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F-actin in conifer roots

(7-nitrobenz-2-oxa-1,3-diazole–phallicidin fluorescent labeling/cytoplasmic streaming/microfilament bundle)

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ABSTRACT **The distribution of F-actin in the complex tissues of a higher plant organ has been visualized by fluorescence labeling the roots of the conifers *Chamaecyparis obtusa* and *Pseudotsuga menziesii* with F-actin-specific fluorescent dye-conjugated phallicidin. F-actin is present in the parenchymatous cells of the vascular tissue. Some vascular parenchyma cells possess larger numbers of F-actin-containing structures (microfilament bundles) than are known to exist in any other higher plant cell. Tissue type appears to be an important determinant of the presence or absence of F-actin in a cell. For example, in contrast to vascular cells, cortical cells show no indication of fluorescence labeling of F-actin after incubation with fluorescent phallicidin. Cytoplasmic streaming is seen only in vascular cells and in a pattern that reflects the intracellular distribution of F-actin.**

In higher plants, confirmation of the presence of actin has been confined to either macerated systems or studies using electron microscopy techniques (1–5). One intrinsic limitation of this work has been the difficulty of constructing from the results a model of the number and distribution of actin-containing structures in a cell. This difficulty arises because macerated systems leave few vestiges of intracellular organization intact while the serial-section electron microscopy technique needed to visualize an entire cell can be relatively time consuming.

Fluorescence-labeling techniques offer a way of quickly observing the distribution of cellular components in large numbers of intact cells. One such technique, application of 7-nitrobenz-2-oxa-1,3 diazole–phallacidin (NBD-Ph), has been used to observe F-actin-specific fluorescence labeling of microfilament bundles in mammalian and algal cells (6). This technique is advantageous when compared with heavy meromyosin/subfragment-1 labeling procedures in which the possibility of ambient G-actin polymerization exists during the extended glycerination treatment (2, 4). In the NBD-Ph technique, the specimen is first fixed with paraformaldehyde, thereby stabilizing the proteinaceous cell components during the subsequent fluorescence-labeling procedure. Another limitation of previous ultrastructural studies is that they have used easily accessible or exterior tissues such as epidermis of leaves (5), root hairs (4), pollen tubes (1), and endosperm cells (2). No data are available with regard to the presence and distribution of F-actin in internal tissues of major plant organs such as leaves, stems, and roots. These tissues contain the majority of cells found in a higher plant and produce, transport, and store the bulk of all plant metabolites. Descriptive data on the distribution of F-actin and its supramolecular assemblies in internal tissues of higher plants should elucidate the wide range of functions in which actin might have some role (7).

Some cells of conifer roots possess several attributes that make them favorable experimental objects for visualizing the distribution of F-actin in the internal tissues of higher plants by fluorescence labeling. Previous ultrastructural observations have shown that the highly elongate vascular parenchyma cells of these roots contain 40 or more linear microfilament bundles, in contrast to adjacent cortical cells that contain few if any microfilament bundles (8–10). The microfilament bundles are distinctively oriented in that they lie parallel to the longitudinal axis of the cell, except at oblique end walls where they appear to continue to follow wall contours (8).

The study of the function of microfilament bundles in higher plant cells is in a relatively early stage of development. Our current understanding is based on extrapolations from studies of Characean algal cells, which have shown that microfilament bundles are composed of F-actin and play an essential role in the generation of cytoplasmic streaming (11–14). This idea seems relevant to higher plants, based on studies showing that (*i*) microfilament bundles are present in streaming cells (5, 15); (*ii*) the direction of streaming is parallel to the longitudinal axis of the microfilament bundles; and (*iii*) streaming is inhibited by cytochalasin B (5, 16–18), a drug known to affect microfilament bundles (19), by the ionophore A23187 (20), and by the chelating agent EGTA (5), chemicals known to affect the distribution of ions important to the generation of streaming in F-actin-containing systems (12).

Here we test the hypothesis that microfilament bundles in higher plant cells are composed of F-actin and investigate the configuration and relevance of these F-actin structures.

