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Rhodamine 123 Labelling of Mitochondria in Sieve Elements I. Localization with Confocal Microscopy in Plant Tissue Culture Phloem

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ABSTRACT

Phloem sieve elements are the specialized cells of higher plants responsible for conducting photoassimilates from regions of production (sources) to regions of utilization or storage (sinks). The translocation of photoassimilates is believed to be the resutl of the ATP-dependent membrane transport of sugars across the plasma membrane of the sieve element or associated companion cell and the subsequent generation of a hydrostatic pressure due to osmosis. During the development of the sieve element many cellular organelles are lost from the cell, including the nucleus and the vacuole. Mitochondria, however, are retained in the mature sieve element. Here we show that many of the particles visible in sieve elements from a plant tissue culture are strongly labelled by Rhodamine 123, a probe that is specifically accumulated by mitochondria in plant and animal cells. These observations suggest that sieve element mitochondria are metabolically active and that they may contribute to the generation of the ATP required for phloem loading.

KEY WORDS:
Phloem, sieve element, mitochondria, rhodamine 123, plastids

INTRODUCTION

The development of a mature phloem sieve element is characterized by several unusual events, including the loss of the nucleus, ribosomes, Golgi bodies and the vacuole. Changes in the cell walls between sieve elements result in the conversion of plasmodesmata into larger, membrane-lined sieve area pores and the joined sieve tubes form a series of conducting elements, the sieve tube (5). The cytoplasmic changes associated with sieve element differentiation can be understood from the standpoint of converting a precursor cell into a segment of the transporting tube. The cell becomes essentially free of organelles in its center to facilitate assimilate flow, and develops large openings at its ends to allow unimpeded element-to-element movement of water and assimilates. The organelles and membrane systems that do persist at maturity, including the sieve element reticulum (SER) and mitochondria, are only found at the periphery of the cell, next to the plasma membrane. Persistent plastids are also found in the mature sieve element, although it is not clear how they are kept from being swept "downstream" in the flow of assimilates since they are not always restricted to the periphery of the cell.

Because phloem cells are located internally in plants, it is difficult to observe sieve elements in the light microscope without dissecting or sectioning the plant organs that contain them. In most cases, cutting the plant to allow phloem observation also induces phloem damage due to the release of the hydrostatic pressure in the sieve tube. The injury to the sieve elements disrupts the normal structure of the cell and causes a release of small particles into the lumen of the sieve tube (4). In the light microscope the small particles in the sieve elements have been observed to display a rapid Brownian movement. The size, number and movement of these particles is highly specific for sieve elements and is often used as a means of identifying the phloem cells in a section of living plant material (1,6,7). Most authors have identified these small particles as starch grains that are released from sieve element plastids

when the cell is injured (1,4,11). At least two reports (6,7), however, have noted that some of the particles which serve as markers of sieve elements in the light microscope are able to accumulate Janus green B. a vital dye used to stain mitochondria and not plastids.

We report here observations made with a confocal laser scanning microscope of living sieve elements from a plant tissue culture. A previous study of conifer sieve elements (8) visualized with confocal microscopy was based on the fluorescence of a dye that is accumulated by the endoplasmic reticulum, DiOC (3,3' -dioxacarbocyanine iodide). As a means of determining the nature of the marker particles associated with Brownian movement in sieve elements, we have used a fluorescent dye, Rhodamine 123 (Rh123), that is only accumulated by functioning mitochondria (3) and here we compare the fluorescent images of cells labelled with Rh123 to images of the same cells obtained by phase contrast confocal microscopy.

METHODS AND MATERIALS

Callus tissue of ***Streptanthus tortuosus*** (Brassicaceae) was grown as previously described (11) except that the cultures were maintained in the dark. ***Elodea canadensis*** plants were grown in a pool in the greenhouse and were harvested just before use. Leaves of ***Elodea*** and small (0.5 mm) pieces of ***S. tortuosus*** callus tissue were incubated in an aqueous solution (10 µg/ml) of Rh123. A stock solution of RH123 was made using 95% ethanol (1 mg/ml) and an aliquot of the stock solution was diluted to 10 µg/ml with water or culture medium before use. The leaves and callus tissue were incubated in RH123 for 20 minutes at room temperature and were washed 2 times with water or culture medium before examination. The leaves were mounted directly on a slide with a cover glass while the callus tissue was first teased apart on a slide with a needle and then slightly squashed by applying pressure on a cover glass placed over the tissue. Observations were performed using a Bio-Rad MRC 600 laser scanning confocal microscope. Rhodamine was excited usign the 488 line of a Krypton-Argon laser with a 488DF10 excitation filter, a 510 dichroic mirror, and a 515LP emission filter. The images were recorded on a Sony 5000 thermal printer.

