Changes in Tubulin Protein Expression in Guard Cells of *Vicia faba* L. Accompanied with Dynamic Organization of Microtubules during the Diurnal Cycle

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We previously reported that the organization of microtubules (MTs) in guard cells of *Vicia faba* L. shows dynamic diurnal changes [Fukuda et al. (1998) *Plant Cell Physiol.* 39: 80]. Here, we report a method to directly extract total proteins from guard cells to investigate the biochemical changes in guard cells of *Vicia faba* L. during the diurnal cycle. Electrophoretic profiles of total proteins of guard cells showed distinct patterns with the time of extraction. Immunoblot analysis also demonstrated changes in α-tubulin and β-tubulin contents with the diurnal cycle. Both tubulins were abundant at 6:00 h and 12:00 h but were almost undetectable at 24:00 h. Although treatment with either actinomycin D or cycloheximide at 18:00 h inhibited neither radial organization of cortical MTs nor stomatal opening, that at 6:00 h inhibited both. These results suggest that the dynamic diurnal changes in the organization of MTs in guard cells and stomatal movement of *Vicia faba* L. may be, at least partly, regulated by de novo synthesis and decomposition of tubulin molecules in guard cells.

Key words: Diurnal cycle — Guard cell — Microtubule — Protein — Tubulin — *Vicia faba* L.

Stomata function as biological valves which regulate the rates of photosynthesis and transpiration in response to a large number of external and internal signals. Stomatal movement is mainly regulated by changes in the turgor of guard cells (Meidner and Mansfield 1968, Raschke 1975), which is also under the control of the mechanics of the cell wall of guard cells (Raschke 1975, Wu and Sharpe 1979). Most studies on the mechanism of stomatal movement and on signal transduction pathways in guard cells have been based on solute transport (Assman 1993), and only a few have focused on the nature of the cell wall of guard cells (Stevens and Martin 1977, Meidner 1982). The curve direction of guard cell expansion during stomatal movement is controlled by the orientation of cellulose microfibrils, which are deposited at right angles to the elongation axis of guard cells (Sharpe et al. 1987), and by differential thickening and extensibility of the cell wall (Palevitz 1981). Numerous studies have also shown that cortical microtubules (MTs) control the developmental process that leads to the formation of functional stomata (Sack 1987, Mineyuki et al. 1988, Palevitz and Mullinax 1989, Marc et al. 1989a, b, Marc and Palevitz 1990).

Since the finding that the cell wall of mature guard cells actively changes in elasticity and metabolism in response to exogenously applied abscisic acid (Kondo and Maruta 1987, Takeuchi and Kondo 1988a, b), the study of MT events has been focused not only on stomatal differentiation but also on stomatal movement in the mature state (Jiang et al. 1996). Recently, we reported that the organization of MTs in mature guard cells of *Vicia faba* L. changes dynamically with the diurnal cycle, and suggested that such changes may be involved in the regulation of stomatal movement (Fukuda et al. 1998).

As guard cells of *Vicia faba* L. have a solid cell wall, and therefore protein extraction from them is difficult, protoplasts of guard cells are commonly isolated by enzymatic digestion of the cell wall prior to protein extraction (Gotow et al. 1984, Ohya and Shimazaki 1989). In the present study, to analyze the dynamic MT rearrangements in mature guard cells of *Vicia faba* L. during the diurnal cycle, we modified the glass-bead extraction method used for extraction of proteins and DNA from microorganisms, to extract proteins from guard cells of *Vicia faba* L. without isolation of guard cell protoplasts. SDS-PAGE of the proteins extracted from both guard cells and mesophyll cells of *Vicia faba* L. revealed a marked change in the composition of the total proteins with the diurnal cycle in guard cells but not in mesophyll cells. Concomitantly, diurnal changes in tubulin levels, as determined by an immunological technique, were also found only in guard cells. We also investigated the effects of inhibitors of RNA
and protein syntheses on MT organization and stomatal movement. The significance of these findings on our understanding of the mechanism of stomatal movement during the diurnal cycle is discussed.

Materials and Methods

Plant material—Vicia faba L. cv. Otafuku was grown in an environment-controlled glasshouse in Tsukuba, Japan, under natural light conditions, a day/night temperature of 20°C/15°C, a relative humidity of 70%, and under hydroponic culture (1 g liter\(^{-1}\) Hyponex (65 μg liter\(^{-1}\) of N, 60 μg liter\(^{-1}\) of P, and 190 μg liter\(^{-1}\) of K; Hyponex Japan, Tokyo, Japan) supplemented with 30 mg liter\(^{-1}\) Fe-ethylene glycol-bis (β-aminooethyl-ether) N,N',N'-tetraacetatic acid (EGTA)]. The fully-expanded, young bifoliate leaflets of 5- to 7-week-old plants, sampled on fine days in August, were used.

