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ORIGINAL ARTICLE

Immunocytochemical determination of the subcellular distribution of ascorbate in plants

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Abstract Ascorbate is an important antioxidant in plants and fulfills many functions related to plant defense, redox signaling and modulation of gene expression. We have analyzed the subcellular distribution of reduced and oxidized ascorbate in leaf cells of *Arabidopsis thaliana* and *Nicotiana tabacum* by high-resolution immuno electron microscopy. The accuracy and specificity of the applied method is supported by several observations. First, preadsorption of the ascorbate antisera with ascorbic acid or dehydroascorbic acid resulted in the reduction of the labeling to background levels. Second, the overall labeling density was reduced between 50 and 61% in the ascorbate-deficient Arabidopsis mutants *vtc1-2* and *vtc2-1*, which correlated well with biochemical measurements. The highest ascorbate-specific labeling was detected in nuclei and the cytosol whereas the lowest levels were found in vacuoles. Intermediate labeling was observed in chloroplasts, mitochondria and peroxisomes. This method was used to determine the subcellular ascorbate distribution in leaf cells of plants exposed to high light intensity, a stress factor that is well known to cause an increase in cellular ascorbate concentration. High light intensities resulted in a strong increase in overall labeling density. Interestingly, the strongest compartment-specific increase was found in vacuoles (fourfold) and in plastids (twofold). Ascorbate-specific labeling was restricted to the matrix of mitochondria and to the stroma of chloroplasts in control plants but was also detected in the lumen of thylakoids after high light exposure. In summary, this study reveals an improved insight into the subcellular distribution of ascorbate in plants and the method can now be applied to determine compartment-specific changes in ascorbate in response to various stress situations.

Keywords Arabidopsis · Ascorbate · High light · Immunocytochemistry · *Nicotiana* · Transmission electron microscopy · Vitamin C

Abbreviations

BSA	Bovine serum albumin
ER	Endoplasmic reticulum
H_2O_2	Hydrogen peroxide
NADPH	Nicotinamide adenine dinucleotide phosphate
ROS	Reactive oxygen species
Vtc	Vitamin C

Introduction

Ascorbate is a multifunctional metabolite in plants that is essential for plant development and growth. It fulfills crucial roles as an antioxidant in plant defense against abiotic and biotic stress and it is important for the regulation of enzymatic activities. It is also involved in redox signaling and in the modulation of gene expression (Noctor and Foyer 1998; Noctor 2006; Foyer and Noctor 2009). Changes in ascorbate levels can be observed during different environmental stress situations such as exposure to high light, ozone, heavy metals and pathogen attack (Vanacker et al. 1998; Ratkevicius et al. 2003; Bartoli et al. 2006;

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Giacomelli et al. 2006; Collin et al. 2008; Sandermann 2008; Chamseddine et al. 2009).

Ascorbate occurs in a reduced form (ascorbic acid) and two oxidized forms (mono and dehydroascorbic acid). Ascorbic acid is able to detoxify reactive oxygen species (ROS) either by direct chemical interaction or through reactions catalyzed by ascorbate peroxidases. Through these processes large amounts of oxidized ascorbic acid can be produced, especially during environmental stress situations. Mono- and dehydroascorbic acid are reduced to ascorbic acid through mono- and dehydroascorbate reductase, respectively. Both processes consume NADPH and the latter process also involves the oxidation of glutathione (Noctor and Foyer 1998; Noctor 2006; Foyer and Noctor 2009). Thus, ascorbate homeostasis in different organs, tissues and cell compartments is controlled by a complex network of metabolic and environmental factors. Ascorbate synthesis is one of these factors. In Arabidopsis the only known pathway of ascorbate synthesis is the L-galactose pathway (Wheeler et al. 1998; Dowdle et al. 2007). The last enzyme of this pathway (galactonolactone dehydrogenase) is physically associated with the mitochondrial complex I rendering mitochondria the main and only center of ascorbate synthesis in Arabidopsis. Additionally, ascorbate synthesis is functionally linked to the respiratory electron transport chain as galactonolactone dehydrogenase uses oxidized cytochrome c as an electron acceptor (Bartoli et al. 2000; Millar et al. 2003).

The compartmentation of ascorbate is well established but compartment-specific levels of ascorbate remained unclear as it was not possible so far to simultaneously quantify subcellular ascorbate levels in all cell compartments. Two methods were mainly used to detect ascorbate in different cell compartments. Ascorbate was detected with biochemical methods in the apoplast of barley, birch and poplar leaves (Vanacker et al. 1998; van Hove et al. 2001; Kollist et al. 2001) where it is considered to be the only significant redox buffer (Pignocchi and Foyer 2003; Foyer and Noctor 2009) and to play an important role in the protection against ozone (Conklin and Barth 2004; Baier et al. 2005; Sandermann 2008). Additionally, ascorbate was detected in isolated chloroplasts (Noctor et al. 2002), mitochondria and peroxisomes (Jiménez et al. 1997, 1998). Ascorbate also occurs in vacuoles (Takahama 2004). Ascorbate was histochemically detected in roots of *Cucurbita* plants and was localized at the nuclear membrane, in nucleoli, along the plasma membrane but was surprisingly absent in the apoplast and in vacuoles (Liso et al. 2004). It was not possible with this method to obtain more information about the localization of ascorbate in smaller organelles such as plastids, mitochondria, dictyosomes and endoplasmic reticulum (ER) and to quantify compartment-specific differences (Liso et al. 2004).

In this study, we describe a method that allows the detection of oxidized and reduced ascorbate within subcellular compartments of leaf cells at a high level of resolution. This method is based on immunocytohistochemistry combined with computer supported transmission electron microscopy. In order to verify the accuracy of the obtained signal, several negative controls were performed. Additionally, ascorbate-specific labeling was determined in the ascorbate-deficient Arabidopsis *vtc1-2* and *vtc2-1* mutants, respectively. Both mutants have defects in enzymes involved in ascorbate synthesis, thus leading to a strong decrease in ascorbate levels (Conklin et al. 2000; Giacomelli et al. 2006; Olmos et al. 2006; Müller-Moule 2008; Foyer and Noctor 2009). Subcellular ascorbate labeling was also investigated in plants exposed to high light intensity that is known to increase ascorbate contents in plants (Müller-Moule et al. 2004; Bartoli et al. 2006; Giacomelli et al. 2006; Haghjou et al. 2009; Foyer and Noctor 2009). The aim of the present study was to analyze the subcellular distribution of ascorbate in plants and to study changes in compartment-specific ascorbate levels in plants exposed to high light intensities.

