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Visualization of microtubules in living cells of transgenic *Arabidopsis thaliana*

Rapid communication

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Summary. Microtubules (MTs) were visualized in living cells of several tissues in transgenic *Arabidopsis thaliana.* The transformed Arabidopsis plant was obtained by infecting it with *Agrobacterium tumefaciens* carrying the GFP-TUA6 plasmid. The fluorescence of the MTs was due to the fluorescence of GFP-TUA6 that was polymerized into the MTs. The distribution patterns of the visualized MTs in the living epidermal cells of leaves was similar to that in fixed epidermal cells. The actual destruction of MTs by oryzalin was observed in a living cell. Cytochalasin B exerts no effect on the distribution pattern of MTs. The fluorescence intensity of MTs was different among cells in different tissues.

Keywords: Cytoskeleton; Epidermal cell; GFP-TUA6 fusion protein; Microtubule; Transgenic plants; Tubulin.

Introduction

Microtubules (MTs) play an essential role in fundamental cellular processes, such as cell division, morphogenesis, and intracellular transport. Preprophase bands, centrosomes, spindle fibers, and phragmoplasts, all of which consist of MTs, are concerned with cell division, and cortical MTs are concerned with morphogenesis (for reviews see Gunning and Hardham 1982, Lloyd 1987, Fosket and Morejohn 1992, Shibaoka 1994, Cyr and Palevitz 1995).

Many investigations have been carried out on the relationship between MTs and these biological processes. The visualization of MTs is one of the first things that is needed for such investigations. To visualize MTs, antitubulin antibodies are usually applied to the cells followed by the application of secondary antibodies coupled with a fluorochrome. MTs in the cells treated with such procedures fluoresce under a fluorescence microscope, because the secondary antibodies conjugate to the MTs through the primary antibodies in such experimental procedures. Although immunohistochemistry has extensively elucidated the function of MTs, it also has some shortcomings. The procedures are rather complicated and delicate and generally use fixed cells. Changes in the movement and arrangement of MTs are difficult to trace in fixed cells in real time. If MTs can be visualized in living cells and their behavior can be traced throughout some biological phenomenon, the role of MTs on that phenomenon would be more deeply understood.

Several attempts have been made to visualize MTs in living cells. Previously, tubulin that was conjugated to a fluorochrome such as rhodamine or fluorescein, was microinjected in the cells to insert tubulin-fluorochrome into MTs (Shelden and Wadsworth 1993, Tanaka and Kirschner 1995, Zhang et al. 1990). As a matter of course, only the microinjected cells could later be examined in this case, so that the cells to be investigated were rather limited in number due to the time-consuming procedure of microinjection.

Recently, expressed fusion proteins with the green fluorescent protein (GFP) from *Aequorea victoria* have been widely used to detect specific proteins in cells. Various proteins related to MTs and coupled

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with GFP have been examined in their location in cells. These include microtubule-associated proteins, MAP2c and Tau34 (Ludin et al. 1996) and MAP4 (Olson et al. 1995, Marc et al. 1998), microtubule motor proteins, kinesin (Romberg et al. 1998) and dynein (Shaw et al. 1997). In yeast cells, MTs were visualized by fluorescence microscopy through the expression of GFP-tubulin. Straight et al. (1997) successfully observed the spindle elongation and chromosome separation in living yeast cells with the expressed fusion protein GFP-α-tubulin. Carminati and Stearns (1997) used a construction of GFP-TUA3 (α-tubulin) in the study of yeast MTs and observed the dynamic behavior of MTs in the cell cycle.

Marc et al. (1998) constructed a GFP-MAP4 reporter gene and induced transient expression of the GFP-MAP4 protein in epidermal cells of fava bean. To our knowledge, there have been no studies that have visualized MTs in the stable transformant of higher plants. If we can examine the MTs in the living cells of such higher plants, the biology of MTs should greatly advance. We report here that we have created such transgenic plants in which GFP-TUA6 fusion proteins are expressed and incorporated into MTs without interference with normal plant life. We also report some observations of MTs in living cells of transgenic plants.

