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Responses of Chrysanthemum Cells to Mechanical Stimulation Require Intact Microtubules and Plasma Membrane–Cell Wall Adhesion

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Abstract

Plant cells are highly susceptible and receptive to physical factors, both in nature and under experimental conditions. Exposure to mechanical forces dramatically results in morphological and microstructural alterations in their growth. In the present study, cells from chrysanthemum (*Dendranthema morifolium*) were subjected to constant pressure from an agarose matrix, which surrounded and immobilized the cells to form a cell-gel block. Cells in the mechanically loaded blocks elongated and divided, with an axis preferentially perpendicular to the direction of principal stress vectors. After a sucrose-induced plasmolysis, application of peptides containing an RGD motif, which interferes with plasma membrane-cell wall adhesion, reduced the oriented growth under stress conditions. Moreover, colchicines, but not cytochalasin B, abolished the effects of mechanical stress on cell morphology. Cellulose staining revealed that mechanical force reinforces the architecture of cell walls and application of mechanical force, and RGD peptides caused aggregative staining on the surface of plasmolyzed protoplasts. These results provide evidence that the oriented cell growth in response to compressive stress requires the maintenance of plasmalemma-cell wall adhesion and intact microtubules. Stress-triggered wall development in individual plant cells was also demonstrated.

Key words: Mechanical force; *Dendranthema morifolium*; Plasma membrane-cell wall adhesion; RGD peptides; Microtubules

Introduction

The effects of mechanical perturbations on plant growth, especially alterations in growth and development of plants to adapt to and compensate for mechanical variables, are collectively known as *thigmomorphogenesis*. The word was coined to describe the touch-inducible decreased elongation and enhanced radial expansion of plant shoots (Jaffe 1973). Thigmomorphogenetic responses in plants have been reported in many studies, and several efforts have been made to elucidate the phenomena of the variations of physiology and morphology, as well as the mechanisms underlying

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sensing and transducing of mechanical signals in plants (Biddington 1986; Jaffe 1993; Trewavas and Knight 1994; Coutand and others 2000; Braam 2005). Furthermore, on the basis of identification and quantification of the mechanical stimulus, it has been gradually recognized that perception and response to exogenous mechanical stimuli are likely occurring essentially at the cellular and subcellular levels. Experimental evidence has been obtained in controllable conditions. Pressing a small part of a protonemal cell with a microcapillary induces chloroplast migration away from the site of stimulation (Sato and others 1999), whereas local application of pressure by microneedles elicits nuclei movement toward the point of contact in epidermal cells and distortion of the cell wall in parsley cells (Kennard and Cleary 1997; Gus-Mayer and others 1998).

Morphogenesis of individual cells is also influenced by mechanical perturbations, as validated in tobacco, including centrifugal force and directly compressive force, which lead to the altered alignment of cortical microtubules and preferential orientation of cell elongation (Wymer and others 1996). In addition, such forces have been shown to orient the primary division plane of immobilized regenerating protoplasts (Lynch and Lintilhac 1997).

The form of plant growth is proposed to be associated with coordinated control over directional cell expansion and subsequent cell division and proliferation (Meijer and Murray 2001). The direction of cell elongation is thought to be determined by the organization of cellulose microfibrils in the cell wall (Gertel and Green 1977). In the diffusely growing cells of higher plants, microtubules play a critical role in the aligned deposition of cellulose, and therefore the patterns of cell expansion (Baskin and others 1999). This is evidenced by the co-linearity observed in many microtubules and cellulose microfibrils (Holdaway and others 1995). Numerous reports have indicated that the microtubule system may function as responding elements for environmental stress sensing and effector elements for the potential elongation and orientation of cell division. Experimental evidence has also accumulated suggesting that filamentous actin, which has been reported to exhibit a stress-induced morphological alteration in mammalian cells (Chiu and others 2004), is essential for cell elongation (Baluska and others 2001) and is associated with the determination of the division plane (Lloyd and Traas 1988) in plant development.

It has been suggested that numerous parallels exist between the plant cell wall and the mammalian extracellular matrix (Reuzeau and Pont-Lezica 1995). Communication between the cytoskeleton and the extracellular matrix is one of the most characteristic features of cellular mechanics, and it allows cells to respond effectively to various signals, especially mechanical stimuli (Baluska and others 2003). In mammalian cells, signaling across a dynamic continuum involving the extracellular matrix, the plasma membrane, and the cytoskeleton is maintained via interactions between plasma membrane-bound receptors known as *integrins* and *protein ligands* within the extracellular matrix that contain Arg-Gly-Asp (RGD) motifs (Giancotti and Ruoslahti 1999). Although true homologs of classical adhesion domains known from animals are lacking in plants (Hussey and others 2002), treatment with RGD peptides has been shown to cause a loss of plasma membrane–cell wall adhesion in plasmolyzed *Arabidopsis* cells and a loss of the thin plasma membrane–cell wall connections known as *Hechtian strands* that form during onion cell plasmolysis (Canut and others 1998). It is becoming increasingly clear that the adhesion domains play a role in mechanosensing in eukaryotic cells (Geiger and Bershadsky 2001; Riveline and others 2001). The mechanosensing properties of adhesion domains are appealing explanation for these phenomena, especially for higher plants, which are known to be very sensitive to mechanical signals.

The influence of mechanical perturbations on the whole plant or tissue of chrysanthemum has been systematically investigated in our lab as presented in a recent review (Wang and others 2006). In a previous article (Zhou and others 2006), a system involving a fabricated mechanical loading and testing apparatus was developed for applying controllable mechanical compression to agarose immobilized chrysanthemum protoplasts, which tended to elongate with a preferential axis oriented perpendicularly to the imposed stress tensors. These findings are partly consistent with those reported by Lynch and Lintilhac (1997). However, the presumed mechanical sensing elements in plant cells and the involvement of some functionally important structures in the oriented elongation with respect to the applied forces still remain elusive.