Materials and methods

Plant material and growth conditions

After stratification for 4 days at 4°C seeds of *Arabidopsis thaliana* [L.] Heynh. Ecotype Columbia (Col-0) originally obtained from the European Arabidopsis stock centre (NASC; Loughborough, UK), and the ascorbate–deficient mutant lines *vtc1-2* and *vtc2-1* were grown on soil in growth chambers with 10/14 h day/night photoperiod. Day and night temperatures were 22 and 18°C, respectively. The relative humidity was 60% and the plants were kept at 100% relative soil water content. Light intensity was 150 µmol m^{-2} s^{-1}. Four weeks after stratification, samples from the youngest fully developed rosette leaf were harvested 2 h after the onset of the light period and prepared for electron microscopy. Leaves at this stage were approximately 2 cm in length and 0.7 cm in width. High light conditions were induced by exposing 2-week-old wild-type plants to 700 µmol m^{-2} s^{-1} light for 2 weeks in the same growth conditions as described above. *Nicotiana tabacum* (L.) cv. Samsun nn, obtained from the German resource centre for biological material (DSMZ, Braunschweig, Germany), were grown at constant conditions of day/night temperature 24/20°C, illumination 250 µmol m^{-2} s^{-1}, a photoperiod of 16/8 h light/dark and a humidity of 70%. Six weeks after germination the youngest fully developed leaves (about 10 cm in length and 6 cm in width) were harvested about 2 h after the onset of the light period and

prepared for transmission electron microscopy. As ascorbate contents are influenced by changes in light quality and intensity special care was taken that each harvested leaf sample was fully exposed to the high light condition and that it was cut in a drop of the fixative solution as described below immediately (within a few seconds) after removing them from the growth chambers. Every experiment was repeated at least twice (with similar results) and the results of 4–6 replicate plants out of these experiments were combined for each data set.

Sample preparation for transmission electron microscopy

Small leaf samples from the middle of the leaves close to the middle vein (about 1.5 mm^2) were cut on a modeling wax plate in a drop of 2.5% paraformaldehyde/0.5% glutardialdehyde in 0.06 M Sørensen phosphate buffer (pH 7.2). Samples were then transferred into glass vials and fixed for 90 min at room temperature in the above-mentioned solution. Microwave fixation was performed in the same fixation solution as described above in a Polar Patent PP1000 microwave oven. Samples were fixed two times for 25 s at 300 W microwave irradiation. In between these steps samples were gently cooled off to about 20°C which took about 3 min. The maximum temperature of the solution, which was constantly aerated to reduce the risk of an unevenly heated solution, during fixation in the microwave oven was 30°C. Samples were then rinsed in buffer (4 times 15 min each) after fixation and then dehydrated in increasing concentrations of acetone (50, 70, and 90%) 2 times for 10 min each. Subsequently, specimens were gradually infiltrated with increasing concentrations of LR-White resin (30, 60 and 100%; London Resin Company Ltd., Berkshire, UK), mixed with acetone (90%) for a minimum of 3 h per step. Samples were finally embedded in pure, fresh LR-White resin and polymerised at 50°C for 48 h in small plastic containers under anaerobic conditions. Ultrathin sections (80 nm) were cut with a Reichert Ultracut S ultramicrotome (Leica Microsystems, Vienna, Austria).

High-pressure freezing and freeze substitution was performed according to Zechmann et al. (2005, 2007). Briefly, small samples were high-pressure frozen with the Leica EM Pact (Leica, Microsystems). Freeze substitution was carried out in 0.5% glutardialdehyde in anhydrous acetone in a deep freezer at −80°C (72 h), −65°C (24 h), −30°C (24 h), 0°C (12 h) and 20°C (1 h). After the samples were rinsed twice in anhydrous acetone for 15 min, they were infiltrated with different mixtures (2:1, 1:1, 1:2) of acetone and LR-White resin and pure resin for at least 3 h, each step at room temperature. The embedded samples were then polymerised in pure, fresh LR-White resin for 48 h at 50°C. Ultrathin sections (80 nm) were cut with a Reichert Ultracut S ultramicrotome (Leica, Microsystems).

Cytohistochemical analysis

Immunogold labeling was done with ultrathin sections on coated nickel grids with the automated immunogold labeling system Leica EM IGL (Leica, Microsystems). The ideal dilution of the primary (anti-ascorbate IgG; Abcam plc, Cambridge, UK) and secondary antibody (goat anti rat IgG; British BioCell International, Cardiff, UK) was determined in preliminary studies by evaluating the labeling density after a series of labeling experiments. The final dilution of the primary and secondary antibody used in this study showed a minimum of background labeling outside the sample with a maximum of specific labeling in the sample. Polyclonal antibodies against ascorbate were raised in rat immunized with ascorbic acid conjugated to bovine serum albumin (BSA). Conjugation was performed through a carbodiimide reaction where the carboxyl group of ascorbic acid was conjugated with the amino groups of BSA.

For cytohistochemical analysis samples were blocked with 2% BSA in phosphate buffered saline (PBS, pH 7.2) for 20 min at room temperature. The excess of blocking solution on the grids was removed with filter paper and the samples were then treated with the primary antibody diluted 1:300 in PBS for 2 h. After short rinses in PBS (3 times 5 min) the samples were incubated with a 10 nm gold-conjugated secondary antibody diluted 1:100 in PBS for 90 min. After short washes in PBS (3 times 5 min) and distilled water (2 times 5 min) labeled grids were either immediately observed in a Philips CM10 transmission electron microscope or post-stained with uranyl acetate (2% dissolved in aqua bidest) for 15 s. Post-staining with uranyl acetate was applied to facilitate the distinction of different cell structures enabling a clearer identification of the investigated organelles. The antibody does not discriminate between reduced and oxidized form of ascorbic acid (Abcam plc).