Protein extraction—The abaxial epidermis was peeled from 10 fully-expanded leaves at the indicated time point. The epidermal peels were collected in ice-cold 50 mM Tris-HCl buffer (pH 7.5) and were homogenized with a blender (mill-mixer FM-33A, SUN Corp. Tokyo, Japan) for 30 s at 13,500 rpm. After filtering the homogenate through a nylon mesh net (200 μm) and washing with deionized water, the epidermal tissue remaining on the net was dry blotted. After this procedure, the epidermal tissue was absolutely free from mesophyll contamination and only guard cells were visible. Viability determination was performed by staining with fluorescein diacetate (FDA) according to the method of Larkin (1976). Fluorescence was observed under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany) (Fig. 1). Cellulose microfibrils were visualized using Calcofluor-white ST (DOJIN, Tokyo, Japan) (Fig. 2), which indicated that homogenization almost completely destroyed the cell walls of epidermal cells, al-
though the epidermal peels retained most of their original shape probably because the cuticle still remained. The material was either immediately used for protein extraction or frozen in liquid nitrogen and stored at −80°C until use.

For protein extraction from guard cells, glass-bead milling (Ohta et al. 1992) was employed. The epidermal tissue (ca. 3 g) was transferred to a pre-cooled 50 ml Falcon tube containing a buffer solution consisting of 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 0.1 mM MgCl₂, 2 mM 2-mercapto-ethanol, 0.1% Triton X-100, 1 μg/ml pepstatin A, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 μg/ml leupeptin. Glass beads (0.5 mm; Iwaki Glass Co., Ltd., Chiba, Japan) were added to the buffer solution (1:1, v/v) and the tissue then milled with a micro-tube mixer (MT-360, Tomy Seiko, Tokyo, Japan) for 2 min followed by cooling on ice for 1 min. This milling operation was repeated 4 times. The extract was centrifuged at 22,000 × g for 30 min at 4°C, and the supernatant was then concentrated to ca. 2.0 ml by ultrafiltration with a membrane filter (Centricron Plus-20, Millipore) and dialyzed with cellulose tubing (size 24/32; Sanko Pure Chemical Co., Ltd., Tokyo, Japan) against 50 mM Tris-HCl (pH 7.5) buffer overnight and used for further analysis. For protein extraction from mesophyll cells, leaves, from which the adaxial and abaxial epidermal layers had been peeled, were homogenized with a blender (mill-mixer FM-33A, SUN Corp.) for 3 min at 13,500 rpm in the same buffer solution as above. The extract was centrifuged at 22,000 × g for 30 min at 4°C, and the supernatant was then dialyzed against 50 mM Tris-HCl (pH 7.5) buffer overnight and used for further analysis.

**Determination of protein**—Protein concentration was determined according to Bradford (1976) using a Protein Assay Kit (BIO-RAD Laboratories, Richmond, CA, U.S.A.) with bovine serum albumin as the standard. This extraction method yielded 2.5 mg of total protein (g FW)⁻¹ of peeled epidermis at any sampling time point.

**Electrophoresis and blotting analysis**—SDS-PAGE was performed using 12% polyacrylamide (bisacrylamide : acrylamide = 0.8 : 30.0) slab gels according to Laemmli (1970). For blotting, equal amounts of protein, 10 or 50 μg for each sample depending on the experiment, were separated on an SDS-polyacrylamide gel, and the proteins then transferred to nitrocellulose (NC) membranes according to Towbin et al. (1979), followed by staining with a Biotin-Blot Total Protein Detection Kit (BIO-RAD).

**Immunoblotting**—Proteins separated by SDS-PAGE were transferred to NC membranes (Towbin et al. 1979) which were then blocked for 1 h in 3% gelatin-TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5). Subsequently, α-tubulin, β-tubulin and elongation factor 1α (EF-1α) on the NC membranes were incubated with each specific monoclonal anti-mouse antibody (Amersham International plc, Buckinghamshire, U.K.) and detected with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (BIO-RAD). Non-reacted primary antibodies were removed by washing the NC membranes in the presence of 0.1% Tween 20 and 0.5 mM MgCl₂, 0.03% p-nitro blue tetrazolium chloride and 0.015% 5-bromo-4-chloro-3-indol phosphate p-toluidine salt (pH 9.5), and the reaction was terminated by washing the membranes in deionized water.

