known to be increased by O_3 and is involved in phytohormone cascade signals (Short et al,. 2012). An increase of the amount of salicylic acid (SA) was shown under O_3 fumigation (Koch et al., 2000). At low concentration, SA was shown to uncouple the mETC (Gleason et al., 2011) and to increase AOX expression (Rhoads and McIntosh, 1992), suggesting a potential role for SA (and may be ethylene) to the signaling process regulating mitochondrial electron flux (Rao et al., 2000; Dizengremel, 2001; Heath, 2008). NO production due to O_3 stress could also be an important regulator of electron partitioning between cytochrome and alternative pathways, in reducing and enhancing cytochrome oxidase and alternative oxidase activities, respectively (Ederli et al. 2006). Therefore, NO impact on mETC could initiate a bottom-up regulation starting with the decrease in mETC ATP yield that would require an increase in glycolysis and TCA

cycle activities to fulfill mitochondria ATP demands. Evidence may be seen in the stimulation by O_3 of a mitochondrial phosphate transporter located in the inner membrane, which catalyzes the influx of Pi, essential for the synthesis of ATP in the matrix (Kiiskinen et al., 1997).

5.2. Setting up of detoxifying processes

The enhanced activities of PEPcase as well as of glycolysis and OPP tend to show a plateau for higher doses of O_3, announcing a limit for the capacity of the metabolism to counteract the oxidative stress (Dizengremel et al., 1994; Fontaine et al., 1999; Renaut et al., 2009). With an extended O_3 exposure, the ROS production keeps increasing and more likely starts to accumulate in plant cells. ROS shortly oxidized cell components (e.g. protein, DNA…) among which membrane lipids that are a major target (Rebouças et al., 2017). Lipid peroxidation products are able to inhibit specific TCA cycle enzymes (Millar and Leaver, 2000) and disrupt its functioning (Table 1). As a consequence, TCA cycle would leak intermediates and export isocitrate or OG to the cytosol for amino acid biosynthesis (Dizengremel et al., 2012). This leak in carbon skeletons would also support cytosolic GSH *de novo* biosynthesis, as the enzymes for it have never been reported in mitochondria. GSH can then return to mitochondria through OG-GSH transporters as shown in animal cells (Lash 2006), yet to be described in plants. GSH can therefore support glutathione peroxidase and glutaredoxin activities as well as ASA regeneration through non-catalytic reduction or mediated by DHAR, therefore significantly contributing to antioxidant defense (Fig 1D, Table 1). With ROS accumulation, other antioxidant systems can intervene, as the peroxiredoxin/thioredoxin systems present in mitochondria that could reduce mitochondrial H_2O_2. In addition, it is likely that ASA biosynthesis is also enhanced under O_3 to support HAF functioning. The *de novo* synthesis of ASA however involves additional feeding of the mETC with electrons, increasing even more the reductant pressure on the chain and the risk for endogenous ROS production. In addition, ROS accumulation likely oxidized mETC components as AOX, which is inactive in oxidized form. This runaway effect may lead to increasing endogenous O_2^- production by complexes I, II and III and ROS over-accumulation in mitochondria.

Glutamate issued from OG export from mitochondria can also enter the GABA shunt that leads to the production of succinate in mitochondria, bypassing TCA cycle enzymes inactivated by oxidative stress (Fig 1B)(Table 1)(i.e. OG dehydrogenase and succinyl-CoA synthetase). GABA-shunt issued succinate can then be reoxidized by Complex II in mETC that still drives a strong electron flow from the complex I and NAD(P)H-DH. However, complex II can be inhibited at high concentrations of oxidative stress-induced phytohormones as SA (Gleason et al., 2011; Belt et al., 2017). This would lead to the accumulation of GABA shunt intermediates, as succinic semialdehyde that is toxic compound for plants.

5.3. Towards programmed cell death

When the O_3 uptake lasts and oxidative pressure keeps rising, the antioxidant defense is overwhelmed and ROS accumulates in excess. It is admitted that plant mitochondria could then play a role similar to that of animal mitochondria in the PCD process (Table 1)(Xie and Chen, 2000; Tiwari et al., 2002; Vianello et al., 2007). The decrease in ATP production and release of cytochrome c are the first signals of PCD. The depletion of ATP synthesis, above described as accompanying both UCP and AOX implication, can be accelerated by mitochondrial membrane disturbances associated to ROS. The peroxiredoxin/thioredoxin system can help by supplying reducing power to reductases acting on lipid hydroperoxides and disulfides resulting from protein oxidation, but it become insufficient. A collapse of mitochondrial transmembrane potential $\Delta\Psi m$ would ensue, triggering the opening of the mitochondrial permeability transition pore (Table 1)(Tiwari et al., 2002; Zandalinas and Mittler, 2018).

