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# Organization of interphase microtubules and actin filaments in spruce callus cells after glutathione treatment

Andreja URBANEK, Bernd ZECHMANN, Günther ZELLNIG & Maria MÜLLER

Institute of Plant Physiology, Karl-Franzens-University Graz, Schubertstraße 51, 8010 Graz, Austria. E-mail: andreja.urbanek@stud.uni-graz.at

**Abstract.** Changes in the distribution of microtubules (MT) and actin filaments were examined in suspension-cultured spruce cells [*Picea abies* (L.) KARST.] that were exposed to different concentrations (500 and 1000 μM) of exogenously applied reduced glutathione (GSH). Using fluorescence microscopy the MT were visualized with monoclonal anti-tubulin antibodies and actin filaments were stained with rhodamin labelled phalloidin (RLP). GSH-treated callus cells showed modifications on the form and arrangement of both cytoskeletal elements, when compared to the control.

**Keywords:** Glutathione, spruce callus cells, microtubules, actin filaments

**Abbreviations:** GSH reduced glutathione; MT microtubules, RLP rhodamin labelled phalloidin; DAPI 4', 6-Diamidino-2-phenyl-indol-dihydrochlorid;

## Introduction

The plant cytoskeleton is a membrane-associated structure, which is essential in a variety of cell processes like cell growth and differentiation, cell division and cytoplasmatic streaming (VOLKMANN & BALUŠKA 1999, DAVIES 2001). Callus cells are commonly used for fundamental studies of the cytoskeleton, as they show dynamic changes in their form and arrangement in response to environmental perturbations (SIVAGURU & al. 1999, BARLOW & BALUŠKA 2000).

GSH is a significant member of low molecular weight thiols and is ubiquitous to plants and animals (FOYER & RENNENBERG 2000). The influence of exogenously applied GSH on plant sulfur metabolism and its involvement in defence reactions were previously studied (WINGATE & al. 1988, SÁNCHEZ-FERNÁNDEZ & al. 1997, ZELLNIG & al. 2000, MÜLLER & al. 2001). Positive effects on root growth through cell division rate were observed on *Arabidopsis* (SÁNCHEZ-FERNÁNDEZ & al. 1997). On the other hand, elevated concentrations of GSH in plant tissues are reported to be deleterious. Transformed tobacco lines with elevated GSH-biosynthesis capacity showed increased oxidative stress, stunted phenotypes and leaf necrosis (CREISSEN & al. 1999). Furthermore, a decrease in mitotic index, increased chromosomal aberrations and alterations in the ultrastructure were reported in different spruce tissues after GSH-treatment (ZELLNIG & al. 2000, MÜLLER & al. 2001).

To clarify, whether alterations of the cytoskeleton are responsible for the observed GSH-induced chromosomal aberrations, the effects of exogenously applied GSH on the arrangement of the MT and actin filaments were investigated in spruce callus cells during the interphase.

## Material and methods

### Plant material and GSH-treatment

Callus cultures of *Picea abies* (L.) KARST. were established from cotyledons by using solid MS medium (MURASHIGE & SKOOG 1962) with ingredients according to MÜLLER & al. (2001) [3 % sucrose, 1 % agar, 1-naphthalenacetic acid (3mg/l) and 6-benzylaminopurine (1mg/l), pH 5.8]. The callus cultures were cultivated in a growth chamber (21°C, 12 h photoperiod, light intensities between 33 and 43.7 μmol $m^{-2}s^{-1}$) and were sub-cultured on fresh MS medium every two weeks.

For our experiments callus tissue was divided into small pieces (500 ± 50 mg fresh weight) and transferred to flasks containing 50 ml sterile liquid MS medium supplemented with the same hormone composition as described above and with either 500 μM GSH, 1000 μM GSH or no GSH (control). Four flasks of suspension culture were used for each treatment. The GSH treated material and controls were maintained for 48 hours under the same light and temperature regimen as the cultures on solid medium and remained unshaken to make sure that the GSH was not oxidized. This procedure was repeated five times and about 600 callus cells were recorded.