In the present study we attempt to answer the question of whether there is a correlation between the direction of stress tensor and the orientation of isolated, but intact plant cells. The orientation under stress conditions has often been demonstrated in intact tissues and organs, but only rarely in individual plant cells, where the influence of turgor pressure within tissues could be separated. Furthermore, if the cytoskeleton and plasma membrane-cell wall continuum is disturbed, do the cells retain the ability to

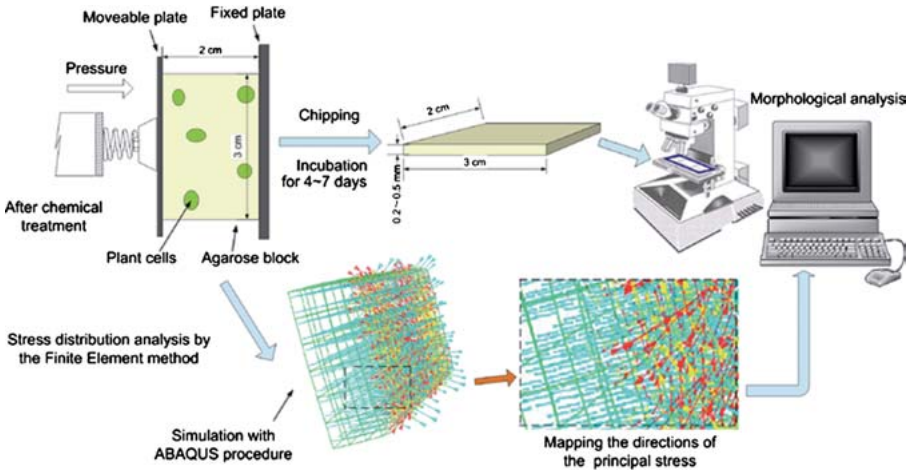

Figure 1. Simple schematic diagram of the mechanical loading and response analyzing system. Individual plant cells were embedded in the agarose block. The loading apparatus involved a force-feedback control circuit coupled to a microchip, by which a pre-defined and controlled stimulus was delivered.

respond to directional mechanical stresses? The latter problem has been addressed by interrupting this continuum with physical or chemical treatments, but this conclusion lacks decisive demonstrations among various plant species.

Materials and Methods

Cell Suspensions

Stems from chrysanthemum (*Dendranthema morifolium*) plants were excised and inoculated on solid basal Murashige and Skoog (MS) medium supplemented with 0.35% (w/v) agar, 3% (w/v) sucrose, 1.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.3 mg l^{-1} zeatin (ZT), and 0.2 mg l^{-1} 6-benzyladenine (6-BA) for callus induction. Cell suspension cultures were established by transferring approximately 1 g fresh weight of the proliferated friable callus into 250-ml Erlenmeyer flasks containing 50 ml ½MS basal liquid medium (adjusted to pH 5.8 using NaOH) supplemented with 1.5 % (w/v) sucrose, 1.0 mg l^{-1} 2,4-D, 0.3 mg l^{-1} ZT, 0.2 mg l^{-1} 6-BA, and 1.0 g l^{-1} polyvinylpyrrolidone (PVP), which dispersed the cells to avoid clumping. Flasks were placed on a gyratory shaker at 120 rpm in the dark at 25° ± 1°C. After the cultures acquired a homogeneous appearance, subcultures were conducted every 14 days by fivefold dilution of the cells into fresh medium.

Embedding and Mechanical Loading

The suspension was centrifuged at 600 rpm for at least 5 min, and deposited cells were harvested and resuspended in fresh liquid medium, as described above, to an appropriate concentration. The suspended cells were then immobilized by gently swirling them into 2.0% preheated low-melting-point (33 ± 1.5°C) agarose (FMC Bioproducts, USA) in MS medium supplemented with 0.3 mol l^{-1} mannitol, 0.1% (w/v) 2-(N-morpholino)-ethanesulfonic acid (MES), 2% (w/v) sucrose, 0.5 mg l^{-1} 2,4-D, 0.05 mg l^{-1} GA, 0.5 mg l^{-1} 6-BA, 1.0 g l^{-1} PVP, and 2.0 g l^{-1} CasSein hydrolysate, maintaining pH 5.8. This concentration of agarose was experientially determined to provide a strength sufficient to prevent the gel from being crushed under stress conditions. Caution was taken to cool the agarose medium close to its gelling point before mixing the cells. This cell–agarose suspension was rapidly poured into a handcrafted cubical mold made from 1.0-mm-thick heat-resistant plastic sheeting at a size of 2.0 × 3.0 × 5.0 cm, and began to gel at approximately 34°C, a temperature that presumably will not cause a significant heat shock to living cells. When solidified, the agarose block was removed from the mold by external air pressure produced by injecting air into the bottom of the chamber.

A previously developed mechanical loading system for *in vitro* cultured cells, based on a principle similar to that reported by Lintihac and Vesecky (1984) and Lynch and Lintilhac (1997), but with some modifications, was used. Figure 1 shows the simple mechanism of the system, combined with imaging and analyzing functions. The freshly obtained block embedded with living cells was relaxed laterally and squeezed between two stainless-steel plates of the loading apparatus mentioned above. One side of the plate was fixed while the other moved back and forth along a guideway. As a result, continuous uniaxial compressive force was imposed on the two opposite sides of the agarose block, lasting for at least 24 h at 25°C in the dark. The block was then half-submerged in MS medium containing 0.2 mol l^{-1} mannitol, 2% (w/v) sucrose,

and 0.5 mg l^{-1} 2,4-D, and incubated for 4–7 days in the dark. Before morphologic observation, the block was removed and then carefully chipped into slices of approximately 0.2–0.5 mm thickness in a direction parallel to the applied pressure, by using a self-fabricated manual sectioning machine. Sterile technique was used during the experimental processes.

Finite Element Simulation of Compression Experiment

To map the presumed stress distribution field in the test specimen, a finite element model of the mechanical loading experiment was constructed using the commercially available finite element software ABAQUS (version 6.5, Hibbitt, Karlsson and Sorensen Inc., Pawtucket, RI, USA) for theoretical predictions. In the simulations, the initial geometry of the model was 5 cm long, 2 cm wide, and 3 cm high, and the ABAQUS command ELASTIC was chosen to define the linear elasticity of the material (Young's modulus = 50 kPa (Bonn and others 1998), Poisson's ratio = 0.5 (Normand and others 2000)). A boundary condition imposing a displacement restriction of a side surface of the model was used to stimulate the immobilizing effect of the fixed plate. Mechanical loading was simulated by using the PRESSURE command for the selected type of load, and a surface of the model toward the restricted side was chosen to be exposed to the predefined pressure. Distribution of mesh was performed in MESH step, using hexahedral elements entirely.

Microscopy Imaging

An inverted microscope (LX71, Olympus Optical Co. Ltd., Japan) equipped with phase contrast optical accessories, fluorescence illumination, and a color digital camera (C-5050, Olympus Optical Co. Ltd., Japan) was used for image acquisition and digitization. For cell elongation analysis in the gel slices of each experimental condition, digital images of at least 50 cells were stochastically recorded and analyzed with an image program (Scion Image, National Institutes of Health, Bethesda, MD, USA) for conducting morphological comparisons. Samples stained with fluorescent dyes were viewed under fluorescence illumination with different excitation and emission filters.

Anticytoskeletal Agents Treatment

A 10 mg ml^{-1} stock solution of cytochalasin B (Sigma Chemical Co. St. Louis, USA) prepared in 0.5% dimethylsulfoxide solvent in PBS (pH 7.4) was used at a final concentration of 50 μg ml^{-1}. Colchicine (Sigma Chemical Co. St. Louis, USA) was dissolved in sterile deionized distilled water as a stock solution of 500 mM and then diluted with PBS to a concentration of 5 mM. The cells were incubated in each solution for 2 h before embedding. Subsequently, the mixtures were centrifuged at 600 rpm for 5 min to remove the cytoskeletal-inhibiting drugs contained in the supernatant. The appropriate concentrations of solvent or water were used as controls.