Cytochrome *c*, a mobile protein attached to the cytosolic face of the inner mitochondrial membrane, acts as an electron carrier between complexes III and IV and is implied in the last step of ASA synthesis. Cytochrome c is labile and can be easily detached from the inner mitochondrial membrane followed by its release into the cytosol through a permeabilization of the outer membrane due to oxidative stress (Table 1)(Dizengremel, 1983; Sun et al., 1999). A release of cytochrome c was observed upon O_3 exposure of tobacco plants (Pasqualini et al., 2003). The release of cytochrome c activates caspases-like proteases that are involved in the execution of the cell death program (Tiwari et al., 2002; Vacca et al., 2006; Welchen and Gonzalez, 2016). A Ca^{2+} overload, admitted to occur upon O_3 exposure, would trigger the entire PCD process (Xiong et al., 2006; Welchen and Gonzalez, 2016). The increased permeability of mitochondria to solutes leads to mitochondrial swelling (Vianello et al., 2007; Zancani et al., 2015) which was early observed in response to O_3 (Lee, 1967, 1968). This ends by overall mitochondria rupture and subsequent cell death.

6. Conclusions and perspectives

In the literature, the role of mitochondria facing O_3 stress is often mentioned as being important but the available information is disseminated. A gathering of these information and a clearer picture of the sequential events following an increasing oxidative pressure is proposed in this review article, based on results mostly obtained with O_3 itself and some others deduced from general oxidative stress (Table 1). As soon as O_3 enters the leaf, delivering ROS, the entire catabolic system is mobilized, partly to compensate the damages and also for providing redox compounds, organic acids and secondary metabolites. These changes allow to optimize detoxification activity and minimize ROS accumulation, in modulating TCA cycle activity and NAD(P)H production, balancing electron transport within mETC, and increasing antioxidant pool in the matrix. In the most severe cases, this wide

reorganization of mitochondria metabolism can be insufficient and mitochondria will be overwhelmed by oxidative pressure, leading its disruption through a PCD-like process.

Even though this scenario is plausible, the action of O_3 on some of the steps needs to be definitively confirmed. The important role of AOX (and potentially other alternative pathways) must be emphasized. AOX has been recently proposed as a functional marker for breeding stress tolerant plant varieties (Polidoros et al., 2009). On the other hand, an elaborate network of anterograde and retrograde signalling between the mitochondria and nucleus is at work in plant cells with redox status and ROS as important actors (Leister, 2005; Rhoads and Subbaiah, 2007; Ng et al, 2014; Farooq et al., 2019). Moreover, it was recently demonstrated that the Ca^{2+}-dependent changes in gene expression observed in response to O_3 differ markedly from Ca^{2+} signatures of other oxidative stress, such as H_2O_2 and cold (Short et al., 2012). A more deeply insight concerning the metabolic and signalling networks under O_3 stress thus needs to be studied. In this context, the ambiguous role of hormones raises questions, particularly for ethylene, which is largely implicated in leaf senescence as well as O_3 damage (Moeder et al., 2002; Nunn et al., 2005; Heath, 2008). Finally, the role of antioxidants to cope with O_3 stress is undisputable but very technically complex to assess relative to each intracellular pool. It is therefore essential to develop new methods to estimate the relative contribution of each pool and their potential exchanges within the cell.

Conflicts of interest

None declared

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Author Contribution

All authors contributed equally to the writing and editing of the manuscript.

