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ORIGINAL ARTICLE

Subcellular compartmentation of glutathione in dicotyledonous plants

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Abstract This study describes the subcellular distribution of glutathione in roots and leaves of different plant species (*Arabidopsis*, *Cucurbita*, and *Nicotiana*). Glutathione is an important antioxidant and redox buffer which is involved in many metabolic processes including plant defense. Thus information on the subcellular distribution in these model plants especially during stress situations provides a deeper insight into compartment specific defense reactions and reflects the occurrence of compartment specific oxidative stress. With immunogold cytochemistry and computer-supported transmission electron microscopy glutathione could be localized in highest contents in mitochondria, followed by nuclei, peroxisomes, the cytosol, and plastids. Within chloroplasts and mitochondria, glutathione was restricted to the stroma and matrix, respectively, and did not occur in the lumen of cristae and thylakoids. Glutathione was also found at the membrane and in the lumen of the endoplasmic reticulum. It was also associated with the trans and cis side of dictyosomes. None or only very little glutathione was detected in vacuoles and the apoplast of mesophyll and root cells. Additionally, glutathione was found in all cell compartments of phloem vessels, vascular parenchyma cells (including vacuoles) but was absent in xylem vessels. The specificity of this method was supported by the reduction of glutathione labeling in all cell compartments (up to 98%) of the glutathione-deficient *Arabidopsis thaliana rml1* mutant. Additionally, we found a similar distribution of glutathione in samples after conventional fixation and rapid microwave-supported fixation. Thus, indicating that a redistribution of glutathione does not occur during sample preparation. Summing up, this study gives a detailed insight into the subcellular distribution of glutathione in plants and presents solid evidence for the accuracy and specificity of the applied method.

Keywords *Arabidopsis* · Compartmentation · Electron microscopy · Glutathione · Phloem · Xylem

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Introduction

The subcellular distribution of glutathione in plants is of great importance due to the importance of glutathione in plant metabolism and plant defense. As an antioxidant, it detoxifies reactive oxygen species either directly or through the ascorbate-glutathione cycle (Noctor and Foyer 1998; Tausz et al. 2004a; Foyer and Noctor 2009; Szalai et al. 2009). Additionally, glutathione is involved in the detoxification of xenobiotics, herbicides (Edwards et al. 2005; DeRidder and Goldsbrough 2006), heavy metals such as cadmium (Zawoznik et al. 2007; Ammar et al. 2008; DalCorso et al. 2008; Dučić et al. 2008; Nocito et al. 2008), and protects proteins from oxidation by a process called glutathionylation (Hurd et al. 2005a, b). Glutathione is also involved in stress signaling and defense gene expression (Foyer et al. 2001; Maughan and Foyer 2006; Foyer and Noctor 2009; Szalai et al. 2009). Therefore, subcellular changes in glutathione contents especially during environmental stress situations provide a deeper insight into compartment-specific defense reactions and reflect the occurrence of compartment-specific oxidative stress. Such information can be used as a subcellular stress marker and can be very helpful to clarify the importance of the protective roles of glutathione during stress situations in plants on the cellular level.

Despite the importance of glutathione within plant cells, its detection on the subcellular level is technically challenging. There are different approaches available that have given a more detailed insight about the subcellular distribution of glutathione in different plant species. Glutathione can be measured with biochemical methods (high performance liquid chromatography) in different organelles after isolation or fractionation (Vanacker et al. 1998a, b, c; Jiménez et al. 1997, 1998; Kuźniak and Sklodowska 2001, 2004, 2005; Ohkama-Ohtsu et al. 2007; Krueger et al. 2009). With these methods, glutathione was detected in mitochondria, chloroplasts, peroxisomes, apoplast, and vacuoles of different plant species. Nevertheless, the isolation of organelles can lead to contamination problems of non-organelle-specific glutathione and, because of the lengthy procedure, it is unclear how well the obtained results reflect the in vivo situation (Noctor et al. 2002; Chew et al. 2003; Krueger et al. 2009). Additionally, glutathione could have not been detected with these methods in small cell compartments (endoplasmic reticulum (ER), dictyosomes) and nuclei. With light microscopical methods after monochloro- or monobromobimane staining (fluorescent dyes that bind specifically to the SH-group of thiols) glutathione could have been detected in nuclei and the cytosol (Fricker et al. 2000; Meyer and Fricker 2000; Meyer et al. 2001; Müller et al. 2005). However, this method is limited by the resolution of the light microscope (200 nm) and by the ability of the stain to infiltrate certain cell compartments (Fricker and Meyer 2001; Hartmann et al. 2003). Therefore, glutathione could not be clearly detected with this method in organelles such as chloroplasts, peroxisomes, mitochondria, ER, and dictoyosomes. Glutathione could also be indirectly visualized by light microscopical methods using a ratiometric redox-sensitive GFP expressed in *Arabidopsis thaliana* (Meyer et al. 2007; Gutscher et al. 2008). With these methods, it was possible to detect glutathione also in smaller organelles such as the ER.

