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Rapid reorganization of microtubular cytoskeleton accompanies early changes in nuclear ploidy and chromatin structure in postmitotic cells of barley leaves infected with powdery mildew

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Summary. Post-mitotic epidermal cells of barley leaves were found to contain, in addition to cortical microtubules (CMTs), distinct arrays of endoplasmic microtubules (EMTs). These encircle nuclei and continuously merge into the CMT arrays that underly the plasmalemma. Detailed three-dimensional reconstruction of both types of MTs during fungal infection showed that profound and very rapid MT rearrangements occurred especially in the case of incompatible (resistant) barley–powdery mildew genotype combination. The most early MT responses, followed by their subsequent complete disintegration, were recorded around nuclei. These events might be relevant for the induction of such nuclear processes as onset of DNA synthesis and nuclear chromatin condensation. Observed pattern of early infection events, as well as less prominent responses in the case of compatible (susceptible) barley–powdery mildew genotype combination, both findings suggest that rapid reorganization of the MT cytoskeleton could be involved in recognition of the fungus by host cells and in the initiation of resistance responses in barley leaves. We hypothesize that the integrity and dynamics of the MT cytoskeleton, especially of its perinuclear part, might participate in control mechanisms involved in activation of resistance genes.

Keywords: Barley leaves; Chromatin condensation; Confocal microscopy; Microtubules; Nuclear events; Ploidy levels; Powdery mildew.

Abbreviations: CMTs cortical microtubules; EMTs endoplasmic microtubules; MT microtubules; PI propidium iodide; SC sensitive combination; RC resistant combination.

Introduction

The host–parasite interaction between barley and powdery mildew fungus (*Erysiphe graminis* f. sp.

hordei Marchal) represents an interesting experimental system which has been comprehensively studied for several decades. The sequence and timing of all events involved are already well described and understood at several levels. In the compatible host–parasite combination, when host cells (only epidermal leaf cells are colonized with the fungus) do not possess a powerful and effective defense mechanism, conidium germination is closely followed by formation of an appressorium which gives rise to a penetration hypha. This structure, after its successful penetration through the host's cell wall, further develops into a ramified vesicle (haustorium), which is known to be the most essential step in the chain of events necessary for successful colonization of host epidermal cells (Bushnell and Bergquist 1975).

However, in the incompatible host–parasite combination, the fungus either fails to penetrate the cell walls of the barley leaf's epidermis, or more often, fails to develop into a primary haustorial body. An intriguing feature of importance for resistance of barley leaves during the incompatible interactions appears to be prominent cytoplasmic aggregations around the penetration site of the host cell wall, where germinated hyphae attempt to accomplish their penetrative growth. These aggregations of cytoplasm are much more pronounced in incompatible (resistant) interactions when compared with compatible (susceptible) interactions, and have been shown to be involved in the localized deposition of cell wall-like structure –

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the so-called papillae (Russo and Bushnell 1989). This protective structure, which is deposited between the cell wall and the plasmalemma subjacent to the penetrating site in the cell wall, represents a significant component of the host cell resistance to powdery mildew attack (Bushnell and Bergquist 1975, Aist et al. 1988).

In an earlier paper, we described an apparently continuous network of endoplasmic MTs (EMTs) encircling the nucleus and connecting it with the cortical array of MTs (CMTs) (Baluška et al. 1992). We hypothesized that the continuity of these two MT arrays might be important in providing the plant cell with a signalling system responsible for the transfer of information between the cell wall and nucleus. This premise ensues from the dynamic instability of individual MTs (Kirschner and Mitchison 1986) which confers specific properties on the whole MT cytoskeleton enabling it to act as a means for quick and reversible communications between one part of the cell and another (Kirschner and Mitchison 1986). An intriguing aspect of this unique structural “information channel” is that the organization of MT arrays is not directly determined by genomic information but rather, due to the specific instability of MTs, by the physical and chemical properties of the endocellular and exocellular milieu (Schilstra et al. 1991, Cyr 1992, Baluška et al. 1993).

If the putative MT-based informational system should influence nuclear events, which are known to be essentially involved in developing the resistance response (Lawton and Lamb 1987, Schmelzer et al. 1989), we could expect that disintegration of the MT cytoskeleton will have a significant impact on the nucleus. Exactly this was found when depolymerization of MTs in metabolically inactive and slowly cycling cells of quiescent centre in maize root apex induced the stimulation of their traverse through the cell cycle interphase (Baluška and Barlow 1993). Moreover, irrespectively of the anti-MT treatment applied (colchicine, oryzalin, and low temperature), complete disintegration of the MT cytoskeleton immediately elicited nuclear enlargement and decondensation of the chromatin complex in cells of the quiescent centre. Our latest findings (Baluška et al. unpubl.) indicate that, after the same anti-MT treatments, very similar nuclear changes occur in actively dividing nuclei of the apical meristem. More importantly, stabilization of the MT cytoskeleton with taxol results in the opposite nuclear responses.

