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Subcellular distribution of ascorbate in plants

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The compartment specific distribution of ascorbate in plants is of great importance for plant development, growth and defense as this multifunctional metabolite plays important roles in the detoxification of reactive oxygen species (ROS), redox signaling, modulation of gene expression and is important for the regulation of enzymatic activities. Even though changes in ascorbate contents during plant growth and various stress conditions are well documented and the roles of ascorbate in plant defense during abiotic stress conditions are well established, still too little is known about its compartment specific roles during plant development and defense. This mini-review focuses on the subcellular distribution of ascorbate in plants and describes different methods that are currently used to study its compartment specific distribution. Finally, it will also briefly discuss data available on compartment specific changes of ascorbate during some abiotic stress conditions such as high light conditions and exposure to ozone.

Ascorbate is one of the most important antioxidants in plants and animals. It detoxifies reactive oxygen species (ROS) either directly or through the glutathione-ascorbate cycle (**Fig. 1**) and is involved in redox signaling, modulation of gene expression and the regulation of enzymatic activities (extensively reviewed in ref. 1 and 2). Ascorbate occurs in a reduced form (ascorbic acid) and two oxidized forms (mono- and dehydroascorbic acid). The ratio between reduced and oxidized ascorbate is essential for the ability of the plant to fight oxidative stress. During environmental stress situations when ROS are formed inside the cell, large amounts of dehydroascorbic acid can be formed by oxidation of ascorbic acid which shifts the ascorbate pool more towards the oxidative state and diminishes the antioxidative capacity of the plant. Additionally, environmental stress situations can change total ascorbate contents in plants which makes ascorbate an important stress marker during abiotic and biotic stress situations.[3-11] Ascorbate contents are typically measured biochemically in individual plant organs or tissues and the obtained values represent a combination of the ascorbate status of all individual organelles. As many environmental stress conditions induce highly compartment specific stress responses changes of ascorbate contents in individual organelles might not be detected when ascorbate is measured in whole organs or tissues. This is crucial as data obtained about the antioxidative status from individual organs are often used to interpret the stress response of the whole plant to the exposed stress conditions. Thus, in order to gain a deeper insight into the defense response of plants it is essential to measure changes in the subcellular distribution of these components during environmental stress situations.

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Subcellular Distribution of Ascorbate in Plants

The detection of ascorbate on the subcellular level is technically challenging as it can be easily washed out and/or redistributed during sample preparation which is also due to the fact that sample preparation itself can be seen as a stress to the plant. Currently, there are several approaches available that have been used to study the subcellular distribution of ascorbate in plants. Despite their advantages and disadvantages, which are not discussed here, they have all given valuable insights into the subcellular distribution of ascorbate in plants. With biochemical measurements ascorbate was detected in isolated chloroplasts,[12] mitochondria and peroxisomes[13,14] and also occurs in vacuoles.[15] Additionally, ascorbate was found in the apoplast of barley, birch and poplar leaves.[3-5,16,17] With hiostochemical methods, ascorbate was detected in roots of Cucurbita plants and was localized at the nuclear membrane, in nucleoli and along the plasma membrane.[18] These earlier reports have been confirmed and extended by a recently developed method that enabled the subcellular detection of ascorbate by transmission electron microscopy in all cell compartments simultaneously in one experiment on a high level of resolution.[19] With this method ascorbate could be localized in different concentrations in mitochondria, chloroplasts, nuclei, peroxisomes, the cytosol, vacuoles and along the membranes of the ER[19] (**Fig. 2**) within mesophyll cells. A similar subcellular distribution of ascorbate was found in epidermis cells as well as in vascular bundle cells. In the latter ascorbate could be detected in companion cells, in sieve elements and also in xylem vessels[19] indicating that ascorbate can be transported within the plant and exchanged between different organs through vascular bundle cells. In both Arabidopsis and Nicotiana plants highest levels of ascorbate specific labeling were found in the cytosol followed by peroxisomes and nuclei. Vacuoles contained the lowest levels of ascorbate whereas mitochondria and chloroplasts contained intermediate levels (**Fig. 3**). Within chloroplasts and mitochondria, ascorbate was only detected in the stroma and matrix, respectively, but not within the lumen of thylakoids or cristae.[19] This situation changed during high light stress as described in the next section.