Recently, we have established a cytohistochemical approach that allowed the detection of glutathione on a high level of resolution in plant cells of roots and leaves (Müller et al. 2004; Zechmann et al. 2006, 2008). With this method, it was possible to detect glutathione in all cell compartments simultaneously in one experiment. We could show that mitochondria always contained the highest levels of glutathione whereas plastids contained lowest levels with intermediate glutathione contents in nuclei, peroxisomes, and the cytosol. None or only very little glutathione could be detected in vacuoles, cell walls, and intercellular spaces. Additionally, we have proven that it is possible to detect changes in the subcellular distribution of glutathione during abiotic and biotic stress situations (Zechmann et al. 2005a, 2007a; Zechmann and Müller 2008; Kolb et al. 2010). Nevertheless, in all of these studies, we have not paid detailed attention on the subcellular distribution of glutathione within organelles (e.g., mitochondria, chloroplasts, and dictyosomes) and vascular bundle cells. Such information is quite important considering that high amounts of reactive oxygen species can be formed in the lumen of cristae (Murphy 2009; Kowaltowski et al. 2009) and in thylakoids (Asada 2006) during respiration and photosynthesis, respectively. Additionally, information about the distribution of glutathione within vascular bundle cells would help to clarify in which compartments of the vascular bundle glutathione is transported in leaves.

In the present study, we fill this gap and describe the subcellular distribution of glutathione in roots and leaves in three different plant species (*A. thaliana*, *Nicotiana tabacum*, and *Cucurbita pepo*) in more detail. These plant species were chosen as they are important model plants in many fields of plant sciences. Additionally, they are widely used to study different physiological aspects in plants during abiotic and biotic stress conditions. Considering the importance of glutathione in the protection against environmental stress situations it is essential to study the subcellular compartmentation of glutathione in these plants in order to understand its role in growth, development, and stress defense in plants. In this study, we present data of the distribution of glutathione within (1) organelles, (2) vascular bundle cells, and (3) the glutathione deficient *A. thaliana* root meristemless mutant (*rml1*) which contains between 90% and 97% less glutathione levels in comparison to the wildtype (Vernoux et al. 2000; Cairns et al. 2006). *rml1* mutants have a mutation of the gene that encodes γ-glutamyl-cysteine synthetase which produces the enzyme necessary for the first step of glutathione synthesis. This correlates with lower glutathione levels and a disturbed phenotype that (a) does not develop a root meristem, thus leading to very short roots (1-2 mm), (b) develops short shoots, and (c) develops smaller rosettes, inflorescence, and flowers (Cheng et al. 1995; Vernoux et al. 2000; Cairns et al. 2006). Additionally, we compared the labeling pattern between samples that were rapidly fixed with the support of microwave irradiation with the situation in conventionally fixed samples. Microwave irradiation can be used to strongly reduce sample preparation time to a few minutes without concurring negative effects on the ultrastructure of the samples (Zechmann and Zellnig 2009a, b) when compared to conventionally prepared samples which can take up to 90 min (Zechmann et al. 2005b, 2007b). Thus, another aim of the present study was to investigate whether differences in labeling pattern occur between conventionally and microwave-fixed samples which could clarify whether glutathione gets relocated during the relatively long fixation time during conventional fixation.

Materials and methods

Plant material

Plants were cultivated in growth chambers under defined conditions. After stratification for 4 days at 4 °C seeds of *A. thaliana* [L.] Heynh. ecotype Columbia (Col-0) were grown on soil in growth chambers with 14/10 h day/night photoperiod. Day and night temperatures were 22 °C and 18 °C, respectively, the relative humidity was 60% and the plants were kept at 100% relative soil water content. Light intensity varied between 110 and 140 µmol $m^{-2}s^{-1}$. Additionally, seeds of *A. thaliana* accession Col-0 and the mutant line *rml1* (*root meristemless1*; Cheng et al. 1995; Vernoux et al. 2000; Cairns et al. 2006) were grown on two-fifth Murashige-Skoog medium (Murashige and Skoog 1962) at a light intensity of 120 µmol $m^{-2}s^{-1}$. Therefore, seeds were surface sterilized with 70% ethanol for 5 min, washed twice with distilled, sterile water (for 2 min each) and placed on the nutrient medium solidified with 1% phytagel. Plates were placed at 4 °C for 4 days and then transferred into growth chambers with 9/15 h day/night period at 22 °C day and 18 °C night temperature.

