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### ► **To cite this version:**

Anthony Gandin, Pierre Dizengremel, Yves Jolivet. Integrative role of plant mitochondria facing oxidative stress: The case of ozone. *Plant Physiology and Biochemistry*, 2021, 159, pp.202 - 210. 10.1016/j.plaphy.2020.12.019 . hal-03493025

**HAL Id: hal-03493025**

**<https://hal.science/hal-03493025>**

Submitted on 2 Jan 2023

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1 **Integrative role of plant mitochondria facing oxidative stress: the case of**  
2 **ozone**

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

5 **Anthony Gandin<sup>a</sup>, Pierre Dizengremel<sup>a,\*</sup> and Yves Jolivet<sup>a</sup>**

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8 **Abstract**

9  
10 Ozone is a secondary air pollutant, which causes oxidative stress in plants by producing reactive  
11 oxygen species (ROS) starting by an external attack of leaf apoplast. ROS have a dual role, acting as  
12 signaling molecules, regulating different physiological processes and response to stress, but also  
13 inducing oxidative damage. The production of ROS in plant cells is compartmented and regulated by  
14 scavengers and specific enzyme pathways. Chronic doses of ozone are known to trigger an important  
15 increase of the respiratory process while decreasing photosynthesis. Mitochondria, which normally  
16 operate with usual levels of intracellular ROS, would have to play a prominent role to cope with an  
17 enhanced ozone-derived ROS production. It is thus needed to compile the available literature on the  
18 effects of ozone on mitochondria to precise their strategy facing oxidative stress. An overview of the  
19 mitochondrial fate in three steps is proposed, i) starting with the initial responses of the mitochondria  
20 for alleviating the overproduction of ROS by the enhancement of existing antioxidant metabolism  
21 and adjustments of the electron transport chain, ii) followed by the setting up of detoxifying  
22 processes through exchanges between mitochondria and the cell, and iii) ending by an accelerated  
23 senescence initiated by mitochondrial membrane permeability and leading to programmed cell death.

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

26 *Abbreviations* – AOX: alternative oxidase, APX: ascorbate peroxidase, ASA: ascorbate, DHA:  
27 dehydroascorbate, DHAR: dehydroascorbate reductase, GABA:  $\gamma$ -Aminobutyric acid, GR: glutathione  
28 reductase, GSH: glutathione, HAF: Halliwell-Asada-Foyer cycle, MDHAR: monodehydroascorbate  
29 reductase, mETC: mitochondrial electron transport chain, O<sub>3</sub>: ozone, OAA: oxaloacetate, OG: 2-  
30 oxoglutarate, OPPP: oxidative pentose phosphate pathway, PCD: programmed cell death, PEP:  
31 phosphoenolpyruvate, PEPcase: phosphoenolpyruvate carboxylase, TCA: tricarboxylic acid, ROS:  
32 reactive oxygen species, SA: salicylic acid, SDH: succinate dehydrogenase, UCP: uncoupling protein.

33

## 34 1. Introduction

35 In photosynthetically active cells, both photosynthesis and respiration produce energy in the form  
36 of ATP via photophosphorylation and oxidative phosphorylation, respectively in chloroplasts and  
37 mitochondria. Both organelles share the provision of energy whose the balance is tightly regulated  
38 under stress conditions especially (Gardestrom and Igamberdiev, 2016). Tropospheric ozone ( $O_3$ ) is a  
39 secondary air pollutant, mainly produced by the photochemical dissociation of nitrogen oxides in the  
40 presence of organic compounds issued from car exhaust and industrial fuel combustion.  $O_3$   
41 concentrations increased fourfold during the last century reaching yearly average values of around 50  
42 to 60 ppb but with peak values commonly observed between 100 and 250 ppb (Renaut et al., 2009).  
43 These concentrations have harmful impact on crops and forests (Wittig et al., 2009; Ainsworth et al.,  
44 2012; Matyssek et al., 2012; Jolivet et al., 2016; Cailleret et al., 2018; Emberson et al., 2018; Franz et  
45 al., 2018; Grulke and Heath, 2019). Plants exposed to moderate  $O_3$  concentrations during prolonged  
46 time (chronic  $O_3$  exposure) commonly exhibit a reduction in photosynthesis and photorespiration and  
47 an increase in respiration (Reich, 1983; Dizengremel, 2001; Bohler et al., 2007; Jolivet et al., 2016).  
48 The cellular changes induced by chronic  $O_3$  injury were associated to an accelerated senescence in cell  
49 leaves (Pell et al., 1997; Pleijel et al., 1997; Miller et al., 1999; Gielen et al., 2007; Heath, 2008;  
50 Yendrek et al., 2017; Emberson et al., 2018; Vollenweider et al., 2019), the chloroplasts being early  
51 degraded while mitochondria remain intact until rather late (Sutinen et al., 1990; Holopainen et al.,  
52 1996). This delayed response of mitochondria thus confers a prominent role to this organelle in the  
53 mechanism of cell response to  $O_3$  injury.