Ascorbate was not detected in the apoplast with microscopical methods.[18,19] Nevertheless, with biochemical methods it could be

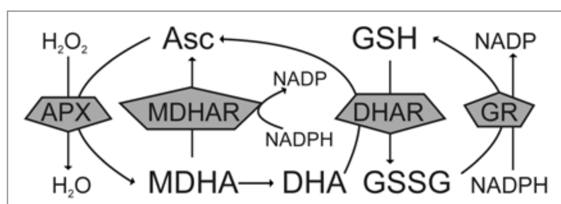

Figure 1. Ascorbate-glutathione cycle in plants. Hydrogen peroxide (H_2O_2) within the plant cell can be detoxified by ascorbate peroxidase (APX). In this reaction the reduced form of ascorbate (Asc) is oxidized to monodehydroascorbate (MDHA). MDHA is then either reduced by monodehydroascorbate reductase (MDHAR) to Asc or, since very unstable, reacts to dehydroascorbate (DHA). DHA is reduced by dehydroascorbate reductase (DHAR) to Asc. In this reaction the reduced form of glutathione (GSH) is oxidized to glutathione disulfide (GSSG). GSSG is then reduced by glutathione reductase (GR) to GSH. The electron acceptor NADP is regenerated during the reduction of MDHA and GSSG by the respective enzymes. Asc and GSH are additional able to detoxify reactive oxygen species by direct chemical interaction. Thus, besides the total ascorbate level their redox state (reduced vs. oxidized state) which depends on the activity of the described enzymes (grey boxes) is also very important for successful plant protection.

demonstrated that ascorbate is also present in the apoplast where it is considered to be the only significant redox buffer[2,20] and to play an important role in the protection against ozone.[10,21,22] Thus, it seems that either the concentrations of ascorbate in the apoplast are extremely low—too low to be detected with histochemical methods—or that the levels detected with biochemical methods represent contaminations from the cytoplasm. With electron microscopical approaches ascorbate could be detected in the apoplast of vascular parenchyma cells facing xylem vessels[19] but not in other cell types within the leaf (**Fig. 2**). As ascorbate was found at very low concentrations in apoplastic extracts[3-5,16,23] with biochemical methods (around or less than 10% of the total ascorbate content in leaves) it could be possible that the data obtained from these biochemical studies could partly represent the ascorbate pool of the apoplast of vascular bundle cells. Thus, in order to further analyze the significance and importance of ascorbate in the apoplast it will be necessary to study its subcellular distribution by immunogold labeling and quantitative transmission electron microscopy during stress situations that are well known to increase apoplastic ascorbate contents such as the exposure to ozone.[10,21,22]

Changes of Subcellular Ascorbate Contents during Abiotic Stress

Many studies have documented changes of ascorbate contents in plants during abiotic stress conditions such as excess light, high ozone levels, heavy metals and UV-radiation.[6-8,11,21,22,24-26] Nevertheless, most of these studies have investigated the situation in whole organs or tissues, rather than in single cells and organelles and therefore very little data is available about changes on the subcellular level during these abiotic stress conditions. Considering that most of the above mentioned stress situations induce highly compartment-specific stress before the whole organ or plant is affected this is essential to understand plant defense on the cellular level.

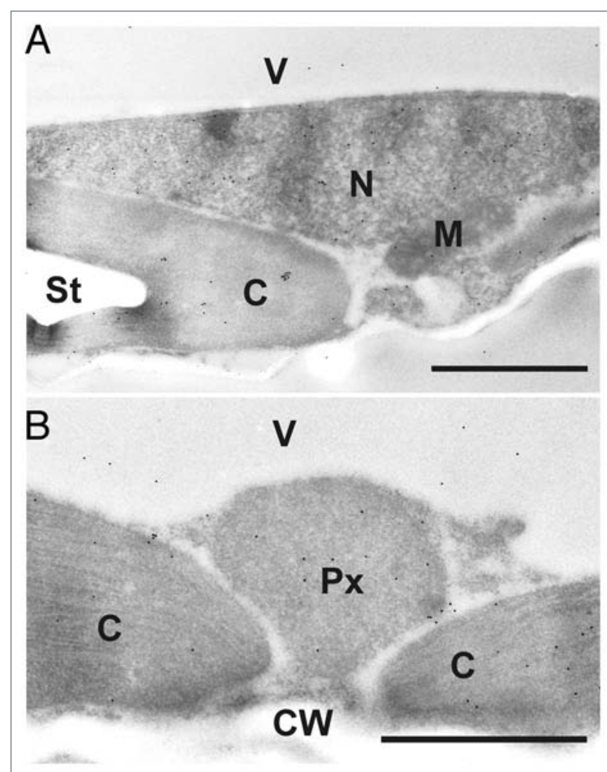

Figure 2. Transmission electron micrographs showing the subcellular distribution of ascorbate in plants. In mesophyll cells of leaves from (a) *Nicotiana tabacum* (L.) cv. Samsun nn and (b) *Arabidopsis thaliana* Col-0 ascorbate specific immunogold labelling was found in mitochondria (M), chloroplasts (C), nuclei (N), peroxisomes (Px), vacuoles (V), the cytosol but not in cell walls (CW) and intercellular spaces (IS). St = starch; Bars = 1 μm.