Interference with Plasma Membrane-Cell Wall Adhesion

A stepwise plasmolysis procedure was carried out by exogenous application of sucrose solutions to the suspensions immediately before embedding, inducing a step-up in sucrose concentrations from 0.3 M to 0.5 M. After that, 5 mmol l^{-1} stock solutions of pentapeptides Gly-Arg-Gly-Asp-Ser (GRGDS) or its inverted sequence, Ser-Asp-Gly-Arg-Gly (SDGRG) (both obtained from Sigma Chemical Co. St. Louis, MO, USA) dissolved in sterile deionized distilled water was added, resulting in a final concentration of 40 μmol l^{-1}. In succession, the hyperosmotic condition was removed by dilution with sterile water. Control experiments were performed by replacement of different solutions with sterile water under the same conditions. Afterwards, the agarose immobilization procedure was processed as mentioned above.

For irreversible plasmolysis of cells embedded in agarose, gel slices containing cells were mounted on microscope slides and subsequently were overlaid with 1 M sucrose solution. The effect was monitored in real time for at least 15 min.

Fluorescent Visualization of Cell Wall

Cell wall development in gel-immobilized cells was examined with calcofluor white, a fluorescent stain specific for polysaccharides with $\beta(1–4)$ linkage, such as cellulose and chitin (Maeda and Ishida 1967; Nagata and Takebe 1970). Each sample of agarose slice was mounted on a microscope slides and stained with 2–3 drops of 0.35% calcofluor white working solution diluted with Tris/HCl buffer (pH 9), and then incubated in the dark for 15 min at room temperature. Microscopic observation was performed after 2–3 PBS washes.

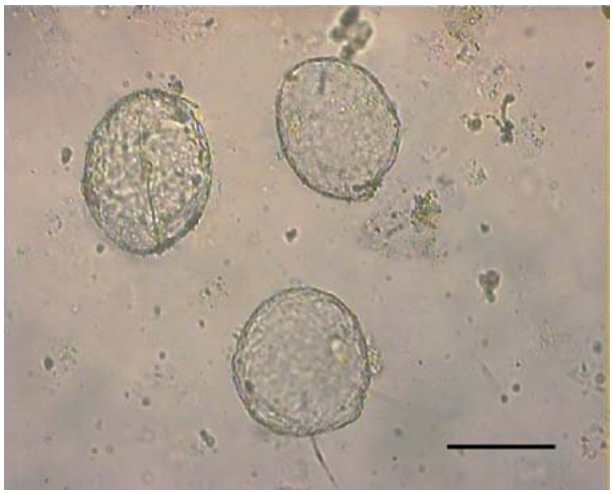

Figure 2. Light micrographs of chrysanthemum (*Dendranthema morifolium*) cells with intact wall. The cells were almost spherical and have no preferentially elongated growth axis. Bar = 50 μm.

Results

Preferential Orientation of Cell Elongation and Division in Response to Mechanical Forces

Cells individually selected from the 2nd and 3rd passages of a suspension culture exhibited ellipsoidal or ellipsoid-like profiles in both liquid and agarose-solidified medium (Figure 2). To facilitate observation of cell elongation, the concentrations of growth factors in the agarose medium were adjusted to reduce cell mitosis and promote cell expansion. Immediately after embedding and exposure to experimental unidirectional compressive forces of about 300 mN for 24 h, the cells exhibited no obvious plastic deformation, as demonstrated in Table 1. This phenomenon is in accordance with the morphological characteristics of chrysanthemum protoplasts before and after mechanical loading in our previous report. Followed by continuous incubation in culture medium for 4–7 days, there was a significant preference for perpendicular elongation (an axis of elongation oriented 60° to 90° to the principal stress vector mapped by the finite element procedure, compared with the unloaded control, where the cells showed a random distribution in orientation of elongation (Figure 3A, B; Figure 4A). These results are consistent with the alignment phenomena observed in wall-regenerating protoplasts in previous studies.

For indirect detection of the putatively oriented division, the gel-immobilized cells experiencing elongation (both loaded and unloaded) were treated with a hyperosmotic condition induced by 1 M sucrose solution. Real-time monitoring revealed that detachment of the plasma membrane from the cell wall was first observed after approximately 1 min of immersion in sucrose solution, after which the protoplasts responded almost instantly to the hyperosmotic stress. The delayed response of the cells was probably due to the lower penetrability of gels when compared to liquid media. Under the experimental conditions, the protoplast spheres were arranged along the major axis of some of the elongated cells (Figure 5). This appearance strongly suggests that the elongation phenomenon in these cells reflects division events in linear organization rather than unidirectional expansion limited by the rate of volume increase. Therefore, it is reasonable to consider that there are some new partition walls that orient in a direction transverse to the growth axis. Based on the preferential orientation of the cells mentioned earlier, these results imply that compressive forces also have a dramatic effect on the orientation of cell division.

Requirement of Plasma Membrane–Cell Wall Adhesion in Oriented Elongation Responses

The mild hyperosmotic treatment of suspended cells with a final concentration of 0.5 M of sucrose in liquid solution (Merllersh and Heath 2001) instantly induced plasmolysis. Most of the cells displayed convex or concave plasmolysis; under both conditions the protoplasts became partially detached from the cell wall, resulting in cytoplasmic masses within rigid cell walls (Figure 6A). However, the plasma membrane-cell wall connections in some cells could be re-established with a deplasmolysis procedure that involved replacement of the plasmolytic solution with distilled water. During deplasmolysis the protoplasts re-expanded their volume and finally the plasmalemma re-attached on the inside of the cell wall (Figure 6B). This reversible plasmolysis-deplasmolysis cycle was broken by exogenous addition of the peptide GRGDS to the medium. Deplasmolysis failed to take place in the presence of GRGDS but not its inverted sequence, SDGRG, which caused no detectable modifications in cell morphology as compared to untreated controls (Figure 6C and D). In a combination of GRGDS and deplasmolytic conditions the volume of the protoplast rapidly expanded, whereas the plasmalemma seemed to be aberrant and was not localized to the cell wall. These observations demonstrate that peptides containing the RGD motif specifically

Table 1. Effects of 24 h Mechanical Force on the Percentage of Spherical Cells per 100 Counted Cells, and the Sum of the Length (mm) of the Major Axis of Counted Cells Before and After Loading

Observed features	Before mechanical loading	After mechanical loading
Spherical cells (%)	15.6 ± 3.0[a]	15.4 ± 3.6
Sum of length of major axis (mm)	16.2 ± 5.3[b]	15.1 ± 4.1

[a]*These values (± SD) are not significantly different from corresponding groups after mechanical loading, p = 0.927 > 0.05.*
[b]*These values (± SD) are not significantly different from corresponding groups after mechanical loading, p = 0.715 > 0.05.*