RESULTS

Living leaves of ***Elodea*** contain abundant chloroplasts that are easily visualized in phase contrast with the confocal microscope. Figure 1 shows the chloroplasts in leaf cells that have been incubated in RH123. The cell walls and chloroplasts are easily visualized, but mitochondria are not seen here. When the same cell is visualized using 488 nm excitation, many particles with a diameter much less that of the plastids are seen to fluoresce. The plastids observed with phase contrast are not visualized in the fluorescent image. When a sieve element of ***S. tortuosus*** callus is observed by phase contrast microscopy (Fig. 3), many small particles are observed. The same particles are also seen when the illumination is changed (Fig. 4) to the 488 nm laser line that excites fluorescence of Rh123. Although some fluorescing particles appear to be larger than others (Fig. 4), the larger images appear to be due to the fluorescence of separate particles that are in contact (Fig. 3).

A small sieve tube is shown in figure 5 as a phase contrast image and again as a fluorescent image in figure 6. The two images are also seen as a single, overlapped image in figure 7. A sieve plate (SP) is seen between two of the sieve tube elements in figure 5. As seen in phase contrast, the small particles that are visible in the sieve element vary in size and in density. Some are dark and some appear white in phase contrast. When the same cell is visualized as the fluorescence excited by the 488 nm laser illumination (Fig. 5), many of the particles seen in phase contrast are observed to fluoresce. When the phase contrast image and the fluorescence images are projected as a single image in the computer (Fig. 7), some particles show fluorescence (green color) while others are imaged only as the phase contrast (red color) image. Comparing the images of figures 5 and 7 demonstrates that only some of the particles visible in the sieve element are excited to fluoresce by the 488 nm laser beam.

DISCUSSION

Rhodamine 123 is a lipophilic cation that is selectively and specifically accumulated by mitochondria in plant and animal cells, When used for the time periods and the concentrations we employed, the dye has been shown to be non-toxic to ***Elodea*** cells and to plant cells grown in culture (12). The accumulation of Rh123 is dependent on the proton gradient generated across mitochondrial membranes and, as such, the accumulation of Rh123 within a cell can be interpreted as an indicator of mitochodrial membrane potential and mitochondrial function. Ionophores that cause the collapse of the

mitochondrial membrane potential, such as dinitrophenol (DNP), block the uptake of Rh123 (3). Under the condition used for our study *Elodea* leaves are seen to accumulate Rh123 in particles that appear to be mitochondria. Chloroplasts of *Elodea* were not labelled by the Rh123 fluorescence. Although we did not test for the effects of DNP or other ionophores in our system, the images that we obtained are consistent with those reported in many other studies of Rh123 and we feel confident that the uptake of Rh123 seen in our study is restricted to mitochondria. The fluorescence images of particles seen here can be used, therefore, to determine if a given particle is a mitochondrion rather than a plastid.

When sieve elements are injured many small particles are observed in the lumen of the cells and the particles exhibit a rapid movement that resembles Brownian motion (6). Currier et al (4) demonstrated that the particles that exhibited Brownian motion were starch grains that were released from broken plastids following phloem damage. The breakdown of plastids and the release of starch grains that show rapid, random motion has also been reported by Barclay et al (1) and Barclay and Johnson (2). In an earlier paper in this journal one of us (11) reported that the presence of rapidly moving starch grains in a cell viewed in the light microscope was routinely used as a marker for identifying sieve elements in *S. tortuosus* tissue cultures. We, and many other investigators, have routinely assumed that the rapidly moving particles are plastids or starch grains released from them after injury.

