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# Recent Developments in Methods Intracellulary Localizing Glutathione within Plant Tissues and Cells (a Minireview)

By

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K e y w o r d s : Glutathione, immunolabeling, monochlorobimane, plant tissues, ultrastructure.


S u m m a r y

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This article reviews the current knowledge of the intracellular distribution of glutathione (GSH and t-GSH) in different plants and plant tissues under various abiotic and biotic stress situations and artificial modulations of glutathione contents. Monochlorobimane fluorescence of GSH is described for light microscopical investigations allowing GSH in cytoplasm and in the nucleus to be distinguished. Further, a specific antibody, which recognizes t-GSH, was used in order to demonstrate the distribution of glutathione in different cell compartments for light and electron microscopical investigations. The labeling shows different glutathione contents in the cell compartments with high staining intensity in mitochondria.


## I n t r o d u c t i o n

Plants are exposed to a variety of biotic and abiotic environmental factors, which cause e.g. diminished photosynthetic metabolism, increased photoreduction of molecular oxygen, increased photorespiration, and dissipation of excitation energy at photosystem II (ASADA 1999, ORT & BAKER 2002, BALL & al. 2004). All these processes lead to an increased formation of reactive oxygen species (ROS). To detoxify ROS plants react by the coordinated regulation of a network of defense and antioxidant systems (WILLEKENS & al. 1997, ROSSEL & al. 2002, PNEULI & al.

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2003). Glutathione is an important component of the antioxidative network. It is a significant member of low molecular weight thiols and is considered to have a broad spectrum of functions in the metabolism of plants. It is known to be of great importance in sulfur uptake, assimilation, storage and transport and is also a crucial part of the antioxidative system in plants and animals by scavenging ROS (e.g. NOCTOR & al. 1997, 2002). Besides its role as a major antioxidant, the glutathione pool is also involved in signaling biological stress by the regulation of gene expression and protein function (MAY & al. 1998, FOYER & NOCTOR 2003). Glutathione is present in roots, stems and leaves in different concentrations between these organs or within the same organ at different developmental stages (FOYER & RENNENBERG 2000). Intercompartmental variations in glutathione concentration are supposed to be crucial in signaling processes. Nevertheless, until recently very little was known about the precise subcellular distribution and localization of glutathione in plant cells. The reason therefore, is that most of the data about the subcellular distribution of glutathione had been collected by isolating specific organelles followed by the measurement of glutathione contents in the obtained soluble fraction by HPLC or spectrophotometry. The results of these methods were influenced by the possible exchange of GSH between different cell compartments during the extraction method and by possible contaminations of cytosolic glutathione (KLAPHECK & al. 1987, NOCTOR & al. 2002). Therefore, varying concentrations of glutathione e.g. in chloroplasts were reported, which were found between 8 % and 50 %, of the total leaf glutathione depending on the isolation media and the methods and plants (NOCTOR & al. 2002). The recent developments of various light and electron microscopical techniques in localizing glutathione within cells allowed a better insight into the subcellular distribution of glutathione and provided a new tool to study changes in the subcellular distribution of glutathione during various stress situations. This review is aimed to give a short survey of different microscopical approaches for the determination of intracellular glutathione contents in different plant tissues during glutathione modulation and abiotic and biotic stress situations.

Abbreviations: BmCl: monochlorobimane, BSO: buthionine sulphoximine, GSH: reduced glutathione, GSSG: oxidized glutathione, t-GSH: total glutathione, OTC: L-2-oxothiazolidine-4-carboxylic acid

## Determination of Reduced Glutathione (GSH)

The technique of histochemical GSH tracing by monochlorobimane (BmCl) in conventional fluorescence microscopy and laser scanning microscopy was developed for different tissues in various plants. Glutathione was demonstrated in single cell layers such as epidermis cells of *Allium cepa* (Fig. 1, MÜLLER & al. 1999a-c) and rhizodermis of *Arabidopsis thaliana* roots (MEYER & FRICKER 2000), in suspension cell cultures and root protoplasts of *Allium* (MÜLLER & al. 2000) as well as leaf protoplasts of *Picea abies* (Fig. 2). Further, the method was used for the determination of glutathione biosynthesis in *Arabidopsis* trichome cells