N. tabacum (L.) cv. *Samsun* nn, obtained from the German resource center 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%. Plants were kept in pots with soil and were watered adequately. Seeds of *C. pepo* L. subsp. *pepo* var. *styriaca* Greb. (Styrian oil pumpkin), received from Saatzucht Gleisdorf (Plant Breeding Company, Gleisdorf, Austria), were germinated on a humid Perlite cloth. Plants were grown individually in soil-filled pots with a photoperiod of 12 h and a light intensity between 130 and 170 µmol $m^{-2}s^{-1}$. Day and night temperatures were 22 °C and 18 °C, respectively, and the relative humidity was 70%.

Harvesting of *Arabidopsis* plants (Col-0) was performed 4 weeks after stratification. Therefore, root tips and samples from the youngest fully developed rosette leaf were harvested 2 h after the onset of the light period. Leaves at this stage were approximately 2 cm in length and 0.7 cm in width. Harvesting of *Arabidopsis* Col-0 and the *rml1* seedlings both grown on solidified nutrient medium was performed 10 days after stratification 2 h after the onset of the light period. *Nicotiana* and *Cucurbita* plants were harvested about 4 weeks after germination 2 h after the onset of the light period. Samples of the youngest fully developed leaves (about 10 cm in length and 6 cm in width) were taken and prepared for transmission electron microscopy.

Sample preparation for transmission electron microscopy and immunogold labeling

Preparation of samples for transmission electron microscopy and immunogold labeling of glutathione was done with ultrathin sections on nickel grids as described in Zechmann et al. 2006, 2008. Small samples of the youngest fully developed leaves (about 1.5 mm^2) and root tips from at least three different plants were fixed in 2.5% paraformaldehyde/0.5% glutardialdehyde in 0.06 M phosphate buffer (pH 7.2) for 90 min at room temperature (RT). Microwave fixation was performed in the same fixation solution as described above in a Polar Patent PP1000 microwave oven. Samples were fixed for two times 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 fixed conventionally and with the help of microwave irradiation were then rinsed in buffer and dehydrated in increasing concentrations of acetone (50%, 70%, and 90%) at RT for 20 min at each step. 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%) and finally embedded in LR White resin and polymerized at 50 °C for 48 h in small plastic containers.

Ultrathin sections (80 nm) of the samples were blocked with 2% bovine serum albumine (BSA) in phosphate buffered saline (PBS, pH 7.2) and then treated with the primary antibody (anti-glutathione rabbit polyclonal IgG; Millipore Corp., Billerica, MA, USA) diluted 1:50 in PBS containing 1% goat serum for 2 h at RT. After a short rinse in PBS, samples were incubated with a 10 nm gold-conjugated secondary antibody (goat anti-rabbit IgG, British BioCell International, Cardiff, www.british-biocell.co.uk) diluted 1:50 in PBS for 90 min at RT. After a short wash in PBS and distilled water, labeled grids were either immediately observed in a Philips CM10 transmission electron microscope or post-stained with uranyl-acetate (15 s).

The specificity of the immunogold-labeling procedure was tested by several negative controls. Negative controls were treated either with: (1) pre-immune serum instead of the primary antibody, (2) gold-conjugated secondary antibody (goat anti rabbit IgG) without the primary antibody, (3) non-specific secondary antibody (goat anti mouse IgG), and (4) primary antibodies pre-adsorbed with an excess of glutathione for 2 h at RT prior to labeling of the sections. For the latter, a solution containing 10 mM of glutathione was incubated with 0.5% glutardialdehyde for 1 h. The excess of glutardialdehyde was then saturated by incubation for 30 min in a solution of 1% (*w*/*v*) BSA. The resulting

solution was used to saturate the glutathione-antibody for 2 h prior to its use.

Micrographs of randomly photographed immunogold-labeled sections 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). For statistical evaluation, at least four different samples were examined. A minimum of 20 (peroxisomes and vacuoles) to 60 (other cell structures) sectioned cell structures of at least 15 different cells were analyzed for gold particle density per sample. The obtained data were statistically evaluated using Statistica (Stat-Soft, USA, 2002).

Results

The subcellular distribution of glutathione was found to be similar in all plant species. Samples prepared with the help of microwave fixation and conventional prepared samples showed a similar subcellular distribution of glutathione (Fig. 1a-h and Table 1). In leaves, highest levels of glutathione were found in mitochondria, followed by nuclei, cytosol, and peroxisomes. Lowest levels were always found in plastids (Fig. 2). Levels in plastids and nuclei of *Nicotiana* plants for example were about 87% and 53% lower when compared to mitochondria. Within mitochondria, glutathione was restricted to the matrix, but could not be detected in the lumen of cristae (Fig. 1f). Within chloroplasts, gold particles bound to glutathione were detected in the stroma but could not be detected in the lumen of single thylakoids nor in inter- and intrathylakoidal spaces within grana thylakoids (Fig. 1g). Within nuclei, glutathione was equally distributed in hetero and euchromatin (Fig. 1a-e) and also occurred in nucleoli (Fig. 1c). Glutathione was also detected along the membranes and in the lumen of the endoplasmic reticulum (Fig. 1h). Gold particles bound to glutathione were found to be associated with the trans and cis side of the golgi apparatus, but did not occur inside the cisternae (inset in Fig. 1b). Gold particles bound to glutathione were either absent or occurred in very low amounts in vacuoles, cell walls and intercellular spaces (Fig. 1a-e) of mesophyll and epidermal cells.