In this report, we describe the entry of NBD-Ph into permeabilized plant tissues and the identification of microfilament bundles as the stained F-actin-containing organelles. F-actin staining occurs only in vascular tissues. The presence of F-actin in these cells and tissues correlates strongly with the presence of cytoplasmic streaming. These results broaden our understanding of the potential for metabolite transport in the root. We also report that F-actin in plant vascular parenchyma cells, as in Characean algal cells (21), does not bind DNase I, in contrast to mammalian (rat) F-actin (22).

MATERIALS AND METHODS

Seedlings of *Chamaecyparis obtusa* and *Pseudotsuga menziesii* that were 3–10 months old were washed in running water to remove soil from the roots. Vigorously growing white roots were selected. The distal 2-cm portions were excised in water and placed in 2–4% paraformaldehyde (methanol-free formaldehyde prepared from a paraformaldehyde powder; Electron Microscopy Services)/30 mM Pipes (Sigma), pH 6.8, for 10–60 min at room temperature. While bathed in a drop of the above solution, the basal 1.5-cm portion of each root was dissected into thin vascular and cortical tissue pieces using forceps and a dis-

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Abbreviation: NBD-Ph, 7-nitrobenz-2-oxa-1,3-diazole–phallacidin.

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secting microscope. The tissue pieces were transferred to a drop of 0.6 μM NBD-Ph (prepared as in ref. 23) in the same buffer for 10–60 min and then to water and viewed with a Nikon Optiphot microscope equipped for phase and fluorescence optics with a mercury lamp, epi-illumination, a blue (495-nm) excitation filter, and a 515-nm barrier filter. Images were recorded on Tri-X film (Kodak) using identical manually timed exposures and developed in fresh batches of Rodinal (Agfa) at 68°C. The following variations of the procedure gave similar results. A 3.7% formalin solution (Mallinckrodt) in Pipes (pH 6.3–6.8) or 0.1% Triton X-100 (Sigma) could be substituted for paraformaldehyde. Although 0.6 μm NBD-Ph provided optimal contrast, concentrations between 0.125 and 30 μM could be used. However, at the lower concentration, contrast was lost quickly, possibly due to incomplete labeling, while, at the higher concentrations, there was a relatively intense background emission.

To demonstrate specific labeling of F-actin, cells were exposed to a NBD-Ph/phalloidin competition mixture (24) containing 0.6 μM NBD-Ph, 20 μM phalloidin (Boehringer Mannheim Biochemicals), and 30 μM Pipes (pH 6.8). Tissue pieces incubated in this solution for 30–60 min at room temperature were stained for viewing in the same solution. Acetone and methanol (Mallinckrodt AR) were used at room temperature. Tissue pieces were transferred directly into and out of the 100% acetone or methanol solutions after being incubated for 20 min.

The DNase I-rhodamine was prepared by Nothnagel (21) as an indiscriminate stain for G-actin, F-actin, and oligomers in animal cells. Tissue pieces were treated with 3% paraformaldehyde/30 mM Pipes, pH 6.8; washed with buffer for 10 min, with buffered 0.1% Triton X-100 for 20 min, and with buffer for 20 min; incubated with DNase I-rhodamine for 30 min at 23°C; washed for 30 min with buffer; and viewed with the Nikon microscope using a 580-nm barrier filter and a green (546-nm) exciter filter.