**Effects of actinomycin D (AD) and cycloheximide (CHX)** on stomatal movement and microtubules in guard cells—To examine the possible participation of de novo mRNA and protein synthesis in stomatal movement and MT organization, 0.1 μM AD (Sigma Chemical Co., St. Louis, MO, U.S.A.) or 1 μM CHX (Sigma Chemical Co.), in a buffer solution consisting of 5 mM piperazine-N,N’-bis-(2-ethanesulfonic acid)-KOH (pH 6.8), 10 mM KCl and 10 μM CaCl₂ (PCK buffer), was used. Epidermal strips were prepared at 6:00 h, 12:00 h or 18:00 h, and floated on the PCK buffer, with or without either test chemical for 1 h, followed by incubation for 2 h at 25°C. The samples prepared at 6:00 h and 12:00 h were illuminated with a 300 W tungsten lamp (Eye Lamp; Iwasaki Electric Co., Tokyo, Japan) during incubation. The light was passed through a 5-cm-thick layer of water to cut off heat radiation, giving a final light intensity of ca. 400 μmol m⁻² s⁻¹. The samples prepared at 18:00 h were kept in the dark during incubation. Before and immediately after each treatment, 45–60 stomata in each strip were photographed using FUJI instant film (FP-3000B; Fuji Photo Film Co., Tokyo, Japan) with a light microscope (BH-2; Olympus, Tokyo, Japan). Stomatal pore size was directly measured from the photographs using slide calipers (Digimatic Mini-Processor DF-1 HS; Mitutoyo, Tokyo, Japan). Aperture size is expressed as the ratio of the inner width to the inner length of the stomatal pore.

**Immunofluorescence visualization of MTs** was performed according to Fukuda et al. (1998). Stained preparations were examined under a microscope equipped with epifluorescence illumination (Carl Zeiss) and recorded on FUJI Super PRESTO 1600 film (Fuji Photo Film Corp.). By the procedures of fixation of guard cells and staining of MTs, stomatal pore size tended to decrease. Thus, the stained preparations could not be used for measurement of stomatal pore size.

**Results**

**Changes in total protein composition and in the levels of tubulins in guard cells**—Equal amounts of protein from guard cells and mesophyll cells were subjected to SDS-PAGE, electrotransferred to NC membranes, and ana-
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alyzed by biotin-blotting (Fig. 3). The protein composition differed significantly with the time of sampling in the guard cells (Fig. 3, left) but not in the mesophyll cells (Fig. 3, right). Extracts from mesophyll cells showed distinct bands of the large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), but extracts from guard cells much less dense bands, at the corresponding positions, indicating that this extraction method did not result in noticeable protein degradation.

The protein composition of the guard cells at 18:00 h (Fig. 3, left d), when stomata are closing and MTs in guard cells begin to become disorganized from a radial pattern to a fragmental pattern, showed a markedly different profile from those at other time points. At 18:00 h, bands 3, 4 and 5, as indicated by triangles (Fig. 3), were markedly dense, whereas band 2 was much less dense. Band 1 was prominently dense at 24:00 h (Fig. 3, left a), but could not be detected at 12:00 h or 18:00 h (Fig. 3, left c and d). These results suggest that the high molecular weight bands 1 and 2 may disappear or decrease, whereas the low molecular weight bands 3, 4 and 5 begin to increase at 18:00 h (Fig. 3 left d).

As shown in Fig. 4, both the α- and β-tubulin bands were prominent at 6:00 h and 12:00 h, but were extremely weak or almost undetectable at 24:00 h in the guard cells. In contrast, the amounts of these tubulins showed little variation during the day in mesophyll cells. The level of EF-1α, used as a control, showed little variation with time in both guard cells and mesophyll cells. Tubulins thus seem to have been synthesized before stomata begin to open at dawn and continue to be present until stomata become completely open at noon, and then to be degraded from evening when stomata begin to close until midnight when the stomata close completely. Therefore, not only the disassembly and assembly of MTs, but also the turnover of tubulin, are probably involved in diurnal MT organization in guard cells.