A similar situation was found in roots (Fig. 3a). In this organ, mitochondria always contained the highest levels of glutathione labeling density (Figs. 2 and 3a). Mitochondria of *Arabidopsis* Col-0 plants for example contained about 430% more gold particles than the compartment with the lowest labeling (plastids). In *Arabidopsis* roots, the cytosol showed the second highest labeling density followed by nuclei and plastids. In *C. pepo* roots, plastids contained the second highest labeling density followed by the cytosol and nuclei. In *N. tabacum* roots, second highest labeling was found in the cytosol followed by plastids and nuclei (Fig. 2).

The subcellular distribution of glutathione was also studied in vascular bundle cells of leaves. Gold particles bound to glutathione were not detected in xylem vessels (Fig. 4a). High labeling of glutathione was found in phloem cells (Fig. 4b). Companion cells showed high glutathione labeling in mitochondria and to a lower extent in the cytosol. Additionally, glutathione was detected in these cell compartments in vacuoles (Fig. 4b). A similar situation was found in vascular parenchyma cells were glutathione was detected in all cell compartments including vacuoles (Fig. 4a, b). Gold particles bound to glutathione were also observed within sieve elements (Fig. 4b). Glutathione labeling was absent in cell walls and intercellular spaces (Fig. 4a, b).

The accuracy and specificity of the obtained labeling was verified by the result of the negative results and by the data obtained from the mutants. Labeling of glutathione was absent in sections treated as negative controls (Fig. 3c, d). Additionally, glutathione labeling in the roots of the *rml1* mutant was strongly decreased when compared to the wildtype (Fig. 3a, b). *rml1* showed a strong decrease in glutathione labeling in mitochondria (−98%), nuclei (−97%), the cytosol (−97%), and plastids (−96%) when compared to the wildtype grown under the same conditions.

Discussion

The subcellular distribution of glutathione was similar in all plant species, even though differences were found in the overall labeling density (e.g., *Cucurbita* showed much lower labeling in roots and leaves when compared with *Arabidopsis* and *Nicotiana* plants). Mitochondria always showed highest amounts of gold particles bound to glutathione, whereas plastids contained the lowest amounts of glutathione. No considerable amounts of glutathione were found in vacuoles and the apoplast of mesophyll (and epidermis) cells of leaves and roots. Similar results were obtained in previous studies which have additionally revealed that mitochondria maintain high and stable levels of glutathione even in situations of temporary and permanent glutathione deficiency (Zechmann et al. 2006, 2008). Thus, high and stable levels of glutathione in mitochondria seem to be important in situations when glutathione levels drop in other cell compartments, e.g., during environmental stress situations which indicates an important role of glutathione in mitochondria for cell survival. Support for this hypothesis comes from studies with animal tissue where glutathione contents and especially its redox state in