Material and methods

GFP-tubulin construct and transformation

An Arabidopsis α-tubulin TUA6 cDNA in pZL1 (GIBCO BRL) was subcloned into pBluescript KS$^-$. (Stratagene) between *Sal*I and *Not*I sites, and an *Nae*I site was introduced in front of the translation initiation Met by PCR (polymerase chain reaction) amplification. The stop codon of a soluble-modified red-shifted version of GFP (smRS-GFP; Davis and Vierstra 1996) was replaced with an *Nae*I site by PCR amplification. The cDNAs of TUA6 and smRS-GFP were then cut with *Nae*I and *Sac*I, and with *Bam*HI and *Nae*I, respectively, and ligated together between *Bam*HI and *Sac*I sites in pBluescript KS$^-$. The resulting plasmid contained an in-frame fusion between smRS-GFP and TUA6, connected by an Ala-Gly linker encoded in the *Nae*I site. The fusion construct was completely sequenced to verify that no unintentional mutations were introduced. The GFP-TUA6 fusion sequence was then inserted between the *Sma*I and *Sac*I sites in pBI 121 (Clontech) so that the fusion protein is expressed under the 35S promoter.

A. thaliana ecotype Columbia plants with a *gl1* marker were vacuum-infiltrated with the *Agrobacterium tumefaciens* pGV2260 strain containing the above construct as described by Bechtold et al. (1993). T1 seedlings were screened for kanamycin resistance (30 mg/l). Transgenic lines homozygous for kanamycin resistance were selected at T2 and T3 generations and used for analysis. Plants were grown either on soil or on *Arabidopsis* mineral nutrient agar (Haughn and Somerville 1986) under standard conditions (23 °C, 16 h illumination per day at 80 μmol/m^2 · s) in an incubator.

Fluorescence microscopy

A laser microscope, Zeiss LMS 510, was used to observe fluorescence of GFP in the MTs with 488 nm excitation and a 505 nm cut-off filter. For detection of rhodamine in the secondary antibodies, 543 nm excitation light and a 560 nm cut-off filter were used.

Immunohistochemistry

For immunohistochemical detection of α-tubulin, leaf petioles were fixed with 4% formaldehyde in PHEM [60 mM piperazine-N,N′-bis (2-ethanesulfonic acid], 25 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 10 mM ethyleneglycol-bis[β-aminoethylether]-N,N,N′,N′-tetraacetic acid, and 2 mM MgCl$_2$, pH 7.4) for 1 h at 4 °C, washed with 20 mM phosphate-buffered saline (PBS) for 30 min, and sectioned with a vibratome, 100 μm in thickness. The sections were transferred in 1% Triton X-100 for 30 min and immersed in 20 mM PBS containing 1% bovine serum albumin for 30 min. Then, they were treated with 1/200 diluted anti-α-tubulin antibodies (Amersham) for 2 h, washed with 20 mM PBS containing 0.03% Tween 20, and further treated with 1/30 diluted sheep anti-mouse IgG antibodies conjugated with rhodamine. After washing with 20 mM PBS for 20 min, sections were mounted in 50% glycerol including 0.05% propyl gallic acid and observed with a laser microscope.

Results and discussion

To visualize microtubules in living plant cells, a modified GFP was fused to the amino terminus of Arabidopsis α-tubulin TUA6 because moderate overexpression of α-tubulin (but not of β-tubulin) is tolerated in yeast cells (Weinstein and Solomon 1990), and an amino-terminal (but not carboxy-terminal) fusion of GFP to a yeast α-tubulin TUB3 complements a yeast *TUB3* null mutant (Carminati and Stearns 1997).