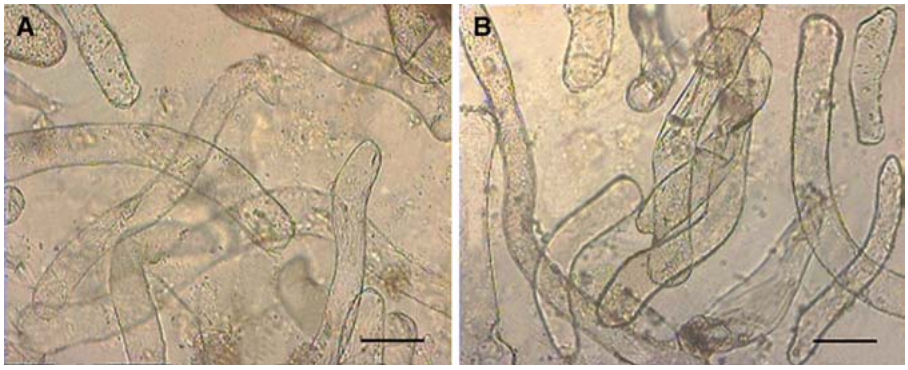

Figure 3. Micrographs of chrysanthemum cells cultured for 4 days in agarose-solidfied medium in the absence (A) and presence (B) of mechanical loading. The direction of compressive force is from the left to the right for (B). Bar = 100 μm.

disrupt plasma membrane–cell wall adhesion in individual plant cells. This appearance reveals that plasma membrane–cell wall adhesion may be irreversibly disrupted by plasmolysis and the application of peptides containing the RGD motif.

As mentioned above, application of directional mechanical forces may elicit a preference for elongation of gel-immobilized cells perpendicular to the principal stress vector. Noticeably, pre-plasmolyzed cells cultured for 4 days after embedding and mechanical loading in the presence of the GRGDS peptide exhibited a random distribution in the direction of the growth axis (Figure 4B and Figure 7A); in the presence of SDGRG peptide, however, the preferential orientation of cell elongation was still conserved (Figure 4C). These findings suggest that RGD-mediated plasma membrane-cell wall adhesion might be necessary for the preferentially oriented elongation of plant cells in response to unidirectionally applied forces.

Involvement of Microtubule Cytoskeleton in Oriented Elongation Responses

The hypothesis that RGD-mediated plasma membrane-cell wall adhesion is required for the compressive force-induced expression of orientation-associated performance suggests that the induction of the performance probably needs some form of communication between the cell wall and the cytoplasm. To test the requirement of a functional cytoskeleton in the stress-induced responses, cells were treated with drugs known to depolymerize microfilaments (cytochalasin B) or disrupt microtubules (colchicine) before mechanical loading (Merllersh and Heath 2001; Komis and others 2002; Sato and others 1999).

Cytochalasin B is a potent actin-depolymerizing drug that has been reported to cause the microfilaments to shorten and eventually dissolve, with the exception of some small spots and rods in various plant species. Treatment with this drug, in both the mechanically loaded and unloaded groups, caused a drastic reduction in cell expansion, expressed as average length of the major axis of elongated cells (Table 2) measured by the Scion Image program, when compared to the corresponding untreated groups. It also resulted in relatively small and unexpanded cells (Figure 7B). However, cytochalasin B treatment failed to produce any detectable alteration in the oriented elongation behavior in the loaded groups (Figure 4E). These results indicate that microfilaments do not play a critical role in the stress-inducible orientation responses demonstrated in our studies. In contrast, the antimicrotubule agent colchicine had a distinct effect on orientation responses at the sufficient chemical concentration of 5 mM. Colchicine treatment appeared to cause a

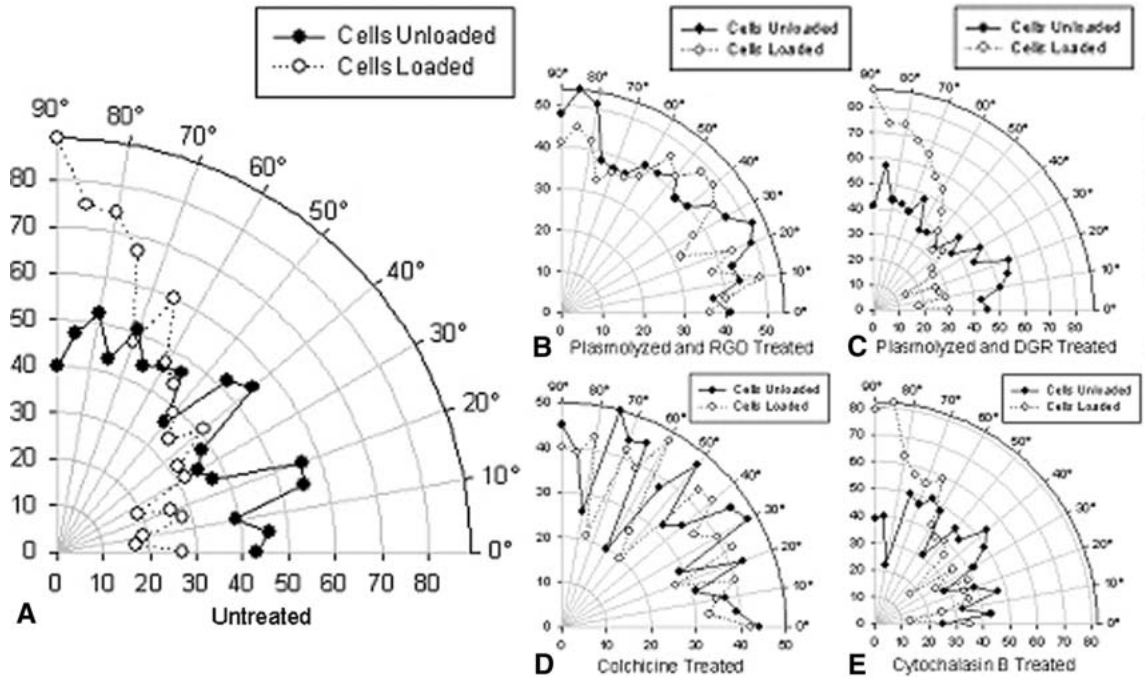

Figure 4. Typical polar representations of the quantitative analysis of alterations in the orientation of cell elongation relative to the compressive stress tensor. For each experimental agarose block, 10 pieces of slices were chosen, and for each slice, digital images of 100 cells were recorded. Cell orientation is defined as the angle between the direction of the major axis of cell growth and the principal stress. Data represent the numbers of cells oriented from 0 to 90° at 5° intervals of total counted cells. Solid circles (●) connected by solid lines represent the data obtained without loading. Open circles (○) connected by dashed lines represent the data obtained after loading. Both the loaded and unloaded cells were analyzed after culture for 4 days. (A) Untreated cells. (B) Plasmolyzed and RGD treated cells. (C) Plasmolyzed and DGR treated cells. (D) Colchicine treated cells. (E) Cytochalasin B treated cells.