(GUTIERREZ-ALCALA & al. 2000) and green suspension culture cells (MEYER & FRICKER 2002) as well as for the localization of glutathione in whole organs e.g. in roots of *Arabidopsis* (FRICKER & al. 2000) and *Brassica oleracea* (MÜLLER & al. 2002) and also in whole *Populus tremula x P. alba* leaves (HARTMANN & al. 2003). The method relies on conjugation of BmCl to GSH within intact tissues resulting in a fluorescent GS-bimane conjugate. The GS-bimane fluorescence levels are well known as an indicator of GSH in living mammalian and animal tissues (cf. FERNANDEZ-CHECA & KAPLOWITZ 1990, BRIVIBA & al. 1993, MILLIS & al. 1997, SEBASTIA & al. 2003) and are also described for plants, especially for non-green tissues (SÁNCHEZ-FERNÁNDEZ & al. 1997, FRICKER & al. 2000, MEYER & FRICKER 2000, MEYER & al. 2001).

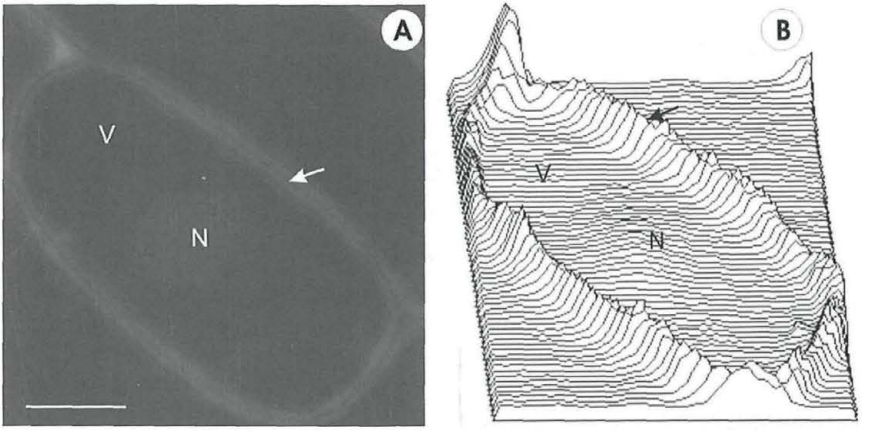


Fig. 1. Epidermal cell of *Allium cepa* L. BmCl Fluorescence image (A) and appropriate luminescence profile (B). (A, B) Epidermal cell showing positive fluorescence in cytoplasm and nucleus (N); no fluorescence could be observed in cell wall (arrow) and vacuole (V). Bar = 20 µm.

Further studies dealt with the changes of the intensity of the fluorescence that was observed after modifying the glutathione status of the cells, either by application of oxidants as for instance $H_2O_2$ (MÜLLER & al. 1999c), by inhibiting glutathione synthesis by BSO (MÜLLER & al. 1999a) or by using 1-chloro-2,3-dinitrobenzene to eliminate the labeling of glutathione (HARTMANN & al. 2003). In addition, tissue- and subcellular glutathione concentrations in sections of *Brassica* roots were manipulated by $H_2S$ fumigation (MÜLLER & al. 2002). In roots of *Brassica,* a sulfur demanding plant, the $H_2S$ treatment resulted in an increased fluorescence in cytoplasm and nuclei of meristematic cells, implying enhanced glutathione contents in the cytoplasm as well as in the nucleoplasm. These results showed the sensitivity of this method by making it possible to distinguish glutathione contents not only between different tissues but also within the cell at the light microscopic level in non-green tissues.

Still, the question arose as to whether it was also possible to stain the entire cellular glutathione pool in green tissues by using BmCl techniques. The data obtained by experiments for *Populus* leaves (HARTMANN & al. 2003), as well as for *Picea* protoplasts, clearly showed that the cytosolic pool was labeled without any positive results for chloroplasts being observed (Fig. 2). HARTMANN & al. 2003 discussed the lack of labeling by the absence of an appropriate glutathione S-transferase in the chloroplasts rather than lack of penetration of the highly membrane-permeant BmCl dye. For obtaining further information concerning GSH pools in chloroplasts it was indispensable to elaborate on additional staining techniques at the cellular level.