At least two other reports, however, have also shown that some of the particles that serve as markers of sieve elements are able to take up the vital dye Janus green B. a dye that is taken up by mitochondria (6,7). These studies suggest that some of the marker particles present in sieve elements are mitochondria rather than plastids or starch grains. Mitochondria are known to persist in mature sieve elements (5) and are normally enclosed in membranes of the SER attached to the sieve element plasma membrane (9). The results of our confocal studies show that sieve elements of *S. tortuosus* callus tissue cultures contain numerous particles that can be identified as mitochondria. The confocal microscope and its associated computer is also able to form an image that shows both the phase contrast view of the cell and the image based on Rh123 fluorescence of mitochondria in the same cell. Based on these composite images we are able to conclude that the majority of the rapidly moving particles observed in these sieve elements are, in fact, mitochondria and not plastids as we have previously assumed. Starch grains and plastids are present in the sieve elements of our cultures, but they are less numerous than are the Rh123-labelled mitochondria.

Our studies also show that the mitochondria in sieve elements of *S. tortuosus* callus tissue accumulate Rh123 to a higher level than do those of the surrounding callus parenchyma cells. Since the uptake of Rh123 is dependent on the plasma membrane potential as well as the potential of the mitochondrial membranes (3), this result can be interpreted to mean that either the sieve element plasma membrane has a higher potential gradient than that of neighboring cells, or that sieve element mitochondria have a stronger membrane potential than mitochondria of adjacent parenchyma cells. Alternatively, both membrane potentials could be greater in sieve elements. Since the sieve element is responsible for the uptake and movement of high levels of assimilates, and since the uptake of the assimilates is thought to be based on the generation of a proton gradient across the plasma membrane (10), a higher level of Rh123 accumulation by sieve elements could reflect a greater level of metabolic activity by these cells. The ATP required to generate the sieve element proton gradient may be produced by sieve element mitochondria. Sjölund et al (10) showed that mitochondria in *S. tortuosus* sieve elements are enclosed in SER membranes that could function to channel the ATP generated by them to the plasma membrane and keep it from being swept down the assimilate stream.

Since our observations are based only on sieve elements from a tissue culture, it may not be possible to extrapolate our findings to the phloem of intact plants. A study of sieve elements in plant organs using Rh123 is in progress.

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RESUMEN

Los elementos cribosos del floema son células especializadas de las plantas superiores, responsables de la conducción de los fotoasimilados desde las regiones de producción (fuentes) hasta las regiones de utilización o almacenamiento (drenajes). La translocación de los fotoasimilados se cree resultante del transporte, ATP-dependiente, de azucares através de la membrana plasmática del elemento criboso o la célula compañera asociada y la subsecuente generación de una presión hidrostática debida a ósmosis. Durante el desarrollo de el elemento criboso se pierden la mayoría de organelas celulares, incluyendo el núcleo y la vacuola. Sin embargo, las mitocondrias son retenidas en el elemento criboso maduro. En este trabajo demostramos que muchas de las partículas visibles en elementos cribosos provenientes de cultivos de tejidos vegetales, son marcadas fuertemente por Rodamina 123, un compuesto que se acumula específicamente en las mitocondrias de células vegetales y animales. Estas observaciones sugieren que las mitocondrias del elemento criboso están metabolicamente activas y ellas pudieran contribuir a la generación de ATP requerido para el llenado del floema.