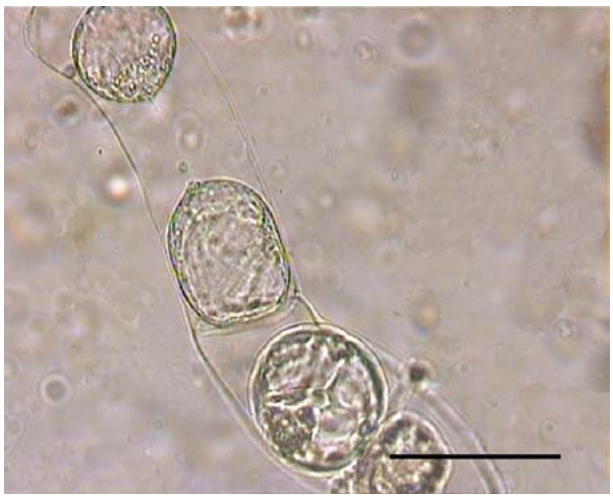

Figure 5. Plasmolyzed protoplasts in the rigid wall of the elongated cell under sucrose-induced hypersomotic conditions. Bar = 50 μm.

loss of directionality in cell expansion and to lead to more isotropic growth (Figure 7C). The majority of treated cells presented an irregular form with prominent protrusions. Specifically, under stress conditions, the treated cell retained the ability to expand without a preferential growth axis, but untreated cells did not (Figure 4D). The effects of colchicine on the preferential orientation of cell elongation is in agreement with recent evidence that cortical microtubules are important for protoplasts to respond to the inductive effects of mechanical centrifugal force (Wymer and others 1996). The present data support the hypothesis that microtubules are involved in the transduction of the unidirectional force into the growth responses and might be part of the mechanotransduction system.

Effects of Mechanical Forces and Interference with Plasma Membrane–Cell Wall Adhesion on Cell Wall Development

To find further evidence that mechanical forces and the absence of plasma membrane-cell wall connection could contribute to the wall-associated alteration in morphology, a direct examination for the development of cell wall after agarose-immobilization and culture for 7 days was initiated using the fluorescent $\beta(1\text{–}4)$ glucan-specific stain calcofluor

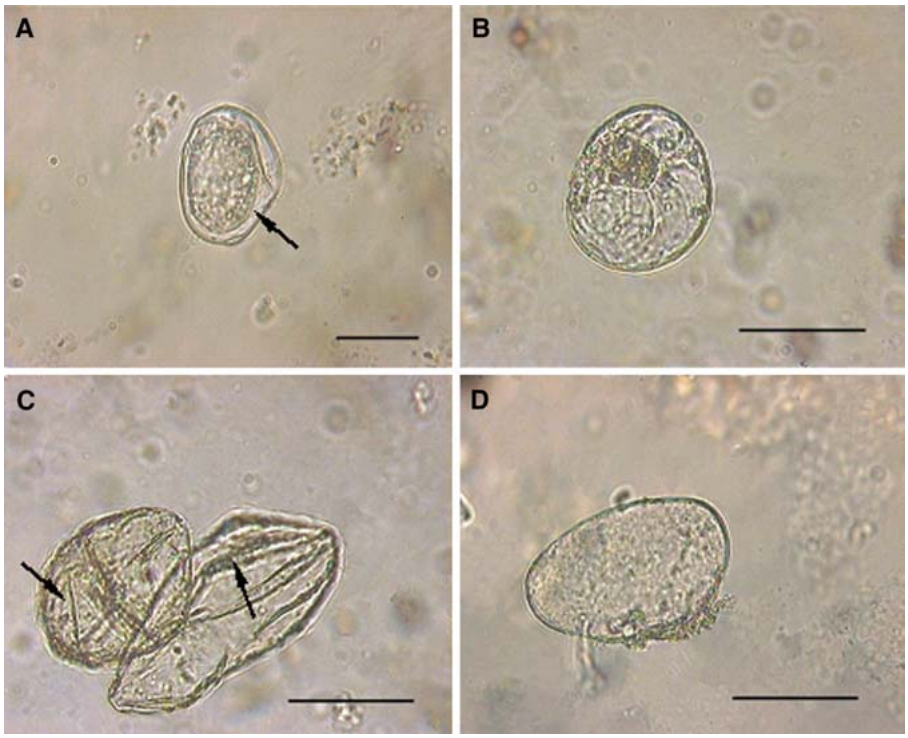

Figure 6. Micrographs of chrysanthemum cells during the plasmolysis-deplasmolysis cycle in the absence and presence of GRGDS or SDGRG peptides. (A), Plasmolyzed cell in liquid medium. (B), Deplasmolyzed cell without application of peptides. (C), Plasma membrane-aberrant and plasma membrane-cell wall nonlocalized cells in the presence of GRGDS peptides under hyposomotic conditions. (D), Deplasmolyzed cell in the presence of SDGRG peptides. Arrows indicate plasma membrane dissociated from wall (A) or shrunken (c). Bar = 50 μm.

white. Results for the effects of mechanical loading and interference with plasma membrane-cell wall adhesion on cell staining are shown in Figure 8 and summarized semiquantitatively in Table 3. As observed under light field microscopy, loaded cells had an appearance similar to that of unloaded cells, both under the plasmolyzed and RGD treated conditions and the non-plasmolyzed and untreated conditions (Figure 8A, 8C, 8E, and 8G). Not surprisingly, no detectable morphological changes of the cell wall in single cells were induced by mechanical forces. Moreover, under fluorescence microscopy, most of the cells not treated with RGD were uniformly stained by calcofluor white throughout the cells (Figure 8B and 8D). More intense staining could be observed at the division sites transverse to the elongated cells. In contrast, calcofluor white staining in mechanically loaded and RGD-treated cells was only slightly localized to the cell wall. Instead, staining appeared to aggregate on the surfaces of plasmolyzed protoplasts. In particular, mechanically loaded and cells not treated with RGD showed intensive fluorescence of the cell walls. This phenomenon was not detected in other stress conditions and treatments. This morphological evidence suggests mainly that, on the one hand, mechanical loading with intact plasma membrane-cell wall adhesion may be responsible for the enhancement of wall structure. On the other hand, plasmolysis and RGD treatment may lead to synthesis of wall-like components on the unattached surfaces of protoplasts inside the rigid walls. Furthermore, this synthesis may be promoted by mechanical loading.

Discussion

From the data presented here, we conclude that (1) a unidirectional mechanical force induces the preferential orientation of cell elongation and division; (2) RGD peptides specifically disrupt plasma membrane–cell wall adhesion, and these force-induced responses require maintaining this adhesion; (3) pharmacological microtubule depolymerization dramatically randomizes the direction of cell orientation in stressed cells, whereas microfilament responses do not change; (4) mechanical force reinforces the architecture of cells wall, and the application of mechanical force and RGD peptides causes synthesis of wall-like cellulose components on the surface of plasmolyzed protoplasts. The findings of the present study thus show clearly that intact microtubules and plasma membrane–cell wall adhesion in chrysanthemum cells are intimately involved in the mechanism by which the cells regulate growth under mechanical stress conditions. Some of the phenomena mentioned above will be discussed in the following sections.