### Fluorescence microscopy

**Indirect immunofluorescent labelling of α-tubulin:** Control samples and GSH-treated material were processed according to the method described by WICK & al. (1981) and APOSTOLAKOS & GALATIS (1999). Briefly, callus tissue was fixed in 4 % paraformaldehyde in a MT stabilising buffer (MSB: 50 mM PIPES in 0.1 M KOH, 5 mM EGTA, 2 mM $MgSO_4$, pH 6.8) for 45 minutes at room temperature (RT), washed in MSB, and digested in 2 % cellulase and 1 % macerozyme in MSB for 45 minutes at RT. The samples were then washed in MSB for 30 minutes and extracted with 2 % Triton X-100 in phosphate-buffered saline (PBS, pH 7,3) for 120 minutes. After washing in PBS, squashed cells were incubated with the primary antibody (monoclonal mouse IgG1 anti- α-tubulin clone B-5-1-2, Sigma) diluted 1:150 in PBS and blocked in 1 % bovine serum albumine for 40 minutes in a moist chamber at 37°C. The specimen were washed several times in PBS and incubated with the fluorochrom-labelled secondary antibody [alexa fluor 488 goat anti-mouse IgG (H+L) conjugate, Molecular Probes] diluted 1:30 in PBS for 60 minutes at 37°C in a moist chamber. After extensive washing in PBS, sections were mounted on slides in an antifade agent (Citifluor PBS solution AF3, Gröpl).

**Staining of actin filaments:** For actin filaments staining a modified method according to OLYSLAEGERS & VERBELEN (1998) was used. The plant material was incubated on slides in actin buffer (100 mM PIPES in 0.2 M KOH, 10 mM EGTA, 5 mM $MgSO_4$ and 0.2 M mannitol, pH 6.9), containing 2 % glycerol and 66 nM RLP for 1 hour.

**Staining of nuclei:** Additionally, after the labelling of MT and actin filaments selected material was stained by applying 5 ml of a DAPI solution (4', 6-Diamidino-2-phenyl-indol-dihydrochlorid, 10 mg DAPI/ml distilled water) to 100 ml of corresponding buffer.

**Equipment:** A Zeiss Axioskop equipped with a 100 W mercury arc lamp was used to obtain digital images with a 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-CLR-VP and colour recording module AM-CLR-VP). Optimas 6.5.1 (BioScan Corp.) was used as image analysis software. Fluorescence images were obtained through a Plan-Neofluar 63x dry objective (n. a., 0.95) and a Plan-Apochromat 100x oil immersion objective (n. a., 1.4).

MT were investigated with a 450–490 nm excitation and 520 nm emission filter block. Rhodamine labelled actin was visualised with a 546/12 nm excitation and 590 nm emission filter block. Chromosome labelling was obtained at 365/12 nm excitation and the fluorescence was imaged through a 397 long pass filter.

## Results

In GSH-exposed callus cells alterations in the organization of interphase MT were observed. In all callus cell types treated with 500 µM and 1000 µM GSH the cortical MT appeared as shortened and tortuous structures (Fig. 1 a). Additionally, MT showed severe alterations in form of tubulin-positive dots in cortical cell areas and in association with the nuclei at both glutathione concentrations (Fig. 1 b). In contrast, such abnormal structures were not observed in control cells. They exhibited an intact cortical MT network characterized by arrays, which showed different organization depending on the cell form. In oblique callus cells with polar cell extension the MT were observed throughout the cell cortex arranged perpendicular to the cell growing axis and parallel to each other (Fig. 1 c). Whereas, disorganized cortical MT were observed in spherical callus cells of control material. In all types of control cells MT were also found extending from the nucleus towards the plasma membrane (Fig. 1 d). Furthermore, they formed a thick network around plastids connecting with other organelles and the cell membrane.