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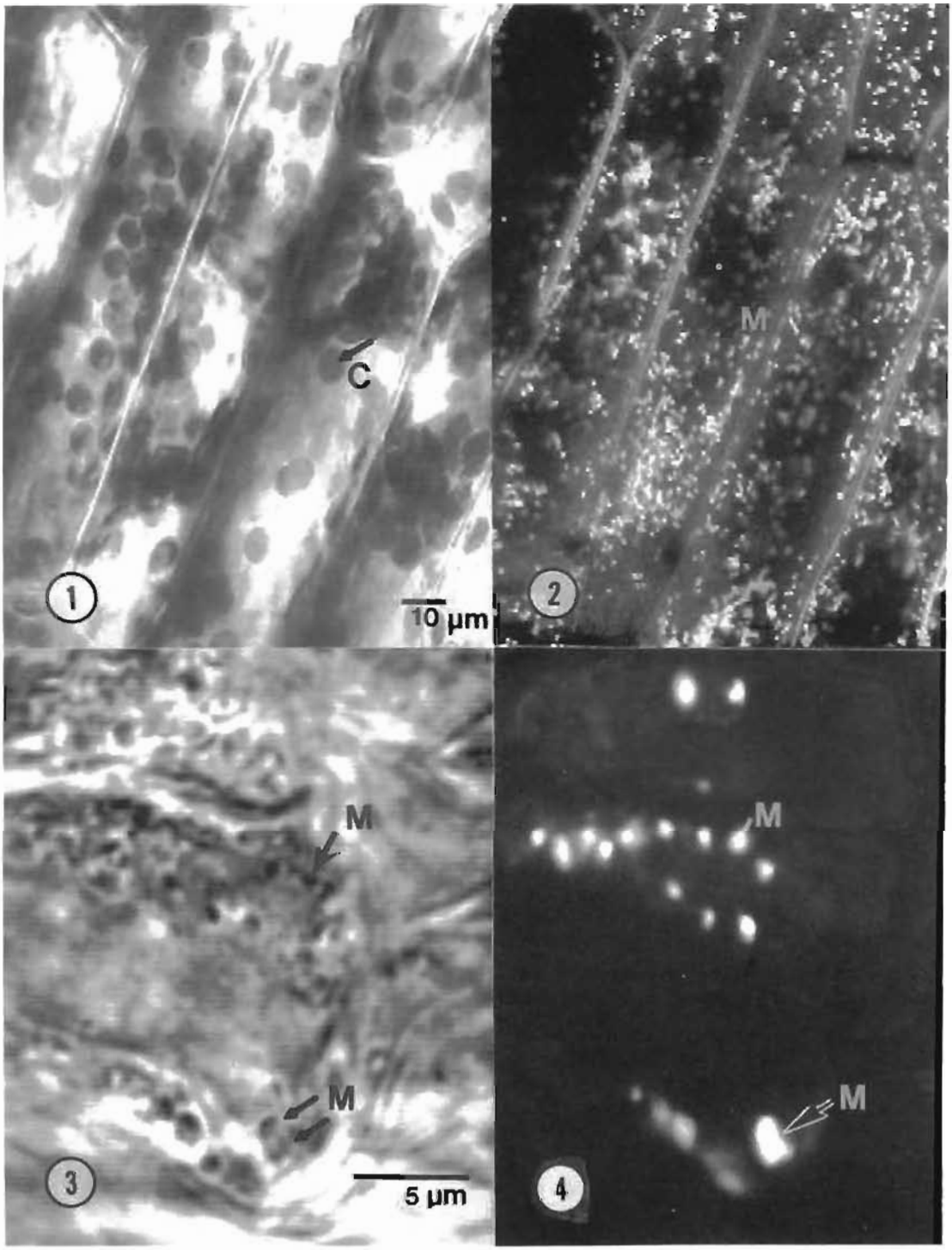

Fig. 1 Phase contrast image of *Elodea* leaf cells. Many large chloroplasts (C) are visible but no mitochondria are seen.

Fig. 2 The same *Elodea* cells shown in Fig. 1, but now imaged as the fluorescence resulting from Rh123 excitation at 488 nm. The chloroplasts are not visible, but many mitochondria (M) are seen to fluoresce.

Fig. 3 A sieve element from a tissue culture of *S. tortuosus* showing the marker particles that exhibit Brownian movement as imaged with phase contrast microscopy. Particles identified as mitochondria (M) are visible.

Fig. 4 The same sieve element shown if Fig. 3, but now imaged as the fluorescence of Rh123. The mitochondria (M) fluoresce strongly. The large area of fluorescence (large arrow) is formed by the overlap of fluorescence from the two mitochondria labelled with a double arrow in Fig. 3.