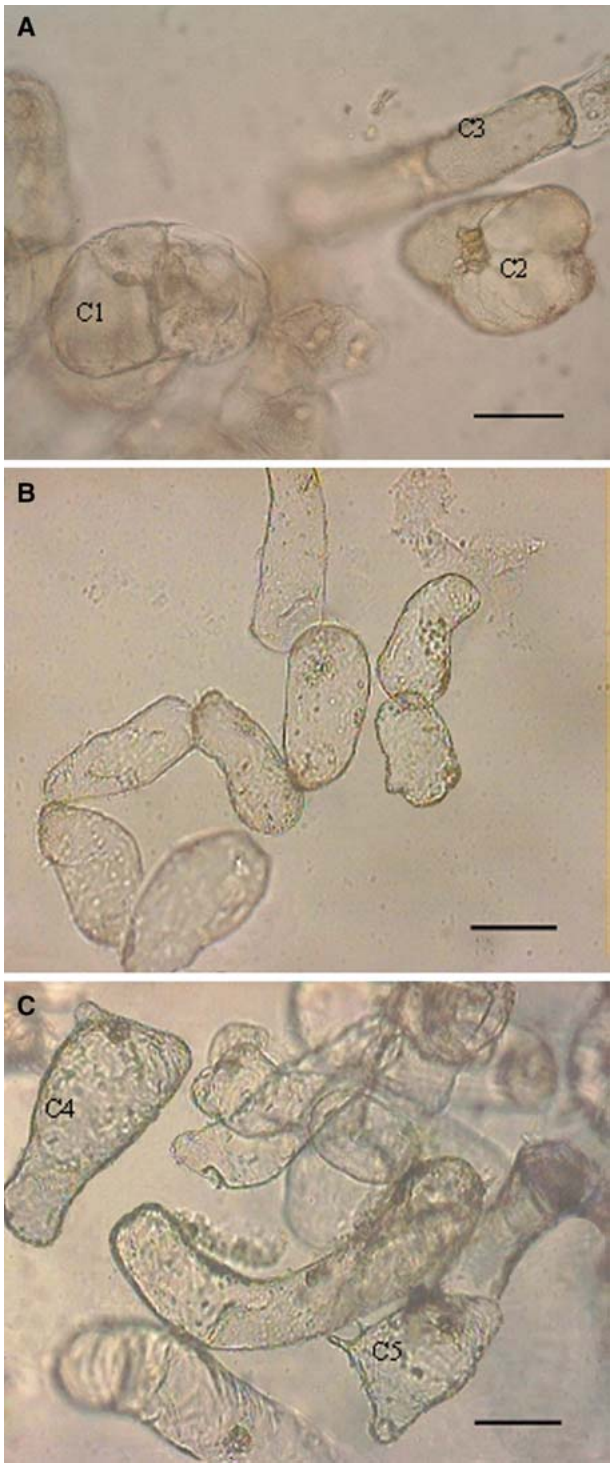

Figure 7. Micrographs of mechanically loaded chrysanthemum cells in the presence of GRGDS peptides (A), Cytochalasin B (B), or Colchicine (C). The direction of compressive force is from the left to the right. Note that the cells C1 and C2 in (A) have an anomalous appearance both in their surfaces and shapes, and C3 has a misaligned elongation axis related to the stress tensor. Cells in (B) maintain the ability to elongate in response to mechanical force, but the extent of elongation is dramatically reduced. Cells C4 and C5 in (C) exhibit an irregular form and expand with no preferential growth directionality. Bar = 100 μm.

Mechanical Force Induces the Preferential Orientation of Cell Elongation and Division

The induction of preferential elongation in mechanically stressed protoplasts described in tobacco (Wymer and others 1996; Lynch and Lintilhac 1997) and other plant species has been considered a common response of plant cells to mechanical forces. In our previous work, the morphological changes in response to mechanical stresses were investigated in chrysanthemum protoplasts, which tended to elongate with a preferential axis oriented perpendicular to the compressive stress direction. What should not be ignored is that, in the plant kingdom, the mechanical properties of the cell wall appear to withstand forces imposed on the cells through shape alteration and volume regulation (Peters and others 2000; Martin and others 2001). So the presumptive involvement of the cell wall in these responses seems to be reasonable. For analyzing the potential role of the cell wall in stress responses, we have provided evidence in the present study that the expression of orientation-associated plant responses is also exhibited in both the elongation and putative division behaviors in intact cells. Important support for this conclusion comes from the finding that some of the cells, when encountering hyperosmotic conditions, disintegrate into a series of linear-organized protoplasts, which actually indicated the existence of transversally aligned new partition walls along the longitudinal cells. This plasmolysis procedure was dynamically monitored by a CCD camera. The images reveal that the formation of a string of protoplasts occurs almost simultaneously, rather than as a sequence of events forming one protoplast followed by numerous subdivisions, yielding a series of subprotoplasts. These phenomena indicate that exerting a force of compression on the cells has dramatic effects on the orientation of both cell elongation and cell division. Although it could be argued that this latter effect of preferential division may be caused by alteration in cell shape rather than mechanical stress signal, there is no precedent for this in the literature. It is still to be determined what is primarily perceived by cells and is directly responsible for the direction of cell division.

Table 2. Effect of Stress Conditions and Cytochalasin B Treatment on the Average Length (mm) of the Major Axis of Elongated Cells after Culture for 4 Days

Stress conditions[a]	Drug treatment[b]	
	Cytochalasin B treated	Cytochalasin B untreated
Loaded	0.163 ± 0.077	0.306 ± 0.141
Unloaded	0.144 ± 0.098	0.331 ± 0.144

Factor [a]: F = 0.014, p = 0.905 > 0.05, the effect of factor [a] is not significant; these values (± SD) of loaded cells are not significantly different from corresponding unloaded cells.
Factor [b]: F = 38.834, p = 0.000 < 0.001, the effect of factor [b] is significant, these values (± SD) of cytochalasin B treated cells are significantly different from corresponding untreated cells.
Factor [a][b]: F = 0.696, p = 0.407 > 0.05, the interaction of factor [a] and factor [b] is not significant, there was no interactive effect between stress conditions and drug treatment. These values (± SD) represent the length of the major axis of elongated cells.*

Plasmolysis Followed by RGD Peptide Treatment Specifically Disrupts the Plasma Membrane–Cell Wall Adhesion

Protoplast plasmolysis has often been used to investigate plasma membrane–cell wall interactions in plants (Lee-Stadelmann and others 1984; Oparka 1994). During the plasmolysis of plant cells the plasmalemma is detached from the cell wall and the protoplast volume is significantly reduced while the architecture of plasma membrane–cell wall connection experiences mechanical perturbations due to tensile stresses (Komis and others 2002). However, cytosol staining with fluorescein diacetate followed by confocal imaging has revealed that large numbers of thin plasma membrane–cell wall connections known as *Hechtian strands* still remain (Mellersh and Heath 2001). One of the fundamental functions of the Hechtian strands is suggested to be mediation of the interaction between the plasma membrane and the cell wall, and accordingly, ensuring re-incorporation of this connection after replacement of the plasmolytic solution with hypoosmotic or isotonic solutions. In the present work the GRGDS peptides, in contrast to control peptides lacking the specific RGD motif, were shown to disrupt this reversible plasmolysis-deplasmolysis cycle.