Similar impacts of the GSH-treatment (500 µM and 1000 µM) were observed on cortical actin filaments of interphase cells as already described on MT. The actin filaments remained as small, tortuous fragments or bright fluorescent dots in the cell cortex. Mostly only a diffuse fluorescence was noticed. The transvacuolar actin cables could not be recognised. The actin baskets around the nuclei persisted, however, they were amorphous in appearance and only seldom observed as arrays that emerged from the nucleus to the plasma membrane. They ended blind in the cytoplasm (Fig. 1 e). Only a diffuse fluorescence was seen around the plastids; an anchoring of plastids at the plasma membrane was lacking. On the other hand, control callus cells showed very well preserved cortical actin filaments (Fig. 1 f). Also transvacuolar actin cables and actin arrangements in the association with nucleus and organelles were clearly evident within control cells.

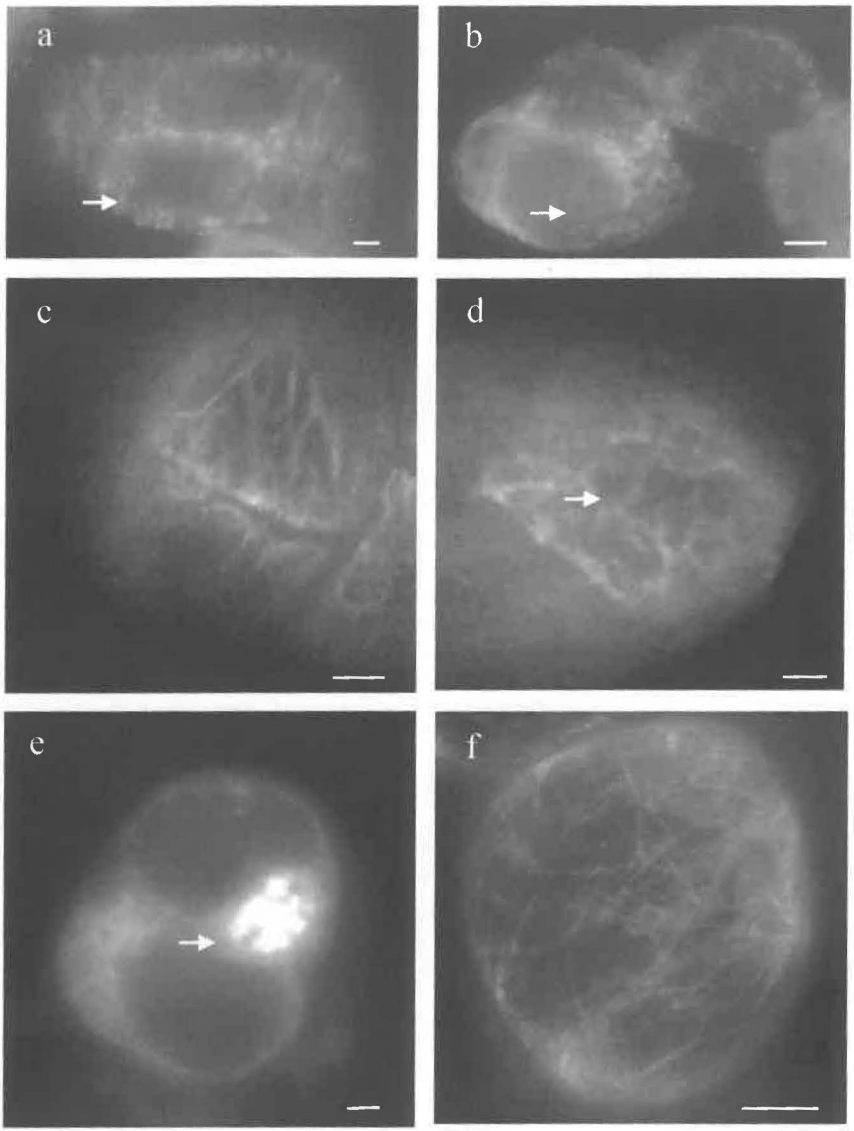


Figure 1: (**a–d**) Arrangement of MT in GSH-treated (1000 μM GSH) spruce callus cells (**a, b**) and control callus cells (**c, d**) during interphase. (**a**) Shortened and tortuous MT (arrow) observed in the cell cortex of GSH-treated callus cells. (**b**) Cells with tubulin-positive dots in cortical cell area and in association with the nucleus (arrow). (**c**) A cortical MT network with arrays arranged parallel to each other and perpendicular to the cell-growing axis. (**d**) MT radiation from the nucleus (arrow) towards the plasma membrane in oblong callus cell.
Distribution of actin filaments in GSH-treated (1000 μM GSH) callus cells (**e**) and in control callus cell (**f**). (**e**) Amorphous actin-positive fluorescence was noticed in association with nucleus (arrow) (staining with DAPI). (**f**) Thin cortical filaments visualized in control callus cell. Bars = 10 μm.

## Discussion

The study of the cytoskeleton in spruce callus cells reported in this paper has revealed the appearance of modified MT and actin filaments after GSH-treatment. Since cytoskeletal elements are involved in mitotic cycle (NICK 1999), the disruption of MT and actin filaments could be related to recently reported chromosomal damages in GSH treated cell tissues (ZELLNIG & al. 2000, MÜLLER & al. 2001). After GSH-treatment shortened and tortuos MT were observed in spruce callus cells during the interphase. Moreover, GSH-treated cells were noticed, containing tubulin-positive dots in the cortical cell area. A similar phenomenon was described in colchicin treated cells of *Vigna sinensis* (APOSTOLAKOS & al. 1990) and in cryptogein and oligogalacturonide treated cells of *Nicotiana tabacum* (BINET & al. 2001).