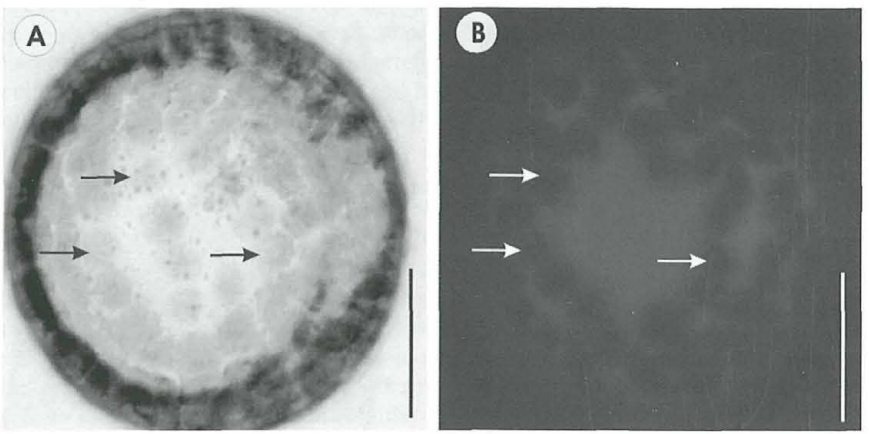


Fig. 2. Leaf protoplast of *Picea abies* L. Bright field (A) and BmCl fluorescence image (B). (A) Bright field image showing roundish protoplast with chloroplasts (arrows). (B) Fluorescence picture showing positive fluorescence in cytoplasm. Chloroplasts are not stained (arrows). Bars = 20 µm.

## Determination of Total Glutathione (t-GSH)

For the detection of t-GSH in plant cells indirect immunoassays for light- and electron microscopy were developed. Light microscopical investigations were performed with leaf protoplasts of *Cucurbita* (Figs. 3, 4). Specimen were labeled with a primary antibody against glutathione (MÜLLER & al. 2003, 2004) and stained with fluorochrome-conjugated secondary antibody (Alexa Fluor 488, Figs. 3A, B). For testing the sensibility of this immunohistochemical method in light microscopy, the glutathione status of the cells was manipulated either by stimulating glutathione biosynthesis by OTC that is a synthetic cysteine precursor and a