54  
55 Although occurring in normal conditions, the generation of reactive oxygen species (ROS) at the level  
56 of the mitochondrial electron transport chain (mETC) is strongly increased under chronic  $O_3$  which  
57 induces oxidative stress (Baier et al., 2005). However, the increase in the respiratory process raises  
58 many questions even though it is usually associated to repair, detoxification and synthesis of defense  
59 compounds (Dizengremel, 2001; Ainsworth et al. 2012). Respiration not only provides energy to plant  
60 cells in the form of ATP and NAD(P)H but also contributes to the production of carbon skeletons and  
61 the maintenance of redox balancing. These three functions co-exist, could overlap and thus need to be  
62 coordinated to regulate plant respiration (O'Leary et al., 2019). A range of papers describe different  
63 aspects of the implication of the mitochondria in the  $O_3$ -induced increase of respiration, but a  
64 coordinated synthesis of the results is lacking. This review deepens the role of mitochondria in the  
65 response to  $O_3$ , highlighting its ability to catabolize carbon intermediates, balance reducing power and  
66 induce antioxidant systems. The time course of mitochondrial events under  $O_3$  exposure, from a  
67 successful scavenging of ROS to the initiation of senescence, will be proposed. The integration of the  
68 mitochondrial behavior in a coordinated cellular response to  $O_3$  will be discussed.

69

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

71 The increase in respiration observed in plants submitted to chronic O<sub>3</sub> exposure is associated to an  
72 increased activity of a set of enzymes belonging to the glycolytic pathway and the oxidative pentose  
73 phosphate pathway (OPPP)(Dizengremel et al., 1994; Sehmer et al. 1998; Dizengremel et al., 2009;  
74 Dghim et al., 2012). This increase provides additional reducing power in the form of NADH and/or  
75 NADPH, which are useful for detoxification mechanisms (Fig 1.) (Dizengremel, 2001; Dizengremel et  
76 al., 2008). It must also be emphasized that several metabolites branch off from glycolysis or/and OPPP  
77 (Plaxton and Podesta, 2006) to initiate biosynthetic pathways (Fig 1.). Thus, phosphoenolpyruvate  
78 (PEP), end-product of the glycolysis, has a central role under O<sub>3</sub> stress (Dizengremel et al., 2012), and  
79 can bind to erythrose-4-phosphate from OPPP, allowing an increased functioning of the  
80 phenylpropanoid pathway essential to the production of defense compounds. Furthermore, the  
81 carboxylation of PEP into oxaloacetate (OAA) via phosphoenolpyruvate carboxylase (PEPcase) could  
82 in part mitigate the loss of CO<sub>2</sub> fixation by Rubisco under O<sub>3</sub> (Fontaine et al., 1999; Renaut et al.,  
83 2009). Furthermore, an increase of pyruvate kinase activity, providing more pyruvate to the  
84 tricarboxylic acid (TCA) cycle, was also observed under O<sub>3</sub> exposure (Dizengremel et al., 1994,  
85 2009). Another role of PEP will be however emphasized here in the context of an increased  
86 mitochondrial respiration under O<sub>3</sub> stress. OAA produced from PEPcase can be transported into the  
87 mitochondrion or produce malate in the cytosol (Fig 1.). Malate can thus either produce pyruvate  
88 through the O<sub>3</sub>-increased activity of the cytosolic NADP-malic enzyme (Dghim et al., 2012; Chen et  
89 al., 2019), providing additional NADPH for detoxification purposes, or be transported into the  
90 mitochondrion. It cannot be excluded that malate could thus be decarboxylated to pyruvate within the  
91 mitochondrion via NAD(P)-malic enzyme (Møller and Rasmusson, 1998; Dizengremel et al., 2012).  
92 An increasing offer in OAA, malate and/or pyruvate, would then replenish the TCA cycle  
93 (Dizengremel et al., 2012).

94

## 95 **3. Deciphering the role of mitochondria under ozone stress**

96 During the first thirty years following the observation of damaging effects of O<sub>3</sub> on plants (Haagen-  
97 Smit et al., 1952), few studies were devoted to the effect of O<sub>3</sub> specifically on plant mitochondria  
98 (Dizengremel and Citerne, 1988). The concentration of O<sub>3</sub> used was often acute (1 ppm delivered for  
99 several hours) and the resulting effect was mainly an inhibition of the oxidative phosphorylation (Lee,  
100 1967). More recent works using chronic O<sub>3</sub> exposure explored the responses of the different  
101 mitochondrial pathways contributing to the oxidative degradation of the organic acids.

102

### 103 *3.1. A noncyclic anabolic function of TCA to enhance antioxidant defense*

104 The TCA cycle operates in the matrix of mitochondria by fully decarboxylating the organic acids;  
105 however, a noncyclic functioning of TCA can also occur (Tcherkez et al., 2009; Sweetlove et al.,  
106 2010) allowing the leak of some organic acids (citrate, isocitrate, oxoglutarate) towards amino acid

107 synthesis (Fig. 1A, O’Leary et al., 2011). This anaplerotic pathway would be solicited under chronic  
108 O<sub>3</sub> exposure (Dizengremel et al., 2012). Citrate is exported from the mitochondrion to the cytosol to  
109 produce 2-oxoglutarate (OG) via cytosolic isocitrate dehydrogenase (IDH), a reaction that provides  
110 NADPH and is increased under O<sub>3</sub> stress (Dghim et al., 2012). Mitochondrial OG can also be directly  
111 exported after being produced from isocitrate by the mitochondrial NAD(P)-IDH. OG may thus be  
112 integrated into the glutamine synthetase 2 / glutamate oxoglutarate aminotransferase or into the  
113 glutamine synthetase 1 (GS1) / aspartate aminotransferase cycles to give glutamate in the chloroplast  
114 or cytosol, respectively (Fig 1A). The activity of GS1 was shown to increase in plants fumigated with  
115 O<sub>3</sub> (Galant et al., 2012). This could allow to cope with a diminished NH<sub>4</sub><sup>+</sup> remobilisation due to O<sub>3</sub>-  
116 decreased photorespiratory activity (Bagard et al., 2008). Interestingly, a higher supply of cytosolic  
117 glutamate could also support *de novo* glutathione (GSH) synthesis by  $\gamma$ -glutamylcysteine synthetase  
118 and glutathione synthetase and therefore contributes to cell ROS detoxification. In animal cells, a  
119 specific OG transporter has been described in the mitochondrial inner membrane (Lash, 2006). This  
120 transporter carries out OG to the cytosol in exchange for cytosolic GSH (Fig. 1A). The presence of  
121 such a transporter still needs to be investigated in plants in which GSH biosynthesis only occurs in  
122 cytosol and chloroplast. Such transporters would therefore insure mitochondrial import of cytosolic  
123 GSH during stress. The enhancement of noncyclic TCA and anaplerotic pathway under O<sub>3</sub> would thus  
124 feed cytosolic antioxidant synthesis in carbon skeletons and simultaneously import antioxidants in  
125 mitochondria.

