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Importance of Detoxification Processes in Ozone Risk Assessment: Need to Integrate the Cellular Compartmentation of Antioxidants?

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The correct integration of the detoxification processes in a risk assessment model for ozone damage on plants remains challenging. In particular, the intracellular compartmentation of antioxidant metabolites could play a role, since each compartment presents its own sensitivity to ROS and metabolite set. For each compartment, we tried to provide both qualitative and quantitative information on the metabolites present as well as the putative transporters implied. When they are known, the modifications caused by O₃ or oxidative stress are presented. Clearly, under O₃ exposure, integrative data which would allow to improve predictive models for O₃ risk assessment are missing.

Keywords: ozone, ROS, antioxidants, cellular compartmentation, risk assessment models

INTRODUCTION

Ozone (O₃) is a phytotoxic air pollutant known to negatively impact crop and forest productivity (Wittig et al., 2009; Jolivet et al., 2016; Li et al., 2017). In order to determine the critical level above which O₃ damage on plants occurs, the POD_Y (Phytotoxic Ozone Dose above a threshold flux of Y nmol.m⁻².s⁻¹) was derived from flux-based methods to improve dose-response relationships including the detoxifying capacity of leaf tissues (Musselman et al., 2006; Dizengremel et al., 2009). Flux-based models use a range of cut-off thresholds (Y) indicative of varying detoxification capacities. However, the considerable uncertainties to determine the contribution of each metabolite to the cellular antioxidant potential make the estimation of the threshold difficult to assess. This difficulty can be explained by the differences in concentrations and redox status of these metabolites between compartments. Here, we resumed recent highlights on the spatial distribution of the main defense metabolites to decipher their possible roles in response to O₃ and point out which importance the occurrence of this compartmentation might have in estimating the detoxification threshold.

DEAL WITH A DIVERSITY OF ANTIOXIDANT METABOLITES

Different classes of molecules serve as antioxidants amongst which ascorbate (AsA), glutathione (GSH), and phenolic compounds as flavonols are considered as the most ubiquitous according to their presence in several cell compartments (Foyer and Noctor, 2011; **Figure 1**), including vacuole and the extracellular space (Zhao and Dixon, 2009; Agati et al., 2012). Sugars such as