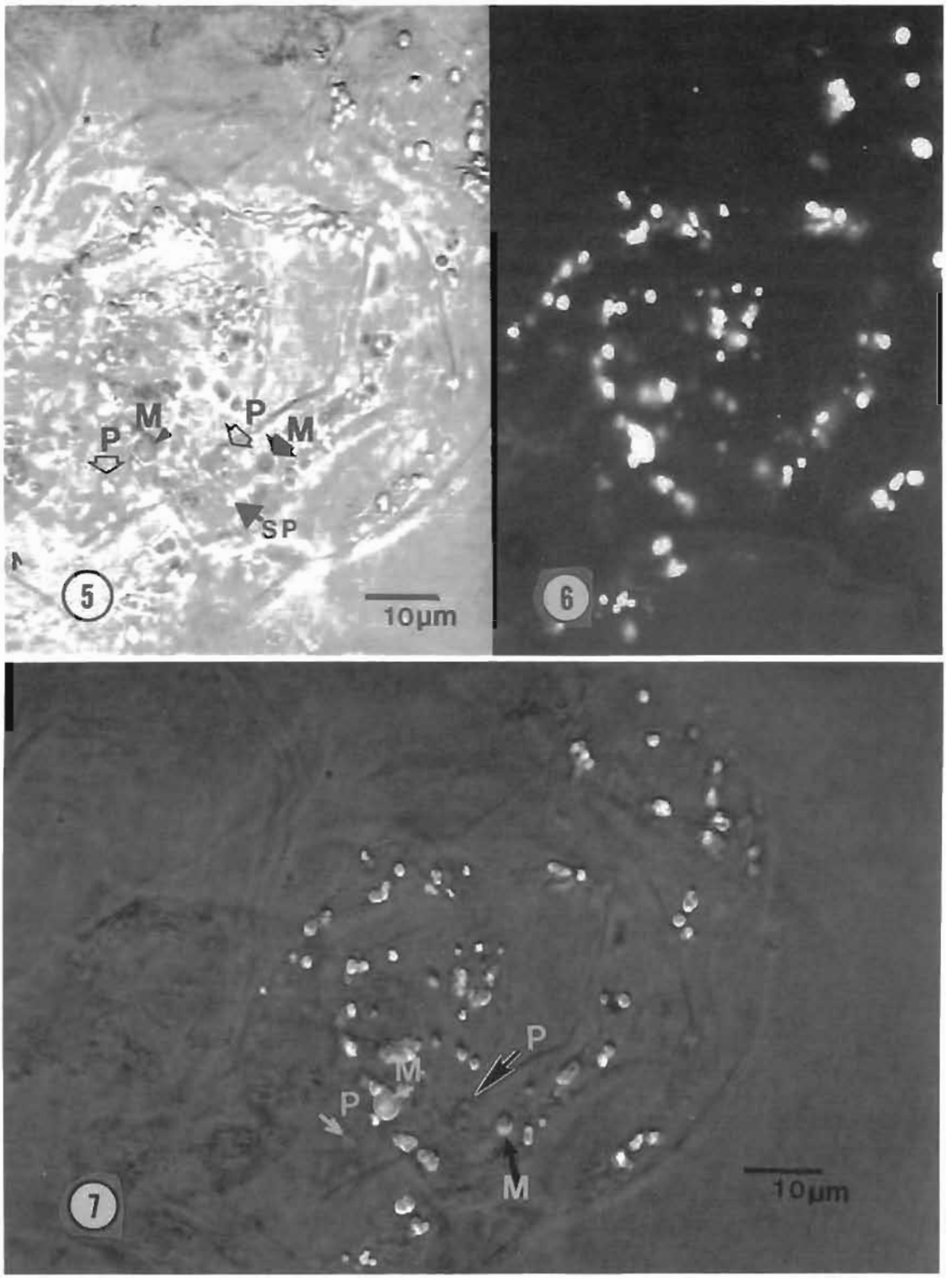

Fig. 5 A lower magnification view of several sieve tube elements and the surrounding parenchyma cells. Note that in this phase contrast image the sieve elements can be identified on the basis of the many small particles present in the cells. A sieve plate can be seen (SP) between two cells. Particles that can be identified as either starch grains from plastids (P) or mitochondria (M) are labelled.

Fig. 6 The same cells as seen in Fig. 5, but now imaged as the fluorescence of Rh123.

Fig. 7 A computer-generated composite of the phase contrast image from Fig. 5 and the Rh123 fluorescence image of Fig. 6. Some of the particles (M) show fluorescence (green color) while others (P) are seen only as a phase contrast image (red color).