Recently, important new findings on the early infection events during plant–fungus interactions were published (Kobayashi et al. 1992, Bacigálová and Hauskrecht 1993, Gross et al. 1993) which propose that depolymerization of the MT cytoskeleton might be an inherent part of sequence events leading to the activation of multiple defence mechanisms. Prominent re-arrangements of whole MT cytoskeleton were found to be accomplished during the cytoplasmic aggregation in epidermal cells of barley leaves under fungal attack of pathogen *Erysiphe graminis* f. sp. *hordei* and non-pathogen *Erysiphe pisi* (Kobayashi et al. 1992). These changes in the MT organization were more pronounced in the resistant, i. e., incompatible combination, when barley leaves were inoculated with non-pathogen *E. pisi*. Also in suspension-cultured parsley cells inoculated with the fungus *Phytophthora infestans* (Gross et al. 1993), rapid translocation of plant cell cytoplasm to the fungal penetration site was shown to be associated with a local disintegration of MTs as well as with a subsequent activation of defence genes.

A preliminary report indicated that barley powdery-mildew infection might elicit relatively rapid changes in DNA contents and chromatin structure in epidermal cells of barley, especially in the resistant host–parasite combination (Bacigálová and Hauskrecht 1993). A major aim of the present work was to test the attractive hypothesis that the early sequential changes in the organization of the MT cytoskeleton and the nucleus might be directly related to the expression of host resistance at later stages of the host–parasite interactions. As the first step in doing this, we set out to obtain comprehensive data on time course relationships between MT and nuclear responses to fungal infection. In order to obtain this information, we have performed here a detailed temporal cytophotometric analysis of such nuclear features of host epidermal cells as ploidy levels and chromatin structure in combination with corresponding temporal three-dimensional investigations of EMTs and CMTs using confocal microscopy. As experimental material, we have used young primary leaves of barley inoculated with conidia of two powdery mildew pathotypes differing in their virulence.

Materials and methods

Plant material

Seedlings of barley (*Hordeum distichon* L.) cv. Ricardo were grown in a growth chamber under controlled conditions as reported earlier. (Frič and Wolf 1992). The first leaves of 7-day-old seedlings were

densely inoculated with vital freshly-harvested conidia of *Erysiphe graminis* f. sp. *hordei* Marchal (powdery mildew), pathotypes 176 and 410. The resistance of cv. Ricardo seedlings towards powdery mildew is controlled by the *ml-a3* allele (Dreiseitl 1991). According to Dreiseitl's test-assortment, this resistance is overcome by the pathotype 410 (susceptible combination, SC), but not by the pathotype 176 (resistant combination, RC).

The inoculated primary leaves were used for further cytophotometric and immunofluorescent analysis at appropriate time intervals for both types of host–parasite combinations. For this purpose, epidermal strips were isolated from abaxial leaf surface and were mechanically fastened between two slides, stuck together using pincers. This simple technique enabled us to follow changes in large number of intact cells throughout the epidermal tissue without the need of applying the harsh treatments which are usually necessary for preparation of sectioned plant material. The technique is also particularly suitable for confocal microscopy studies as the thickness of epidermal cells (approximately 50–70 nm) is well within the range which can be three-dimensionally reconstructed.

Indirect immunofluorescent microscopy

To prevent any avoidable changes in the MT organization during isolation of epidermal strips, whole leaves were excised into 9 ml of MT-stabilizing buffer (MTSB: 50 mM PIPES buffer, 50 mM MgSO₄, 50 mM EGTA, pH 6.9) mixed with 1 ml of dimethyl sulphoxide (DMSO). After this, excised leaves were incubated for 15 min at room temperature and then fixed with 4% paraformaldehyde in the MTSB and DMSO mixture for 1 h at room temperature. Following a brief rinse in MTSB, barley leaves were prepared for isolation of epidermal strips and their subsequent mechanical mounting on slides as described above. This original immunofluorescent technique combined with confocal microscopy proved to be extraordinarily powerful and enabled us to inspect the intact MT cytoskeleton three-dimensionally throughout the epidermal tissues. To facilitate penetration of antibodies into epidermal strips, these were treated for 15 min with mixture of 1% hemicellulase, 1% driselase, 1% cellulase in 0.5 M EGTA, 0.4 M mannitol, 1% Triton X-100, 0.3 mM phenylmethylsulphonyl fluoride (all dissolved in MTSB). The enzymatic digestion was stopped by transferring the slides to MTSB for 15 min followed by 1% Triton X-100 in MTSB for 10 min. After rinsing for 20 min in MTSB, the strips were incubated with mouse monoclonal antibody raised against chick brain α -tubulin (Amersham, Buckinghamshire, U.K.), diluted 1 : 200 in PBS for 60 min at 37 °C. After a further rinse with MTSB, they were stained with fluorescein isothiocyanate-(FITC-) conjugated anti-mouse IgG raised in goat (Sigma Chemical Co, St. Louis, MO, U.S.A.), diluted 1 : 200 in PBS for 60 min at 37 °C. Following a rinse in PBS, the stained strips were treated with 0.01% Toluidine Blue in PBS for 10 min to diminish the autofluorescence of the tissues. Anti-tubulin-stained strips were finally mounted in anti-fade mountant containing *p*-phenylenediamine.