126  
127 O<sub>3</sub> enters the leaf through stomata and rapidly generate various ROS (Kangasjarvi et al., 1995) that  
128 cause the peroxidation of membrane lipids (Ranieri et al., 1996; Loreto and Velikova, 2001). In this  
129 respect, the leak of compounds out of TCA cycle may result from inactivation of some downstream  
130 enzymes of the cycle by the lipid peroxidation products, as 4-hydroxy-2-nonenal, which has been  
131 shown to specifically inhibit oxoglutarate dehydrogenase and pyruvate dehydrogenase complexes by  
132 about 80% (Millar and Leaver, 2000). To maintain the rate of respiration, part of the TCA cycle can be  
133 by-passed, as do the  $\gamma$ -aminobutyrate shunt (GABA shunt) for succinate synthesis in bypassing  
134 oxoglutarate dehydrogenase and succinyl-coA-synthetase (Fig. 1B, Rhoads et al., 2006; Dizengremel  
135 et al., 2012). GABA synthesis is catalyzed by glutamate dehydrogenase and glutamic acid  
136 decarboxylase, which both increased in O<sub>3</sub>-treated rice leaves (Cho et al., 2008). Increased  
137 concentrations of GABA were generally observed in response to stress, conferring on the GABA shunt  
138 an adaptive mechanism (Michaeli and Fromm, 2015). Recently, Che-Othman et al. (2019) concluded  
139 that increase in GABA shunt activity in wheat leaves overcome the inhibition of the TCA cycle key  
140 enzymes, providing an alternative carbon source to support the stress-dependent increase in  
141 mitochondrial respiration. In response to O<sub>3</sub>, GABA could also act as a transmembrane signal

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

144

145 The exchange of metabolites between chloroplast and mitochondria allows balancing the cellular  
146 redox within plant cell (Hoefnagel et al., 1998; Gardestrom and Igamberdiev, 2016; O'Leary et al.,  
147 2019). This is mediated via a system, often called malate valve, which couples a malate/OAA redox  
148 shuttle based on mitochondrial NAD-malate dehydrogenase to a chloroplastic NADP-malate  
149 dehydrogenase (Hoefnagel et al., 1998; Scheibe, 2004). When the NADPH/NADP ratio is high, malate  
150 is transported from the chloroplast into the mitochondria where it is transformed in OAA, resulting in  
151 the transfer of chloroplast NADPH in excess to mitochondrial NADH (Fig 1.)(Scheibe, 2004, Yoshida  
152 et al., 2007). This system allows to minimize chloroplast endogenous ROS production due to an overly  
153 reduced photosynthetic reaction system and a lack of NADP (Finnegan et al., 2004). This could  
154 happen under O<sub>3</sub> stress when a transient stromal over-reduction due to damaged Calvin cycle occurs  
155 before any impairment of the light reactions (Dizengremel, 2001; Dizengremel et al., 2009). In  
156 addition, O<sub>3</sub> is well known to induce stomatal closure (Wittig et al., 2007; Hoshika et al., 2015) that  
157 would also drive a NADPH accumulation in chloroplast by reducing CO<sub>2</sub> levels. Malate valve  
158 contributes to import reducing power in mitochondria and therefore to detoxification capacity in  
159 supporting mitochondrial antioxidant regeneration and to minimize chloroplastic ETC imbalance  
160 resulting in ROS generation.

161

### 162 *3.2. Role of the mitochondrial electron transport chain*

163 The mETC is a major site of ATP synthesis, but is also a source of ROS (Fig. 1C) through complex I  
164 (NADH: ubiquinone oxidoreductase) and complex III (UQH<sub>2</sub>: cytochrome c oxidoreductase;  
165 cytochrome bc<sub>1</sub> complex) (Murphy, 2009). More recently, it was shown that complex II (SDH) can  
166 also produce O<sub>2</sub><sup>-</sup> at the FAD-binding site through the monovalent electron reduction of O<sub>2</sub> (Jardim-  
167 Messeder et al., 2015; Huang et al, 2019). Endogenous ROS production by mETC can however be  
168 mitigated by the use of alternative pathways. The plant mETC possesses an alternative non-proton  
169 pumping pathway, by-passing complexes III and IV, and mediated by alternative oxidase (AOX) (Fig  
170 1C)(Lance et al., 1985; Vanlerberghe and McIntosh, 1997; Millar et al., 2011). AOX might act to  
171 maintain a basal ubiquinone pool reduction state, preventing over-reduction of upstream electron-  
172 transport components and lowering ROS production (Purvis and Shewfelt, 1993; Rhoads et al., 2006 ;  
173 Mittler, 2002; Umbach et al., 2005; Saha et al., 2016). It has been stated that the participation of the  
174 alternative oxidase is maintained very low under standard conditions by repressing its gene expression,  
175 potentially because of its wasteful nature (Selinski et al. 2018). However, the activity of AOX can be  
176 enhanced by several parameters acting at different levels (transcriptional, translational, post-  
177 translational) in response to a large range of stressors. The AOX gene expression was shown to be  
178 increased by H<sub>2</sub>O<sub>2</sub> itself (Vanlerberghe & McIntosh, 1997) or through specific transcription factors

