Plant Cell Growth Responds to External Forces and the Response Requires Intact Microtubules

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Microfibril deposition in most plant cells is influenced by cortical microtubules. Thus, cortical microtubules are templates that provide spatial information to the cell wall. How cortical microtubules acquire their spatial information and are positioned is unknown. There are indications that plant cells respond to mechanical stresses by using microtubules as sensing elements. Regenerating protoplasts from tobacco (Nicotiana tabacum) were used to determine whether cells can be induced to expand in a preferential direction in response to an externally applied unidirectional force. Additionally, an anti-microtubule herbicide was used to investigate the role of microtubules in the response to this force. Protoplasts were embedded in agarose, briefly centrifuged at 28 to 34 g, and either cultured or immediately prepared for immunolocalization of their microtubules. The microtubules within many centrifuged protoplasts were found to be oriented parallel to the centrifugal force vector. Most protoplasts elongated with a preferential axis that was oriented 60 to 90° to the applied force vector. Protoplasts treated transiently with the reversible microtubule-disrupting agent amiprophos-methyl (applied before and during centrifugation) elongated but without a preferential growth axis. These results indicate that brief biophysical forces may influence the alignment of cortical microtubules and that microtubules themselves act as biophysical responding elements.

Shape is a basic characteristic of all cells. In plants, shape is determined by a rigid cell wall that surrounds the protoplasm. The cell wall is a composite of crystalline cellulose microfibrils, an assortment of other carbohydrate polymers, and proteins (Carpita and Gibeaut, 1993). Cellulose has a high tensile strength and a high elastic modulus (Wainwright et al., 1976), which allows it to resist deformation. The pattern of microfibril deposition within the cell wall, much like hoops around a barrel, influences cell shape by resisting certain directions of cell expansion during turgor-driven growth (Green, 1980). The pattern of cellulose deposition in most plant cells is influenced by cortical microtubules (Hogetsu and Shibaoka, 1978; Williamson, 1991; Cyr, 1994; Cyr and Palevitz, 1995) that are located throughout the cortical cytoplasm in close proximity to the plasma membrane (Ledbetter and Porter, 1964; Hardham and Gunning, 1978). Extensive evidence supports the hypothesis that cortical microtubules exert their influence on microfibril deposition by orienting the cellulose-synthesizing complexes (Ledbetter and Porter, 1964; Heath, 1974; Hardham and Gunning, 1978; Hogetsu and Shibaoka, 1978; Green, 1980; Staehelin and Giddings, 1982; Williamson, 1991; Cyr, 1994; Cyr and Palevitz, 1995).

The morphology of a cell changes during development. In the apical meristem, most of the cells are small and more or less isodiametric. These cells expand isotropically for a time and then shift to a vectorial growth mode that results in cells that have lengthened primarily in one direction. Cortical microtubules in the cells of the meristem are not always well aligned, but as one examines cells increasingly distal to the meristem, an increasing degree of organization is seen (Sakaguchi et al., 1988, 1990; Marc and Hackett, 1989). Thus, there is a correlation between microtubule alignment and the pattern of cell expansion (and presumably microfibril alignment).

Observations of the patterns of cell expansion and microtubule orientation have led to the hypothesis that there are biophysical forces generated within tissues and organs that ultimately function to align cortical microtubules (Hush and Overall, 1991; Williamson, 1991; Cyr, 1994) and planes of cell division (Lintilhac, 1974b; Green, 1980). Thus, cortical microtubules may function as effector elements (to orient cellulose microfibrils) and additionally as responding elements (to sense physical forces). Tests of the hypothesis that biophysical forces align cortical microtubules or the planes of cell division are complicated because tissues and organs are complex structures in which each cell experiences not only endogenous turgor but also those forces applied from all of its neighbors. To circumvent this difficulty, we used the single-celled protoplast as a model system to study the effect of applied forces on the establishment of the axis of elongation.

The basic pattern of plant cell shape change during normal development is recapitulated in a regenerating protoplast, i.e. the enzymatic removal of the cell wall releases a spherical protoplast containing cortical microtubules hav-

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ABBREVIATIONS: APM, amiprophos-methyl; FMS medium, Fukuda, Murashige and Skoog medium.
Embedding and Centrifugation of Protoplasts