Confocal microscopy

To scan and visualize confocal images of MTs, the preparations were analysed with a Leica confocal microscope, using the 488 nm line of the argon laser for the fluorescence excitation of FITC-labelled anti-tubulin, and the 514 nm line of the argon laser for investigations on DNA stained with propidium iodide (PI). Depending on the preparation, only the FITC fluorescence intensity was recorded (single channel images), or simultaneously FITC and PI (two channel

images). Voxel sizes of 500 × 500 nm lateral, and 500 nm axial (×40 objective lens), or 200 × 200 × 500 (×100 lens) were used. The 3-D images consisted of 8 to 40 optical sections of 256 × 256 voxels each.

For the visualization of the confocal images, a Hewlett Packard/Apollo 425T workstation and Scilimage software (ten Kate et al. 1990) were used. All 3-D data sets were subjected to a contrast-enhancement algorithm to scale the fluorescence intensity values to the maximal possible range (i.e., 0–255). Single channel images were visualized as pairs of black and white superposition images obtained at different viewing angles. The two channel images are represented as the superposition of images in false-colours: FITC fluorescence in green and PI in red colour (yellow colour indicates an overlap of green and red).

Both cortical and perinuclear microtubules were inspected in six different epidermal strips for each time-treatment and parasite–host combination. Cells showed consistently similar patterns throughout the whole epidermal strips in each particular treatment. Differences between individual replicates were found to be negligible.

Cytophotometry

Isolated epidermal strips mechanically fastened to slides were immediately fixed (fixation of isolated epidermal strips allowed their more efficient stripping and did not affect significantly cytophotometric results as we found in preliminary experiments) with ethanol-acetic acid (3 : 1) for 30 min. Then, the strips were washed in running tap water for 30 min and stained with Feulgen reaction (see Bacigálová and Hauskrecht 1993) or by Coomassie Brilliant Blue R (see Wolf and Frič 1981), and the samples were embedded in the Canada balsam and enclosed with cover slips. Cytophotometric investigations of nuclei were done using a light microscope U2 (Carl Zeiss Jena) connected to a cytophotometer Quantanal AMP20 according to Kubica (1981). In each evaluated nucleus, ten points of 0.328 mm diameter were measured by monochromatic light at a wavelength of 546 nm. All points transmitting less than 30% of light were considered to represent heterochromatin. Readings between 30 and 50% of light transmission were taken for condensed euchromatin whereas all points that transmitted more than 50% of light corresponded to decondensed euchromatin. Squash preparations made from meristematic root tip portions were used for the determination of 2C value by measuring the absorption of 100 telophases (2C) and 50 prophase (4C).

For calculation of data, a personal computer was applied using special software ABIO and Quatro-Pro spread sheet Q (Borland, CA, U.S.A.). Six hundred nuclei were measured from each sample and three different epidermal strips (200 nuclei per strip) were evaluated for each time and parasite–host combination. Differences between individual replicates were insignificant.

Results

Rearrangements of the microtubular cytoskeleton

Microtubules in cells of control leaves and their reorganizations in the RC

Cells of control leaves, at any time during the 24 h experimental period, exhibited MT patterns typical for post-mitotic plant cells slowing or ceasing their growth. In particular, dense arrays of CMTs were

Fig. 1 A–H. Confocal stereo-pairs showing organization of the MT cytoskeleton in epidermal cells of control barley leaves and in cells of leaves infected with pathotype 176 of powdery mildew representing the resistant combination (RC). **A** Dense arrays of oblique CMTs in control leaves at the start of experiment (0 h). **B** Higher magnification of the MT cytoskeleton of control leaves. Note arrays of fine EMTs closely appressed to the nucleus (arrowhead), which is located near the cell wall (asterisk). **C** Dense arrays of oblique and longitudinal CMTs in control leaves at the end of the experiment (24 h). **D** and **E** Partially depolymerized EMTs around the nuclei (arrowheads), 15 min after infection. **F** Less dense arrays of CMTs, 2 h after infection. **G** Strongly disintegrated MT cytoskeleton 24 h after infection. Arrowhead indicates the position of nucleus. **H** Developing papilla surrounded by dense arrays of fine MTs located beneath the penetration site, 24 h after infection

arranged in directions oblique or longitudinal with respect to the cell axis (Fig. 1 A, C). Nuclei were closely appressed to the cell walls and showed invariably more or less abundant perinuclear EMTs which

continuously merged into CMTs arrays (Fig. 1 B). This characteristic picture quickly changed in the RC where, as early as 15 min after inoculation, CMTs looked partially affected, especially near nuclei.

Fig. 2 A–H. Organization of the MT cytoskeleton in epidermal cells of barley leaves infected with pathotype 410 of powdery mildew representing the susceptible combination (SC). **A** and **B** Dense arrays of oblique and longitudinal CMTs recorded 15 min after infection. **C** Image from the same treatment showing the nucleus (arrowhead), encircled by a basket-like structure formed from fine EMTs, that was partially liberated from the cortical system of MTs and located deeper in the cell. **D** and **E** Partially affected arrays of CMTs 2 h after infection. **F** 24 h after infection; most cells were characterized by bright CMTs that were sparsely distributed and less ordered, as compared with the control images. **G** However, few cells showed dense arrays of CMTs comparable to those of control cells. **H** MTs in directly attacked cells acquired sometimes arrangements reminiscent of the radial MT arrays found around developing papilla in the RC

These were now often located slightly deeper in the host cell interior and apparently associated with fluorescent spots of irregular shapes, suggesting that EMTs were partially depolymerized (Fig. 1 D, E).