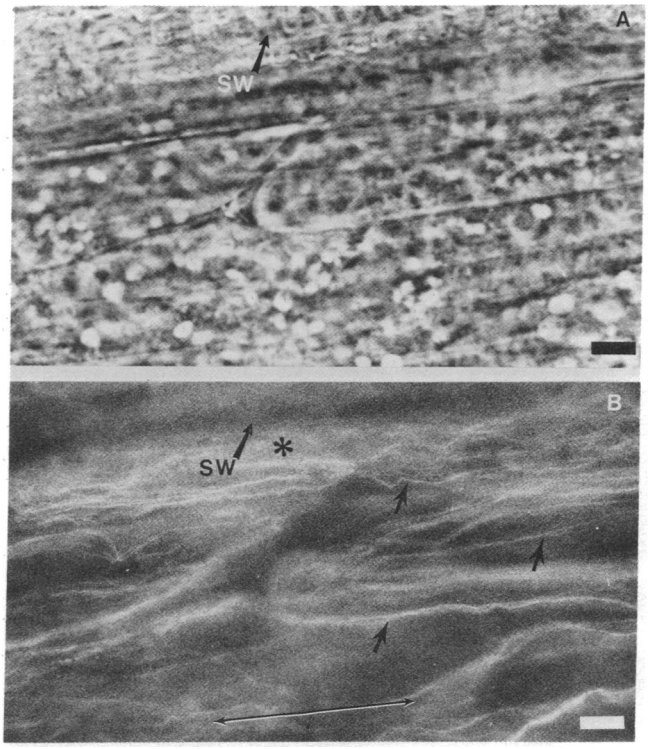

FIG. 1. Vascular tissue of *Pseudotsuga menziesii*. (*A*) Phase-contrast image. (*B*) NBD-Ph fluorescence labeling. Vascular parenchyma cells of this multilayered tissue piece contain numerous linear fluorescent microfilament bundles (→). The adjacent mature tracheary elements that lack cytoplasm show only the autofluorescent properties of their secondary walls (SW). (↔, Orientation of longitudinal axes of cells; *, fluorescent background due to labeled microfilament bundles that are out of focus; bars = 2.68 μm.)

RESULTS

Entry of the NBD-Ph Label into the Tissue. In the initial stages of our investigation, we attempted to observe fluorescence labeling of F-actin in living cells. Carefully dissected and relatively large tissue pieces were incubated in 0.6–30 μM NBD-Ph for 30–60 min. In all cases, these attempts failed. Only autofluorescent components, to be discussed elsewhere, were seen. The viability of vascular parenchyma cells was assured by their active and continuous rotational cytoplasmic streaming. Cortical cells were never observed to stream either in the intact root or when dissected. After further dissection of vascular parenchyma tissue pieces, only cells that had obviously been broken open exhibited fluorescence labeling. To overcome these limitations, tissues used in subsequent experiments were first treated with formaldehyde or detergent to facilitate entrance of NBD-Ph into the cells. Streaming in vascular parenchyma cells was always terminated by such treatment. Figs. 1 and 2 show multilayered tissue pieces after fluorescence labeling of F-actin with NBD-Ph. No such fluorescent structures are seen in control material (Fig. 3*A*).

Autofluorescence adds to the background but is not an operational hindrance because most of it is localized in cell types other than vascular parenchyma. The paraformaldehyde-treated control tissue piece in Fig. 3*A* shows the very low levels of autofluorescence present in the vascular parenchyma cells (Fig. 3*B*).

Location and Identification of the Fluorescent Structures. The abundance and orientation of NBD-Ph labeled F-actin in the vascular parenchyma cells can be seen in Figs. 1 and 2. The generally linear fluorescent structures usually lie parallel to the longitudinal axes of the cells, except at oblique end walls where they seem to follow wall contours. This fluorescence labeling pattern was present in the majority of cells of this type. In cells that had obviously been disrupted by dissection, fluorescent structures were present as tangled masses. Occasionally, when cellular contents had spilled onto the glass slide, arrays of linear and sinuous structures showing intermittent fluorescence were seen. The number of linear fluorescent structures found in each cell in well-preserved tissue pieces was in the 5–30 range. This range is probably an underestimate because the most intensely stained fluorescent structures may have consisted of several individual structures lying close together. In general, the number, orientation, and morphology of the fluorescent structures resemble quite closely structures identified as microfilament bundles in the same cell type with the electron microscope (8, 9).

Cortical cells can be easily distinguished from vascular parenchyma cells by their relatively small dimensions. Fig. 4 shows cortical cells after NBD-Ph labeling. No linear fluorescent structures similar to those seen in vascular parenchyma cells are ever discernable. Some of the cell walls, vacuolar contents, and nuclei display fluorescent components due to autofluorescence, whether or not stained with NBD-Ph. Ultrastructural observations have shown (8, 9) that cortical cells possess few if any microfilament bundles so no NBD-Ph staining would have been expected in cortical cells, as observed. The absence of significant NBD-Ph staining in cortical cells also indicates the scarcity of F-actin in any form in these cells.