Agar medium was used to immobilize the protoplasts during all of the experiments. A low-strength agar matrix containing a buoyant medium was initially used. However, this matrix was found to be incompatible with immunofluorescent procedures. For immunolocalization experiments, a high-strength agar was used. In experiments in which a low-strength agar was used, the protoplasts were suspended in a solution containing FMS medium, 5% Accucenz (to increase medium density; Accurate Chemical and Scientific, Westbury, NY), and 0.6% low-melting-point agarose (catalog No. 5030, Sigma). This concentration of Accucenz was empirically determined to be sufficiently dense to prevent protoplasts from sedimenting at approximately 30g. Caution was taken to cool the agarose close to its gelling point before addition of the protoplasts. The protoplast-agarose suspension was pipetted into molds made from 0.5-mm-thick Silastic sheeting (Dow Corning, Midland, MI) cut in a "U"-shape, sandwiched between 7.5 × 2.5-cm glass microscope slides, and sealed with Scotch Brand 850 silver polyester film tape (3M, St. Paul, MN). The gel molds were placed in 50-mL plastic centrifuge tubes and allowed to solidify at 23°C for at least 30 min prior to centrifugation. The embedded protoplasts were spun at 450 rpm for 15 min in a Sorvall G2-C centrifuge (DuPont-Sorvall), resulting in a centrifugal force ranging from 28 to 35g, depending on the position of the protoplast in the agarose. Following centrifugation, the molds were immediately opened and the agarose sheets placed in Petri dishes, covered with 2 to 3 mL of FMS medium, and cultured at 26°C in the dark.

In experiments in which a high-strength agar was used, the protoplasts were suspended in a medium containing FMS and 0.5% TC agar (Carolina Biologicals, Burlington, NC). While warm, the suspension was gently layered onto glass microscope slides. This higher strength gel did not require a mold; in all other respects, the slides were centrifuged and cultured under identical conditions as described above for the low-strength agar.

Immunolocalization of Microtubules

The slides containing the protoplasts, immobilized in TC agar, were fixed for 45 min in a solution containing 4% formaldehyde (freshly prepared from paraformaldehyde), 0.1% glutaraldehyde, 50 mM sodium phosphate (pH 9.5), and 0.25 mM mannitol. Centrifuged preparations were fixed immediately after the rotor came to a halt. The slides were then treated for 15 min with 0.1% Triton X-100 and blocked for an additional 15 min with 3% BSA and 0.05% Tween 20. The primary antibody was raised in rabbits against Hibiscus tubulin and was applied for at least 60 min. Following a 15-min wash in PBS plus 0.05% Tween 20, a goat anti-rabbit fluorescein isothiocyanate conjugate was applied for at

Analysis of the Axis of Elongation

The axis of cellular elongation was determined after culturing for at least 3 d. Petri dishes containing the agarose-embedded cells were positioned on a Zeiss IM inverted microscope equipped with a Newvicon camera (Panasonic, Secaucus, NJ). Images of representative fields were digitized, and the angle of elongation with respect to the centrifugal force was measured using Image-Pro PLUS image-analysis software (Media Cybernetics, Silver Spring, MD). At least 100 cells were measured for each treatment. The measurements were plotted as histograms (PSI-Plot; Polysoft Software International, Salt Lake City, UT) containing 18 bins of 5° each; the number of cells per bin was expressed as a percentage of the total cell number. Histogram distributions were statistically examined (MINITAB; Minitab Inc., State College, PA) using the $\chi^2$ goodness of fit test, in which the expected value was taken as the mean percentage present in each bin (100/18) with 17 df (bin number – 1).

**MATERIALS AND METHODS**

**Protoplast Isolation**

Protoplasts were obtained from a 3-d-old suspension culture of *Nicotiana tabacum* Bright Yellow-2 (BY-2) cells grown as previously described (Hasezawa and Syono, 1983), with the exception that the cultures were not kept in continuous darkness. Protoplasts were isolated using 1% cellulase Y-C and 0.1% pectolyase Y-23 (Seshin Pharmaceutical, Tokyo, Japan) in 0.35 M mannitol (adjusted to pH 5.5). This mixture was filter sterilized using a 0.45-μm filter (Acrodisc; Gelman Sciences, Ann Arbor, MI). Cells were incubated in the enzymatic solution for 1.5 h at room temperature with gentle swirling, and the liberated protoplasts were filtered through cotton and gently pelleted by centrifuging at 500 rpm for 3 min. The pelleted protoplasts were allowed to incubate in enzymes for another 30 min. The enzymatic solution was replaced with 10 mL of FMS containing a buoyant medium was initially used. However, this matrix was found to be incompatible with immunofluorescent procedures. For immunolocalization experiments, a high-strength agar was used. In experiments in which a low-strength agar was used, the protoplasts were suspended in a solution containing FMS medium, 5% Accucenz (to increase medium density; Accurate Chemical and Scientific, Westbury, NY), and 0.6% low-melting-point agarose (catalog No. 5030, Sigma). This concentration of Accucenz was empirically determined to be sufficiently dense to prevent protoplasts from sedimenting at approximately 30g. Caution was taken to cool the agarose close to

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least 60 min. The slides were washed as before, mounted, and examined using a laser scanning confocal microscope (Zeiss). The presented images represent extended Z-series optical sections that have been added (and interpolated) together using software provided by the microscope manufacturer.