Other parts of these cells also showed slight disturbances of their MT arrays at this stage of infection, especially regarding their density. Two hours after inoculation, CMTs were even less dense and partially

Fig. 3 A–G. Two channelled confocal images represented as the superposition of images in false-colours: FITC fluorescence in green and PI in red colour (yellow colour indicates an overlap of green and red). **A** Nuclei of control cells encircled by perinuclear EMTs. **B** Well-preserved arrays of perinuclear EMTs in the SC, 15 min of infection. **C** Slightly affected (less numerous) arrays of perinuclear EMTs in the RC, 15 min of infection. (Flat red structures at bottom of this panel are stomatal guard cells rich in cytoplasm and therefore well stainable by propidium iodide. Note well preserved MTs in their companion cells.) **D** Image of CMTs and sparse perinuclear EMTs in the SC, 2 h after infection. **E** Strongly affected MT cytoskeleton in the RC, 2 h after infection. **F** Completely depolymerized MT cytoskeleton leaving spots of tubulin monomers around nuclei in the RC, 24 h after infection. Note the increased size of irregularly shaped polyploid nucleus. **G** Appressorium of germinating spore at penetrating attempt. Note the presence of nucleus near the penetration site. **H** Papilla (dark roundish structure below the hypha) beneath the penetration site

Fig. 4 A–D. Proportion of individual nuclear ploidy levels (2C, 4C, 8C) found in epidermal cells of control barley leaves (start of experiment: 0 h, end of experiment: 24 h) and in epidermal cells of barley leaves infected with powdery mildew: **A** resistant combination 0–8 h, **B** resistant combination 10–22 h, **C** sensitive combination 0–8 h, **D** sensitive combination 10–22 h

disoriented throughout the epidermal tissue (Fig. 1 F).

The further course of CMT rearrangements showed a particularly striking character in those cells which were directly penetrated by fungal hyphae. Here, MTs were completely reorganized and formed very fine and densely radiating networks where obviously terminated near the surface of a papilla (Fig. 1 H). In all other cells, not directly attacked by the fungus, MTs were sparse and poorly organized (Fig. 1 G).

Rearrangements of microtubules in the SC

Epidermal cells of barley leaves which were inoculated with the more virulent pathotype showed a different course of changes in organization of the MT cytoskeleton. First of all, CMTs in the SC preserved their density and orientation during the first 15 min after inoculation (Fig. 2 A, B). Similarly, EMTs retained their integrity and distinctly encircled the nuclei which were, however, often loosened from the cell wall into locations deeper in the cell interior

(Fig. 2 C). Two hours after inoculation, distribution of CMTs in the SC already appeared to be disturbed (Fig. 2 D, E) but not to the extent described for epidermal cells of the RC.

Pronounced differences in rearrangements of the MT cytoskeleton between the RC and SC were also recorded at later stages of fungal infection. In the SC 24 h after inoculation, most cells showed prominent but randomly and sparsely distributed CMTs (Fig. 2 F). Directly attacked cells displayed strands of MTs radiating from near the penetration site (Fig. 2 H). Their distribution, although reminiscent, was not so well organized as that observed in the case of RC. Very few epidermal cells of the SC showed even well organized arrays of dense CMTs as late as 24 h after inoculation (Fig. 2 G).

Two-channelled confocal images

This technique enabled us to visualize, in more detail, interactions between EMTs and nuclei, as well as between germinating conidia and host cell structures

Fig. 5 A–D. Chromatin structure expressed as proportion of nuclear volume occupied by heterochromatin (*H*), condensed euchromatin (*CE*), or decondensed euchromatin (*DE*) in epidermal cells of control barley leaves (start of experiment: 0 h, end of experiment: 24 h) and in epidermal cells of barley leaves infected with powdery mildew: **A** resistant combination 0–8 h, **B** resistant combination 10–22 h, **C** sensitive combination 0–8 h, **D** sensitive combination 10–22 h

such as the nucleus or papilla. Using this method, we confirmed and extended our results obtained from single-channelled confocal images. Nuclei of control cells were found to be encircled by fine EMTs (Fig. 3 A). These arrays of EMTs retained their density in epidermal cells of the SC 15 min after inoculation, but they acquired a slightly blurred appearance (Fig. 3 B). In contrast, these perinuclear MTs quickly appeared to vanish in leaf epidermal cells of the RC. As early as 15 min after their inoculation, rather less numerous perinuclear EMTs were detected in most epidermal cells. A typical example of this is shown in Fig. 3 C.

Many epidermal leaf cells of the SC still showed clear images of sparse EMTs and CMTs even 2 h after the inoculation (Fig. 3 D). On the other hand, strongly affected arrays of perinuclear EMTs were found in cells of the RC at this stage of the host–parasite interaction, when only traces of fluorescent filaments were visible near most nuclei (Fig. 3 E). Completely depoly-

merized perinuclear EMTs were found in many epidermal leaf cells of the RC 24 h after inoculation. Characteristic picture was represented by numerous fluorescent spots of tubulin monomers which surrounded polyploid nuclei of irregular shapes (Fig. 3 F).

The two-channelled confocal microscopy proved to be useful regarding various host–pathogen structural interactions. For example those between the appressorium of germinating spore and the host cell nucleus translocated to its vicinity (Fig. 3 G), or between the penetration hypha and the adjacent developing papilla (Fig. 3 H).