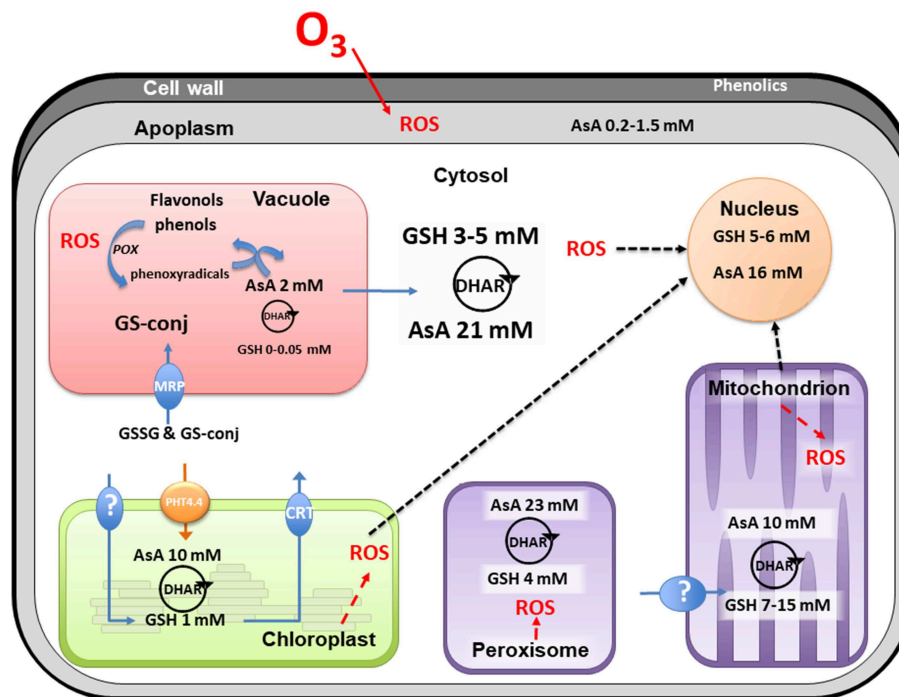


FIGURE 1 | Compartmentation of plant oxidative system. This non-exhaustive figure summarizes current knowledge from plant subcellular localization of antioxidants in non-stress conditions. In view of the multiplicity of all enzyme isoforms, the figure only mentions the presence of the HAF cycle in each compartment where the dehydroascorbate reductase (DHAR) has been characterized. AsA, total ascorbate; GSH, total glutathione; GSSG, glutathione disulphide; GS-conj, glutathione conjugates; HAF, Halliwell-Asada-Foyer; MRP, Multidrug Resistance associated Protein; CRT, Chloroquine Resistance Transporter; PHT, phosphate transporter; POX, phenol peroxidases; ROS, reactive oxygen species. See the text for references.

sucrose, RFOs (raffinose family oligosaccharides) and fructans are known to directly quench ROS in different organelles and thus contribute to antioxidant defense (Keunen et al., 2013). Another type are lipophilic antioxidants, such as α -tocopherol or carotenoids located in organelle membranes particularly in plastids (Das and Roychoudhury, 2014). Subcellular compartments also possess various enzymes able either to act as ROS scavengers or to support regeneration of the reduced form of the antioxidants (Noctor et al., 2018). The first group includes enzymes trapping the superoxide ion (such as superoxide dismutase, SODs) and hydrogen peroxide with catalases (specifically located in the peroxisome) or several peroxidases (AsA, GSH, or thioredoxin dependent) whose cell location has already been detailed in several reviews (Rouhier and Jacquot, 2005; Dos Santos and Rey, 2006; Mhamdi et al., 2010b; Rahantaniaina et al., 2013; Noctor et al., 2018). The second group, responsible for the regeneration of antioxidants, is ascribed to the close redox coupling of AsA and GSH pools *in vivo* (HAF as Halliwell Asada Foyer cycle). The presence of dehydroascorbate reductase (DHAR) seems to be sufficient to consider the presence and the functioning of the HAF cycle within a specific cell compartment (Figure 1; Rahantaniaina et al., 2013). It is also important to consider that the HAF cycle functioning needs to be linked with a correct enzyme regeneration through NAD(P)H reducing power, e.g., under O_3

stress (Dizengremel et al., 2008; Dghim et al., 2013), in cytosol as well as in organelles. *In fine*, detoxification results from the combined actions of all these mechanisms, which therefore explains the difficulty to choose key parameter(s) in modeling the detoxifying capacity of leaf tissues.

TOTAL POOL OF ANTIOXIDANTS: NOT SO SATISFYING

One major issue when considering the total pool of antioxidants is the difficulty to evaluate it as a whole, taking into account the diversity of the metabolites it contains. Indeed, the methods and kits used are disputable (Noctor et al., 2016). For example, in order to take into account the total antioxidant capacity in plants, some authors used the FRAP assay (ferric reducing ability of plasma given in micromoles of Fe^{2+} per gram in dry matter). However, the significance of the FRAP test is questionable since it takes antioxidants into consideration that do not necessarily react to the oxidative load generated by O_3 (Severino et al., 2007). To solve this issue, researches focused on specific metabolites considered as cue to explain differences in O_3 tolerance. AsA is one of the main examples of a potential factor in O_3 tolerance (Burkey et al., 2000; Conklin and Barth, 2004) at least for some species. To support this fact, studies with

AsA deficient mutants and clones (Conklin et al., 1996, 2000; Veljovic-Jovanovic et al., 2002) showed that low concentrations of AsA in the plant tissue limit O₃ tolerance. However, in numerous examples and particularly in ligneous species (e.g., *Populus* genotypes or *Quercus* sp.), differences in AsA content were not sufficient to explain the differences in O₃ tolerance (Dusart et al., 2019; Pellegrini et al., 2019). Other antioxidants such as GSH and/or phenolic compounds could play a more important role in some species or genotypes. In addition, changes at the leaf scale could hide more subtle changes at the scale of a cell compartment that is crucial for cell homeostasis. The redox state of each metabolite also varies, as some compartments differ from others with lower reduction states. Thus, the apoplast and vacuole were endowed with a lower level of reduction for AsA and GSH (Noctor and Foyer, 2016). In this respect, both the antioxidant content and the ability of cell compartments to regulate the redox levels of the molecules have to be considered.