RGD-dependent cell adhesion constitutes a versatile recognition system providing cells with anchorage, traction for migration, signals for polarity, position, differentiation, and possibly growth (Ruoslahti and Pierschbacher 1987). Except for the process of cellular locomotion, this description may equally well define the role of the cell wall in the growth and development of plant cells and bacteria (Schindler and others 1989). Microscopic examinations have revealed that the mechanism by which the application of RGD peptides decreases this adhesion involves disruption of the Hechtian strand connections to the cell wall (Mellersh and Heath 2001). As shown in our experiments, deplasmolysis fails to take place in the presence of GRGDS. Even though the volume of protoplast rapidly increased under hypoosmotic conditions, the plasmalemma appeared to be aberrant and did not localize at the cell wall. This appearance indirectly supports the hypothesis that exogenous RGD peptides may interfere with plasma membrane-cell wall adhesion, and the mechanism is probably associated with the existence of an RGD binding protein-based RGD-recognition system in plants and the RGD-induced disruption of Hechtian strand connections mentioned above.

Mechanical Force-induced Responses Require the Maintenance of RGD–dependent Plasma Membrane–Cell Wall Adhesion

Exogenous application of peptides containing the RGD sequence has been proven to specifically disrupt responses in animals involving integrin-mediated communication between the ECM and the cell interior (Ruoslahti 1996). Indeed, the general membrane-matrix recognition principle may transcend species and kingdom, and the presumptive RGD-recognition system may exist in higher plants, perhaps playing a role in responses of plants to environmental stresses. Morphological evidence presented in this study shows a direct correlation between the stress-induced responses of cell growth and the interaction in plasma membrane and cell wall communications. Experimental support for this correlation comes from the fact that, apart from the effects on plasma membrane–cell wall adhesion, peptides containing RGD but

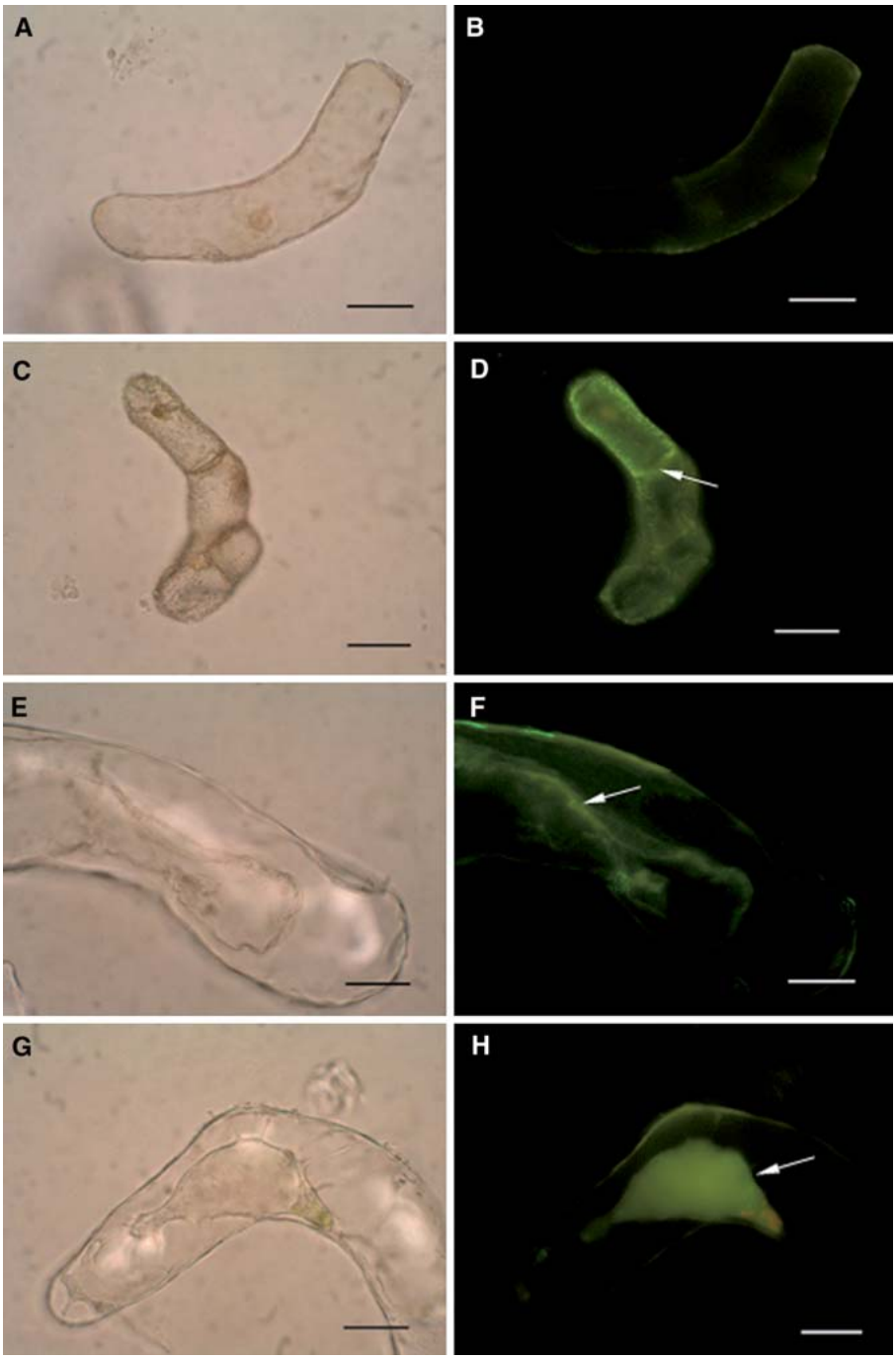

Figure 8. Light and fluorescent micrographs of cells with and without mechanical loading in the absence or presence of GRGDS peptides. Samples were stained with Calcofluor White. (A) and (B), Unloaded and untreated cell. (C) and (D), Mechanically loaded and untreated cell. (E) and (F), Unloaded and RGD treated cell. (G) and (H), Mechanically loaded and RGD treated cell. Arrows indicate the intense staining at the division sites in (D) or the retracted plasma membrane in (F) and (H). Bars in (A) to (D) =100 μm, and in (E) to (H) =100 μm.

not peptides lacking this specific motif are capable of interfering with the expression of preferential orientation. Although the interference may be caused by the perturbation on cellular processes rather than disruption of adhesion, there is no evidence for the hypothesis that the peptides can cross the plasma membrane intact. However, the molecular mechanism of coordination between plasma membrane-cell wall adhesion and stress-induced responses has remained elusive.