Preadsorption experiments and negative controls

Several negative controls were made to support the specificity of the immunogold procedure. Negative controls were treated either with (1) gold-conjugated secondary antibody (goat anti rat IgG) without prior incubation of the section with the primary antibody, (2) non-specific secondary antibody (goat anti-rabbit IgG), (3) preimmune serum instead of the primary antibody and (4) primary antibody preadsorbed with an excess of reduced and oxidized ascorbate for 2 h prior to labeling of the sections. For the latter a solution containing either 10 mM of ascorbic acid or dehydroascorbic acid was incubated with or without 0.5% glutardialdehyde for 1 h. When glutardialdehyde was used then its excess was saturated by incubation for 30 min in a solution of 1% (w/v) BSA. The resulting solutions were both used in independent experiments to saturate the

anti-ascorbate antibodies for 2 h prior to its use in the immunogold labeling procedure described above.

Quantitative analysis of immunogold labeling

Micrographs of randomly photographed immunogold labeled sections of mesophyll cells were digitized and gold particles were counted automatically using the software package Cell D with the particle analysis tool (Olympus, Life and Material Science Europa GmbH, Hamburg, Germany) in different visually identified cell structures (mitochondria, plastids, nuclei, peroxisomes, the cytosol, vacuoles). Due to the low amount of gold particles found in cell walls, ER and dictyosomes, no statistical evaluation of the gold particle density was made for these compartments. For statistical evaluation at least three different samples were examined. A minimum of 20 (peroxisomes) to 60 (other cell structures) sectioned cell structures of at least 15 different cells throughout the blocks were analyzed for gold particle density. The obtained data were presented as the number of gold particles per μm^2. Unspecific background labeling was determined on 30 different sections (outside the specimen) from 5 different samples and subtracted from the values obtained in the sample. Unspecific background labeling was around 0.3 gold particles per μm^2. For all statistical analyses the non-parametric Kruskal–Wallis test followed by a post hoc comparison according to Conover was used; $P < 0.05$ was considered as significant.

Results

Immunocytochemical methods were used to determine the subcellular distribution of ascorbate in leaves of Arabidopsis and *Nicotiana tabacum*. Arabidopsis accession Col-0 and the Arabidopsis mutants *vtc1-2* and *vtc2-1* with globally decreased ascorbate accumulation and wild-type plants exposed to high light as stress factor were used in these studies. Various negative controls were performed in order to support the specificity of the obtained signal. The subcellular distribution of ascorbate and the ratio between cell compartments was similar at the edge of the samples, which get in contact with the fixative immediately, when compared to levels throughout the samples. Additionally, conventionally prepared samples showed similar ascorbate-specific labeling when compared to samples prepared with the help of microwave fixation and after high-pressure freezing and freeze substitution (Supplemental Figs. S1, S2; Table 1). As conventional fixation is widely used, easy to apply in the lab and in the field, does not require expensive equipment and gives fast and reliable results, we have performed all further experiments with a standardized protocol widely used for immunocytohistochemical investigations.

Specificity tests for ascorbate labeling

Ascorbate labeling in plants raised at light intensity of 150 $\mu mol\ m^{-2}\ s^{-1}$ was present in all cell compartments except cell walls and intercellular spaces (Fig. 1a). The accuracy and specificity of the observed labeling was supported by several control experiments (Fig. 1b–d, g). The preadsorption of the primary antibody with an excess of either ascorbic acid or dehydroascorbic acid and the treatment of sections with preimmune serum instead of the primary antibody (Fig. 1b–d) reduced the labeling density to background values thus indicating that the observed immunogold labeling appears to be solely linked to ascorbate. Additionally, two ascorbate-deficient mutants showed a

Table 1 Ratio of ascorbate between organelles

	Ratio of ascorbate between organelles					
	Mitochondria	Chloroplasts	Nuclei	Peroxisomes	Cytosol	Vacuole
Arabidopsis						
Conventional fixation	12.2	12.8	19.4	27.1	25.8	2.7
Microwave fixation	12.5	13.9	19	25.9	24.8	3.9
Edge of the sample	11.4	13.7	20.5	25.2	25.8	3.4
High-pressure freezing	12.9	10.9	22.2	25.4	24	4.6
Nicotiana						
Conventional fixation	11.9	10.7	31.9	10	31.4	4.1
Microwave fixation	11.3	12.2	30.3	10.2	30.4	5.6
Edge of the sample	11.2	10.5	33.8	9.4	31.1	4
High-pressure freezing	10.7	11.3	31.5	11.3	29.6	5.6

Ratio (in %) of the distribution of gold particles bound to ascorbate (total amount of gold particles per μm^2) between cell compartments of plants prepared with conventional and microwave fixation and at the edge of the sample after conventional fixation and after high-pressure freezing and freeze substitution