COMPARTMENTATION OF ANTIOXIDANTS

The different cell compartments are not equal in terms of antioxidant concentrations (Zechmann, 2017) and redox state (Foyer and Noctor, 2016) and the main differences between cell compartments are discussed in this section. We also highlight some specific features related to the transport of these metabolites through intracellular membranes, which also contributes to the extent of antioxidant content and redox status in each compartment.

Apoplast

The apoplast comprises the cell wall and the fluid in the intercellular spaces as such constitutes the first barrier encountered by O₃ after entering the leaf through the stomata (Laisk et al., 1989). AsA contained in the apoplastic fluid was often considered as the first line of defense against O₃ and, consequently, its content may increase with the beginning of the exposure (Riikonen et al., 2009). In this context, it has been integrated in different models (Polle et al., 1995; Ranieri et al., 1996; Plöchl et al., 2000; Burkey and Eason, 2002; Conklin and Barth, 2004; Tuzet et al., 2011). In fact, in many species, the importance of apoplastic AsA regarding O₃ tolerance is still unclear (D'Haese et al., 2005; Booker et al., 2012; Dai et al., 2018). For example investigated poplar clones showed no relation between apoplastic AsA content and O₃ sensitivity of clones (Van Hove et al., 2001; Di Baccio et al., 2008). Moreover, the efficiency of apoplast detoxification by AsA depends on the export of the oxidized form to the cytoplasm where it then has to be regenerated (Luwe and Heber, 1995). Finally, despite an efficient transmembrane exchange between apoplast and symplasm, the AsA concentrations (around 0.2–1.5 mM) in the apoplast are 10–30 times lower than in the cytosol (Moldau et al., 1997; Plöchl et al., 2000; Van Hove et al., 2001) with higher oxidation rates (Booker et al., 2012). The transport proteins responsible for DHA uptake and ascorbate efflux have not yet been identified, even though the ascorbate efflux mechanism possibly occurs via an anion channel (Smirnov, 2018). It should also be considered that the presence of ascorbate oxidase in the apoplast could maintain

the AsA pool in a more oxidized state than the intracellular pool (Smirnov, 2018).

There are furthermore other metabolites with antioxidant activity being present in the apoplast. Considering GSH, its content in the apoplast has been found to be very low (Zechmann, 2014), limiting its ability to scavenge ROS or contribute to ASA regeneration. Apoplastic GSH is therefore expected to be rather involved in signaling than in detoxification (Zechmann et al., 2008). Phenolic compounds (Grace, 2007) may be widespread in the apoplastic fluid of plants and in some cases were shown to increase in response to O₃ (Langebartels et al., 1991; Eckey-Kaltenbach et al., 1993; Booker et al., 2012). However this increase was considered to have little effectiveness in scavenging O₃ in *Arabidopsis* (Booker et al., 2012). Cell wall bound phenolics may also scavenge ROS (Vreeburg and Fry, 2007) but their contribution to O₃ detoxification is yet unknown. In the cell wall of foliar and stem cells, O₃ exposure resulted in an increase in lignin biosynthesis in a dose-dependent manner suggesting a role in detoxification (Cabane et al., 2012). The contribution of phenolics and more widely secondary metabolites in apoplastic O₃ detoxification has not been sufficiently studied so far and should be further investigated especially because of their species-specific diversity.

Cytosol

Cytosol is an important determinant in the antioxidant capacity of the cell, acting as a hub for the production/transportation to other cell compartments and between compartments (Zechmann, 2017). Using labeling techniques (Zechmann, 2011), showed that the highest concentration of AsA was found in this compartment (**Figure 1**). Moreover, isoenzymes related to HAF cycle localized in the cytosol seem to play an important role in O₃ tolerance (Yoshida et al., 2006; Di Baccio et al., 2008; Mhamdi et al., 2010a; Rahantaniaina et al., 2017; Dusart et al., 2019). A good example of the cytosol interface is the GSH biosynthesis taking place both in cytosol and chloroplast (Rausch et al., 2007). Interestingly, although chloroplasts synthesize GSH, this metabolite is also readily taken up by intact chloroplasts (Foyer et al., 2001). For the other compartments, unable to carry out GSH biosynthesis, transport from cytosol must also be efficient (Rausch et al., 2007). While these carriers are far from being characterized, their regulation under oxidative stress is fully unknown.

