Orientation of cortical microtubules correlates with cell shape and division direction

Immunofluorescence of intact epidermis during development of *Graptopetalum paraguayensis*

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**Summary.** Cortical microtubules in the epidermis of regenerating *Graptopetalum* plants were examined by in situ immunofluorescence. Paraepidermal slices of tissue were prepared by a method that preserves microtubule arrays and also maintains cell junctions. To test the hypothesis that cortical microtubule arrays align perpendicular to the direction of organ growth, arrays were visualized and their orientation quantified. A majority of microtubules are in transverse orientation with respect to the organ axis early in shoot development when the growth habit is uniform. Later in development, when growth habit is non-uniform and the tissue is contoured, cortical microtubules are increasingly longitudinal and oblique in orientation. Microtubules show only a minor change in orientation at the site of greatest curvature, the transition zone of a developing leaf. To assess the role of the division plane on orientation of arrays, the pattern of microtubules was examined in individual cells of common shape. Cells derived from transverse divisions have predominately transverse cortical arrays, whereas cells derived from oblique and longitudinal divisions have non-transverse arrays. The results show that, regardless of the stage of development, microtubules orient with respect to cell shape and plane of division. The results suggest that cytoskeletal function is best considered in small domains of growth within an organ.

**Keywords:** Cortical microtubules; Cytoskeleton; Organ growth; Immunofluorescence; *Graptopetalum.*

**Abbreviations:** DMSO dimethylsulfoxide; EGTA ethylene glycol-bis-(β-aminoethyl ether)-N, N', N'-tetra acetic acid; FITC fluorescein isothiocyanate; MTSB microtubule stabilizing buffer; PBS phosphate buffered saline.

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**Introduction**

The microtubule cytoskeleton is important during plant development because of its dual role in cell growth and division. Past research has focused primarily on the structure and function of microtubule arrays during growth in single cells or protoplasts using immunofluorescence microscopy (Wick et al. 1981, Gunning 1982, Clayton 1985, Lloyd et al. 1985), or in whole organs using electron microscopy (Hardham et al. 1980, Lang and Green 1984). The technical difficulty of viewing microtubule arrays in situ by immunofluorescence microscopy has prevented the study of microtubules during organ development until recently (Roberts et al. 1985; Hogetsu and Oshima 1986; Sakaguchi et al. 1988a, b; Mineyuki et al. 1989). Visualized by these new methods, cortical microtubules appear to be quite dynamic and variable in their overall orientation. Microtubules are generally transverse to the organ axis in elongating epidermal cells of cylindrical organs such as root tips or stem internodes (Roberts et al. 1985, Hogetsu and Oshima 1986). Microtubules shift from transverse to oblique when growth slows (Hogetsu and Oshima 1986, Laskowski in prep.) or when cells change shape due to hormone or microtubule inhibitor treatment (Takeda and Shibaoka 1981, Steen and Chadwick 1981, Lang et al. 1982, Mita and Shibaoka 1983, Roberts et al. 1985). These observations support the suggestion of Lloyd (1984) that microtubule arrays exhibit dynamic changes in orientation during cell growth.
It is still not fully understood how cortical microtubules influence organ growth. A current hypothesis proposes that cortical microtubules align perpendicular to the direction of growth, thereby orienting microfibrils in a reinforcing direction (Green 1980). This hypothesis is based on evidence in single non-dividing cells that (1) microtubules in the cell cortex orient cellulose microfibrils in the cell wall (Green 1963, Hepler and Pal-evitz 1974, Robinson and Quader 1982) and (2) cellulose is generally aligned perpendicular to the direction of growth (Green 1980, Lloyd 1984). The hypothesis is further supported by evidence from polarized light microscopy showing that cellulose is circumferentially aligned perpendicular to the direction of growth of the whole organ (Green and Brooks 1978).

Alignment of microtubules and reinforcement by cellulose microfibrils may also be important during the process of organ initiation, when both cell division and growth occur. A change in alignment of both microtubules and microfibrils occurs at sites of organ emergence (Hardham et al. 1980). Lang and Green (1984) showed that cortical microtubules reorient after shifts in division plane at the apex of Graptopetalum. In addition, Sakaguchi et al. (1988a, b) showed that microtubule alignment correlates with cell position on the apex. That is, microtubules are randomly aligned in cells at the center of a plant apex but are more concentrically aligned in cells at the periphery where leaf primordia emerge.

The present study investigates the orientation of cortical microtubules during early stages of leaf development of Graptopetalum before cell division ceases but after initiation of the primordium. At this stage of development, the direction of organ growth is primarily longitudinal with respect to the long axis of the plant. Characteristically, however, three-dimensional changes in organ shape accompany the early differentiation of leaf and stem from the primordium. Our aim is to define the role that microtubules play during these changes in organ shape. To accomplish this, we have characterized the patterns of microtubule orientation in the epidermis of Graptopetalum with respect to the longitudinal direction of growth and at the sites of organ shape change. Evidence that tangential tensions on the plant surface are important during growth (Green 1980, Pearce and Penny 1983) suggests that microtubules in epidermal cells may have a significant role in the final stages of the change in organ shape. Although inner tissues also influence the mechanical properties involved, the present study is confined to orientation of microtubules in the epidermis.

To examine microtubule patterns, we have developed a method of in situ immunofluorescence microscopy. The method uses hand-carved paradermal sections. The sections are subjected to mild enzymatic digestion of the cell wall, which permeabilizes the cells to tubulin antibodies without dissociating the tissue. This method permits observation of microtubule patterns in expanses of whole tissue.

Two issues are addressed in the study. The first issue concerns the orientation of microtubules during an overall change in shape from a cylindrical leaf primordium to contoured leaf and stem organs. For quantitative analysis, microtubules in the epidermis are viewed as a single cohesive unit enclosing the whole organ. The question asked is: does the orientation of microtubules correspond with the overall direction of organ growth in the predicted reinforcing direction? The second issue concerns the pattern of microtubules within individual cells of the organ during its development. For this analysis, microtubule orientations within single cells of common shape are compared. The question asked is: what morphological factors influence the alignment of microtubules within individual cells in relation to their neighbors? By combining electron microscopy and in situ immunofluorescence microscopy, the orientation of cortical microtubules is analyzed with respect to division planes and the overall direction of organ growth.

Materials and methods

Preparation of plants

Young leaves of the succulent Graptopetalum paraguayense E. Wallther were removed from plants grown in lathouses at the Carnegie Institution of Washington, Department of Plant Biology, Stanford University. The residual meristem on the adaxial side of the leaf scar was exposed by removing overlying tissue from the leaf as described by Green and Brooks (1978). Excised leaves were grown at room temperature in far-red light for up to 2 weeks. Growth of the residual meristem was classified into three stages: (1) growth of the shoot primordium, (2) differentiation of the leaf and stem, and (3) continued growth of the leaf and stem.

Scanning electron microscopy (SEM)

Regenerates at each stages were processed for scanning electron microscopy following the method of Williams et al. (1987). In brief, a dental impression material of medium viscosity (Kerr Reflect, single mix vinyl polysiloxane elastomeric impression kit, Patterson Dental Supply, Sunnyvale, CA) was spread on the plant meristem and allowed to set at room temperature for 5–10 min. The negative mold of the specimen was peeled from the plant surface, filled with Spurr’s resin (Spurr 1969) and polymerized at 70°C overnight. The resin replicas, or casts, were mounted on Philips SEM stubs and sputter coated with gold (approx 20 nm) in a Polaron E 5400 high resolution coating system. The specimens were examined using a Philips 505 SEM operating at an accelerating voltage of 15 kV.