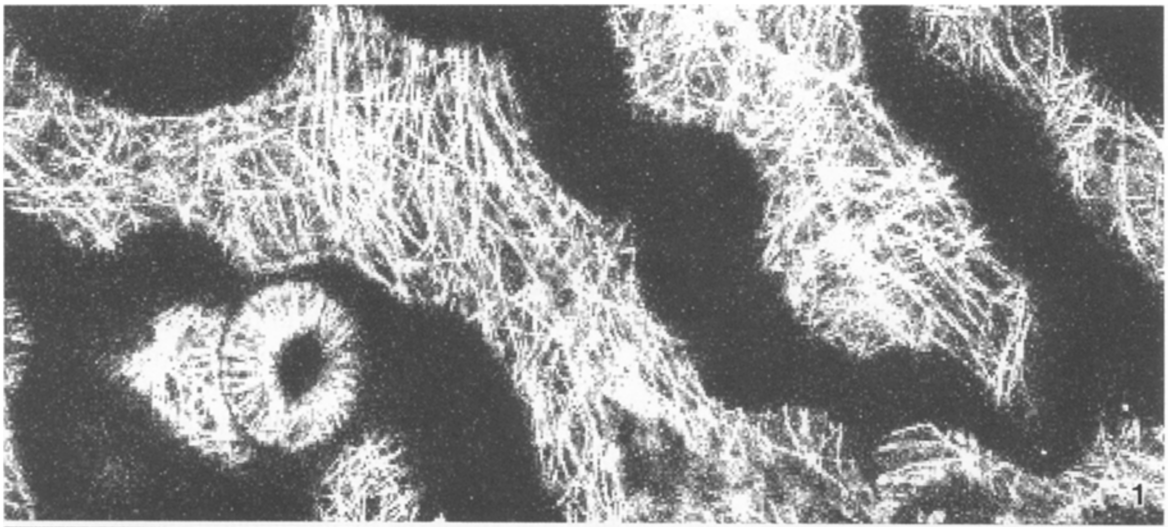
The GFP-TUA6 fusion protein was expressed under

Fig. 1. Cortical MTs in the living ordinary and guard cells of leaf epidermis. × 1,000

Fig. 2 a, b. Cells treated with the antibodies against α-tubulin. **a** Fluorescence of MTs for GFP; **b** fluorescence of MTs for rhodamine. × 920

Fig. 3 a, b. Oryzalin treatment of living epidermal cells. **a** Before treatment with oryzalin; **b** 30 min after treatment with 10 μM oryzalin. Most MTs in the cells have been disorganized. Arrows point to the fluorescent nodes of the faintly fluorescent network. × 750