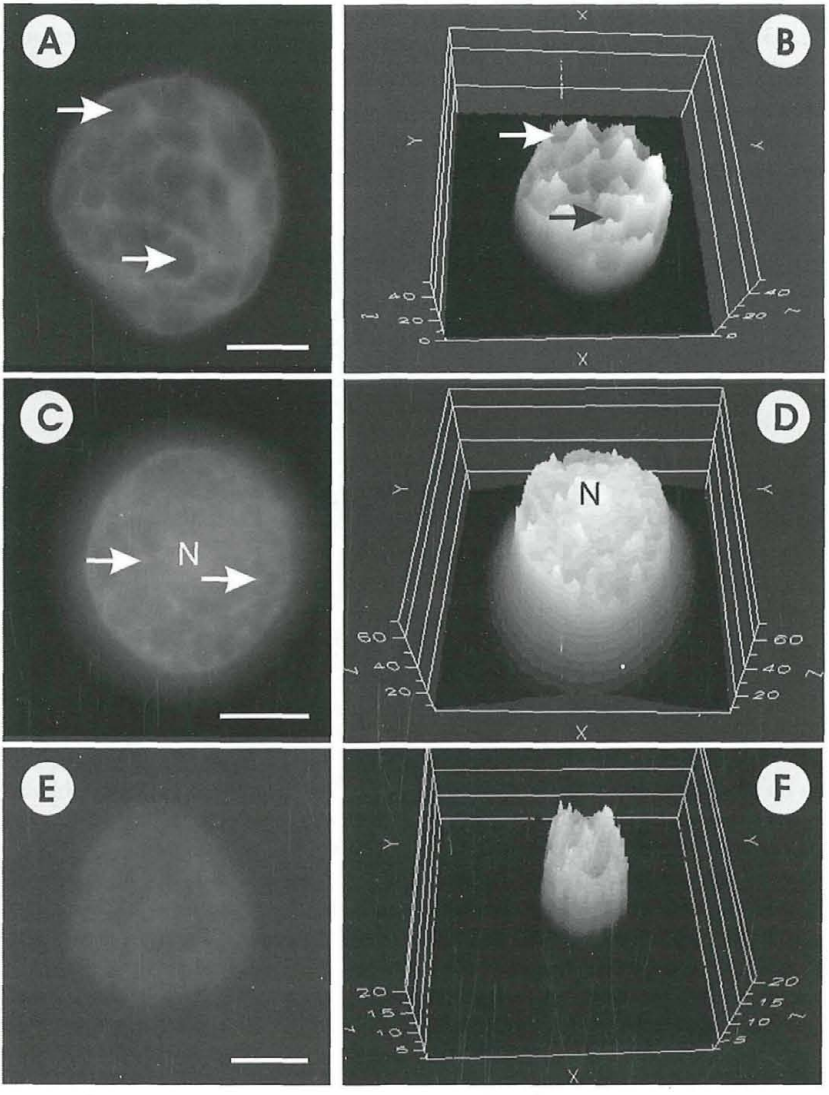


Fig. 3. Leaf protoplasts of *Cucurbita pepo* L. Immunofluorescence images (A, C, E) and appropriate luminescence profiles (B, D, F). (A, B) Untreated protoplasts showing positive fluorescence in cytoplasm. Chloroplasts are not stained (arrows). (C, D) 1 mM OTC treated cell is characterized by stronger fluorescence in cytoplasm and in nucleus (N). Mitochondria are visible as bright fluorescence dots (arrows). (E, F) Treatment with 1 mM BSO leads to very poor fluorescence image. Bars = 20 μm.

prolin analogue (Figs. 3C, D), or by inhibiting glutathione biosynthesis by BSO (Figs. 3E, F). The effects of these treatments could be observed in an increase (in case of OTC) or a decrease of t-GSH in investigated leaf protoplasts (Figs. 3, 4).

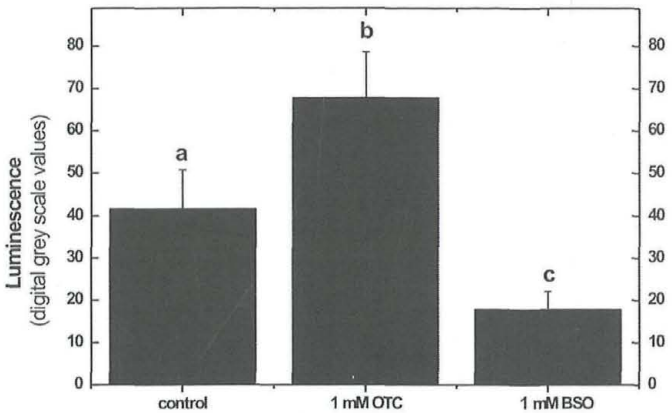


Fig. 4. Quantification of t-GSH in control, 1mM OTC and 1 mM BSO treated *Cucurbita pepo* L. leaf protoplasts. Luminescence, lead back to fluorescence, is represented as means of 20 to 30 replicate samples ± standard deviation. Different letters (a, b, c) indicate significant differences ($P<0.01$) analyzed by two-way analysis of variance (ANOVA).