Requirement of de novo syntheses of RNA and protein—The diurnal changes in radially-organized cortical MTs in guard cells (Fukuda et al. 1998) were in accordance with the above-described changes in α- and β-tubulin contents. In order to determine whether de novo synthesis of mRNA and/or protein is required for stomatal opening and MT reorganization in guard cells, especially in the morning, the effects of AD and CHX on stomatal opening and MT organization were examined. As shown in Fig. 5, the treatment with either 0.1 μM AD or 1 μM CHX starting at 6:00 h completely inhibited stomatal opening during the 2-h incubation in light (Fig. 5A). However, the stomata opened to the level similar to the control level within 30 min after transfer of the epidermal peels to the buffer without these chemicals in light (data not shown). Immediately before the incubation in light, cortical MTs in most guard cells (ca. 75%) were in a fragmental pattern similar to those in Fig. 5C and D. The MTs in ca. 90% of guard cells sampled at 6:00 h and not exposed to the inhibitors were in a radial pattern after the 2-h incubation in light (Fig. 5B), but the treatment with these inhibitors prevented the radial organization of cortical MTs. MTs in more than 90% of guard cells treated with these inhibitors remained in a fragmental pattern even after 2 h of incubation in light (Fig. 5C, D). In contrast, neither AD nor CHX showed notable inhibitory effects on stomatal movement (Table 1) or MT organization in guard cells sampled at 18:00 h (data not shown). But AD and CHX did suppress the slight increase in stomatal aperture in epidermal strips sampled at 12:00 h. These results indicate that the synthesis of mRNA and proteins is an essential prerequisite for stomatal opening and MT reorganization in guard cells in the morning. Taken together with the results of immunodetection (Fig. 4), the de novo synthesis of tubulin seems to be important for MT reorganization at dawn. However, in the evening, de novo protein synthesis may not be required for MT disorganization and stomatal closure. At noon, de novo protein

![Fig. 4](https://academic.oup.com/pcp/article-abstract/41/5/600/1830556/fig4)

**Fig. 4** The levels of α-tubulin, β-tubulin and EF-1α in guard cells and mesophyll cells at various time points. A 20 μg aliquot of proteins from each sample source, obtained at the time point and from the cell type indicated in Fig. 3, was subjected to SDS-PAGE and immunoblot detection.
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**Figure 5** Effects of actinomycin D and cycloheximide on stomatal opening and MT organization in guard cells. A, effects of actinomycin D (AD) and cycloheximide (CHX) on stomatal movement. •, control; ○, 0.1 μM AD; ●, 1 μM CHX. Averages of 60 stomatal apertures ± SEs are shown for each time point. Experiments were repeated three times with comparable results. The epidermis was peeled and immediately used at 6:00 h. Immunofluorescence micrographs showing typical patterns of cortical MTs in guard cells either after 2-h in light without treatment (B), or following 2-h treatment with 0.1 μM AD (C) or 1 μM CHX (D).

**Table 1** Effects of actinomycin D and cycloheximide on stomatal movement at various times of day

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Treatment time (min)</th>
<th>Stomatal aperture a (Time of day h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6:00</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.46 ± 0.04</td>
</tr>
<tr>
<td>AD b</td>
<td>0</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.23 ± 0.05</td>
</tr>
<tr>
<td>CHX c</td>
<td>0</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.25 ± 0.04</td>
</tr>
</tbody>
</table>

Epidermis was peeled at 6:00 h, 12:00 h and 18:00 h. Each chemical treatment started separately within 12 min after peeling.

a Stomatal aperture is expressed as the ratio of inner width to inner length of the stomatal pore. Data are shown as the mean values with standard errors (n = 30).

b 0.1 μM actinomycin D.

c 1.0 μM cycloheximide.
synthesis may not be required for maintaining the radial organization of MTs, although synthesis may be necessary for stomatal opening.

**Discussion**

In the present study, we developed a direct and rapid method to extract total proteins from guard cells in order to investigate the biochemical aspects of MT reorganization during the diurnal cycle. In guard cell protoplasts isolated from *Vicia faba* L., the most heavily stained bands represented polypeptides of low molecular weight, estimated to be 12.5, 15 and 16 kDa (Ohya and Shimazaki 1989). On the other hand, molecular weights of the total proteins extracted by the present method were broadly distributed from 12 kDa to 200 kDa, and showed distinct profiles with the time of day. Since proteins were extracted rapidly and directly from guard cells without enzymatic treatment in the present study, the extent of protein degradation was minimized during protein preparation. The efficacy of extraction was demonstrated by the levels of the large and small subunits of RuBisCO, which were found to be much less in extracts from guard cells than in those from mesophyll cells, which is consistent with a previous report that RuBisCO is present in guard cells but at a considerably lower level than in mesophyll cells (Reckmann et al. 1990).