Interphase cells of control spruce material, showed an intact cortical MT network delineating the plasma membrane (NICK 1999). In callus cells, which undergo cell differention in a polar manner, parallel MT arrays arranged perpendicular to the cell-growing axis were found to be typical. Whereas, in spherical callus cells cortical MT are laying unorganized, as previously described in suspension culture cells of *Solanum tuberosum* (COLLINGS & EMONS 1999).

In GSH-treated spruce material similar changes in arrangement of cortical actin filaments were observed as described on cortical MT network of interphase cells. Alterations were also visible on transvacuolar actin cables, nucleus- and plastid-associated filaments. They were observed in form of short stretches or were noticed as diffuse fluorescent structures. Arrays emerging from the nucleus to the plasma membrane were seldom observed. Otherwise, control callus cells were characterized by very well preserved actin bundles, which were similar to actin organizations previously described in other papers (JUNG & WERNICKE 1991, CLEARY 1995, LAZZARO 1996, DE RUIJTER & EMONS 1999, KANDASAMY & MEAGHER 1999).

The arrangement of the cytoskeleton in the interphase is of great importance in a variety of cell processes including cell growth and cell morphogenesis (KOST & al. 1999, VOLKMANN & BALUŠKA 1999, DAVIES 2001). Based on our results, we could postulate that GSH-induced disruption of cortical MT and MF network in spruce callus cells caused limited cell elongation or disabled a cleary polar cell elongation. Furthermore, the alterations on nucleus-associated actin network, found in the present study, may indicate possible abnormalities during the cell division, since actin filaments are involved in the transport of the nucleus to the cell centre prior to the cell division (NICK 1999).

## Conclusion

Although GSH is an essential substance in plant metabolism, our experiments demonstrated that the plant cytoskeleton reacts sensitively to exogenously applied GSH. Severe alterations in the form and arrangement of the cytoskeleton in GSH-treated spruce callus were observed during interphase.

The impacts of GSH-treatment on interactions among cytoskeletal elements and the correlation between changes in patterns of actin filaments and MT require further investigations.

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