APM Experiments

The involvement of microtubules in the elongation response was tested by depolymerizing the microtubules with APM (donated by Miles Inc., Kansas City, MO). Protoplasts were manipulated as described above, with the exception that 10 μM APM (from a 10 mM stock solution in ethanol) was added to the protoplasts before the low-strength agarose was added (final APM = 5 μM). The protoplasts were incubated in the APM for a total of 60 min from the time of APM addition to the end of the centrifugation. This treatment results in virtually complete depolymerization of the microtubules (Falconer and Seagull, 1987), and this was confirmed in the cells used here (data not shown). APM was removed from the agarose by incubating the agarose (gel volume = 300 μL) in three rinses of 10- to 20 mL of FMS medium during a 2-h period. The effect of APM on microtubules is reversed by this treatment (Falconer and Seagull, 1987; data not shown). There was no apparent effect of ethanol on the growth or the response of protoplasts.

RESULTS

Protoplasts that were exposed to a brief centrifugal force of 28 to 35 g for 15 min do not exhibit any obvious plastic deformation immediately after centrifugation (Fig. 1). However, after 72 h of culturing there was a significant preference for elongation perpendicular to the force vector as compared with noncentrifuged, control protoplasts (Figs. 2 and 3, A and B). More specifically, 21% had an axis of elongation oriented 45° to 60° to the force, and 54% had this axis oriented 60° to 90° to the force. That the elongation is accompanied by ordering of cellulose microfibrils is indicated by the positive birefringence that elongating BY-2 cells exhibit (data not shown). These results indicate that a brief, unidirectionally imposed force is sufficient to affect the subsequent elongation axis of cultured protoplasts.

Cortical microtubules of cultured protoplasts are known to reorient prior to cellular elongation (Hasezawa et al., 1988). Therefore, we hypothesized that the centrifugal conditions that affected the elongation axis of these cells would also alter the orientation of cortical microtubules in a predictable manner, namely, the microtubules would reorient parallel to the imposed force and at right angles to the future elongation axis. To test this hypothesis, protoplasts were embedded in agar, centrifuged, and prepared for immunolocalization of the microtubules. Many of the centrifuged protoplasts showed a nonrandom distribution of their cortical microtubules compared to noncentrifuged controls (in Fig. 4, compare A and B to C and D). The orientation of these microtubules was approximately parallel to the direction of the applied force. This result indicates that the organization of cortical microtubules is affected by a brief application of centrifugal force.

The finding that cortical microtubules within protoplasts reorient after exposure to an external force suggests that microtubules might be part of the mechanosensory system. To test the hypothesis that microtubules are involved in the transduction of the centrifugal force into the growth response, the protoplasts were treated with the reversible microtubule-depolymerizing agent APM and then centrifuged. APM acts by binding to tubulin, thereby preventing its addition to the polymer (Morejohn and Fosket, 1984; Morejohn, 1991; Murthy et al., 1994), causing dynamic microtubules to depolymerize. This effect is readily reversible, and microtubules return rapidly when the drug is removed (Falconer and Seagull, 1987; data not shown).

Protoplasts that were pretreated with APM (45 min), centrifuged in the presence of APM (15 min), and then cultured in the absence of APM elongated in a random direction (Figs. 2C and 3D). The failure of APM-treated protoplasts to elongate in a preferential direction was not due to residual toxic effects of APM in the agarose because...
Figure 2. The axis of elongation is influenced by centrifugal force in the absence, but not the presence, of APM. The direction of the centrifugal force is toward the bottom of the page for B and C. Protoplasts were treated in the following ways and then cultured for 3 d: A, Not centrifuged; B, centrifuged for 15 min at 28 to 35g; C, centrifuged as in B but in the presence of APM. All cells were cultured in the absence of APM. Bar = 20 μm.

APM-treated protoplasts appeared to elongate to the same extent as control (untreated) cells (Fig. 2C). Protoplasts cultured in the presence of APM did not elongate at all (data not shown). These results support the hypothesis that cortical microtubules are necessary for a cell to respond to a unidirectionally applied, exogenous force.