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Table

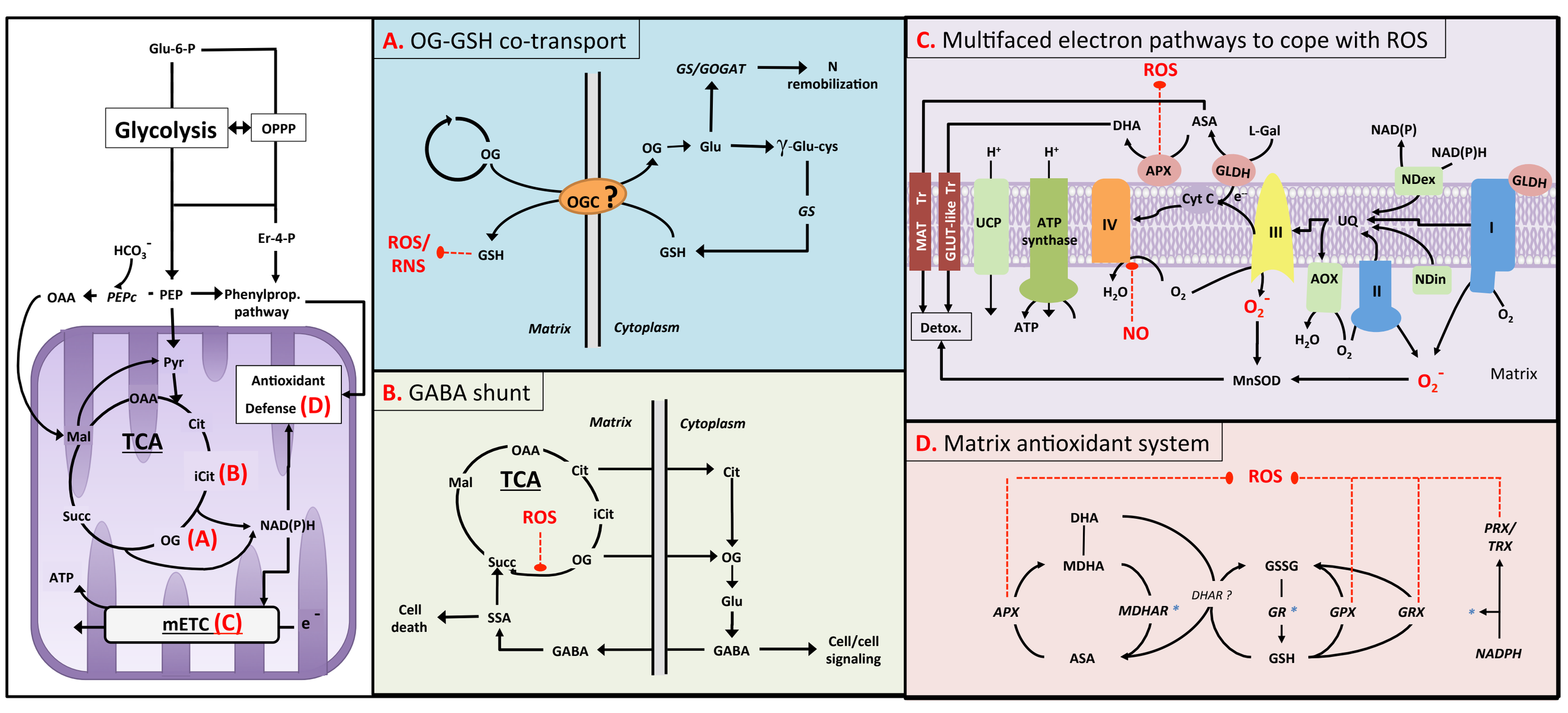
Table 1.

Proposed sequential response of plant mitochondria to O_3 stress (see the text and Fig 1. for a complete explanation); *in italics: events inferred from general oxidative stress.*

Successive steps	**Increasing Phytotoxic Ozone Dose**
Mobilisation of pre-existing mechanisms to cope with ROS	↓
Cytosol	
- Enhancement of the catabolic pathways and PEPcase	
- Supply of organic acids and redox power (NADPH)	
- Maintenance of a high detoxification	
- Increase in the phenylpropanoid metabolism	
Mitochondria	
- Increased supply of NAD(P)H to ETC	
- Adjustment of electron flow path in ETC by balancing between high substrate supply (organic acid and NAD(P)H) and ATP requirement	
• Increased activity of UCP and AOX to lower reduction pressure	
• Ca^{2+}-dependent activation of the NAD(P)H-ND_{ext} and ND_{int}	
• SA-mediated uncoupling of respiratory chain	
• Increased gene expression of AOX	
Setting up of detoxifying processes	↓
- TCA cycle inhibition by peroxidation products	
• Enhancement of anaplerotic pathway	
• Leak of organic acids to amino acids synthesis	
• Increase synthesis of glutathione and *import in the matrix*	
• Induction of GABA shunt	
- Conversion of superoxide by Mn-SOD	
- Induction of antioxidant defense	
• Increased *de novo* synthesis of ascorbate	
• Increased activity of HAF cycle and APX/GR gene expression	
• *Enhancement of peroxyredoxin/thioredoxins systems*	
- Inactivation of AOX	
- Complex II inhibition by high level of SA	
• *Accumulation of toxic intermediates of GABA shunt*	
Towards programmed cell death (PCD)	↓
- Increasing damage to fatty acids and proteins of mitochondrial membranes	
- ATP-synthesis decrease due to uncoupling of electron flow	
- Decrease in ascorbate synthesis	
- *Possible occurrence of Mitochondrial Permeability Transition*	
- Release of cytochrome c in the intermembrane space	
• *Activation of caspases-like proteases*	
- Disruption of the outer mitochondrial membrane	
- Swelling of mitochondria	
- Cell death	