Fig. 4 a, b. Cytochalasin B treatment of epidermal cells. **a** Before treatment with cytochalasin B; **b** 30 min after treatment with 10 μM cytochalasin B. MTs in the cells remain almost unchanged in arrangement. × 750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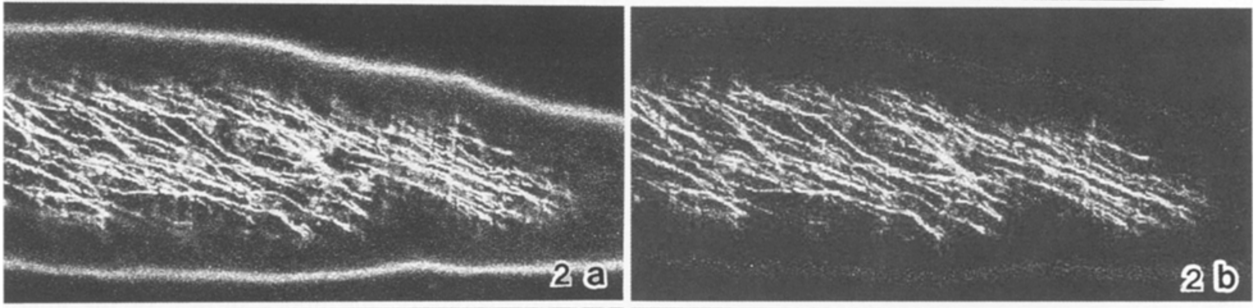
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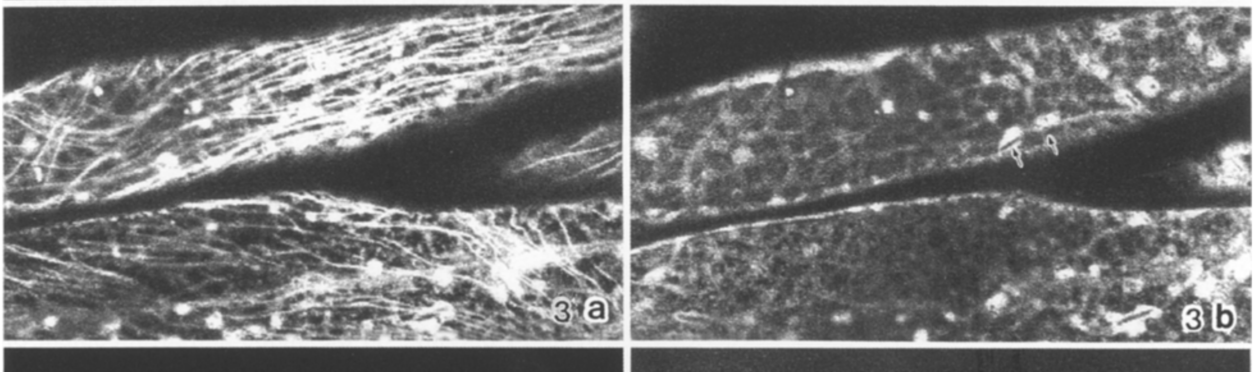
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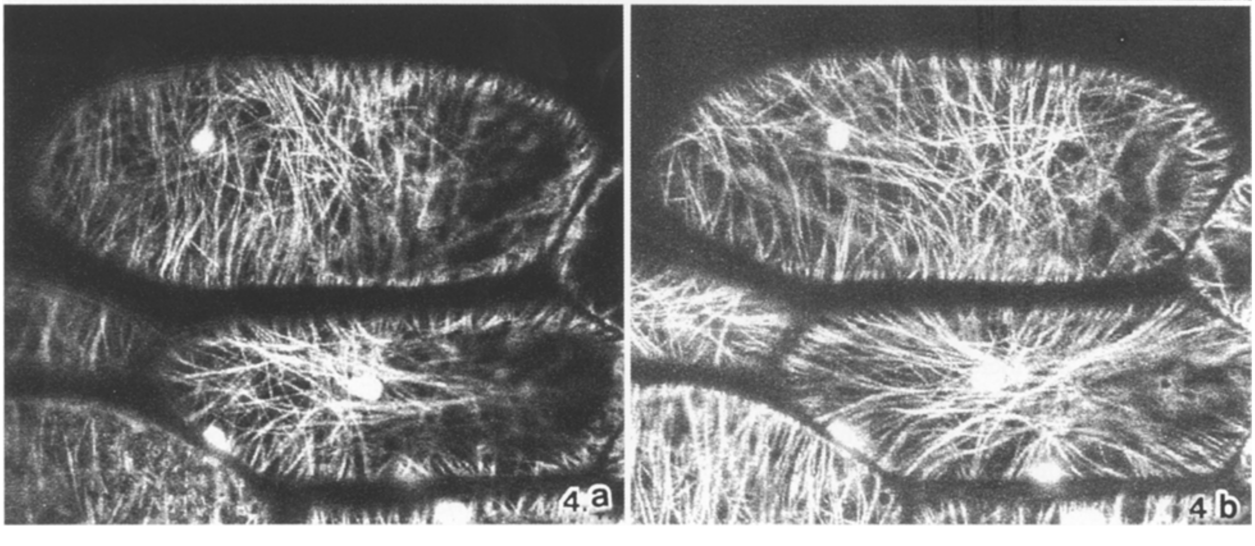
4 a
4 b

Table 1. GFP-TUA6 expression intensity in various types of cell

Organ/tissue	GFP-TUA6 expression
Root	
root cap	±
primary meristem	++[a]
cortex	–
pericycle	++[a]
root hair	–
Hypocotyl	
epidermis	++
cortex	+
pericycle	++[a]
Leaf	
epidermis	++
mesophyl	+
Flower	
calyx	+
pollen tube	–

[a] Cells fluoresced green, but MTs were not recognized

the control of the constitutive CaMV 35S promoter in transgenic Arabidopsis plants. Four independent transgenic lines were generated that fluoresce strongly with 488 nm excitation. One line (line 4) had a smaller seed set, while the other three lines were completely fertile. The growth and morphology of all lines were mostly normal. The only noticeable morphological features were anticlockwise twisting of petals in line 4, and anticlockwise twisting of cotyledon petioles in all four lines. One of the lines (line 13) was analyzed in the present work.

Many green-fluorescent fibers could be detected in various kinds of cell by the laser microscope with 488 nm excitation (Figs. 1, 2 a, 3 a, and 4 a). These fibers were identified as microtubules, because they emitted yellow fluorescence from rhodamine when they were treated with the anti-α-tubulin antibody followed by the treatment with the secondary antibody conjugated with rhodamine (Fig. 2 b). When the same portion of the cell as in Fig. 2 b was irradiated with 488 nm light, green fluorescent fibers became visible (Fig. 2 a) in place of the yellow fluorescent fibers. All fibers that emitted green fluorescence also emitted yellow fluorescence, but no fibers emitted only one sort of fluorescence, green or yellow. In the wild-type cells, no green fluorescent fibers were seen under irradiation of 488 nm light. The green fluorescent fibers were clearly due to the GFP that was expressed as the GFP-TUA6 fusion protein and incorporated into MTs simultaneously along their entire length. Probably, the expressed GFP-TUA6 may be incorporated randomly in the MTs without being discriminated from the original component, α-tubulin.

