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Histochemical Localization of Glutathione in Plant Tissues: A Comparison of Two Fluorescence Staining Methods

By

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S u m m a r y

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Two fluorescent dyes monochlorobimane and mercury orange that label glutathione (GSH) were used as a tool for measuring the intracellular distribution of GSH in living epidermal cells of *Allium cepa*. Both agents gave repeatable staining which could be subjected to quantitative image analysis. Blockage of thiol groups with N-ethylmaleimide (NEM) decreased fluorescence of both dyes to a similar extent demonstrating specifity to –SH groups.

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

Glutathione (GSH) is found in most prokaryotic and eukaryotic cells. In plant cells numerous physiological functions have been attributed to GSH (FOYER & al. 1997), as regulation of the inter-organ sulfur allocation (e.g. HERSCHBACH & RENNENBERG 1991, 1994, LAPPARTIENT & TOURAINE 1996), action as a regulator of gene expression (e.g. WINGATE & al. 1988), it is the precursor of phytochelatins, it is a substrate for the GSH S-transferases etc. Glutathione may also be involved in the redox regulation of the cell cycle (e.g. SÁNCHEZ-FERNÁNDEZ & al. 1997) and often GSH has been considered to play an important role in defence of plants against oxidative stress (ALSCHER 1989, GRANT & al. 1996).

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Glutathione contents in plants are known to be affected by various conditions, including oxidative stress, exposure to heavy metals, atmospheric pollution etc. Effective control of for instance foliar GSH synthesis could be mediated by subcellular compartmentation (NOCTOR & al. 1998). Glutathione is found in cytosol and chloroplast, but precise determination of compartment concentrations is confounded by the possible exchange between the different compartments during subcellular fractionation (KLAPHECK & al. 1987). For animal and mammalian tissues different methods have been used for measuring the intracellular distribution of glutathione. To the best of our knowledge and to data from the literature (COLEMAN & al. 1997, SÁNCHEZ-FERNÁNDEZ & al. 1997) nearly no comparable data exist for plants.

The objective of this study was to trace the subcellular distribution of GSH with two different fluorescence techniques in an in vivo model plant system.

Materials and Methods

Plant material

Epidermal cells of *Allium cepa* L. were used as an in vivo model system.

Staining with monochlorobimane and mercury orange

Monochlorobimane (BmCl; Molecular Probes Inc.) was dissolved in methanol and diluted in phosphate buffer pH 8.3 to obtain the required concentration. The epidermis samples were stained in this solution at room temperature for different times. After removing the staining solution the preparations were thouroughly washed with buffer for 30 sec to remove excess staining solution (modified according to RICE & al. 1986, MIRABELLI & al. 1992).

Mercury orange [1(4-chloromercuryphenyl-azo-2-naphthol); Sigma] was dissolved in acetone and adjusted to the required concentration in 9:1 (v/v) acetone-water and kept at 4°C. The epidermal cells were stained in this solution at 4°C for different times. After removing the staining solution the preparations were thouroughly washed with acetone-water (9:1, v/v) for 5 min to remove excess staining solution, and then washed twice for 5 min with distilled water (modified according to LAURRAURI & al. 1987, THOMAS & al. 1995).

Blocking of thiol-groups by N-ethylmaleimide (NEM, Sigma) was performed to verify the selectivity of the staining regarding thiol groups. NEM stock solution was prepared in 2-propanol and diluted to the required concentration with phosphate buffer pH 8.3. After blocking of thiol groups in this solution, specimen were thoroughly rinsed in buffer to remove excess NEM.

Quantitative fluorescence microscopy

Equipment: fluorescence microscope (Zeiss, Axioskop) with a 50 W mercury arc lamp, 3-chip-colour video camera (Sony DXC 930 P with Sony-control-system), a frame grabber (ITI MFG-3M-V, Imaging Technology Inc., with variable scan module AM-VS-VP and colour recording module AM-CLR-VP). The frame grabber has a resolution of 1024 x 1024 pixels with 24 Bit true colour (24 + 4 Bit), 4 Bit overlay, 0.5 MB overlay memory, 3 MB image memory, 4 MB program and data memory. The image-CPU is a 40 MHz TI 34020. The central computer is a 66 MHz i486 DX/2 AT (R+R Inc.) with a Cirrus VGA-board, two 17" monitors Flexscan F550i-w (Eizo Inc.) and a 1 GB harddisk. The computer works under the operating system Windows 95 (Microsoft Inc.) Image analysis software is Optimas (BioScan Corp.).

Each fluorescent image was obtained through a 20x dry objective and digitized into a 512x480 image frame onto 256 levels. For BmCl fluorescence, cells were excited at 365 nm and the fluorescence was imaged through a 395 nm dichroic mirror and a 420 nm long pass filter. For

mercury orange fluorescence, the excitation wave-length was 510-560 nm and the images were acquired by using a 580 nm dichroic mirror and a 590 nm long-pass barrier filter.