Figure legend

Figure 1. Representative schema of metabolic network involved in plant mitochondria response to O_3-induced oxidative stress. Four main focuses of mitochondria response are presented to highlight (A) the oxoglutarate-glutathione co-transport between cytoplasm and matrix that allow to replenish matrix GSH pool jointly to the leak of OG out of mitochondria; (B) the GABA-shunt bypassing TCA cycle enzymes once they are inhibited by ROS and its potential role in cell signalling and/or death; (C) the multifaced electron pathway which is modulated to minimize endogenous ROS production and its close relationship to ascorbate synthesis and import; (D) the antioxidant system reported in mitochondria matrix to scavenge ROS. Dark lines and arrows represent mitochondria-related metabolic links. Red lines represent the connectivity between mitochondrial metabolism and ROS production or scavenging. ASA: ascorbate, APX: ascorbate peroxidase, AOX: alternative oxidase, Cit: citrate, Cyt C: cytochrome C, Er-4-P: erythrose-4-phosphate, GABA: gamma-aminobutyric acid, γ-Glu-cys: L-gamma-glutamylcysteine, GLDH: L-galactono-1,4-lactone dehydrogenase, Glu: glutamate, GLUT-like Tr: glucose transporter, Glu-6-P: glucose-6-phosphate, GS/GOGAT: glutamine synthetase/glutamate synthase, GPX: glutathione peroxidase, GR: glutathione reductase, GRX: glutathioredoxine GSH: reduced glutathione, GSSG: oxidized glutathione, iCit: isocitrate, Mal: malate, MAT Tr: mitochondrial ascorbic acid transporter, (M)DHA: (mono)dehydroascorbate, (M)DHAR: (mono)dehydroascorbate reductase, mETC: mitochondrial electron transport chain, MnSOD: Mn-dependent superoxide dismutase, NDin/ex: internal/external NAD(P)H dehydrogenases, OAA: oxaloacetate, OG: oxoglutarate, OPPP: oxidative pentose phosphate pathway, PEPc: phosphoenolpyruvate carboxylase, PRX/TRX: peroxiredoxin/thioredoxin, Pyr: pyruvate, ROS: reactive oxygen species, SSA: succinic semialdehyde, Succ: succinate, UCP: uncoupling protein


Glu-6-P
Glycolysis
OPPP
Er-4-P
HCO3-
OAA
PEPc
PEP
Phenylprop. pathway
Pyr
OAA
Cit
Mal
TCA
iCit (B)
Succ
OG (A)
NAD(P)H
Antioxidant Defense (D)
ATP
mETC (C)
e-
A. OG-GSH co-transport
GS/GOGAT
N remobilization
OG
OG
Glu
γ-Glu-cys
GS
OGC ?
ROS/ RNS
GSH
GSH
Matrix
Cytoplasm
B. GABA shunt
Matrix
Cytoplasm
OAA
Cit
Mal
TCA
Cit
iCit
ROS
Succ
OG
OG
Glu
Cell death
SSA
GABA
GABA
Cell/cell signaling
C. Multifaced electron pathways to cope with ROS
ROS
DHA
ASA
L-Gal
NAD(P)
NAD(P)H
APX
GLDH
NDex
GLDH
MAT Tr
GLUT-like Tr
UCP
H+
H+
ATP synthase
IV
Cyt C
e–
III
UQ
I
AOX
II
NDin
H2O
O2
O2-
H2O
O2
O2
Detox.
ATP
NO
MnSOD
O2-
Matrix
D. Matrix antioxidant system
ROS
DHA
MDHA
APX
MDHAR *
DHAR ?
GSSG
GR *
GPX
GRX
PRX/ TRX
*
ASA
GSH
NADPH