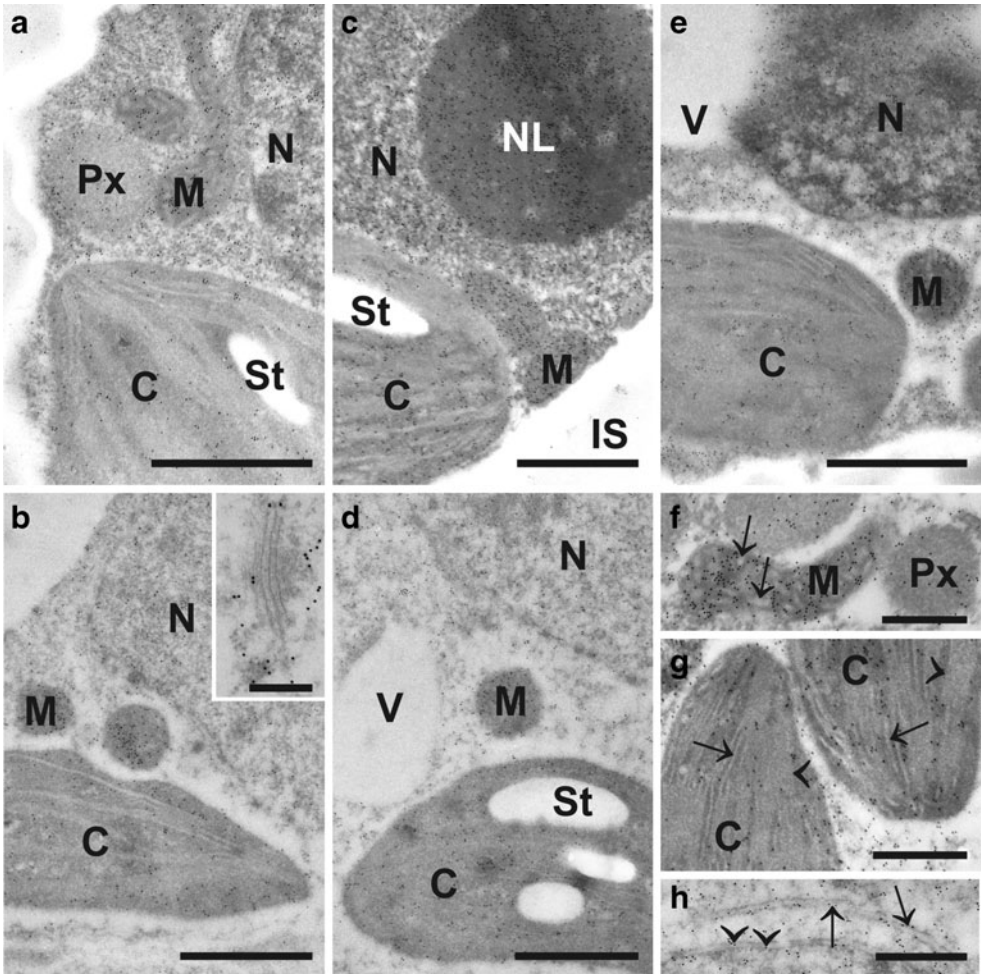

Fig. 1 Transmission electron micrographs showing the subcellular distribution of glutathione in leaf cells from *C. pepo* (**a**, **b**, **f**, **h**), *A. thaliana* Col-0 (**c**, **d**) and *N. tabacum* (**e**, **g**) plants after conventional fixation (**a**, **c**, **e**, **f**, **g**) and microwave-assisted fixation (**b**, **d**, **h**). Gold particles bound to glutathione could be found in chloroplasts (*C*), mitochondria (*M*), nuclei (*N*), nucleolus (*NL*), peroxisomes (*Px*), and the cytosol but not in vacuoles (*V*), cell walls, and intercellular spaces (*IS*). Within mitochondria, glutathione was detected in the matrix but not in the lumen of cristae (*arrows in f*). Inside chloroplasts, glutathione was detected in the stroma but not in starch grains (*St*) or the lumen of single (*arrows in g*) and grana thylakoids (*arrowheads in g*). Glutathione was also detected along the membranes (*arrows in h*) and inside the lumen (*arrowheads in h*) of the endoplasmic reticulum. *Bars* 1 μm in a-e, 0.5 μm in f-h and 0.25 μm in inset of *1b*

mitochondria were found to be associated with apoptosis (Circu and Aw 2008).

The distribution of glutathione in chloroplasts and mitochondria was restricted to the stroma and the matrix, respectively. Glutathione was not detected in the lumen of single thylakoids, nor within inter- and intrathylakoidal spaces. Additionally, glutathione was not detected inside the lumen of cristae. The same situation was found during biotic stress situations (Zechmann et al. 2007a), for the *pad2-1* mutant (Zechmann et al. 2008) and the *rml1* mutant

Table 1 Ratio of glutathione between organelles

Ratio of glutathione between organelles

	Mitochondria	Chloroplasts	Nuclei	Peroxisomes	Cytosol
Arabidopsis					
Conventional fixation	40.9^{a}	6^{f}	24.2^{b}	12.3^{e}	16.6^{d,e}
Microwave fixation	41.4^{a}	5.1^{f}	22.3^{b,c}	13.2^{e}	18^{c,d}
Nicotiana					
Conventional fixation	44.8^{a}	6.1^{e}	23.9^{b}	12.2^{c}	13^{c}
Microwave fixation	44.4^{a}	7.4^{e}	20.2^{b}	14.2^{c}	13.9^{c}
Cucurbita					
Conventional fixation	53.6^{a}	7.9^{d}	17.2^{b}	12.2^{c}	9.1^{c,d}
Microwave fixation	51.4^{a}	9.5^{c,d}	16.2^{b}	12.4^{c}	10.5^{c,d}

Ratio (in%) of the distribution of gold particles bound to glutathione between cell compartments of plants prepared with conventional and microwave fixation. $P<0.05$ was regarded significant, analyzed by the Kruskal-Wallis test, followed by post hoc comparison according to Conover. Significant differences among samples are indicated by different lowercase letters. $N>20$ for peroxisomes and $n>60$ for all other cell structures