With this background, it was of interest to collect further and more precise data on t-GSH localization and t-GSH contents in plant cells of *Cucurbita* by using transmission electron microscopy. The subcellular localization of glutathione was investigated with an indirect immunogold-labeling method (MÜLLER & al. 2003, 2004). On all investigated sections gold particles bound to glutathione were present after the immunoreaction, but the labeling intensity varied over a wide range between the compartments. Gold labeling could be observed in the cytosol and nearly all cell organelles; the highest intensities were detected in mitochondria; very low or an absence of gold labeling was observed in ER, vacuoles and in the cell walls. The density of gold particles bound to glutathione in chloroplasts was lower than expected from biochemical data (NOCTOR & al. 2002). Since plastids are thought to be the main center of glutathione synthesis those results indicated a relocation of glutathione after its production from the chloroplasts, into the cytosol where it is then transported into other compartments like mitochondria (FOYER & al. 2001, NOCTOR & al. 2002). Glutathione contents were found to be higher in nuclei than in the cytosol after BmCl staining (Fig. 1, MÜLLER & al. 2002) and immunohisto- (Fig. 3) and immunocytochemical investigations (Fig. 5, MÜLLER & al. 2004). It was not possible so far to detect glutathione in vacuoles with biochemical methods and the microscopical techniques described in this paper (Figs.

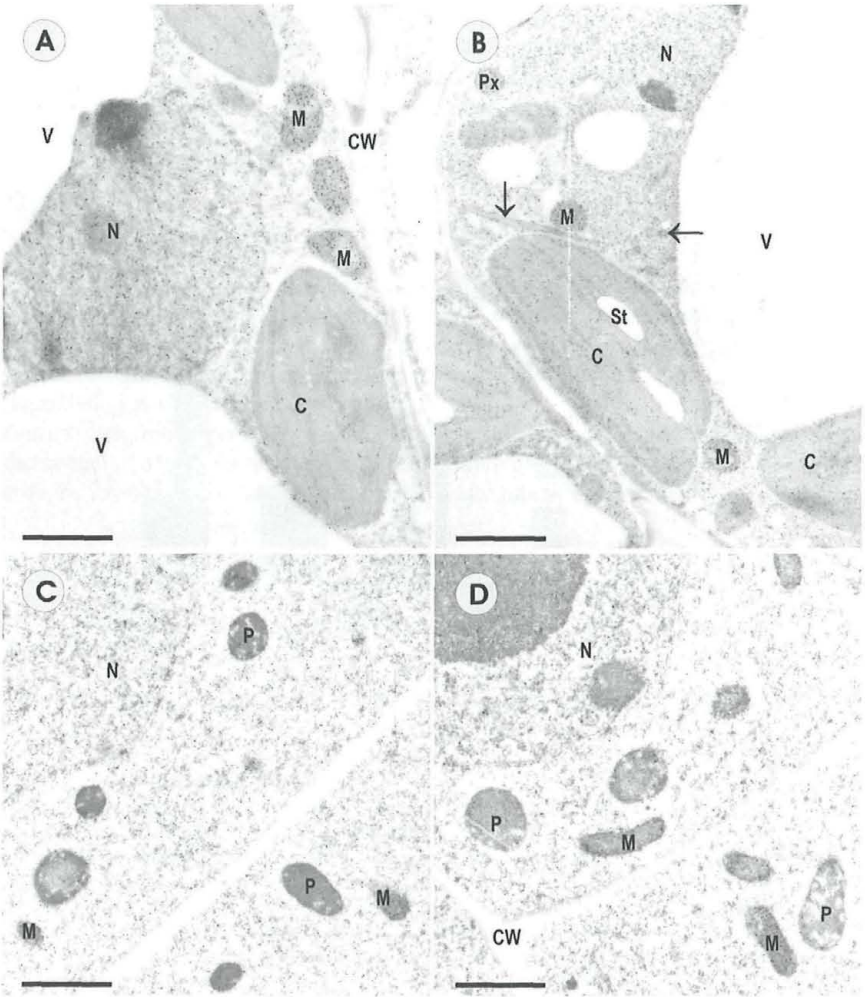


Fig. 5. Transmission electron micrographs of leaf mesophyll and root tip cells with immunogold labeling of glutathione. Cells from sections, taken from the center of the leaves and from root tips fixed for immunogold labeling and post stained for 15 sec. with uranyl acetate. Bars=1 µm. A) Control cells of younger leaf showing gold particles in chloroplasts (C), mitochondria (M), the nucleus (N) and the cytosol. B) ZYMV-infected cells of younger leaf with cylindrical inclusions (arrows) in the cytosol and gold particles in mitochondria (M), peroxisome (Px), the nucleus (N) and chloroplasts (C) with starch (St). C) Control root tip cells showing gold particles in mitochondria (M), plastids (P), the nucleus (N) and within the cytosol. D) ZYMV-infected root tip cells with gold particles in plastids (P), mitochondria (M), the nucleus (N) and the cytosol. In general no or only few gold particles bound to glutathione were found in cell walls (CW) and vacuoles (V) within root tip and leaf mesophyll cells.