Vacuole

In vacuole, the AsA concentration is often considered not to exceed 2 mM, making it the lowest within the plant cells (**Figure 1**; Zechmann, 2017). However, its level was 2- and 4-times increased during drought and high light stress, respectively, which represents the strongest increase among all subcellular compartments (Rautenkranz et al., 1994; Zechmann, 2017). Changes in AsA concentration under O₃ are not known. This raises the question whether vacuolar AsA could significantly contribute to cell defense against oxidative stress. When facing stress conditions, a large amount of H₂O₂ is transported and accumulated in the vacuole, which might act as a sink for ROS (Michalak, 2006; Koffler et al., 2014).

It is expected that the increase in vacuolar AsA contributes to delocalize the detoxification of H₂O₂ and thus to avoid cytosol redox imbalance. AsA seems to contribute to ROS scavenging in the vacuole, either directly or by coupling with phenolics and phenol oxidase. In fact, vacuoles are also well-known to hold large amounts of polyphenols, especially anthocyanins, flavan-3-ol monomers, proanthocyanidins, and glycosylated flavonols. Vacuolar flavonols have been suggested to contribute to H₂O₂ detoxification in this compartment, by giving electrons to phenol peroxidases (POX) (Sakihama et al., 2002). Furthermore, it has been shown that vacuolar AsA can reduce phenoxy radicals produced by POX and then regenerate phenolic compounds (Takahama and Oniki, 1997). Therefore, an alternative AsA/phenolics/POX mechanism might efficiently contribute to the cellular defense arsenal against ROS, jointly to the “classic” HAF cycle. On this point, none or very little free GSH (lower than 0.5 mM) has ever been identified in the vacuole, limiting the potential regeneration of ascorbate by this way in this compartment (Zechmann et al., 2008; Zechmann, 2014). In addition, DHAR was reported in the vacuole (Zhang et al., 2015) but HAF cycle does not seem to be functioning due to the lack of glutathione reductase isoform in this compartment, therefore leading to glutathione disulphide (GSSG) accumulation (Queval et al., 2011). In any case, oxidized AsA (DHA and monodehydroascorbate) can cross the tonoplast to be regenerated in the cytoplasm (Rautenkranz et al., 1994). In case of oxidative stress, vacuoles also act as a sink for oxidized GSH or GSH conjugates formed in the cytosol and transferred to the vacuole via the action of one or more ABC transporters of the MRP (Multidrug Resistance associated Protein) subclass (Queval et al., 2011; Koffler et al., 2014). Despite extensive investigation of tonoplast transporters, the role of MRPs in the transport of GSSG to the vacuole is less clear (Bachhawat et al., 2013) as well as the final fate of this oxidized form in the vacuole.

Chloroplast

Within the chloroplast, the electron transport chain is, in addition to its role as major energy producer, one of the main sites of endogenous ROS generation (Asada, 2006; Tripathy and Oelmüller, 2012). Being triggered and increased by apoplastic ROS, the chloroplastic ROS function as amplifiers of signals from outer cell compartments to the nucleus where they modify the nuclear gene expression (Shapiguzov et al., 2012; Foyer and Noctor, 2016; Kleine and Leister, 2016). The presence of AsA (10 mM) and GSH (1 mM) has been previously reported in the chloroplast where they represent a significant part of the cell antioxidant pool (Figure 1; Queval et al., 2011; Zechmann, 2011). Interestingly, abiotic stresses lead to a major increase in AsA and GSH contents in the chloroplast (Heyneke et al., 2013). Furthermore, although lacking under normal conditions, both antioxidants also accumulated inside the thylakoid lumen under stress, thus, demonstrating the particular dependence of the chloroplast on these antioxidants when facing harmful abiotic stress (Heyneke et al., 2013). Concerning AsA and GSH transport, chloroplast and cytosol are tightly connected by the presence of many transporters. AsA uptake by chloroplasts is mediated by a member of a phosphate transporter family, named

PHT4;4 (Ferne and Tóth, 2015; Miyaji et al., 2015). An active uptake of cytosolic GSH also occurred across the chloroplast envelope (Noctor et al., 2002), even though the molecular identity of the transporter(s) is still unknown (Bachhawat et al., 2013). In addition, three proteins belonging to the CRT (Chloroquine Resistance Transporter)-like transporter family were found to be chloroplastic and responsible for glutathione efflux from the chloroplast to the cytosol in *Arabidopsis* (Bachhawat et al., 2013).