Ploidy levels

In epidermal cells of 7-day-old primary leaves of barley, 2C nuclei prevailed (81%) while 4C and 8C nuclei were present in lower amounts, 16% and 3%, respectively (Fig. 4 A): A slightly increased propor-

tion of 4C nuclei (34%) was recorded when control leaves were processed 24 h later. This occurred at the expense of 2C nuclei (61%) whereas 8C nuclei did not change their amount significantly (5%).

However, inoculation of barley leaves with powdery mildew conidia rapidly elicited prominent nuclear response regarding their ploidy levels. This was especially quick and pronounced in resistant host–pathogen combination (RC). As early as 2 h after inoculation, the proportion of 4C and 8C nuclei increased (44% and 18%, respectively) at the expense of 2C nuclei (38%) (Fig. 4 A). When DNA contents were analysed at further 4 and 6 h after inoculation, the proportion of 8C nuclei slightly increased again (25% and 28%, respectively) and the proportion of 4C nuclei decreased proportionally (37% and 34%, respectively). In contrast, the proportion of 2C nuclei remained unchanged (38%) (Fig. 4 A). Between further 6 and 16 h after inoculation, no significant changes in ploidy levels were recorded. Then, at 22 h after inoculation, the proportion of 4C nuclei increased (51%) at the expense of 2C nuclei (22%) while the proportion of 8C nuclei remained unchanged (Fig. 4 B).

In contrast to the RC, ploidy levels in the SC increased less prominently during the first 2 h (Fig. 4 C). Furthermore, ploidy levels in the SC preserved their lower values (when compared with those recorded in the RC) also at the later stages of infection. The first signs of ploidy levels comparable to the RC occurred in the SC only at 22 h after inoculation, when the proportion of 2C nuclei decreased to 17% and the proportion of 4C and 8C nuclei increased to 52% and 31%, respectively (Fig. 4 D).

Chromatin condensation

As with the ploidy levels, changes in chromatin condensation showed quicker and more prominent changes in the RC when compared with the SC. For instance after 2 h of infection, amount of condensed chromatin (heterochromatin and condensed euchromatin) increased from 68% to 96% in epidermal nuclei of barley leaves in the RC (Fig. 5 A). This highly condensed character of nuclear structure remained stable during the first 16 h of infection and then slightly decreased to 69% at 22 h of infection (Fig. 5 B). The reason for this marked increase in density of nuclear chromatin was that replication of DNA during 2C–4C and 4C–8C endo-S phases was not accompanied with adequate nuclear enlargement (data not shown). As a result, a higher amount of DNA was present per unit

of nuclear volume, resulting in a higher density of the chromatin complex.

In contrast to this prominently changed pattern of nuclear structure found during RC interactions, nuclei displayed rather stable structure during first 16 h of infection in the case of SC (Fig. 5 C). A slight increase in the proportion of condensed chromatin in these nuclei was recorded only between 16 and 22 h, when condensed chromatin represented 74% of total chromatin (Fig. 5 D).

Discussion

Plant cells respond to fungal pathogen inoculation by activating several specific endocellular processes that have been implicated as mechanisms of disease resistance (Aist and Bushnell 1991). The latest progress in this field has unequivocally confirmed that crucial events, which determine the character of further advancement of host–pathogen interactions, are accomplished very early after the initiation of fungal infection. This picture is in accordance with results of the present study, when epidermal leaf cells of the RC, compared to those of the SC, showed quicker and more pronounced responses to powdery mildew inoculation. As early as 15 min after inoculation of vital powdery mildew conidia on the surface of barley leaves, partial depolymerization of the MT cytoskeleton, especially of its perinuclear part, was characteristic for host cells only of the RC. Additionally, 2 h after inoculation, most host cell nuclei showed doubled DNA contents in the RC whereas insignificant increases of nuclear DNA content were observed in the SC at this stage of infection.

When discussing the very rapid response of the MT cytoskeleton elicited by the fungal inoculation, we must recall the widely accepted view according to which non-dividing plant cells should be lacking any perinuclear EMTs. In higher plant cells, MTs are generally believed to be present around nucleus only during S and G2 phases of the mitotic cycle (Wick 1985, Hasezawa and Nagata 1991). However, introduction of new more gentle sectioning technique using Steedman's wax challenged this belief, at least for maize root cells nuclei of which were found to be invariably surrounded by a specific set of EMTs (Baluška and Barlow 1993). This finding suggests that the failure of other authors to detect, at the light-microscope level, the perinuclear EMTs of post-mitotic plant cells is a methodological problem. For instance, EMTs ramifying between nuclear envelope and plasmalemma might be extremely suscepti-

ble, and thus easily destructable. In support of this, we have reported that EMTs are the first to be disintegrated in cells of maize root apex exposed to low temperature (Baluška et al. 1993) or MT-poisons (Baluška and Barlow 1993).