Optical sectioning of multilayered tissue pieces stained with NBD-Ph shows that the background emission in Figs. 1 and 2

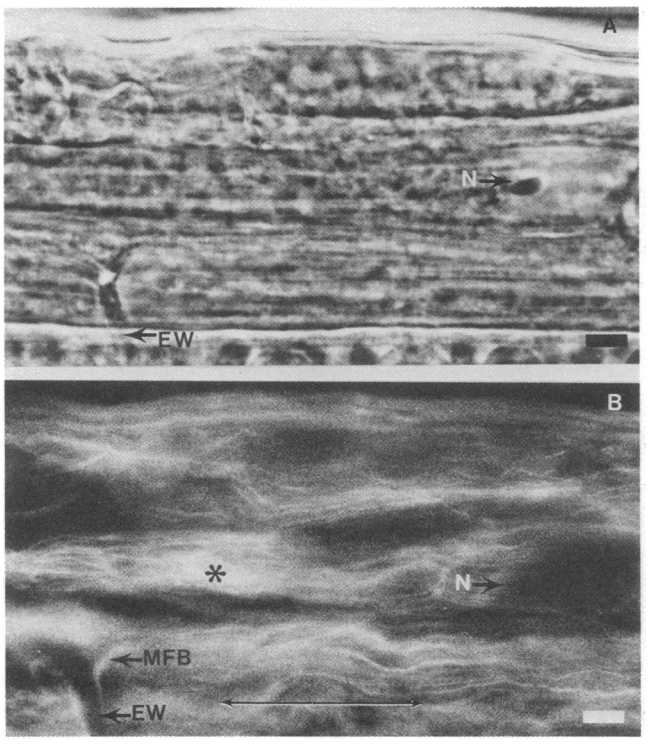

FIG. 2. Vascular tissue of *Chamaecyparis obtusa*. (*A*) Phase-contrast image. (*B*) NBD-Ph fluorescence labeling. Note the lack of fluorescence in the nucleus (N) of a vascular parenchyma cell in this multilayered tissue piece. Also note the turning of an intensely fluorescent microfilament bundle(s)? (MFB) as it follows the contour of the end wall (EW). (↔, Orientation of longitudinal axes of cells; *, fluorescent background due to labeled microfilament bundles that are out of focus; bars = 2.68 μm.)

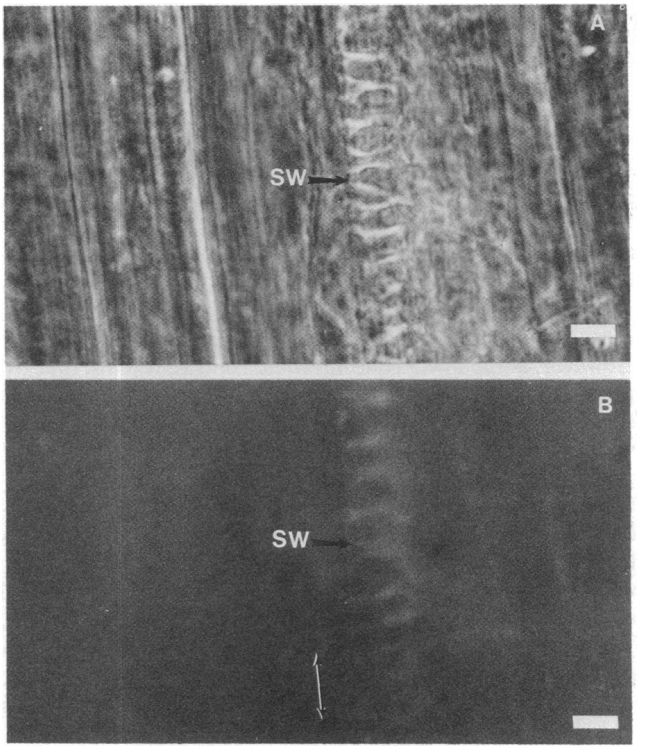

FIG. 3. Vascular parenchyma of *Chamaecyparis obtusa*. (*A*) Phase-contrast image. (*B*) Fluorescence image after incubation with paraformaldehyde. Only the autofluorescence of the secondary wall material (SW) in a tracheid of this multilayered tissue piece is visible. The vascular parenchyma cells on either side of the tracheid show little if any autofluorescence. (↔, Orientation of longitudinal axes of cells; bars = 2.68 μm.)