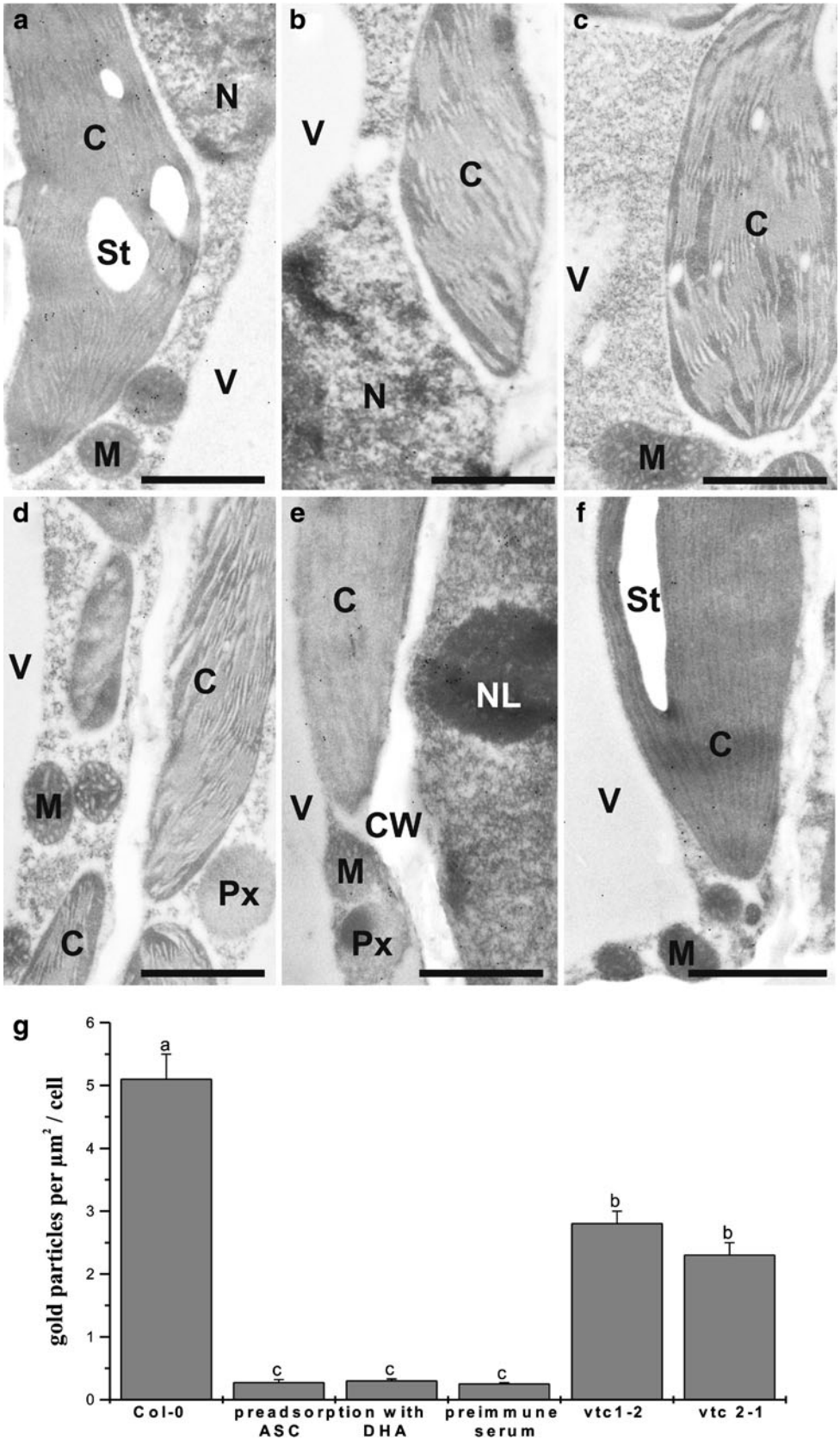

Fig. 1 Specificity of immunocytochemical ascorbate labeling. Transmission electron micrographs (**a**–**f**) and quantitative analysis (**g**) showing the overall distribution of gold particles bound to ascorbate in leaf cells of Arabidopsis Col-0 plants (**a**–**d**) and the Arabidopsis mutants *vtc1-2* (**e**) and *vtc2-1* (**f**). **a** Ascorbate-specific labeling detected in different cell compartments of Arabidopsis Col-0 plants such as chloroplasts (*C*), mitochondria (*M*), nuclei (*N*), and vacuoles (*V*). Negative controls include preadsorption of the anti-ascorbate antisera with ascorbic acid (ASC, **b**), dihydroascorbate (DHA, **c**), and the treatment of sections with preimmune serum instead of the primary antibody (**d**). Very few or no gold particles were present on these sections. Gold particle density was significantly lower in cells of the *vtc1-2* (**e**) and *vtc2-1* (**f**) mutants. *CW* cell walls, *NL* nucleoli, *Px* peroxisomes, *St* starch. *Bars* 1 µm. **g** Significant differences between the samples are indicated by *different lowercase letters*; samples which are significantly different from each other have no letter in common. $P < 0.05$ was regarded significant analyzed by the Kruskal–Wallis test, followed by post hoc comparison according to Conover

strong decrease in ascorbate-specific labeling in all cell compartments. Generally, the *vtc2-1* mutant showed lower levels than the *vtc1-2* mutant (Figs. 1e–g, 2). A decrease in ascorbate within leaves of the *vtc1-2* and the *vtc2-1* mutant was observed in mitochondria (44 and 76%), chloroplasts (44 and 50%), nuclei (54 and 65%), peroxisomes (70 and 76%), and vacuoles (43 and 52%). On a global level immunogold labeling of ascorbate decreased about 50 and 61% in *vtc1-2* and *vtc2-1* mutants, respectively. These data correlated well with a decrease in total ascorbate content measured with HPLC of about 46 and 55% in *vtc1-2* and *vtc2-1* mutants, respectively when compared to the wildtype (Supplemental Fig. S3). In similar studies a decrease of about 50 and 70% was found with biochemical investigations in *vtc1-2* and *vtc2-1* mutants (Conklin et al. 2000; Olmos et al. 2006; Müller-Moule 2008; Foyer and Noctor 2009).

Subcellular distribution of acorbate in leaves of Arabidopsis and *Nicotiana tabacum*

In both Arabidopsis and *N. tabaccum*, the highest concentration of ascorbate-specific labeling was found in the cytosol, the lowest ones in vacuoles. No ascorbate was detected in cell walls (Figs. 3, 4). Arabidopsis peroxisomes contained similar levels as the cytosol (Fig. 4). Intermediate labeling density was observed in mitochondria and plastids (Fig. 4). Arabidopsis mitochondria and plastids contained a 53 and 50%, respectively, lower ascorbate concentration than the cytosol. The situation was similar in *N. tabacum* where mitochondria and plastids contained about 62 and 66% less gold particles bound to ascorbate than the cytosol (Fig. 4). Nuclei contained about the same amount of gold particles bound to ascorbate as the cytosol in *N. tabacum*. Within mitochondria ascorbate was detected in the matrix but not in the lumen (Fig. 3b). Within chloroplasts of control plants ascorbate was detected in the stroma, and along the outside of the thylakoid membranes, but gold particles were absent in the lumen of thylakoids (Fig. 3c).