The present study, which was undertaken on non-sectioned cells of intact epidermal tissue using confocal microscopy, reveals the existence of very sensitive perinuclear EMTs also in post-mitotic leaf cells. Therefore, the system of perinuclear EMTs, which merges into the CMT arrays at the cell periphery, seems to be a general feature of non-dividing plant cells physically connecting the cell nucleus with cell wall structure that directly face the extracellular environment. Specific properties of this dynamic structural system, as we have already mentioned in the Introduction, as well as their extreme sensitivity, both features support its possible involvement in transmitting various signals or messages in both directions. As a consequence of this, the MT cytoskeleton could be hypothesized to play the role as a universal cytoplasmic sensor that translates extracellular messages into endocellular responses.

We reported that depolymerization of EMTs, in cells of the maize root apex, by three different anti-MT treatments immediately activated G1 nuclei of the quiescent centre and these quickly entered S phase (Baluška and Barlow 1993). A very similar response was found in inoculated barley leaves of the RC where onset of S phase was accomplished even quicker, indicating that their cells were held probably at, or very near, the G1/S border. This crucial transition point is of major importance for progression of the cell cycle. As no cell divisions occurred later, and also the number of polyploid cells increased, we conclude that endo-S phases were activated in barley leaves.

Several studies indicate that the MT cytoskeleton may fulfill a restrictive role in the regulation of interphase progression, particularly at the G1/S boundary, in both plant and animal cells. For example, quicker progression through G1 phase was described in plant cells treated with the MT poison colchicine (Davidson 1979). Similarly, colchicine was found to promote the growth-factor-driven cell cycle traverse in animal cells (Teng et al. 1977). Transient disintegration of the MT cytoskeleton was reported by several authors to be necessary for the completion of the G1/S transition and subsequent initiation of DNA synthesis in non-transformed animal cells (Vasiliev et al. 1971, Crossin and Carney 1981a, Chou et al. 1984,

Shinohara et al. 1989). Other authors observed that depolymerization of MTs enhanced DNA synthesis in mouse fibroblasts stimulated with peptide growth factors (Friedkin et al. 1979, 1980). In different experimental systems, disorganization of MTs was reported to be a sufficient trigger for mitogenic activation of some quiescent animal cells without the need for otherwise essential growth factors (Chou et al. 1984, Miura et al. 1987, Tsuji et al. 1992). On the other hand, stabilization of MTs with taxol was found to prevent the colchicine-induced enhancement of the G1/S transition (Crossin and Carney 1981b, Chou et al. 1984, Shinohara et al. 1989, Tsuji et al. 1992), as well as the stimulation of DNA synthesis by cytomegalovirus (Ball et al. 1990). All these findings support the notion that stable MTs are important for expression of negative control over interphase progression, especially during final stages of the G1 phase, and in this way they seem to participate in execution of the crucial G1/S check-point.

Interestingly, similar early local depolymerization of EMTs around the nucleus, as we observed here for the RC, was also described near the penetration site in suspension-cultured parsley cells inoculated with the phytopathogenic fungus *Phytophthora infestans* (Gross et al. 1993). These authors did not follow nuclear DNA contents, and the depolymerization of MTs was considered to be relevant only for the rapid translocation of cytoplasm and nucleus towards the penetration site. However, they found that maximal transcription of defence genes temporarily coincided with early local depolymerization of MTs around the host cell nucleus which had already been translocated to the infection site. It is well known that replication of DNA can activate previously inactive genes due to a disruption of both active and repressed chromatin structures by replication forks (Svaren and Chalkey 1990) and thus provide the cell with a good opportunity for reprogramming its genome (Wolffe 1991). Therefore, it might be speculated that the disintegration of perinuclear EMTs might be somehow relevant not only to the induction of nuclear DNA synthesis but also, rather indirectly, to the subsequent stimulation of defence gene expression. Transcriptional reprogramming of host cell genome and subsequent de novo production of various defence compounds, for instance phytoalexins, are of fundamental importance for the development of resistance to pathogen attack (Tani and Yamamoto 1978, Lawton and Lamb 1987, Schmelzer et al. 1989).

We have observed that host cell nuclei, especially in the RC, quickly changed their structure and the proportion of condensed chromatin increased. This finding can be explained by the obvious imbalance between increases in the DNA content and enlargements of nuclei, when the very rapid doubling of DNA content was not associated with an adequate increase in nuclear size (Bacigálová and Hauskrecht 1993). Similar structural changes, when host nuclei became more dense, were also described for host–parasite interactions between *Fusarium solani* and endocarp cells of pea pods (Hadwiger and Adams 1978). Also, these authors found that condensation of nuclear chromatin in host nuclei was a very early event, as it was apparent already within 1 h after the fungal conidia came in contact with the host cell surface. However, in other parasite–host cell interaction systems, especially when root cells were the ultimate target of fungal infection, no induction of DNA synthesis was recorded and here decondensation of chromatin structure was proposed to act as a possible means of activating defence genes (Münzenberger et al. 1992, Berta et al. 1992). Virtually nothing is known about possible rearrangements of the MT cytoskeleton in these fungal infected root cells. But some structural responses of their nuclei which were detected, such as nuclear hypertrophy or obtaining of irregularly shaped nuclear forms (Münzenberger et al. 1992), seem to indicate that perinuclear MTs might be strongly affected in these cells too. The reason for this notion is that very similar nuclear changes were recorded in maize root treated with various anti-MT treatments (Baluška and Barlow 1993, Baluška et al. unpubl.). In addition, changes in nuclear structure were found to precede the penetration event also in roots (Münzenberger et al. 1992), thus favouring the putative sensing role of the host cell MT cytoskeleton in its recognition of the fungal pathogen, as we have hypothesized for barley leaves–powdery mildew interactions.