Young leaves were cut off, mounted in MS culture medium, and directly observed under a laser microscope without cutting into small pieces or peeling off the epidermis. The fluorescence of the MTs in leaf epidermal cells was very intense. All cells contained fluorescent MTs in contrast to 10 to 100 cells per leaf in which the GFP-MAP4 proteins were transiently expressed by particle bombardment (Marc et al. 1998). MTs densely covered the surface of the protoplasm of the irregularly shaped epidermal cells (Fig. 1). The distribution density of the MTs was larger than that visualized by immunohistochemistry (Panteris et al. 1993). The MTs seemed to be grouped into many bundles, in which the MTs ran parallel to each other. Many of them extended in straight lines between different regions of the cell.

Stomatal guard cells are kidney-shaped and can easily be recognized by their specific shape. In young guard cells, cortical MTs appeared to surround cell peripheries in radial directions, not merely radiate in two dimensions on the upper surface of the guard cells (Fig. 1). A radial arrangement of the cortical MTs has been reported in several papers (Marc et al. 1989), Palevitz and Mullinax 1989, Fukuda et al. 1998). It must be emphasized that the shape and arrangement of MTs in living cells are not similar to those of fixed cells, but the shape and arrangement of fixed cells are similar to those of living cells.

Oryzalin is widely used to disorganize MTs in the cells (Braun and Sievers 1994, Giani et al. 1998). The effects of oryzalin on the shape and arrangement of MTs in the epidermal cells of a hypocotyl are shown in Fig. 3. The epidermal cells of the hypocotyl contained cortical MTs that were arranged in one direction in some cases, and in several different directions in other cases (Figs. 3 a and 4 a). When the cells shown in Fig. 3 a were treated with oryzalin for 30 min, most MTs became disorganized (Fig. 3 b). Disorganized GFP-TUA6 proteins formed weakly fluorescent reticular networks with small but intensely fluorescent nodes.

Cytochalasin B is known to disorganize microfilaments, but not MTs (Williamson and Hurley 1985, Tominaga et al. 1997). The arrangement and quantity of MTs in hypocotyl epidermal cells treated with cytochalasin B for 30 min (Fig. 4 b) were almost the same as those in untreated cells (Fig. 4 a).

There were some differences in the intensity of expression of the GFP-TUA6 protein among various kinds of cell (Table 1). Generally, its expression was strong in epidermal cells of various tissues and organs, such as leaves, hypocotyls, petioles, and calyx, and the expressed GFP-TUA6 proteins were incorporated into MTs. In contrast, fluorescent MTs were not seen in the epidermal cells of roots. Root hairs and pollen tubes also did not contain fluorescent MTs. Cells of the root meristem and pericycle in the central cylinder emitted green fluorescence. In both kinds of cells, very small granules fluoresced green and no fluorescent fibrils were visible.

At least six *TUA* and nine *TUB* (β-tubulin) genes have been identified in *Arabidopsis thaliana* (Kopczak et al. 1992, Snustad et al. 1992). Carpenter et al. (1992) reported that *TUA1* transcripts accumulate in stamens and mature pollens, and that *TUA1* transcript accumulation is accompanied by increased *TUA1* promoter activity. Ludwig et al. (1988) described accumulation of *TUA1* transcript in flowers but not in leaves and roots. In maize, different patterns of mRNA accumulation of *TUA1*, *TUA2*, and *TUA3* genes have been reported among different tissues (Joyce et al. 1992, Montoliu et al. 1991). All of the β-tubulin genes in maize were expressed in most tissues, although each gene showed a unique pattern of differential transcript accumulation (Rogers et al. 1993, Villemur et al. 1994). The results of these studies suggested that the different tubulin isotypes have different functions. The difference in expression intensity of *GFP-TUA6* among the tissues shown in Table 1 supports the idea that the *TUA6* gene is preferentially expressed in specific tissues such as the epidermis except the epidermis of the roots.

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