1, 5). Therefore it seems that glutathione is absent from vacuoles and that its distribution within plant cells is limited to the cytosol and to organelles (FOYER & RENNENBERG 2000, RENNENBERG 2001, MÜLLER & al. 2004). Apoplastic glutathione contents were also not detected with the above described microscopical methods.

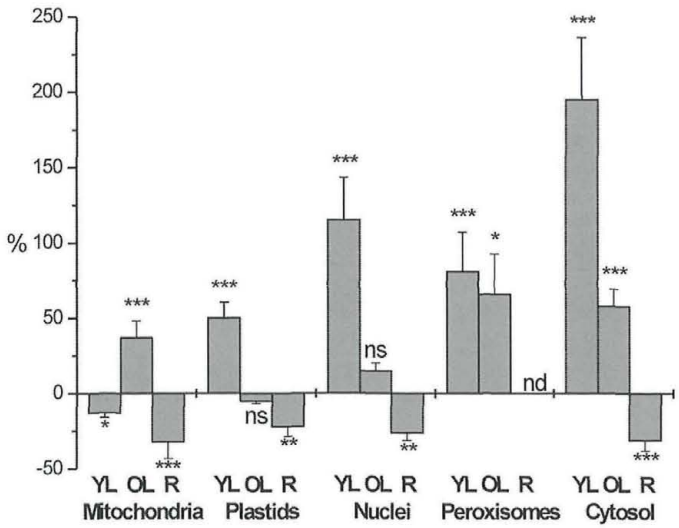


Fig. 6. Statistica graph showing the distribution of t-GSH in ZYMV-infected leaf mesophyll and root tip cells in comparison to the control. Values are means ± standard deviation and document the percentage of increase or decrease in the amount of gold particles bound to glutathione. nd=not determined; n > 20 for peroxisomes and n > 60 for other cell structures. Significant differences between organelles of control and organelles of ZYMV-infected cells were calculated with the Mann Whitney U-test. ns if $p>0.05$, (*) if $p<0.05$, (**) if $p<0.01$, and (***) if $p<0.001$; YL=younger leaves, OL= older leaves and R=roots.

Further data of subcellular localization of glutathione were collected in connection with the influence of Zucchini yellow mosaic virus (ZYMV) infection on *Cucurbita pepo* plants (ZECHMANN & al. 2005). The results showed an increase of gold labeling in all compartments except for mitochondria of younger leaves and chloroplasts, nuclei of older leaves and a decrease in root cells after ZYMV infection compared to uninfected plants (Figs. 5, 6). The data of the leaf cells did not correspond to the biochemical analyses, which showed that glutathione levels of the whole organ remained more or less unchanged during ZYMV infection. The cells, which were investigated for immunogold labeling, were intact mesophyll

cells that were still fighting against ZYMV disease and the observed high glutathione levels especially of mitochondria and peroxisomes could have helped to suppress the ZYMV induced oxidative stress (cf. ZECHMANN & al. 2005). The obtained results showed that during compatible viral-infections local increases in glutathione-contents can occur in intact cells, which will not be detected if a homogenate of a whole or parts of a leaf is investigated.

## Conclusions

We can conclude that with the use of the above described methods it is possible to obtain precise data about the distribution of glutathione contents in different compartments by light and electron microscopy. Nevertheless, to gain a deeper insight into different signaling pathways during glutathione synthesis and degradation it would be necessary to collect and compare data about the distribution of glutathione precursors since there are not any systematic investigations of the subcellular expression of glutathione available for plant tissues thus far. Therefore the investigations have to be continued and intensified in this field.

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