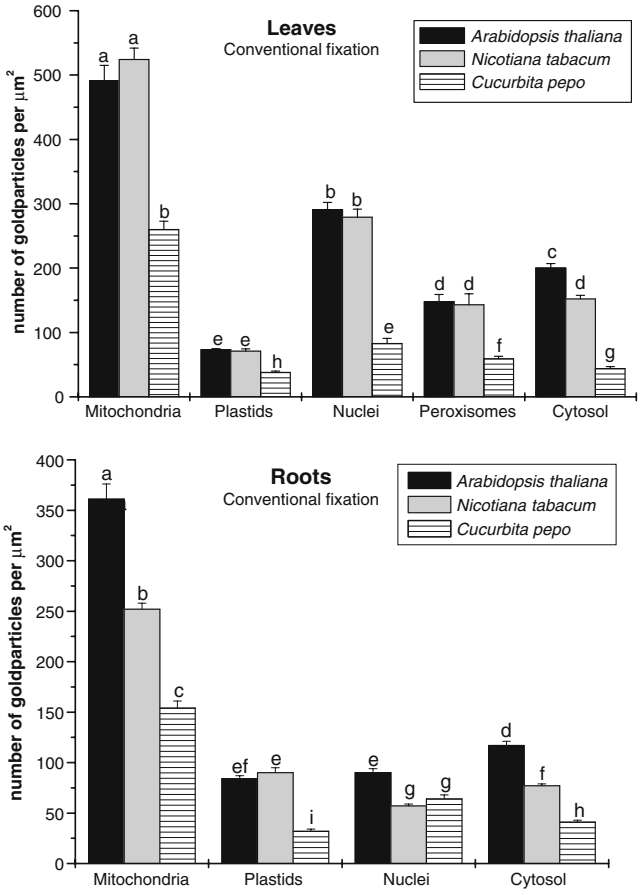

Fig. 2 Graphs show the labeling density of glutathione within different cell compartments of leaves and roots of three different plant species after conventional fixation. Values are means with standard errors and document the amount of gold particles per squared micrometer. Significant differences between 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 $n>60$ for all other cell structures

in this study. These data indicate that the protection of free glutathione against reactive oxygen species produced during photosynthesis and respiration in chloroplasts (Asada 2006; Kim et al. 2008; Pfannschmidt et al. 2009) and mitochondria (Rhoads and Subbaiah 2007; Go and Jones 2008; Oelze et al. 2008; Murphy 2009), respectively, takes mainly place in the stroma and the matrix of these cell compartments. This is interesting because reactive oxygen species especially H_2O_2 are also produced in high amounts in the lumen of cristae (Murphy 2009; Kowaltowski et al. 2009) and in thylakoids of chloroplasts (Asada 2006). Thus, it seems that within the lumen of cristae and thylakoids, either other substances take over the detoxification of glutathione, or that reactive oxygen species and H_2O_2 are transported through the membranes into the matrix or stroma where they are detoxified or that levels of free glutathione are very low in these compartments, especially during non-stressed conditions. Nevertheless, it still needs to be clarified if glutathione can be detected in the lumen of thylakoids and cristae during situations of glutathione accumulation or during stress situations that can lead to compartment specific stress in chloroplasts and mitochondria.

The accuracy and the specificity of the labeling were proven by several observations. First, gold particles bound to glutathione were absent in sections treated as negative controls. Second, microwave fixed samples which were fixed in about four minutes (first fixation step took 25 s) showed a similar distribution of glutathione as samples prepared with conventional fixation (fixation takes about 90 min). Additionally, we have demonstrated in a previous study (Zechmann et al. 2008) that the subcellular distribution at the edge of the sample (which gets in contact with the fixative immediately) is the same as in the middle of the sample (which gets in contact with the fixative last). Thus, these results indicate that glutathione does not get redistributed during fixation and that the detected labeling pattern is as close as possible to the in vivo situation within the cells. Additionally, the accuracy and specificity of the labeling is confirmed by the labeling pattern of the *A. thaliana rml1* mutant. This mutant shows only about 3-10% of glutathione levels when compared to the wildtype which is due to a mutation of the gene that encodes γ-glutamylcysteine synthetase that produces the enzyme necessary for the first step of glutathione synthesis (Vernoux et al. 2000; Cairns et al. 2006). In the present study, glutathione labeling was found to be about 96-98% lower in all cell compartments of the *rml1* mutant when compared to the wildtype. Interestingly, *rml1* showed the strongest reduction of glutathione in mitochondria (−98%). In recent studies, we have shown that glutathione contents within mitochondria of leaves and roots of the glutathione deficient *A. thaliana pad 2-1* mutant remained the same as in the wildtype whereas glutathione was strongly decreased in all other cell compartments that contained glutathione of up to −91%. In opposite to *rml1* which forms very short roots (1−2 mm), small shoots and leaves (Cheng et al. 1995; Vernoux et al. 2000), *pad2-1* mutant has a phenotype similar to the wildtype in non-stressed conditions (Parisy et al. 2007). Thus, the preservation of high levels of glutathione in mitochondria in situation of glutathione deficiency seems to be essential for a "normal" plant development. The *rml1* phenotype can also be achieved by treatment of plants with buthionine sulfoximine, which inhibits glutathione synthesis by inhibiting γ-glutamylcysteine synthetase that produces the enzyme necessary for the first step of glutathione synthesis (Vernoux et al. 2000). Nevertheless, mild BSO-stress did not affect concentrations of glutathione in mitochondria in roots and