Excess light. Excess light is a unique stress to the plant that mainly affects metabolic changes in chloroplasts first before other cell compartments are affected.[27-29] It leads to the production of ROS by overstraining the reactions during photosynthesis.[30-33] The generated radicals are then able to damage many cellular components including the photosystems in chloroplasts which are essential for photosynthesis and therefore for the survival of the plant. An increase in ascorbate contents during high light conditions was commonly found with biochemical methods[2,7,8,24,26] indicating an important role of ascorbate in the protection against excess light stress. Nevertheless, all of these studies were done by looking at ascorbate levels in whole leaves and therefore the antioxidative defense situation on the subcellular level remained unclear.

On the subcellular level ascorbate was found to be strongly increased in most cell compartments during high light conditions (**Table 1**).[19] Surprisingly, the strongest increase in ascorbate specific labelling was found in vacuoles and not in chloroplasts, which opens the question about the importance of vacuoles for ascorbate metabolism during stress conditions. It has been proposed recently that ascorbate plays an important role in the detoxification of H_2O_2 that diffuses into vacuoles especially during environmental stress situations.[15] In this cell compartment ascorbate helps to reduce phenoxyl radicals

(created by oxidation of phenols by H_2O_2) and is oxidized to mono- and dehydroascorbic acid which is then transported into the cytosol for reduction to ascorbic acid.[15] Thus, it can be concluded that ascorbate plays important roles in vacuoles during excess light conditions most probably by direct or indirect detoxification of H_2O_2 produced in chloroplasts and leaking into vacuoles.

A very strong increase in ascorbate contents was also detected in chloroplasts (104%) after the exposure to light conditions of 700 μmol m^{-2} s^{-1} for two weeks (**Table 1**). This data highlights the importance of ascorbate in the antioxidative protection against oxidative stress induced in this cell compartment during high light conditions. Ascorbate could also be detected inside the lumen of thylakoids of chloroplasts exposed to high light stress.[19] This observation is interesting in respect to non-photochemical quenching which decreases the formation of ROS by dissipation of excess absorbed light as heat. One important mechanism for non-photochemical quenching is the formation of zeaxanthin from violaxanthin that is catalysed by the enzyme violaxanthin de-epoxidase. This enzyme is located inside the thylakoid lumen and uses ascorbic acid as a reductant.[34-37] Thus, the detection of ascorbate in the thylakoid lumen of plants exposed to high light conditions and the general increase inside the stroma highlights the importance of high ascorbate contents for the compartment specific protection of chloroplasts during high light conditions.