179 (e.g. NAC17; Ng et al., 2013). Furthermore, differential expression of AOX isogenes can lead to the  
180 production of heterodimers having varying catalytic properties which potentially offer a fine-tune  
181 mechanism of AOX activity in response to stress. Under O<sub>3</sub> exposure, an activation of AOX at both  
182 transcriptional and translational levels was observed in poplar, tobacco and *Arabidopsis* (Jolivet et al.,  
183 1997; Dizengremel, 2001, Ederli et al., 2006; Tosti et al., 2006). In tobacco, the O<sub>3</sub> induced activation  
184 of gene expression of AOX1a was suggested to be NO-dependent, in cooperation with ethylene  
185 signaling, and correlated to an inhibition of cytochrome oxidase by NO (Ederli et al., 2006, Kumari et  
186 al., 2019). This is a good example of a disfunctioning of mETC triggering the expression of a nuclear  
187 gene encoding a mitochondrial protein (retrograde signaling system, Ng et al., 2014).

188  
189 AOX activity can also be regulated by redox state. The reduced form of the AOX dimer is more active  
190 than the oxidized form, suggesting that AOX activity can be modulated depending on reducing power  
191 availability, but may be affected by excessive accumulation of ROS. Thioredoxins belonging to Trxo  
192 and Trxh families are able to render AOX operational by reducing disulfide bonds (Trxo family: Marti  
193 et al., 2009; Umekawa and Ito, 2019; Trxh family: Gelhaye et al., 2004). The redox state activation of  
194 AOX protein is also coupled to ketocarboxylic acids binding that enhance its activity: pyruvate and  
195 OG are very effective activators in lowering Km for UQ substrate (Umbach et al., 2002; Selinski et al.,  
196 2018). These two systems of AOX activation, redox state and metabolite binding, interact with each  
197 other to complete AOX activity regulation. The increased availability of pyruvate under O<sub>3</sub>  
198 (Dizengremel et al., 1994, 2009) could explain the enhancing participation of AOX to the  
199 mitochondrial electron flow; however this activation would require sufficient reducing power  
200 availability to maintain AOX in its reduced form and minimize endogenous ROS production by  
201 mETC. In addition, AOX, that has a lower O<sub>2</sub> affinity than cytochrome oxidase (Km of 1-2.5μm vs.  
202 0.1-0.2μm), allows to maintain a low level of O<sub>2</sub> in mitochondria that therefore contributes to  
203 minimize ROS production (Gupta et al. 2015).

204  
205 Alternative pathways of mETC also include the shunt of complexes I and II, implying one of the four  
206 alternative non-proton pumping NAD(P)H dehydrogenases located either on the outer (NDex) or inner  
207 (NDin) surface of the inner mitochondrial membrane (Fig. 1C). The NDin might largely participate for  
208 the reoxidation of matrix NADH under conditions where its concentration is increased (Møller, 2001),  
209 which could be the case under O<sub>3</sub> stress as mentioned above. Matrix NAD(P)H can be produced via  
210 the NAD(P)-IDH and NAD-malic enzyme or imported through the malate/OAA shuttle. The activity  
211 of the NAD(P)-malic enzyme is increased under O<sub>3</sub> (Dizengremel et al., 2009; Dghim et al., 2012.  
212 NDin is thus probably competing with NAD(P)H-dependent ROS-detoxifying enzymes for matrix  
213 NAD(P)H (Møller, 2001). Although rather inactive in normal conditions, the Ca<sup>2+</sup>-dependent NDex  
214 linked to the outer face of the inner mitochondrial membrane could be activated under stress by an  
215 increase in Ca<sup>2+</sup> cytosolic concentration, as it has been previously shown under O<sub>3</sub> (Heath and Castillo,

216 1988; Clayton et al., 1999). In addition, an increased expression of the NDex was demonstrated to  
217 accompany increased AOX expression under several stress treatments (Rhoads et al., 2006). Under  
218 oxidative stress, alternative dehydrogenases could help decrease ROS production linked to a high  
219 reduction level of mETC, by keeping the cytosolic NAD(P) pool relatively oxidized (Rhoads et al.,  
220 2006). In addition, uncoupling proteins (UCP) cross the inner mitochondrial membrane (Vercesi et al.,  
221 2006) and mildly participate to the modulation of the transmembrane potential (Fig 1C)(Rhoads et al.,  
222 2006). UCP is also able to decrease ROS formation in reintegrating proton and thus bypassing  
223 respiratory control by ATP synthase (Rhoads et al., 2006). While AOX can be inactivated by a strong  
224 oxidative stress (Winger et al., 2005), UCP is able to keep operating when ROS level is much higher  
225 (Considine et al., 2003; Rhoads et al., 2006). The role of the NADH dehydrogenases and UCP under  
226 O<sub>3</sub>-driven oxidative stress must however be confirmed, especially in regard to the role of AOX.