is generally attributable to the large amount of unresolved F-actin-specific fluorescence labeling of linear structures in under- or overlying cells. Thus, discrete fluorescent structures could be observed clearly only in tissue pieces <5 cells thick (50 μm) and were best delineated in monolayer tissue pieces as shown in Fig. 5. Monolayers of cells show the fluorescence-labeled microfilament bundles as highly defined linear and sinuous structures against a black background. In these preparations, we observed segregation of F-actin-containing structures to the parietal areas of the cytoplasm and occasionally in two loosely organized groups (Fig. 5). Their orientation is parallel to the rotational pattern of streaming seen in such cells.

The results of the NBD-Ph/phalloidin competition demonstrated the specificity of NBD-Ph labeling of F-actin in the linear structures. After such competition experiments, the only fluorescence seen in vascular tissues was the autofluorescence of secondary walls. The excess of nonfluorescent phalloidin, which specifically binds to F-actin, apparently prevents NBD-Ph labeling of structures such as those seen in Figs. 1, 2, and 5.

For comparison with organelles known to contain F-actin, NBD-Ph-labeled structures in the vascular parenchyma cells and NBD-Ph-labeled microfilament bundles in normal rat kidney cells were tested for their reaction to organic solvents. As expected, acetone, whether applied before or after NBD-Ph, had no effect on the normal fluorescence labeling pattern of either cell type (6). On the other hand, similar application of methanol completely prevented any fluorescence labeling in both cell types. Neither of the organic solvents affected autofluorescent cell components. The results indicate yet another similarity between known F-actin microfilament bundles and NBD-Ph-labeled structures in the vascular parenchyma.

In contrast to the results obtained with NBD-Ph, fluorescence labeling of F-actin with DNase I-rhodamine was unsuccessful in vascular parenchyma cells. Only autofluorescent components could be visualized. Several techniques were used in an effort to promote labeling including fine dicing of the tissue to directly open these relatively long cells to the bathing medium. Even in cases in which masses of organelles were seen spilled out into the medium, no labeling was detectable. The same batch of DNase I-rhodamine did, however, label microfilament bundles in normal rat kidney cells.

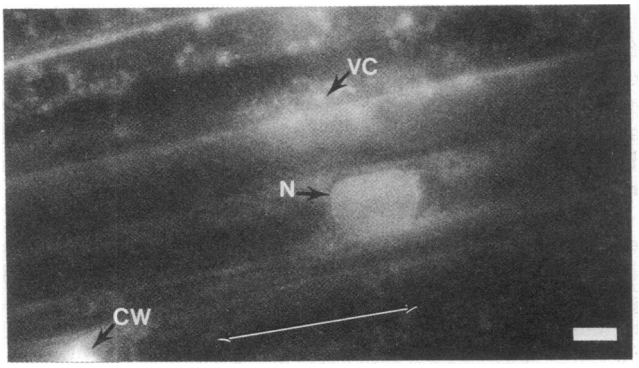

FIG. 4. Cortical tissue of *Chamaecyparis obtusa* after incubation with NBD-Ph. These cells contain autofluorescent nuclei (N), vacuolar contents (VC), and cell walls (CW). However, no microfilament bundles are seen. (↔, Orientation of longitudinal axes of cells; bar = 2.17 μm).