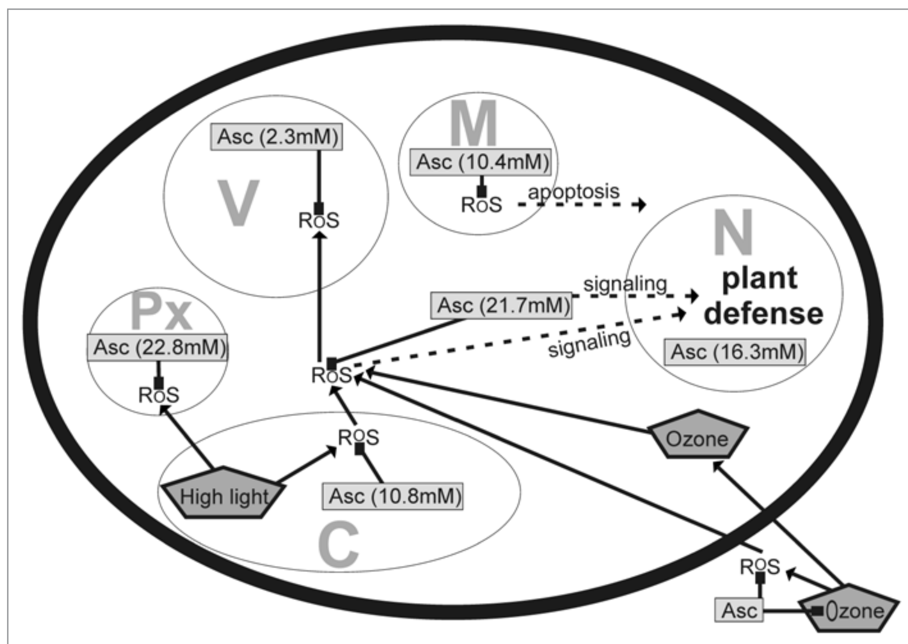

Figure 3. Compartment specific concentrations of ascorbate (Asc) in *Arabidopsis thaliana* accession Col-0 plants and occurrence of reactive oxygen species (ROS) during high light stress and ozone exposure. Compartment specific concentrations of ascorbate were calculated by setting a total ascorbate content of 5.6 μmol/g fresh weight determined by HPLC equal to an estimated average ascorbate content of 5.6 mM per cell. This concentration corresponded to the average labeling density throughout the cell of 5.1 gold particles per μm^2. Thus gold particle density in the individual cell compartments could be correlated with different ascorbate concentrations in mM (for details see ref. 19). C, chloroplast; M, mitochondrium; N, nucleus; Px, peroxisome; V, vacuole.

Ozone. Ozone is highly dangerous to the integrity of plant cells as it is capable to oxidize a variety of cell components such as sulfhydryl groups (e.g., glutathione), double bonds of fatty acids, amino acids and other antioxidants such as ascorbate and tocopherol. In water it can produce ROS such as hydrogen peroxide, superoxides anion radical and hydroxyl radical.[38] Unlike other ROS ozone is not produced inside the plant cell but enters plant organs through open stomata. Thus, the first reaction site is the apoplast and the plasma membrane where the major part of ozone gets detoxified by ascorbate. The remaining ozone degrades into other ROS after it dissolves in the apoplastic fluid or after it enters the cytosol (**Fig. 3**). ROS inside the cell can then be detoxified by ascorbate as well as other antioxidants which is reflected by commonly observed changes in antioxidants during ozone exposure.[10,21,22] Ascorbic acid seems to be the most important antioxidant in the detoxification of ozone in the apoplast and at the plasma membrane. It reacts with ozone to dehydroascorbate and is then transported into the cytosol where it is reduced to ascorbic acid by the help of reduced glutathione. Ascorbic acid is then transported back into the apoplast where it can detoxify ozone again. Despite the proposed role of ascorbate in the protection against ozone in the apoplast the importance of these reactions are challenged by the observations that some ascorbate deficient mutants (*vtc2-2, vtc2-3, vtc3-1, vtc4-1*) which contain only about 30% ascorbate levels of the wildtype are not or only slightly more sensitive to ozone than the wildtype.[39] Additionally, ascorbate could not be detected with immunocytohistochemical investigations in the apoplast of non-stressed and wildtype plants exposed to high light conditions and the ascorbate deficient mutant *vtc1-2* and *vtc2-1*.[19] As ascorbate has been detected in quite low amounts (under 10% of the total ascorbate content in leaves) in the apoplast with biochemical methods it seems that besides ascorbate also other components might be important for the detoxification of ozone in the apoplast. Nevertheless, it remains unclear if ascorbate could also be detected in the apoplast after ozone stress by immunogold labeling of these components and further studies are necessary to clarify the importance of ascorbate in the protection against ozone in the apoplast.

Conclusion and Perspective

Changes in the subcellular distribution of ascorbate in plants are of great importance to understand the compartment specific antioxidative stress response which is responsible for direct detoxification of ROS and defense signaling. As it is possible now to study changes in the subcellular distribution of ascorbate in

Table 1. Changes in the subcelluar distribution of ascorbate in *Arabidopsis thaliana* accession Col-0 plants exposed to excess light conditions of about 700 μmol m^{-2} s^{-1} for two weeks when compared to plants grown at 150 μmol m^{-2} s^{-1},[19]

	Mitochondria	Chloroplasts	Nuclei	Peroxisomes	Cytosol	Vacuoles
% change	35% (*)	104% (***)	35% (*)	-31% (**)	35% (**)	395% (***)

all cell compartments simultaneously in one experiment it will be subject of future investigations to link compartment specific changes of ascorbate with abiotic and biotic stress situations. As ascorbate levels closely interact with antioxidative defense mechanisms it will also be important to correlate such changes with compartment specific changes of other antioxidants (e.g., glutathione) and substances involved in plant defense. Thus, it should be possible in the near future to gain a deeper understanding of the compartment specific importance of antioxidants for plant development, growth and defense.

Acknowledgements

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