227  
228 Beside electron transport, mETC also hosts the final step of ascorbate (ASA) biosynthesis that  
229 converts L-galactono-1,4-lactone to ASA and is catalysed by L-galactono-1,4-lactone dehydrogenase  
230 (Fig 1C)(Ôba et al., 1995; Bartoli et al., 2000). This enzyme is located on the inner mitochondrial  
231 membrane where it is physically associated to complex I and regulated by electron transport.  
232 Furthermore, oxidized cytochrome c is essential to the functioning of L-GLDH in acting as a specific  
233 electron acceptor (Fig 1C)(Bartoli et al., 2000). As an example, L-GLDH activity was decreased by  
234 40% in Arabidopsis knockdown mutants under-expressing cytochrome c (Welchen et al. 2016). The  
235 question remains as the relative contribution of ASA synthesis to respiratory electron flux compared to  
236 that driven by TCA cycle activity and whether this contribution is modulated in O<sub>3</sub> stress conditions.  
237 In any case, produced ASA can thereafter be used by cytosolic and mitochondrial antioxidant systems  
238 to minimize ROS accumulation. Therefore, the coupling of L-GLDH and mETC activities via  
239 cytochrome c could contribute to couple respiration and antioxidant defense to cope with oxidative  
240 stress.

241

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

243 Several antioxidant mechanisms, mainly present in the soluble parts of the mitochondria (i.e.  
244 intermembrane space and matrix), can contribute to mitigate the increased ROS production during O<sub>3</sub>  
245 exposure. A matrix-localized Mn-superoxide dismutase was reported in literature, coping with  
246 endogenous O<sub>2</sub><sup>-</sup> production by mETC, and involved in the protection against ROS produced by O<sub>3</sub>  
247 attack (Fig 1C)(Alscher et al., 2002). To manage superoxide dismutase products, the occurrence of a  
248 Halliwell-Asada-Foyer cycle (HAF, Fig. 1D) has been reported in cytosol, chloroplasts, peroxisomes  
249 as well as in mitochondria (Jiménez et al., 1997; Noctor and Foyer, 1998; Foyer and Noctor, 2011)  
250 even though the presence of dehydroascorbate reductase (DHAR) in mitochondria remains  
251 questionable (Møller, 2001; Noctor et al., 2018). Several ascorbate peroxidases (APX) have been  
252 identified in plant mitochondria, one intermembrane APX bound to the inner mitochondrial membrane

253 (De Leonardis et al., 2000) and two others located in the matrix, either soluble or bound to the inner  
254 membrane (Mittova et al., 2004). As mentioned above, ASA is delivered to the mitochondrial  
255 intermembrane space where it can be oxidized by the intermembrane APX. Dehydroascorbate (DHA)  
256 can then be transported into the matrix via a GLUT-like transporter of the glucose transporter family  
257 (Chew et al., 2003; Szarka et al., 2013). In addition, if the lack of DHAR in the matrix were  
258 confirmed, DHA will have to be reduced in ASA by the glutaredoxin or directly by GSH (Fig  
259 1D)(Ehrhart and Zeevalk, 2003). Recent work also demonstrated the presence of mitochondrial  
260 ascorbate transporter-MAT, a member of the SLC25 family, to directly transport ASA through the  
261 mitochondrial inner membrane (Scalera et al., 2018).

262  
263 The synthesis of GSH only occurs in cytosol and chloroplast (Rausch et al., 2007); however GSH  
264 presence has been reported in mitochondria suggesting a crossing of the inner mitochondrial  
265 membrane via a still unknown transporter in plants (Fig. 1A; Chen and Lash, 1998; Maughan and  
266 Foyer, 2006). The presence of glutathione reductase (GR) was confirmed in mitochondria to allow  
267 GSH regeneration where its activity is NADPH dependent (Fig. 1D, Edwards et al., 1990; Jimenez et  
268 al., 1997). Under O<sub>3</sub>, the synthesis of GSH is increased likely to maintain glutathione redox state  
269 (Dumont et al., 2014) and compensates ROS scavenging by glutathione. Similarly, the activities of  
270 APX, monodehydroascorbate reductase (MDHAR) and GR have been shown to increase under O<sub>3</sub>  
271 (Dusart et al., 2019; Gandin et al., 2019).

272  
273 As previously mentioned, thioredoxins have been reported in plant mitochondria (Sweetlove and  
274 Foyer, 2004; Navrot et al., 2007; Vieira Dos Santos and Rey, 2008). Also, a type II peroxiredoxin  
275 (PrxII F), has been identified in plant mitochondria (Rhoads et al., 2006; Finkemeier et., 2005), where  
276 it could reduce mitochondrial H<sub>2</sub>O<sub>2</sub> by using thioredoxin or GSH (Fig 1D). The latter are then  
277 regenerated by thioredoxin reductase or GR (Sweetlove et al., 2004; Rhoads et al., 2006). The type II  
278 peroxiredoxin/thioredoxin system could also reduce lipid peroxides (Rouhier et al., 2004) and could  
279 therefore contribute to maintain mitochondria membrane integrity under stress. A mitochondrial  
280 protein disulfide isomerase functioning together with thioredoxin and NADPH could also reduce  
281 disulfides resulting from protein oxidation (Sweetlove et al., 2002; Rhoads et al., 2006). In addition to  
282 the above-mentioned effect of thioredoxin on AOX, the association of all these processes would lower  
283 the content of ROS in the matrix in O<sub>3</sub> exposed plants.