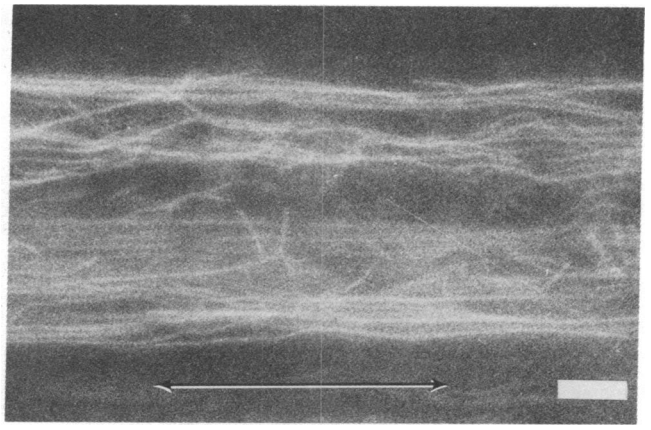

FIG. 5. Vascular parenchyma cells of *Chamaecyparis obtusa* from monolayer tissue pieces after incubation with NBD-Ph. Note the low background and the resulting clearly defined images of the individual microfilament bundles compared with those in the thicker tissue pieces of Figs. 1 and 2. Approximately 20 microfilament bundles are present. (↔, Orientation of longitudinal axes of cells; bar = 2.17 μm.)

DISCUSSION

NBD-Ph fluorescence labeling promises to be of great utility for investigations of F-actin in higher plants. After brief treatment of the material with formaldehyde or detergent, NBD-Ph quickly permeates multilayered plant tissues. There is no necessity for extended application and washing sequences such as are used in antibody-labeling techniques. If mono- or bilayer tissue pieces are used, background emission is quite low, even when tissues are viewed in the original labeling solution. Because of these factors, the use of NBD-Ph greatly expedited our efforts to achieve an overall understanding of the distribution of F-actin in a complex tissue.

Several lines of evidence lead to the conclusion that the fluorescence-labeled F-actin-containing structures in vascular parenchyma cells are the microfilament bundles seen in the same cell type with the electron microscope. They are similar in terms of their intracellular number, orientation, and morphological characteristics (8–10). The fluorescent structures are absent in cell types known not to possess microfilament bundles. These structures, like the microfilament bundles of Characean algae, apparently do not bind DNase I. Finally, these structures resemble microfilament bundles of normal rat kidney cells in their ability to label with NBD-Ph, their general linear morphology, and their similar reactions to acetone and methanol.

In a survey of higher plant cell types containing microfilament bundles (10), the number of bundles per cell was in the 1–5 range. That value is quite small in comparison with the 5–30 range found in our study and the value of 40 or more found in an ultrastructural study of the same cell type (8, 9). The largest number of microfilament bundles known for any other cell type in higher plants appears to be *ca.* 12 in the epidermal cells of *Vallisneria gigantea* (5). Thus, either vascular parenchyma cells of conifers contain more F-actin bundles than other higher plant cells or F-actin is not well preserved in other higher plant cells during processing for electron microscopy. If the roots of conifers do actually possess large numbers of cells that are rich in F-actin, they would be excellent materials from which to isolate higher plant actin for biochemical studies.

The apparently exclusive occurrence of F-actin microfilament bundles in the nucleate vascular cells of the root suggests a possible physiological role. One function attributed to microfilament bundles is a role in the generation of cytoplasmic streaming (refs. 13 and 14; cf. refs. 25 and 26). This hypothesis agrees with our results that indicate that streaming is present in the cell type that possesses microfilament bundles and is absent from those cells that do not have microfilament bundles. Substances move in tissues composed of streaming parenchyma cells at rates faster than can be explained on the basis of diffusion (18, 27). Unlike angiosperm roots, the vascular tissue of the distal-most 3 to 6 mm of conifer roots contains no sieve elements (refs. 28 and 29; unpublished observations) with which to supply the nonphotosynthetic root meristem with organic nutrients. It is conceivable that the streaming phenomenon aids in the supply of these substances to the apex. Thus, the function and distribution of F-actin within this organ could be important.

The observed lack of DNase I-rhodamine fluorescence labeling of microfilament bundles in both conifer root cells and algae suggests that plant actins may have certain biochemical and functional attributes that are not shared with animal actins (22). Given the enormous range of organisms known to possess actin (30), it is possible that this dissimilarity between plants and animals could be used as a tool to elucidate evolutionary relationships.

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