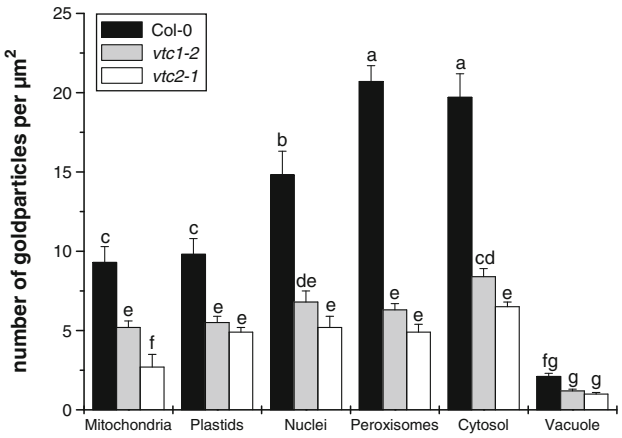

Fig. 2 Quantitative analysis of ascorbate-specific labeling in the ascorbate-deficient mutants *vtc1-2* and *vtc2-1*. *Graph* shows means with standard errors and documents changes in the density of gold particles bound to ascorbate in Arabidopsis leaf cells of the *vtc1-2* and *vtc2-1* mutants in comparison to the wild-type Col-0. Significant differences between the samples are indicated by *different lowercase letters*; samples which are significantly different from each other have no letter in common. $P < 0.05$ was regarded significant, analyzed by the Kruskal–Wallis test, followed by post hoc comparison according to Conover. $n > 20$ for peroxisomes and vacuoles and $n > 60$ for all other cell structures

Occasionally, ascorbate-specific labeling was found to be associated with the membrane of the ER but was never found inside the ER lumen (Fig. 3d). Ascorbate labeling was not associated with dictyosomes (inset in Fig. 3a). Ascorbate was also detected within vascular bundle cells (Fig. 5). Companion cells showed ascorbate labeling in mitochondria, the cytosol and within vacuoles (Fig. 5a). Ascorbate was also detected in sieve elements (Fig. 5a). A similar situation was found for vascular parenchyma cells where gold particles were found in all cell compartments including vacuoles (Fig. 5b). Gold particles bound to ascorbate were also found in plasmodesmata of phloem cells (inset in Fig. 5a) and in the cell walls and lumen of xylem vessels (Fig. 5b).

Total ascorbate contents in leaves of about 5.6 µmol/g fresh weight were determined in Arabidopsis Col-0 plants with HPLC measurements (Supplemental Fig. S3). This corresponds to an estimated average concentration of about 5.6 mM total ascorbate per leaf cell. The average labeling in one cell was calculated to be 5.1 gold particles per μm^2 (Fig. 1g). Hence, total ascorbate contents of 5.6 µmol/g fresh weight correspond to 5.1 gold particles per μm^2 per cell. Thus, compartment-specific labeling density in Arabidopsis Col-0 plants shown in Fig. 4 corresponds to ascorbate concentrations of about 10.4 mM in mitochondria, 10.8 mM in chloroplasts, 16.3 mM in nuclei, 22.8 mM in peroxisomes, 21.7 mM in the cytosol and 2.3 mM in vacuoles.

Effect of high light intensities on the distribution of ascorbate

Exposure of wild-type plants to high light intensity, which is well known to induce an accumulation of foliar ascorbate, caused a strong increase in ascorbate-specific gold particle density on the whole cells (about 2.5-fold) and in most individual cell compartments (Figs. 3e, 6). Surprisingly, the strongest increase was found in vacuoles (395%), followed by plastids (104%), mitochondria, nuclei and the cytosol (about 35% each). A high light induced decrease in ascorbate labeling density was found in peroxisomes (31%). Ascorbate could not be detected in cell walls (apoplast) of leaves exposed to high light (Figs. 3e, 6). In plants exposed to high light conditions, gold particles bound to