DISCUSSION

The effects of mechanical force on the orientation of cortical microtubules have been examined by Hush and Overall (1991), who observed that the orientation of cortical microtubules in pea roots could be altered from transverse to oblique or longitudinal by a compressive force. Similarly, Cleary and Hardham (1993) found that the isotropic force of a pressure chamber could alter microtubule orientation. Finally, Zandomeni and Schopfer (1994) found that microtubules in maize coleoptile epidermal cells changed their orientation when excised segments were bent.

Other groups have also studied the effects of biophysical forces on cellular morphology by examining the placement of cell division planes. Green and his co-workers (Green and Lang, 1981; Green and Poethig, 1982; Green, 1988; Hernandez and Green, 1993) as well as Lintilhac and co-workers (Lintilhac, 1974a, 1974b; Lintilhac and Vesecky, 1984) examined the correlation between the plane of cell division and shear-free planes throughout tissues. They have concluded that it is possible to generate within tissues mechanical forces that predict (and perhaps control) the pattern of cell divisions, which, in turn, influence organ formation.

All of the above studies have been conducted on intact organs. The benefit of examining cells within organs is that intercellular contact is maintained, thus allowing tissue effects to be observed. However, a drawback of such studies is in understanding the magnitudes and directions of the applied forces that any given cell is experiencing. Therefore, we have chosen to simplify the study system to a single, isolated cell (the protoplast) to examine the effect that an applied force of known direction has on the orien-
Hoffmann, 1984; Goodbody et al., 1991; Grabski et al., cytoplasm has tensile and elastic character (Hahne and alignment of cortical microtubules. This assumes that the accumulative, but in some manner they appear to influence the ulative consequences of such organelar movement are spec-

fixed have cortical microtubules that are arranged in a relatively directional during centrifugation. Because the high-strength agar was used, we noted that a portion of cells elongated parallel to the centrifugal force, and experiments are in progress to investigate how the strength of the supporting agarose affects the tendency of cells to elongate parallel, or perpendicular, to the applied force. Regardless of the role that matrix deformation plays in cellular elongation, the current data clearly show that a brief application of force has a demonstrable effect on the alignment of cortical microtubules, with significant effects on subsequent cellular elongation.

It is also noteworthy to consider the events that transpire between centrifugation and the onset of cellular elongation, i.e. although the microtubules reorient during the 15 min of centrifugation, measurable elongation has not been reported to occur until sometime between 48 and 72 h (Kuss-Wymer and Cyr, 1992). This indicates the existence of a mechanism by which microtubule organization can be locked into an organized configuration. Presently, we do not know how this is accomplished, but it is appropriate to consider the role that wall deposition may play in this event because the cytoskeleton and wall appear to form a functional continuum (Cyr, 1994; Shibaoka, 1994). Moreover, BY-2 protoplasts rapidly resynthesize their walls after enzyme removal (i.e. Calcofluor staining fibrils are noted almost immediately after protoplasting enzymes are removed and the cells become osmotically competent within 20 min; C.L. Wymer and R.J. Cyr, unpublished observations). It is therefore possible that the cortical microtubules, realigned by centrifugation, immediately begin to direct the synthesis of cellulose microfibrils, which, in turn, serve to maintain the continued order of the underlying cortical microtubules. Indeed, such an interplay between cortical microtubules and cellulose microfibrils has been proposed to explain the maintenance of order during the synthesis of new walls (Cyr, 1994).

Our data indicate that a centrifugal force mimics a type of developmental induction event that can program a regenerating protoplast to elongate along a preferential axis. If cortical microtubules are necessary for the cell to transduce this inductive information, then their removal should render a protoplast unresponsive to the inducing force. This hypothesis was tested using APM, a herbicide that can reversibly depolymerize the vast majority of microtubules within 60 min (Falconer and Seagull, 1987; data not shown). For example, protoplasts lacking microtu-
bules are centrifuged and then cultured to allow recovery of the cortical microtubules, the resulting cells elongate randomly. This experiment supports the hypothesis that intact microtubules are necessary for the cell to respond to the inductive effects of a mechanical force.

The concept that biophysical forces are used by the cell to align cortical microtubules has been questioned because cortical microtubules have been reported to change alignment in cells within organs that have not undergone any measurable growth (Sakiyama-Sogo and Shibaoka, 1993). However, the data reported herein indicate that a brief, transient force is sufficient to align cortical microtubules. Similarly, transient biophysical forces, acting at the subcellular level within a developing plant organ, may have sufficient magnitude and duration to affect the alignment of cortical microtubules and yet remain undetected in all but the most sensitive kinematic study.

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