284  
285 **5. Suggested steps of the response of mitochondria to ozone: from resistance to cell death**  
286 O<sub>3</sub> being a powerful oxidant, a plausible scenario of the successive mechanisms coping with the  
287 progressively rising intensity of the O<sub>3</sub>-related stress is proposed below (Table 1). The sequential  
288 presentation is probably an idealized view of more intricate relationships between the responses to O<sub>3</sub>.  
289 In addition, ROS, responsible for oxidative damages caused by O<sub>3</sub>, are also known to play an

290 important role in triggering signaling pathways. Thus, there is a permanent fine-tuning of ROS  
291 accumulation to balance beneficial signaling and phytotoxic effect. The steps described below lie on  
292 results obtained from a series of O<sub>3</sub> experiments and, in a minor way, are complement in integrating  
293 probable events derived from general oxidative stress.

294

### 295 *5.1. Mobilisation of pre-existing mechanisms to cope with ROS*

296 The first impact of O<sub>3</sub> on photosynthesis, and especially on the biochemical components, leads to  
297 excessive NADPH accumulation within the chloroplast. A series of metabolic pathways, generally  
298 associated to carbohydrate breakdown as glycolysis and OPP, are also enhanced and result in even  
299 more NAD(P)H produced (Fig1, Table 1). This additional reducing power allows the existing  
300 detoxification processes to function at optimal level. An increase in APX activity has been described  
301 under O<sub>3</sub> and shown to be supported by enhanced ASA regeneration by NADH-dependent MDHAR  
302 (Gandin et al., 2019). In addition, the cytosolic carboxylating enzyme, PEPcase, is requested, either to  
303 cope with the decreased CO<sub>2</sub> fixation by Rubisco and/or to contribute to the anaplerotic pathway  
304 (Dizengremel et al., 2012). Several intermediates of catabolism are therefore favoured, either by  
305 pyruvate kinase (e.g. pyruvate) or/and by PEPcase (e.g. OAA and malate), and have impact on  
306 mitochondria functioning (Dghim et al., 2012). These organic acids will be transported into the  
307 mitochondria to feed TCA cycle and finally provide NAD(P)H to the mETC. Additional reducing  
308 power is also provided by the transfer of redox equivalents (e.g. through malate/OAA shuttle) either  
309 from chloroplasts, in which the redox pressure is transiently high, or from cytosol. However,  
310 increasing feeding of mitochondria with NAD(P)H threaten to over-reduce mETC and induce  
311 endogenous ROS production.

312

313 To maintain the mETC in a sufficiently oxidized state, ATP synthesis and respiratory control could be  
314 balanced by the UCP while the electron flow may use the activated AOX and some of the four  
315 NAD(P)H dehydrogenases located on both sides of the inner mitochondrial membrane (Table 1).  
316 Three NAD(P)H-DH are activated by Ca<sup>2+</sup> which concentration is known to be increased by O<sub>3</sub> and is  
317 involved in phytohormone cascade signals (Short et al., 2012). An increase of the amount of salicylic  
318 acid (SA) was shown under O<sub>3</sub> fumigation (Koch et al., 2000). At low concentration, SA was shown to  
319 uncouple the mETC (Gleason et al., 2011) and to increase AOX expression (Rhoads and McIntosh,  
320 1992), suggesting a potential role for SA (and may be ethylene) to the signaling process regulating  
321 mitochondrial electron flux (Rao et al., 2000; Dizengremel, 2001; Heath, 2008). NO production due to  
322 O<sub>3</sub> stress could also be an important regulator of electron partitioning between cytochrome and  
323 alternative pathways, in reducing and enhancing cytochrome oxidase and alternative oxidase activities,  
324 respectively (Ederli et al. 2006). Therefore, NO impact on mETC could initiate a bottom-up regulation  
325 starting with the decrease in mETC ATP yield that would require an increase in glycolysis and TCA

326 cycle activities to fulfill mitochondria ATP demands. Evidence may be seen in the stimulation by O<sub>3</sub>  
327 of a mitochondrial phosphate transporter located in the inner membrane, which catalyzes the influx of  
328 Pi, essential for the synthesis of ATP in the matrix (Kiiskinen et al., 1997).

329

### 330 *5.2. Setting up of detoxifying processes*

331 The enhanced activities of PEPcase as well as of glycolysis and OPP tend to show a plateau for higher  
332 doses of O<sub>3</sub>, announcing a limit for the capacity of the metabolism to counteract the oxidative stress  
333 (Dizengremel et al., 1994; Fontaine et al., 1999; Renaut et al., 2009). With an extended O<sub>3</sub> exposure,  
334 the ROS production keeps increasing and more likely starts to accumulate in plant cells. ROS shortly  
335 oxidized cell components (e.g. protein, DNA...) among which membrane lipids that are a major target  
336 (Rebouças et al., 2017). Lipid peroxidation products are able to inhibit specific TCA cycle enzymes  
337 (Millar and Leaver, 2000) and disrupt its functioning (Table 1). As a consequence, TCA cycle would  
338 leak intermediates and export isocitrate or OG to the cytosol for amino acid biosynthesis (Dizengremel  
339 et al., 2012). This leak in carbon skeletons would also support cytosolic GSH *de novo* biosynthesis, as  
340 the enzymes for it have never been reported in mitochondria. GSH can then return to mitochondria  
341 through OG-GSH transporters as shown in animal cells (Lash 2006), yet to be described in plants.  
342 GSH can therefore support glutathione peroxidase and glutaredoxin activities as well as ASA  
343 regeneration through non-catalytic reduction or mediated by DHAR, therefore significantly  
344 contributing to antioxidant defense (Fig 1D, Table 1). With ROS accumulation, other antioxidant  
345 systems can intervene, as the peroxiredoxin/thioredoxin systems present in mitochondria that could  
346 reduce mitochondrial H<sub>2</sub>O<sub>2</sub>. In addition, it is likely that ASA biosynthesis is also enhanced under O<sub>3</sub> to  
347 support HAF functioning. The *de novo* synthesis of ASA however involves additional feeding of the  
348 mETC with electrons, increasing even more the reductant pressure on the chain and the risk for  
349 endogenous ROS production. In addition, ROS accumulation likely oxidized mETC components as  
350 AOX, which is inactive in oxidized form. This runaway effect may lead to increasing endogenous O<sub>2</sub><sup>-</sup>  
351 production by complexes I, II and III and ROS over-accumulation in mitochondria.