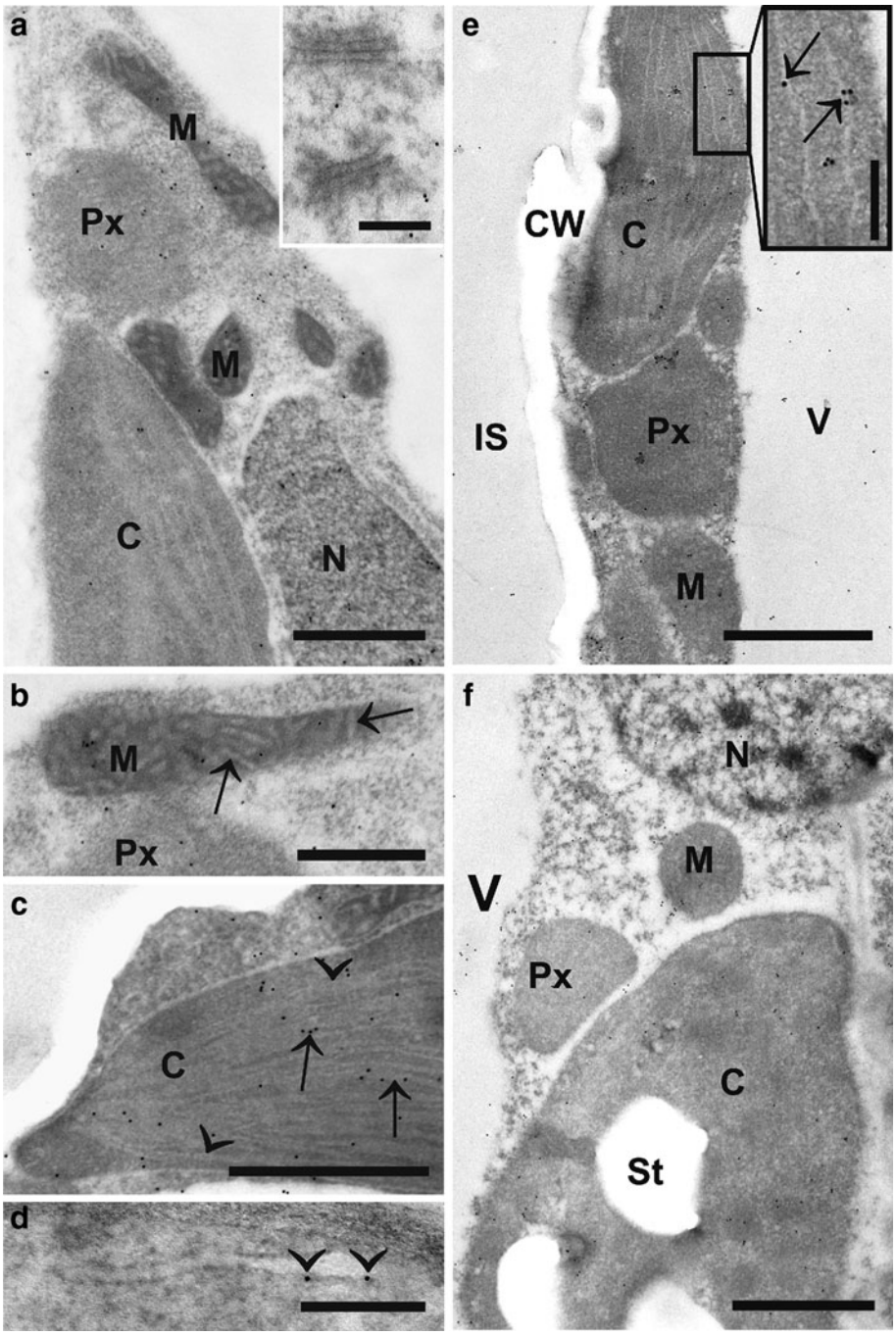

Fig. 3 Transmission electron micrographs showing the subcellular distribution of ascorbate in *Arabidopsis thaliana* Col-0 plants (**a**–**e**), exposed to light intensities of 150 µmol m^{-2} s^{-1} (**a**–**d**) or 700 µmol m^{-2} s^{-1} (**e**), and *Nicotiana tabacum* (**f**) plants. Gold particles bound to ascorbate could be found in different densities within chloroplast (*C*), mitochondria (*M*), nuclei (*N*), peroxisomes (*Px*), vacuoles (*V*) and the cytosol but not in cell walls (*CW*) and intercellular spaces (*IS*). Within mitochondria ascorbate was detected in the matrix but not in the lumen of cristae (*arrows* in **b**). Inside chloroplasts of control plants ascorbate was detected in the stroma and along the outside of the membranes of thylakoids (*arrows*) but not in starch grains (*St*) or the lumen of single and grana thylakoids (*arrowheads* in **c**). In cells exposed to high light conditions gold particles were also detected inside the lumen of thylakoids (*arrows in inset* of **e**). Ascorbate was also detected along the membranes (*arrows* in **d**) but not inside the lumen of the endoplasmic reticulum. Gold particles bound to ascorbate were not associated with dictyosomes (*inset* in **a**). *Bars* 1 µm (**a**, **e** and **f**), 0.5 µm (**b** and **c**), 0.2 µm (*inset* of **a**, **d** and **e**)

ascorbate could also be detected inside the lumen of thylakoids (inset in Fig. 3e) but not in the lumen of cristae.

The compartment-specific relative distribution of gold particles in wild-type and ascorbate-deficient mutants did not change in nuclei, the cytosol and vacuoles. Small changes were observed in mitochondria, plastids and peroxisomes. In comparison to the wild type a higher ratio of ascorbate was found in plastids (3.7 and 6.6% higher in *vtc1-2* and *vtc2-1*, respectively), whereas a decrease occurred in peroxisomes of the *vtc1-2* (8.8%) and *vtc2-1* mutant (7.3%; Table 2), respectively. Exposure of plants to high light caused changes in the ratio of ascorbate labeling between cell compartments only in plastids (+6.5%), peroxisomes (−13.4%) and vacuoles (+7.3%) whereas in all other cell compartment the ratio remained the same (Table 1).

Discussion

The subcellular distribution of ascorbate is important for the understanding of its compartment-specific roles. In this study the compartment-specific distribution of ascorbate

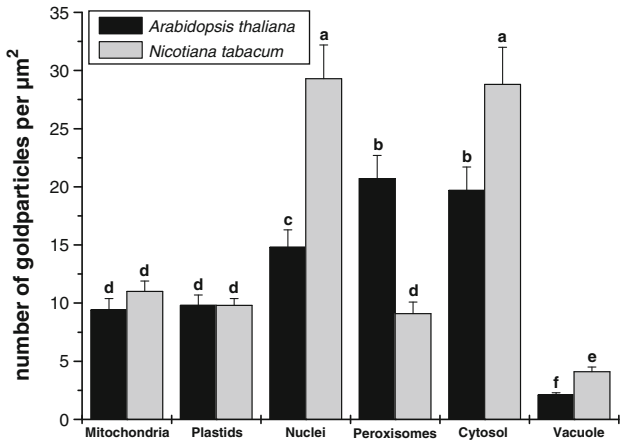

Fig. 4 Quantitative analysis of compartment-specific ascorbate labeling in Arabidopsis and *N. tabacum*. *Graph* shows the labeling density of ascorbate within different cell compartments of leaves from Arabidopsis and *N. tabacum*. Values are means with standard errors and document the amount of gold particles per μm^2. Significant differences between the samples are indicated by *different lowercase letters*; samples which are significantly different from each other have no letter in common. $P < 0.05$ was regarded significant analyzed by the Kruskal–Wallis test, followed by post hoc comparison according to Conover. $n > 20$ for peroxisomes and vacuoles and $n > 60$ for all other cell structures

was analyzed at high resolution by using immunogold cytochemistry in leaf cells of Arabidopsis and *N. tabacum*. The specificity and accuracy of the immunogold labeling approach is supported by several observations. First, preabsorption of the antibody with ascorbic acid or dehydroascorbic acid, and treatment of the sections with preimmune serum instead of the primary antibody reduced the labeling density to background levels. Second, the ascorbate-deficient mutants *vtc1-2* and *vtc2-1* showed a strong decrease in labeling density (of up to 76% in peroxisomes of the *vtc2-1* mutant) when compared to the wild type. Immunogold labeling of ascorbate decreased about 50 and 61% in *vtc1-2* and *vtc2-1* mutants, respectively. These values correlated well with a biochemically determined decrease in total ascorbate in leaf extracts of about 46 and 55%, respectively. A similar decrease (between 50 and 70%) in total ascorbate was previously reported for these mutants (Conklin et al. 2000; Giacomelli et al. 2006; Olmos et al. 2006; Müller-Moule 2008; Foyer and Noctor 2009). Additionally, an accumulation of ascorbate-specific labeling was observed in whole cells and in most cell compartments of wild-type plants exposed to high light. This stress factor is well known to induce an increase in ascorbate contents in plants (Müller-Moule et al. 2004; Bartoli et al. 2006; Giacomelli et al. 2006; Haghjou et al. 2009).