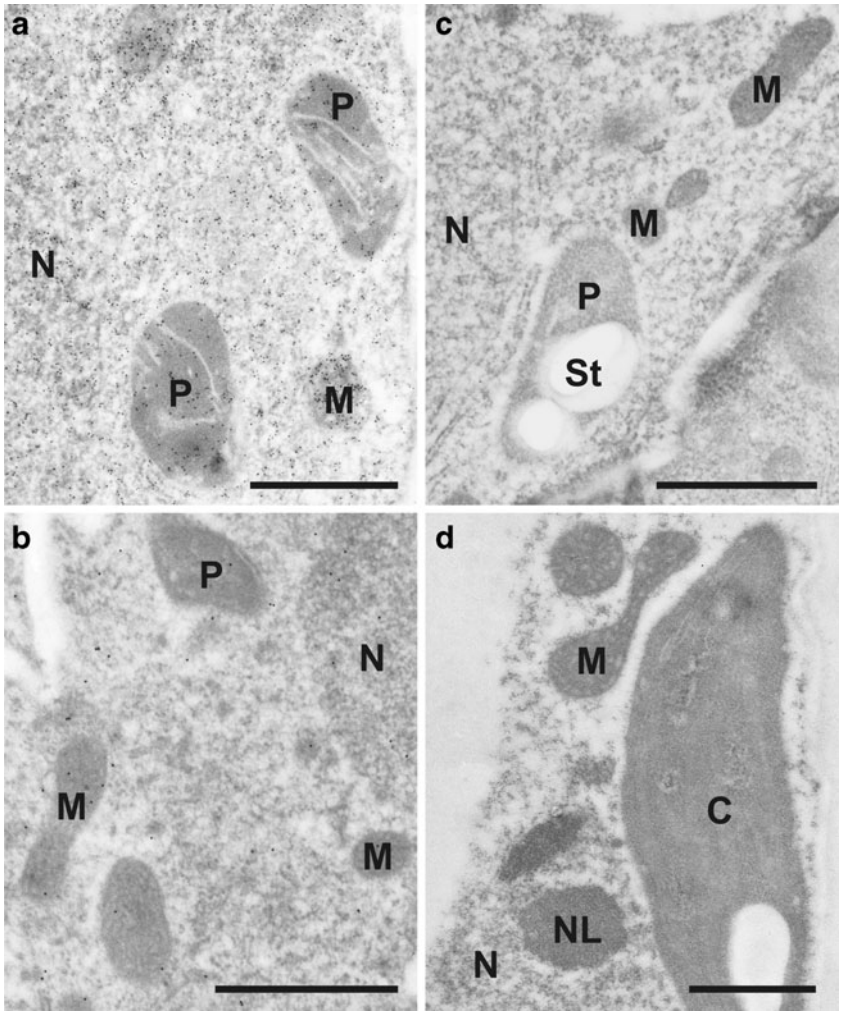

Fig. 3 Transmission electron micrographs showing the subcellular distribution of glutathione in leaf and root tip cells of *A. thaliana* Col-0 (**a**, **c**, **d**) and the *rml1* mutant (**b**) after conventional fixation. In roots of the wildtype (**a**) gold particles bound to glutathione were found in mitochondria (*M*), nuclei (*N*), plastids (*P*), and the cytosol. Note the much lower gold particle density in root tips of the *rml1* mutant (**b**) when compared to the wildtype (**a**). No gold particles could be found in negative controls (**c**, **d**) after the primary antibody was saturated with an excess of glutathione prior to its application (**c**) and after treating sections with pre-immune serum instead of the primary antibody (**d**). *C* chloroplasts, *NL* nucleolus, *St* starch. *Bars* 1 μm

leaves, and does not induce changes in the phenotype (Zechmann et al. 2006). These results demonstrate that high and stable levels of glutathione in mitochondria in plants, especially in situations of permanent and short term glutathione deficiency, play an important role for the development and growth of roots and plants, thus allowing a phenotype similar to the wildtype.