In contrast to data presented here, Kobayashi et al. (1992) reported that the first rearrangements of the MT cytoskeleton, when coleoptile cells of barley are inoculated with non-pathogen *Erysiphe pisi* (which corresponds to the RC in our system), started only 6 h after inoculation while reorientations of actin filaments were accomplished 3 h earlier. However, these authors studied only CMTs and did not pay any attention to possible changes in the distribution of EMTs located around nuclei. Nevertheless, when they inoc-

ulated barley coleoptiles with *Erysiphe graminis* f.sp. *hordei* (which corresponds to the SC in our system), they found, similar to the results of this study, that there were less prominent responses in the arrangement of actin microfilaments and MTs. This supports the putative involvement of cytoskeletal structures in the activation of host cell resistance mechanisms. Their original finding that papilla deposition was associated with a prominent radial organization of all MTs around papilla, while the rest of the cell interior was devoid of MTs, was confirmed in our study. These findings suggest that, in directly attacked cells, all MTs participate in the formation of the papilla – perhaps in the same way as was originally proposed for cell wall synthesis (Giddings and Staehelin 1991) – by providing tracks for MT-based motility (Cai et al. 1993) of putative vesicles (Smart et al. 1982) containing building blocks of these protective cell wall-like structures. Interestingly, we never observed such perfectly arranged MTs around developing papilla in the SC.

In conclusion, our findings showed both here and in our earlier papers (Baluška et al. 1992, Baluška and Barlow 1993) suggest that the MT cytoskeleton connects post-mitotic plant nuclei with cell walls. This coherent structural system seems to provide plant cells with an important sensor which might be capable of bidirectional transduction of various environmental signals and developmental messages between the extracellular micro-environment and the nuclear genome. We are tempted to hypothesize that both the integrity as well as the dynamic of MTs are relevant for the genomic control of specific resistance genes during powdery mildew attack of epidermal cells in barley.

References

- Aist JR, Bushnell WR (1991) Invasion of plants by powdery mildew fungi, and cellular mechanism of resistance. In: Cole GT, Hoch HC (eds) *The fungal spore and disease initiation in plants and animals*. Plenum, New York, pp 321–345
- Gold RE, Bayles CJ, Morrison GH, Chandra S, Israel HW (1988) Evidence that molecular components of papillae may be involved in ml-o resistance of barley powdery mildew. *Physiol Mol Plant Pathol* 33: 17–32
- Bacigálová K, Hauskrecht M (1993) Morphology and chromatin changes of nuclei in epidermal cells in the early stages of barley–powdery mildew interactions. *Agriculture (Bratislava)* 39: 389–398
- Ball RL, Carney DH, Albrecht T (1990) Taxol inhibits stimulation of cell DNA synthesis by human cytomegalovirus. *Exp Cell Res* 191: 37–44
- Baluška F, Barlow PW (1993) The role of the microtubular cytoskel-