Nevertheless, one could argue that ascorbate is redistributed or lost by diffusion during conventional sample preparation, thus leading to a false interpretation of the subcellular distribution of ascorbate in plants. To test this possibility, we have investigated the subcellular distribution of ascorbate at the very edge of the sample, which comes in contact with the fixative immediately and in samples which were rapidly fixed with the help of microwave irradiation (first fixation step takes 25 s). Additionally, we

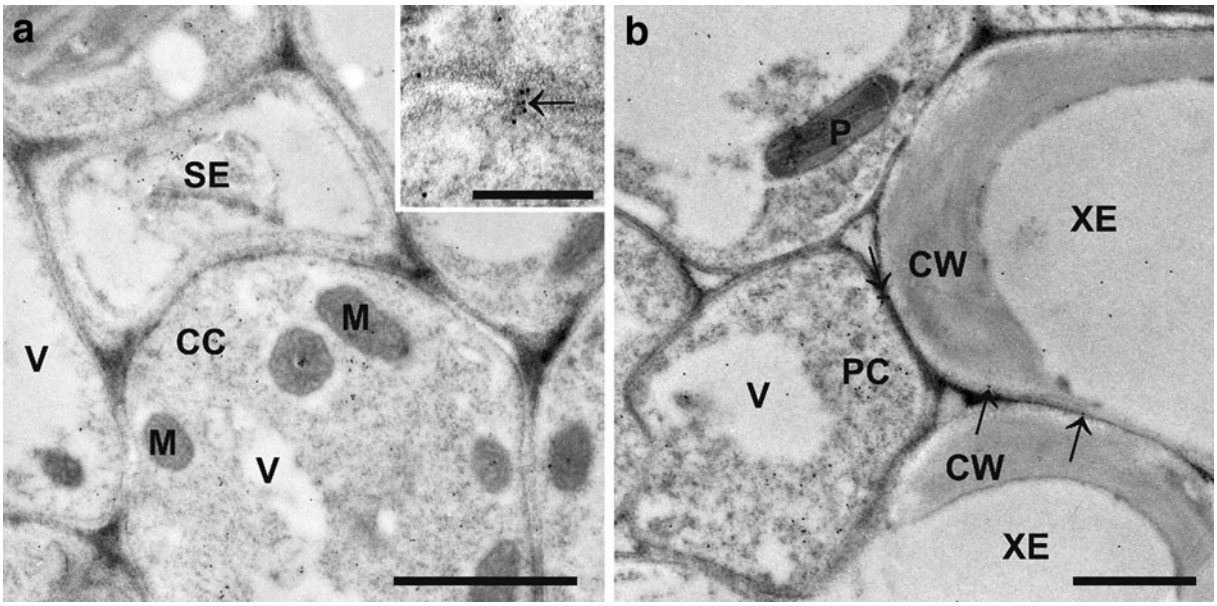

Fig. 5 Transmission electron micrographs of the subcellular distribution of ascorbate in vascular cells of leaves of *Arabidopsis thaliana* Col-0. Gold particles bound to ascorbate could be found in plastids (*P*), mitochondria (*M*), vacuoles (*V*), the cytosol of companion cells (*CC*) and vascular parenchyma cells (*PC*), and inside sieve elements (*SE*). Gold particles bound to ascorbate were found in plasmodesmata (*arrow in inset* in **a**). Gold particles bound to ascorbate could also be detected in xylem elements (*XE*) and their cell walls (*CW*; *arrows* in **b**). *Bars* 1 µm

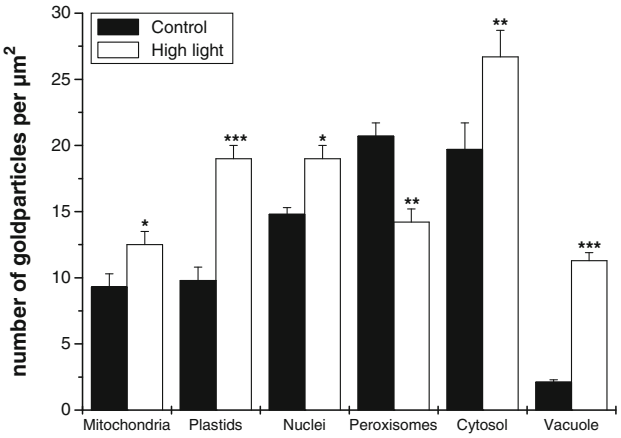

Fig. 6 Effect of high light conditions on the subcellular distribution of ascorbate. *Graph* shows means with standard errors and documents the amount of gold particles per µm^2. Significant differences were calculated using the Mann–Whitney *U* test; *, **, and ***, respectively, indicate significance at the 0.05, 0.01, 0.001 level of confidence. $n > 20$ for peroxisomes and vacuoles and $n > 60$ for all other cell structures

have evaluated ascorbate-specific labeling in high-pressure frozen leaves which freezes samples in milliseconds (Suppl. Fig. S1). In all cases the labeling density and more important the ratio of ascorbate between organelles remained similar compared to samples prepared with conventional fixation (fixation takes 90 min; Table 1). Thus, these results indicate that ascorbate does not get redistributed during conventional fixation.