The distribution of glutathione was also studied in vascular bundle cells. There, glutathione was found in quite high concentrations in phloem cells (companion cells

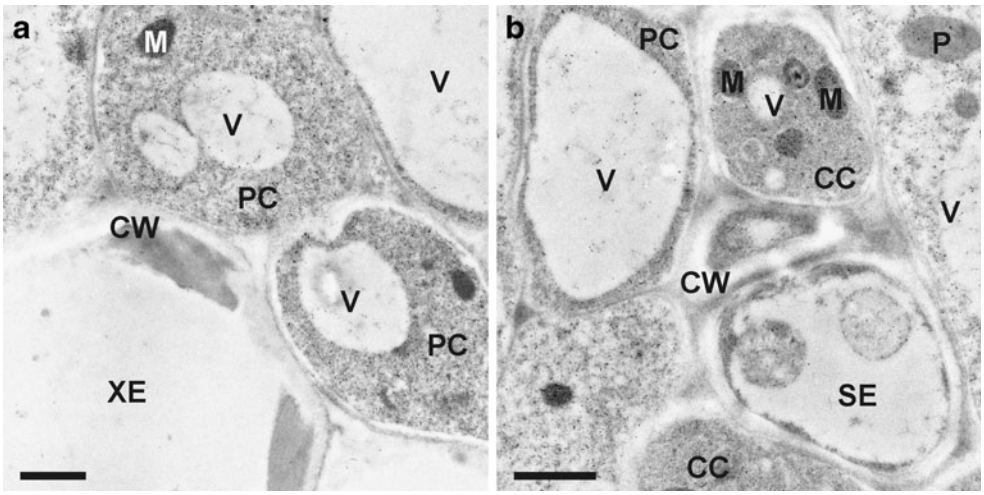

Fig. 4 Transmission electron micrographs showing the subcellular distribution of glutathione in vascular bundle cells of leaves from *A. thaliana* Col-0 plants after conventional fixation. Gold particles bound to glutathione could be found in mitochondria (*M*), vacuoles (*V*), the cytosol of companion cells (*CC*), and vascular parenchyma cells (*PC*), and within sieve elements (*SE*). None or only very few gold particles bound to glutathione could be seen xylem elements (*XE*) and cell walls (*CW*). *Bars* 1 μm

and sieve elements) and vascular parenchyma cells, but not in xylem cells and cell walls. These results clearly demonstrate that glutathione cannot be detected by the immunogold-labeling method used in this study in the xylem; thus, indicating that glutathione contents in xylem vessels are quite low in plants grown under non-stressed conditions. Nevertheless, glutathione was detected in the xylem of different angiosperm and gymnosperm trees with biochemical methods (Köstner et al. 1998; Rennenberg et al. 2007). Additionally, glutathione is most probably transported in xylem vessels from roots to shoots and leaves, as it has been shown previously that the application of high amounts of glutathione to roots led to an increase of glutathione in leaves (Tausz et al. 2004b; Zechmann et al. 2006). Thus, it seems probable that glutathione can occur in the xylem under certain conditions, e.g., when glutathione levels in roots are very high. The occurrence of glutathione in phloem cells, vascular parenchyma cells, and sieve elements indicates that glutathione can be loaded into phloem cells and most probably transported to other (parts of the) organs through sieve tubes. The loading of glutathione into phloem cells takes apparently place through plasmodesmata as glutathione was not found in cell walls or in intercellular spaces.

It is also interesting that glutathione could be detected in vacuoles of companion cells and vascular parenchyma cells but not in mesophyll and epidermis cells. So far, free glutathione could have not been detected in vacuoles of mesophyll cells and protoplasts with light microscopical methods (Müller et al. 2005). With biochemical methods, glutathione has been detected recently in isolated vacuoles of leaves, but the obtained values also contained contamination of the mitochondrial fraction (Krueger et al. 2009). Additionally, whole leaves were used in these studies and it remained unclear if the obtained concentrations of glutathione represented the real contents of glutathione in vacuoles and if these concentrations were the same for cells of different tissues (e.g., epidermis, mesophyll, and vascular bundle). In this study, we were able to show on a higher level of resolution that glutathione solely occurs in vacuoles of vascular bundle cells but not in vacuoles of mesophyll and epidermal cells during non-stressed conditions. However, it still needs to be clarified if glutathione can also occur in vacuoles of mesophyll cells under certain conditions, e.g., high levels of (oxidized) glutathione in the cytoplasm.

Summing up, the present study gave a detailed insight into the subcellular distribution of glutathione in plants. Glutathione can be found in considerable amounts in all cell compartments except vacuoles and the apoplast of root, epidermis, and mesophyll cells, with the highest concentration in mitochondria and lowest ones in plastids. Glutathione does not occur in the lumen of cristae and thylakoids but can be found in the lumen of the endoplasmic reticulum and also along its membranes. Additionally, glutathione is present at the cis and trans side of dictyosomes but not within cisternae. Within vascular bundle cells, glutathione was found in all compartments of phloem vessels and vascular parenchyma cells (including vacuoles) but not in xylem vessels. Studies with the glutathione deficient *Arabidopsis* mutant *rml1* revealed that glutathione contents were decreased in all cell compartments thus indicating that the present approach specifically detects changes in glutathione contents in all cell compartments simultaneously in one experiment.

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