Using the present extraction method, we found changes in the tubulin contents during the diurnal cycle in guard cells (Fig. 4). These results suggest that MT reorganization at dawn could be regulated, at least partly, by tubulin content. Only a few studies have dealt with the changes in tubulin level in association with MT organization in higher plants. Changes in tubulin concentration were found to be accompanied with a reorganization of microtubular arrays in the process of tracheary element differentiation and during cell division of isolated *Zinnia* mesophyll cells (Fukuda 1987, 1989, Yoshimura et al. 1996), as well as during cell shaping in barley leaves (Hellmann and Wernicke 1998) and also during the development of the carrot plant (Hussey et al. 1987). However, here, we identified, for the first time, the diurnal changes in tubulin contents in mature plant cells.

Tubulin accumulation can be controlled either at the level of mRNA synthesis or protein synthesis (Giani et al. 1998). In the present study, however, stomatal opening and MT organization were inhibited by treatment with either AD or CHX at 6:00 h. Therefore, it seems likely that tubulin accumulation in guard cells at dawn might be regulated by de novo syntheses of both mRNA and proteins. MT reorganization, however, is possibly regulated not only by de novo synthesis of tubulin molecules but also by various protein factors that modulate MT reorganization, including microtubule-associated proteins (MAPs), protein kinases and phosphatases as well as their target proteins. Previously, we hypothesized that stomatal opening might be regulated by MT reorganization through synthesis and/or organization of cellulose microfibrils at dawn (Fukuda et al. 1998). According to this hypothesis, the inhibitory effects of AD and CHX on stomatal opening would be due to the inhibition of synthesis of radially-oriented cellulose microfibrils in guard cells, which would have resulted from the inhibition of MT reorganization. Recently, we found that treatment with 2,6-dichlorobenzonitrile, an inhibitor of cellulose synthesis (Hogetsu et al. 1974), suppressed the stomatal opening early in the morning without any effect on MT organization, although the treatment had no effect on stomatal movement later in the day (unpublished data). These results suggest that de novo synthesis of cellulose microfibrils would also be prerequisite for stomatal opening in the morning. However, it remains to be solved whether de novo synthesis of cellulose-synthesizing enzymes could be involved in stomatal opening in the morning. Alternatively, the inhibition of stomatal opening might be caused by the suppression of synthesis of osmoregulation-related proteins, such as ion-channels, H⁺-ATPase and/or other protein factors related to the modulation of these proteins (Li and Assmann 1996, Mori and Muto 1997, Hwang et al. 1997, Kinoshita and Shimazaki 1999).

The composition of total proteins in guard cells at 18:00 h differed markedly from those at other time points. Some forms of proteolytic activity might be activated in the evening so as to specifically degrade proteins with comparatively high molecular weights (Klockenbring et al. 1998). A more plausible possibility, however, is that changes in protein composition reflect metabolic differences in guard cells between the light and dark periods. For example, starch is stored in guard cell chloroplasts when stomata close and that the starch levels decrease when stomata open (Outlaw and Manchester 1979), by degradation into malate, which is driven by phosphoenolpyruvate carboxylase via glycolysis (Schnabl 1980). A part of the malate could be converted to starch via gluconeogenesis when stomata close. Changes in the activities of some enzymes related to starch-malate conversion during stomatal movement have been reported (Schnabl et al. 1982). On the other hand, our previous and present studies strongly suggest that cell walls of mature guard cells are actively metabolized and turn over, to alter the mechanical properties of cell walls in guard cells (Kondo and Maruta 1987, Takeuchi and Kondo 1988a, b). We suppose that cellulose-synthesizing enzymes would be synthesized in the morning and enzymes involved in degradation of cell wall, such as cellulase (endo-1,4-β-glucanase) (Nakamura and Hayashi 1993), would be synthesized in the evening. However, it also remains to be solved whether the synthesis and/or activities of these enzymes change during the diurnal cycle and whether these enzymes are involved in...
stomatal movement.

Previously, we observed that MT organization changed in parallel with stomatal movement (Fukuda et al. 1998). In the present study, the composition of total proteins as well as the levels of tubulin molecules were found to change with the diurnal cycle. Our future research will focus on the diurnal changes in quantity and activity of enzymes related to cell wall metabolism (Hayashi 1989) and to regulation of mechanical properties of cell walls, such as endo-xylanoglucon tranferase (Nishitani and Tominaga 1992). Then, we will study to clarify the regulatory machinery of MT organization, including the involvement of MAPs, protein kinases and phosphatases, and the mechanism by which the guard cell wall structure is regulated, and to clarify the transduction pathways of diurnal signals to cell wall modification via MT reorganization in guard cells.

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References


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