Microtubules and the Microfilament Cytoskeleton Play Diverse Roles in Oriented Cell Expansion

Microtubules and microfilaments have long been known to play key roles in plant cell morphogenesis (Mathur and Hulskamp 2002). The phenomena emerging from drug studies have shown that these roles are the same in both tip-growing and diffuse-growing cells. More specifically, in light of new

Table 3. Semiquantitative Score for Fluorescent Staining with Calcofluor White of Cell Wall in Chrysanthemum Cells (100 cells per treatment)

	Mechanically loaded		Unloaded	
Features[a]	Plasmolyzed and RGD treated	Untreated	Plasmolyzed and RGD treated	Untreated
The wall was uniformly stained throughout the cells	+	+++++	++	++
The wall displayed enhanced fluorescence when compared to (1)	−	++++	−	−
The wall was not stained with Calcofluor White	++	+	+	+
Intense staining was observed at the division sites between the cells	+	++++	++	++
Staining congregated on the plasmolyzed protoplast surface	++++	−	+++	−

(−) None; (+) less than 10% cells; (++) 11%–40% cells; (+++) 41%–70% cells; (++++) 71%–90% cells; (+++++); more than 90% cells.
[a]The five independent categories listed the appearances of cell staining observed under the fluorescent microscope. Particularly, cells in one experimental condition may have more than one of the features described.

evidence showing that a wide variety of proteins bind to microtubules, a broader question can be raised: whether a major function of plant microtubules is in modulating signaling pathways as plants respond to sensory inputs from the environment. Accordingly, the involvement of microtubules, as well as microfilaments, in the elongation response was tested in the present study.

Our data showed that cell expansion was drastically reduced and that relatively small and unexpanded cells were produced after administration of cytochalasin B, a microfilament-specific treatment. The phenomena have been observed by others in *Arabidopsis* (Baluska and others 2001; Hepler and others 2001), and it is believed that a disturbed actin cytoskeleton also causes reduced and/or misdirected delivery of Golgi-derived vesicles to the cell cortex, ultimately resulting in growth reduction (Mathur and Hulskamp 2002). However, actin drug-treated cells showed no defects in stress-induced preference of growth directionality, implying that microfilaments do not appear to be involved directly in establishment or maintenance of orientation in response to mechanical stress. On the contrary, in our experiments, colchicine, a microtubule drug, appeared to cause a loss of growth directionality. The findings of this work support the hypothesis that in chrysanthemum cells the microtubule (MT) network is somehow involved in regulation of directionality-associated growth or shaping. An earlier work by Wymer and others (1996) indicated the importance of MTs for stress-dependent orientation of cell division. Those investigators centrifuged protoplasts with 35 *g* and regenerated cells from them. They found that the axis of elongation had been influenced by the centrifugation. Random orientation resulted when the protoplasts were treated with amiprophos-methyl (APM), a tubulin-specific herbicide. They concluded that cortical MTs are necessary if a cell is to respond to a unidirectionally applied external force. In contrast to the present study, their work involved enzymatic degradation of the cell wall, which will undoubtedly in itself alter the pattern of cytoskeleton organization. Our experiments were designed to maintain an intact cellular structure and the physiological concentrations of nutrients in the cell wall. Our findings confirmed that intact microtubules are necessary for the cell to respond to the inductive effects of a mechanical force.

The diverse behaviors of microtubule and microfilament cytoskeletons in stress-induced responses appear to support the biochemical concept that microtubules are important for establishing and maintaining growth polarity, whereas actin microfilaments deliver the materials required for growth to specific sites (Marthur and Hulskamp 2002). More specifically, transverse microtubules have been recognized to constrain the movement of cellulose synthase complexes to generate transverse microfibrils that are essential for elongation growth. Other interpretations, for instance, that microtubules are intimately associated with cellulose synthesis activity, are also possible (Wasteneys 2004). The correlation between microtubules and mircofibril alignment suggests that microtubules may function to regulate the direction of cell expansion. Moreover, the mechanical model at the molecular level of cell structure based on tensegrity (Ingber 2003) may help to explain how the mechanics of the cytoskeletan are controlled, as well as how cells sense and respond to mechanical forces. In this model cytoskeletal filaments both generate and resist mechanical loads. Particularly,

tensional forces are borne by microfilaments and intermediate filaments, and these forces are balanced by interconnected structural elements that resist compression, most notably, internal microtubule struts and extracellular matrix adhesions. It is relevant to consider that, in plants, microtubules are preferentially pressure-sensitive and should be directly responsible for the cellular responses to our experimental compressive forces. Importantly, although the disruption of microfilaments fails to produce any detectable alterations in the stress-induced orientation, it should not be negated that, actually, the cellular response to stress depends on connectivity with discrete molecular networks that span the cell surface and extend through the cytoplasm, and on cooperative interactions between all the cytoskeletal filament systems.

Mechanical Force Improves Cell Wall Deposition, and This Improvement is Plasma Membrane-Cell Wall Adhesion-Dependent

As shown in Table 3, we have provided evidence that compressive stress can dramatically affect cellulose synthesis detected via calcofluor staining, suggesting that plants can respond to mechanical forces by increasing the mechanical strength of the cell wall. The cell wall–associated development triggered by mechanical force, presumably similar to the phenomenon of callose deposition beneath the cell wall of pea resulting from pathogen penetration (Mellersh and Heath 2001) and more intensive wall thickening detected in the inner tangential wall of the endodermis induced by various abiotic stresses in maize plants (Degenhardt and Gimmler 2000), is dependent on adhesion between the plant cell wall and the plasma membrane, implying that the induction or expression of these responses requires communication between the plant cell wall and the cytosol. These findings in our experiments also prove that the cell wall adaptations to environmental stresses may be retained in individual plant cells. The mechanism involved in the wall-associated responses in plant cells has not been identified.

Disruption of Plasma Membrane–Cell Wall Adhesion Combined with Mechanical Force Causes Calcofluor Staining on the Surface of Plasmolyzed Protoplasts

In the present article, the staining was localized on the surfaces of plasmolyzed protoplasts rather than on the cell wall. It would appear that the organization and synthesis of the cellulose components of the cell wall are considerably affected by the hyperosmotic condition followed by application of exogenous RGD peptides, as well as by the experimental mechanical loading. These RGD-related elements are speculated to be a result of the existence and activity of a plant RGD-binding protein that physically couples the plasma membrane and cell wall. Mechanical forces in these phenomena may function to stimulate defensive wall-like responses.

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