352

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

361

### 362 *5.3. Towards programmed cell death*

363 When the O<sub>3</sub> uptake lasts and oxidative pressure keeps rising, the antioxidant defense is overwhelmed  
364 and ROS accumulates in excess. It is admitted that plant mitochondria could then play a role similar to  
365 that of animal mitochondria in the PCD process (Table 1)(Xie and Chen, 2000; Tiwari et al., 2002;  
366 Vianello et al., 2007). The decrease in ATP production and release of cytochrome c are the first  
367 signals of PCD. The depletion of ATP synthesis, above described as accompanying both UCP and  
368 AOX implication, can be accelerated by mitochondrial membrane disturbances associated to ROS.  
369 The peroxiredoxin/thioredoxin system can help by supplying reducing power to reductases acting on  
370 lipid hydroperoxides and disulfides resulting from protein oxidation, but it become insufficient. A  
371 collapse of mitochondrial transmembrane potential  $\Delta\Psi_m$  would ensue, triggering the opening of the  
372 mitochondrial permeability transition pore (Table 1)(Tiwari et al., 2002; Zandalinas and Mittler,  
373 2018).

374

375 Cytochrome *c*, a mobile protein attached to the cytosolic face of the inner mitochondrial membrane,  
376 acts as an electron carrier between complexes III and IV and is implied in the last step of ASA  
377 synthesis. Cytochrome *c* is labile and can be easily detached from the inner mitochondrial membrane  
378 followed by its release into the cytosol through a permeabilization of the outer membrane due to  
379 oxidative stress (Table 1)(Dizengremel, 1983; Sun et al., 1999). A release of cytochrome *c* was  
380 observed upon O<sub>3</sub> exposure of tobacco plants (Pasqualini et al., 2003). The release of cytochrome *c*  
381 activates caspases-like proteases that are involved in the execution of the cell death program (Tiwari et  
382 al., 2002; Vacca et al., 2006; Welchen and Gonzalez, 2016). A Ca<sup>2+</sup> overload, admitted to occur upon  
383 O<sub>3</sub> exposure, would trigger the entire PCD process (Xiong et al., 2006; Welchen and Gonzalez, 2016).  
384 The increased permeability of mitochondria to solutes leads to mitochondrial swelling (Vianello et al.,  
385 2007; Zancani et al., 2015) which was early observed in response to O<sub>3</sub> (Lee, 1967, 1968). This ends  
386 by overall mitochondria rupture and subsequent cell death.

387

## 388 **6. Conclusions and perspectives**

389 In the literature, the role of mitochondria facing O<sub>3</sub> stress is often mentioned as being important but  
390 the available information is disseminated. A gathering of these information and a clearer picture of the  
391 sequential events following an increasing oxidative pressure is proposed in this review article, based  
392 on results mostly obtained with O<sub>3</sub> itself and some others deduced from general oxidative stress (Table  
393 1). As soon as O<sub>3</sub> enters the leaf, delivering ROS, the entire catabolic system is mobilized, partly to  
394 compensate the damages and also for providing redox compounds, organic acids and secondary  
395 metabolites. These changes allow to optimize detoxification activity and minimize ROS accumulation,  
396 in modulating TCA cycle activity and NAD(P)H production, balancing electron transport within  
397 mETC, and increasing antioxidant pool in the matrix. In the most severe cases, this wide

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

400

401 Even though this scenario is plausible, the action of O<sub>3</sub> on some of the steps needs to be definitively  
402 confirmed. The important role of AOX (and potentially other alternative pathways) must be  
403 emphasized. AOX has been recently proposed as a functional marker for breeding stress tolerant plant  
404 varieties (Polidoros et al., 2009). On the other hand, an elaborate network of anterograde and  
405 retrograde signalling between the mitochondria and nucleus is at work in plant cells with redox status  
406 and ROS as important actors (Leister, 2005; Rhoads and Subbaiah, 2007; Ng et al, 2014; Farooq et al.,  
407 2019). Moreover, it was recently demonstrated that the Ca<sup>2+</sup>-dependent changes in gene expression  
408 observed in response to O<sub>3</sub> differ markedly from Ca<sup>2+</sup> signatures of other oxidative stress, such as  
409 H<sub>2</sub>O<sub>2</sub> and cold (Short et al., 2012). A more deeply insight concerning the metabolic and signalling  
410 networks under O<sub>3</sub> stress thus needs to be studied. In this context, the ambiguous role of hormones  
411 raises questions, particularly for ethylene, which is largely implicated in leaf senescence as well as O<sub>3</sub>  
412 damage (Moeder et al., 2002; Nunn et al., 2005; Heath, 2008). Finally, the role of antioxidants to cope  
413 with O<sub>3</sub> stress is undisputable but very technically complex to assess relative to each intracellular pool.  
414 It is therefore essential to develop new methods to estimate the relative contribution of each pool and  
415 their potential exchanges within the cell.