At the same time, chloroplasts contain large amounts of flavonoid-like ROS defense agents, which they are able to biosynthesize (Hernández et al., 2009; Pollastri and Tattini, 2011). Under severe light, flavonoids complete the scavenging role of the most abundant lipid-soluble antioxidants group(s), the carotenoids (and tocopherols), in chloroplasts (DellaPenna and Pogson, 2006). Due to their ability to remodel lipid membranes, flavonoids might preserve the integrity of the chloroplast envelope and therefore prevent oxidative stress-caused damage (Agati et al., 2012).

Mitochondria

An endogenous production of ROS takes place in mitochondria essentially at the level of the complexes I, II, and III of the respiratory chain which must not be neglected even though the contribution of this organelle to oxidative stress is rather low (Apel and Hirt, 2004; Rhoads et al., 2006; Bettini et al., 2008; Waszczak et al., 2018). Mitochondria are well-supplied with antioxidants and the enzymes of the HAF cycle are present (Jiménez et al., 1997; Foyer et al., 2001; Foyer and Noctor, 2011). Similar as chloroplast, a concentration of 10 mM AsA was determined in mitochondria (Zechmann, 2011; Zechmann et al., 2011). Considering that the last step of ascorbate biosynthesis can take place in the intermembrane space in contact with complex I (Millar et al., 2003), it is now seen that DHA is transported in the matrix to subsequently be reduced by the HAF cycle (Navrot et al., 2007). In this context, a high concentration of GSH (between 7 and 15 mM) has been determined in this organelle (Zechmann et al., 2008; Queval et al., 2011; Zechmann, 2014), despite it is considered to be devoid of GSH synthesis pathway. This implies an uptake of GSH from the cytosol assumed by different transporters (Chen and Lash, 1998). In addition, there is no work mentioning the accumulation of phenols in the mitochondria, these compounds been known as inhibitors of the respiratory activity (Demos et al., 1975). Finally, in spite of a quite large panel of antioxidants, the mitochondria have been pointed out as being more sensitive to O₃ than chloroplasts (Pellinen et al., 1999). Nevertheless, there is no consensus to validate this difference of sensitivity between organelles (Sutinen et al., 1990).

Peroxisome

Due to H₂O₂ production driven by photorespiration, the peroxisome is also provided with antioxidant systems (Corpas et al., 2019). To minimize H₂O₂ accumulation in this compartment, a significant catalase activity has been observed. In addition, the presence of DHAR isoform suggests a functioning HAF cycle (Jiménez et al., 1997) in the peroxisome. A high concentration of AsA (23 mM) was determined in the peroxisome (Zechmann, 2017) while GSH was also detected but

at low level (4 mM) (Zechmann et al., 2008). O₃ increased catalase activity and enhanced the number of peroxisome in tolerant birch leaves (Oksanen et al., 2004). The increase of peroxisome number could be a response to an enhanced requirement for detoxification as photorespiration decreased (Booker et al., 1997; Bagard et al., 2008).

CONCLUSION

This article points out the great diversity of antioxidative systems, scattered in the different cellular compartments of leaves. The data so far published suggest that this diversity must be taken into account in O₃ risk assessment. However, under O₃, there is a lack of information regarding changes in the concentrations of the different antioxidants in each compartment under ozone treatment. As mentioned in previous works, and considering its occurrence in different cell compartments, it is obvious that the HAF cycle has a prominent role in cell detoxification. In addition, phenolic compounds in cell wall, vacuole and chloroplasts might also play a protective role. Subcellular immunocytochemical localization could allow a more precise

identification of the respective contribution of each compartment to the global defense system. The next step would be to get an integrative scheme allowing to improve the modeling for the participation of detoxification to risk assessment. Recently, an attempt to integrate the vacuole in an H₂O₂ metabolism under oxidative constraint model appeared to be promising, especially since transporters were considered (Tuzet et al., 2019). Indeed, the transport of antioxidants between compartments during oxidative stress should be also studied to better understand the role of compartmentation.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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