The highest concentration of ascorbate labeling was detected in nuclei and the cytosol of both plant species, whereas mitochondria, plastids and vacuoles were among the organelles with the lowest ascorbate levels (Fig. 4). As mitochondria are the only compartment in Arabidopsis that are capable of ascorbate synthesis (Bartoli et al. 2000; Millar et al. 2003), these results demonstrate that ascorbate must be transported through the cytosol into other cell compartments. Occasionally, gold particles bound to ascorbate were also associated with the membranes of the ER. No ascorbate was detected in dictyosomes, the cell walls or intercellular space of mesophyll cells suggesting that ascorbate is not secreted. The observed low levels of ascorbate-specific labeling in mitochondria are interesting with respect to previous studies that show that mitochondria contain highest levels of glutathione in different plants (Zechmann et al. 2008; Zechmann and Müller 2010). Even during situations of severe glutathione deficiency (e.g. BSO-treatment; glutathione-deficient *pad2* mutant) the levels of glutathione in mitochondria remained similar to wild-type levels (Zechmann et al. 2006, 2008). This is apparently not the case for ascorbate which was decreased at similar levels in all cell compartments, including mitochondria, in the ascorbate-deficient Arabidopsis mutants. Interestingly, the *vtc1-2* mutant showed significantly elevated glutathione contents in mitochondria, chloroplasts and the cytosol (Fernandez-Garcia et al. 2009). These results indicate that the ascorbate-deficient *vtc1-2* mutant compensates the decrease of ascorbate levels with an increase of glutathione levels in certain cell compartments.

In non-stressed wild-type plants the cytosol and nuclei and not chloroplasts were found as the major site of ascorbate accumulation. Nevertheless, under high light conditions where ROS are mainly produced in the chloroplasts (Asada 2006; Kim et al. 2008; Pfannschmidt et al. 2009), ascorbate-specific labeling strongly increased in chloroplasts reaching levels similar to those observed in the cytosol. These results (Fig. 6) complement previous studies that have demonstrated increased ascorbate levels in high light conditions (Müller-Moule et al. 2004; Bartoli et al. 2006; Giacomelli et al. 2006; Haghjou et al. 2009) and an important function of ascorbate in the detoxification of ROS produced in chloroplasts. Whereas ascorbate was not detected in the lumen of thlyakoids in non-stressed plants, ascorbate-specific labeling could be found there during high light conditions (Fig. 3e, inset). 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 to violaxanthin that is catalyzed by the enzyme violaxanthin de-epoxidase. This enzyme is located inside the thylakoid lumen and uses ascorbic acid as a reductant (Hager 1969; Hager and Holocher 1994; Müller-Moule et al. 2002; Grouneva et al. 2006). Thus, the detection of ascorbate in the thylakoid lumen of plants exposed to high light conditions

Table 2 Ratio of ascorbate between organelles

	Mitochondria	Plastids	Nuclei	Peroxisomes	Cytosol	Vacuoles
Col-0	12.2	12.8	19.4	27.1	25.8	2.7
vtc1-2	15.6	16.5	20.4	18.9	25.0	3.6
vtc2-1	10.7	19.5	20.6	19.4	25.8	4.0
Light stress	12.0	19.3	19.3	13.7	25.7	10.0

Ratio (in %) of the distribution of gold particles bound to ascorbate (total amount of gold particles per µm^2) between cell compartments of wild type, ascorbate-deficient mutants *vtc1-2* and *vtc 2-1* and wild-type plants exposed to high light

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. Ascorbate-specific labeling was exclusively detected in the matrix of mitochondria but not in the lumen of cristae. This is interesting because ROS are also produced in high amounts in the lumen of cristae (Murphy 2009; Kowaltowski et al. 2009). Thus, it seems that within the lumen of cristae either other substances take over the detoxification of ROS, or that ROS are exported into the matrix to be detoxified.

During high light conditions ascorbate labeling strongly increased in vacuoles (Fig. 6). These results open the question about the importance of vacuoles for ascorbate metabolism, especially 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 (Takahama 2004). 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 (Takahama 2004).

It is interesting that nuclei contained high levels of ascorbate similar to cytosol in control plants. These results could reflect the possibility that nuclear pores are freely permeable to ascorbate. Ascorbate was also detected with light microscopy in nuclei (Liso et al. 2004). The possible roles of ascorbate in nuclei are still unclear. Interestingly, high levels of glutathione were also detected in nuclei of different plant species (Zechmann et al. 2006, 2008; Fernandez-Garcia et al. 2009). The presence of antioxidants such as ascorbate and glutathione in nuclei could be important for the protection of DNA against oxidative modifications as demonstrated for mammalian cells (Green et al. 2006). Additionally, antioxidants could be important to maintain reducing conditions, and in the activation of transcriptional factors which control the expression of defense related genes (Mou et al. 2003; Gomez et al. 2004; Foyer and Noctor 2009). Nevertheless, more investigations are necessary to clarify the exact roles of ascorbate in nuclei.

Ascorbate labeling in the apoplast of mesophyll cells was below the level of detection in non-stressed plants and in plants exposed to high light. These results are similar to what was found with light microscopical analysis where ascorbate was not detectable in the apoplast of cucurbit plants (Liso et al. 2004). Nevertheless, with biochemical methods ascorbate was detected in the apoplast of leaves of barley plants (Vanacker et al. 1998), in different poplar trees (van Hove et al. 2001) and leaves of birch trees (Kollist et al. 2001). In these studies ascorbate in the apoplast was present in low amounts in non-stressed plants compared to foliar intracellular levels. It is possible that the apoplastic concentration of ascorbate in mesophyll cells of non-stressed Arabidopsis plants is too low to be detected with the present method. This hypothesis is supported by the observation that ascorbate could be detected in cell walls of vascular bundle cells (Fig. 5b); there ascorbate was present in cell walls of vascular parenchyma cells facing xylem vessels. In other cells ascorbate was not detected in cell walls but could be located in plasmodesmata (Fig. 5a, inset). Thus, it is possible that biochemical measurements of ascorbate in the apoplast in non-stressed plants also reflect the ascorbate pool of vascular bundle cells. Additionally, biochemical measurements involve the extraction of the intercellular washing fluid which could to some extent lead to contamination problems of non-apoplast-specific ascorbate. 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 during stress situations that are well known to increase apoplastic ascorbate contents such as the exposure to ozone (Conklin and Barth 2004; Baier et al. 2005; Sandermann 2008).

In summary, we have established a method based on immunocytochemistry for the detection and quantification of ascorbate at the subcellular level. The method is specific for ascorbic acid and dehydroascorbic acid as demonstrated by various control experiments and can now be applied in future experiments to investigate compartment-specific changes in ascorbate levels in response to different environmental conditions.

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