416

#### 417 **Conflicts of interest**

418 None declared

419

#### 420 **Funding source**

421 This research was supported by the French National Research Agency through the Laboratory of  
422 Excellence ARBRE (ANR-12-LABXARBRE-01).

423

#### 424 **Author Contribution**

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

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785

786 Table

787

788 **Table 1.**

789 Proposed sequential response of plant mitochondria to O<sub>3</sub> stress (see the text and Fig 1. for a complete  
790 explanation); *in italics: events inferred from general oxidative stress.*

791

792 **Successive steps**

**Increasing  
Phytotoxic Ozone Dose**

795 **Mobilisation of pre-existing mechanisms to cope with ROS**

796 Cytosol

- 797 - Enhancement of the catabolic pathways and PEPcase
- 798 - Supply of organic acids and redox power (NADPH)
- 799 - Maintenance of a high detoxification
- 800 - Increase in the phenylpropanoid metabolism

801

802 Mitochondria

- 803 - Increased supply of NAD(P)H to ETC
- 804 - Adjustment of electron flow path in ETC by balancing between high  
805 substrate supply (organic acid and NAD(P)H) and ATP requirement
  - 806 • Increased activity of UCP and AOX to lower reduction pressure
  - 807 • Ca<sup>2+</sup>-dependent activation of the NAD(P)H-ND<sub>ext</sub> and ND<sub>int</sub>
  - 808 • SA-mediated uncoupling of respiratory chain
  - 809 • Increased gene expression of AOX

810

811 **Setting up of detoxifying processes**

- 812 - TCA cycle inhibition by peroxidation products
  - 813 • Enhancement of anaplerotic pathway
  - 814 • Leak of organic acids to amino acids synthesis
  - 815 • Increase synthesis of glutathione and *import in the matrix*
  - 816 • Induction of GABA shunt
- 817 - Conversion of superoxide by Mn-SOD
- 818 - Induction of antioxidant defense
  - 819 • Increased *de novo* synthesis of ascorbate
  - 820 • Increased activity of HAF cycle and APX/GR gene expression
  - 821 • *Enhancement of peroxyredoxin/thioredoxins systems*
- 822 - Inactivation of AOX
- 823 - Complex II inhibition by high level of SA
  - 824 • *Accumulation of toxic intermediates of GABA shunt*

825

826 **Towards programmed cell death (PCD)**

- 827 - Increasing damage to fatty acids and proteins of mitochondrial membranes
- 828 - ATP-synthesis decrease due to uncoupling of electron flow
- 829 - Decrease in ascorbate synthesis
- 830 - *Possible occurrence of Mitochondrial Permeability Transition*
- 831 - Release of cytochrome c in the intermembrane space
  - 832 • *Activation of caspases-like proteases*
- 833 - Disruption of the outer mitochondrial membrane
- 834 - Swelling of mitochondria
- 835 - Cell death

836

837

838 *Figure legend*

839 *Figure 1. Representative schema of metabolic network involved in plant mitochondria response to O<sub>3</sub>-*  
840 *induced oxidative stress. Four main focuses of mitochondria response are presented to highlight (A)*  
841 *the oxoglutarate-glutathione co-transport between cytoplasm and matrix that allow to replenish*  
842 *matrix GSH pool jointly to the leak of OG out of mitochondria; (B) the GABA-shunt bypassing TCA*  
843 *cycle enzymes once they are inhibited by ROS and its potential role in cell signalling and/or death;*  
844 *(C) the multifaced electron pathway which is modulated to minimize endogenous ROS production and*  
845 *its close relationship to ascorbate synthesis and import; (D) the antioxidant system reported in*  
846 *mitochondria matrix to scavenge ROS. Dark lines and arrows represent mitochondria-related*  
847 *metabolic links. Red lines represent the connectivity between mitochondrial metabolism and ROS*  
848 *production or scavenging. ASA: ascorbate, APX: ascorbate peroxidase, AOX: alternative oxidase,*  
849 *Cit: citrate, Cyt C: cytochrome C, Er-4-P: erythrose-4-phosphate, GABA: gamma-aminobutyric acid,*  
850 *γ-Glu-cys: L-gamma-glutamylcysteine, GLDH: L-galactono-1,4-lactone dehydrogenase, Glu:*  
851 *glutamate, GLUT-like Tr: glucose transporter, Glu-6-P: glucose-6-phosphate, GS/GOGAT: glutamine*  
852 *synthetase/glutamate synthase, GPX: glutathione peroxidase, GR: glutathione reductase, GRX:*  
853 *glutathione reductase, GSH: reduced glutathione, GSSG: oxidized glutathione, iCit: isocitrate, Mal:*  
854 *malate, MAT Tr: mitochondrial ascorbic acid transporter, (M)DHA: (mono)dehydroascorbate,*  
855 *(M)DHAR: (mono)dehydroascorbate reductase, mETC: mitochondrial electron transport chain,*  
856 *MnSOD: Mn-dependent superoxide dismutase, NDin/ex: internal/external NAD(P)H dehydrogenases,*  
857 *OAA: oxaloacetate, OG: oxoglutarate, OPPP: oxidative pentose phosphate pathway, PEPc:*  
858 *phosphoenolpyruvate carboxylase, PRX/TRX: peroxiredoxin/thioredoxin, Pyr: pyruvate, ROS:*  
859 *reactive oxygen species, SSA: succinic semialdehyde, Succ: succinate, UCP: uncoupling protein*

860