For the quantification of the fluorescence yield digital images were processed with the help of Optimas. Average luminance values (on a 0-255 scale) of cells were calculated after background subtraction. For mercury orange treatments, digital filtering using a 3x3 median and a color filter was performed before calculation.

Results and Discussion

GSH is an ubiquitous compound that is important in cellular defence mechanisms against free radicals and reactive oxygen species (MEISTER & ANDERSON 1983). For mammalian tissues there is good evidence that subcellular GSH pools exist in cytosol and mitochondria, each with different rates of turnover and depletion (e.g. JOCELYN & CRONSHAW 1985, REED 1990). In order to protect nuclear structures from damage and to participate to DNA synthesis GSH must also be present in the nucleoplasm, but little is known about the nuclear GSH content (cf. THOMAS & al. 1995). There is a dearth of reliable information concerning the intracellular localization of glutathione in plants.

In this paper two cytochemical staining techniques frequently used for mammals and animals have been modified to allow a quantification of cell GSH by fluorescence microscopy and image analysis.

Monochlorobimane produced a strong blue cellular fluorescence in all cells upon the UV excitation (Fig. 1a). The mercury orange staining yielded a bright orange-red fluorescence upon strong green excitation (Fig. 1b). On a qualitative basis, the fluorescence appeared to the same extent in cytoplasm and nucleoplasm. For monochlorobimane the optimum staining conditions defined by the strongest fluorescence were 30 μM for 3 min, while for mercury orange the optimum was 50 μM for 5 min.

A range of conditions was investigated in order to ensure that staining of GSH was saturated while minimising background labelling of protein sulphydryls. The problem of nonspecific staining of protein-SH groups was overcome by utilizing the findings of different work groups, as for instance ASGHAR & al. 1975 that the –SH groups of small molecules, such as GSH and cysteine, react with the SH reagents at fast rate, whereas the spontaneous reaction with other cellular thiols is very slow (e.g. RICE & al. 1986, COOK & al. 1991).

Blocking of GSH by alkylation with NEM decreased fluorescence yield by both dyes. The rest fluorescence yield after blocking by NEM measured by image analysis system was 0 to 5% for the monochlorobimane staining and a maximum 10% for the mercury orange staining. The nuclear and cytoplasmic GSH contents were blocked to a similar extent. The optimum condition for a maximum decrease of fluorescence was 10 mM NEM for 1 min.

The used staining solutions also react rapidly with cysteine, and the methods described here should probably be considered an assay for non-protein sulphydryls, rather than for GSH only. However, cellular cysteine levels are generally much lower than GSH concentrations (RENNENBERG 1997). With the

establishment and further development of these methods open key questions involving intracellular GSH distributions may be addressed (NOCTOR & al. 1998).

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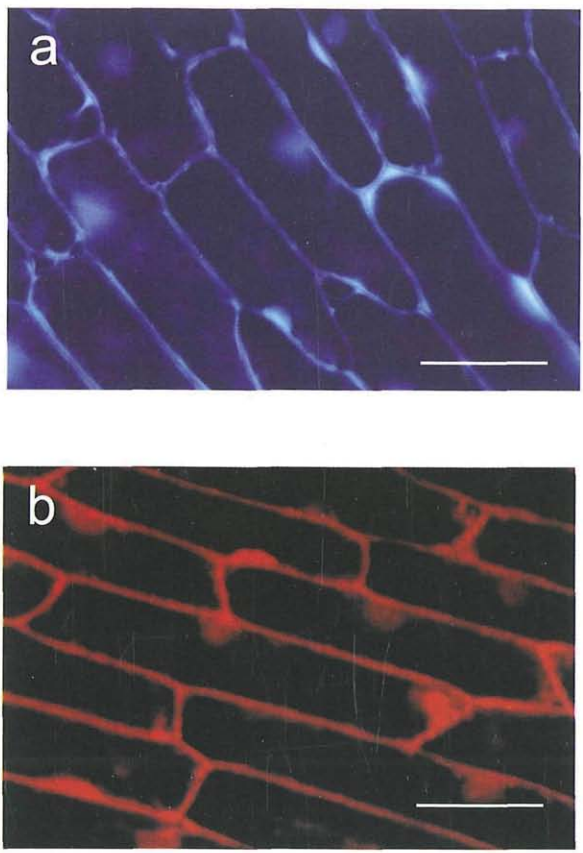

Fig. 1. Intracellular localization of GSH in epidermal cells of *Allium cepa* L. a) Fluorescence image of the intracellular distribution of monochlorobimane-GSH adducts. b) Fluorescence image of the intracellular distribution of mercury orange-GSH adducts. Bars = 50 µm.