- eton in determining nuclear chromatin structure and passage of maize root cells through the cell cycle. *Eur J Cell Biol* 61: 160–167
- Parker JS, Barlow PW (1992) Specific patterns of cortical and endoplasmic microtubules associated with cell growth and tissue differentiation in roots of maize (*Zea mays*). *J Cell Sci* 103: 191–200
 - – (1993) The microtubular cytoskeleton in cells of cold-treated roots of maize (*Zea mays* L.) shows tissue-specific responses. *Protoplasma* 172: 84–96
- Berta G, Sgorbati S, Giaccone P, Gianinazzi-Pearson V (1992) Nuclear morphology and ploidy level in infected and uninfected hair root cells of *Calluna vulgaris* and *Vaccinium myrtillus* mycorrhizae. *Protoplasma* 170: 160–165
- Bushnell WR, Bergquist SE (1975) Aggregation of host cytoplasm and the formation of papillae and haustoria in powdery mildew of barley. *Phytopathology* 65: 310–318
- Cai G, Bartalesi A, Del Casino C, Moscatelli A, Tiezzi A, Cresti M (1993) The kinesin-immunoreactive homologue from *Nicotiana tabacum* pollen tubes: biochemical properties and subcellular localization. *Planta* 191: 496–506
- Chou I-N, Ziegler J, Rapaport E (1984) Imbalance of total cellular nucleotide pools and mechanism of colchicine-induced cell activation. *Proc Natl Acad Sci USA* 81: 2401–2405
- Crossin KL, Carney DH (1981a) Evidence that microtubule depolymerization early in the cell cycle is sufficient to initiate DNA synthesis. *Cell* 23: 61–71
- – (1981b) Microtubule stabilization by taxol inhibits initiation of DNA synthesis by thrombin and by epidermal growth factor. *Cell* 27: 341–350
- Cyr RJ (1991) Calcium/calmodulin affects microtubule stability in lysed protoplasts. *J Cell Sci* 100: 311–317
- Davidson D (1979) Colchicine stimulation of interphase cells: timing the response using 5-aminouracil. *Cytology* 44: 633–638
- Dreiseitl A (1991) A Czechoslovak test-assortment of spring barley cultivars for identifying powdery mildew pathotypes. *Genet Breed* 27: 251–257
- Frič F, Wolf G (1992) Protease activity in powdery mildewed barley leaves. *Biológia (Bratislava)* 47: 307–315
- Friedkin, M, Legg A, Rozengurt E (1979) Antitubulin agents enhance the stimulation of DNA synthesis by polypeptide growth factors in 3T3 mouse fibroblasts. *Proc Natl Acad Sci USA* 76: 3909–3912
- – – (1980) Enhancement of DNA synthesis by colchicine in 3T3 mouse fibroblasts stimulated with growth factors. *Exp Cell Res* 129: 23–30
- Giddings TH, Staehelin LA (1991) Microtubule-mediated control of microfibril deposition: a reexamination of the hypothesis. In: Lloyd CW (ed) *The cytoskeletal basis of plant growth and form*. Academic Press, London, pp 85–99
- Gross P, Julius Ch, Schmelzer E, Hahlbrock K (1993) Translocation of cytoplasm and nucleus to fungal penetration sites is associated with depolymerization of microtubules and defence gene activation in infected cultured parsley cells. *EMBO J* 12: 1735–1744
- Hadwiger LA, Adams MJ (1978) Nuclear changes associated with the host–parasite interaction between *Fusarium solani* and peas. *Physiol Plant Pathol* 12: 63–72
- Hasezawa S, Nagata T (1991) Dynamic organization of plant microtubules at the three distinct transition points during the cell cycle progression of synchronized tobacco BY-2 cells. *Bot Acta* 104: 206–211
- ten Kate TK, van Balen R, Groen FCA, Smeulders AWM, de Boer G (1990) SCILAIM: a multilevel interactive image processing system. *Pattern Recognition Lett* 10: 429–441
- Kirschner M, Mitchison T (1986) Beyond self-assembly: from microtubules to morphogenesis. *Cell* 45: 329–342
- Kobayashi L, Kobayashi Y, Yamaoka N, Kunoh H (1992) Recognition of a pathogen and a nonpathogen by barley coleoptile cells. III. Responses of microtubules and actin filaments in barley coleoptile cells to penetration attempts. *Can J Bot* 70: 1815–1823
- Lawton MA, Lamb CJ (1987) Transcriptional activation of plant defense genes by fungal elicitor, wounding and infection. *Mol Cell Biol* 7: 335–341
- Miura M, Ninomiya-Tsuji J, Tsuji Y, Ibayashi S, Ide T (1987) Colchicine activates cell cycle-dependent genes in growth arrested rat 3Y1 cells. *Exp Cell Res* 173: 294–298
- Münzenberger B, Kottke I, Oberwinkler F (1992) Ultrastructural investigations of *Arbutus unedo*-*Laccaria amethystea* mycorrhiza synthesized in vitro. *Trees* 7: 40–47
- Russo VM, Bushnell WR (1989) Responses of barley cells to puncture by microneedles and to attempted penetration by *Erysiphe graminis* f.sp. *hordei*. *Can J Bot* 67: 2912–2921
- Schilstra MJ, Bayley PM, Martin SR (1991) The effect of solution composition on microtubule dynamic instability. *Biochem J* 277: 839–847
- Shinohara Y, Nishida E, Sakai H (1989) Initiation of DNA synthesis by microtubule disruption in quiescent rat 3Y1 cells. *Eur J Biochem* 183: 275–280
- Schmelzer E, Krüger-Lebus S, Hahlbrock K (1989) Temporal and spatial patterns of gene expression around sites of attempted fungal infection in parsley leaves. *Plant Cell* 1: 993–1001
- Smart MG, Allen NS, Aist JR, Israel HW (1982) Cytoplasmic responses to fungal attack: a fibrillar system related to organelle transport. *Phytopathology* 72: 933
- Svaren J, Chalkey R (1990) The structure and assembly of active chromatin. *Trends Genet* 6: 52–56
- Tani T, Yamamoto H (1978) Nucleic acid and protein synthesis in association with the resistance of oat leaves to crown rust. *Physiol Plant Pathol* 12: 113–121
- Teng MH, Bartolomew JC, Bissell MJ (1977) Synergism between antimicrotubule agents and growth stimulants in enhancement of cell cycle traverse. *Nature* 268: 739–741
- Tsuji K, Ueno A, Ide T (1992) Inhibitory effect of taxol, a microtubule stabilizing agent, on induction of DNA synthesis is dependent upon cell lines and growth factors. *Cell Struct Funct* 17: 139–144
- Vasiliev JM, Gelfand IM, Guelstein VI (1971) Initiation of DNA synthesis in cell cultures by colcemid. *Proc Natl Acad Sci USA* 68: 977–979
- Wick SM (1985) Immunofluorescence microscopy of tubulin and microtubule arrays in plant cells. III. Transition between mitotic/cytokinetic and interphase microtubule arrays. *Cell Biol Int Rep* 9: 357–371
- Wolf F, Frič F (1981) A rapid staining method for *Erysiphe graminis* f.sp. *hordei* in and on the whole barley leaves with a protein-specific dye. *Phytopathology* 71: 596–598
- Wolffe AP (1991) Implications of DNA replication for eukaryotic gene expression. *J Cell Sci* 99: 201–206