Localization of Phytochrome in Oats by Electron Microscopy[1]

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ABSTRACT

Phytochrome was localized by immunoelectron microscopy in cells of the coleoptile tip of etiolated and irradiated oat (*Avena sativa* L., cv. Konata) seedlings. By using ultrathin frozen sections and immunopurified, monospecific antibodies, both the sensitivity and resolution of the immunocytochemical assay were increased. The results with etiolated plants agree with and extend previously published data. A brief red light illumination caused the redistribution of phytochrome from a diffuse to a more particulate appearance. Areas that accumulated phytochrome were identified as small vacuoles into which phytochrome was sequestered following illumination. In seedlings illuminated for several hours and in normal light-grown plants, the cellular distribution of phytochrome is qualitatively similar to that of nonirradiated, dark-grown material, except that in green plants the nucleus shows a positive immunocytochemical reaction.

The distribution of phytochrome in nonirradiated etiolated oat seedlings has been described on both the tissue and the cell level (1, 6). These investigations showed that the photoreceptor was especially abundant in the coleoptile tip. It appeared to be uniformly distributed throughout the protoplast, but was not observed in nuclei. However, within five min of photoconversion to the P_{fr} form, phytochrome was observed to be associated with discrete areas, the nature of which could not be identified (5). In these experiments, the coleoptiles were embedded in hydrophobic media for sectioning and whole antisera were used for immunocytochemical staining. This protocol put severe limits on both the resolution and the structural preservation that could be obtained.

Recently Epel *et al.* (2) introduced cryosectioning and the use of purified, specific antibodies to the problem of phytochrome localization. They improved considerably the resolution of the procedure at the light microscope level. Their results confirmed the earlier observations showing the aggregation of phytochrome following a brief irradiation. In the present paper, we extend the use of this cryosectioning technique to the electron microscope level in order to identify the discrete regions in which phytochrome aggregates.

MATERIALS AND METHODS

Oat (*Avena sativa* L., cv. Konata) grain was either germinated and grown in moist vermiculite in darkness at 25°C or outside in natural day-night conditions. After 3 d, etiolated seedlings were irradiated with red (two Sylvania 15-w red fluorescent lamps at 30 cm distance), far red (low voltage incandescent lamp, the output of which was filtered through a Balzers 730-nm interference filter), or white (white fluorescent lamps) light.

Antibodies. Rabbit antiphytochrome immunoglobulins were immunopurified from whole serum as described elsewhere (3). Rhodamine- and ferritin-conjugated goat antirabbit IgG antibodies were obtained commercially (Miles Laboratories, Inc., Elkhart, IN). For light microscopy, immunoglobulins were diluted to 36 μg/ml and for electron microscopy to 100 μg/ml.

Light Microscopy. Fixation, freeze sectioning, and staining for light microscopy were performed following the procedure as described by Epel *et al.* (2). In brief, the subapical part of the coleoptile (0.5–1 mm beneath the tip) was fixed in 3% (v/v) formaldehyde and 0.5% (v/v) glutaraldehyde for 4 to 5 h. After rinsing, the tissue was reduced with sodium borohydride to eliminate background fluorescence, infused with 1.3 M sucrose for 6 to 9 h, and mounted on a copper block and frozen in liquid N_2. Sections of 1 μm thickness were made with a Sorval MT2B microtome with cryokit at −55 to −60°C. The sections were transferred with a drop of 2.3 M sucrose to a siliconized glass slide (Dessicote-Beckmann). On the slide, the sections were treated first with 2% (w/v) gelatin in PBS[3], then with 0.02 M glycine in PBS and finally rinsed. They were incubated with rabbit antiphytochrome immunoglobulins for 15 min, rinsed several times, incubated with rhodamine-conjugated goat antirabbit IgG antibody, rinsed, and mounted in 90% (v/v) glycerol.

All steps in the staining protocol were performed in 0.1 M Na-phosphate buffer (pH 7.4) except where mentioned. The immunoglobulins were diluted in the same buffer. For light microscopy, a Zeiss Photo-microscope III with Nomarsky optics was used with an epifluorescence attachment equipped for rhodamine fluorescence. Pictures were made using Kodak Ektachrome 400 slide film.

Electron Microscopy. For electron microscopy, the procedure described by Tokuyasu (7) was followed. Fixation was as described for light microscopy and infiltration of the tissue was with 0.8 M sucrose. Ultrathin sections (0.1 μm) were made at −85 to −90°C, picked up with a drop of 2 M sucrose in 0.75% (w/v) gelatin, and transferred to Formvar and carbon-coated grids which were ionized before use. The grid was left for 30 min on the wet surface of a gelatin-agarose block. Afterwards it was floated off with PBS, treated with PBS containing glycine and gelatin, and then washed. The washed grids were floated sequentially on antiphytochrome immunoglobulins, PBS, ferritin-conjugated goat antirabbit IgG antibody, PBS, 2% (v/v) glutaraldehyde, and PBS. Sections were stained with neutral uranyl acetate, washed with water, and stained again for 10 min on 1% (w/v) methylcellulose with 0.01% (w/v) acidic uranyl acetate. After removal of excess embedding medium, the grids were dried on a wire loop.

The sections were viewed with a Philips EM 300 electron

[1] This paper is dedicated to the memory of Dr. Sterling B. Hendricks, one of the truly great scientists of our time.

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[3] Abbreviations: PBS, sodium phosphate-buffered physiological saline solution.

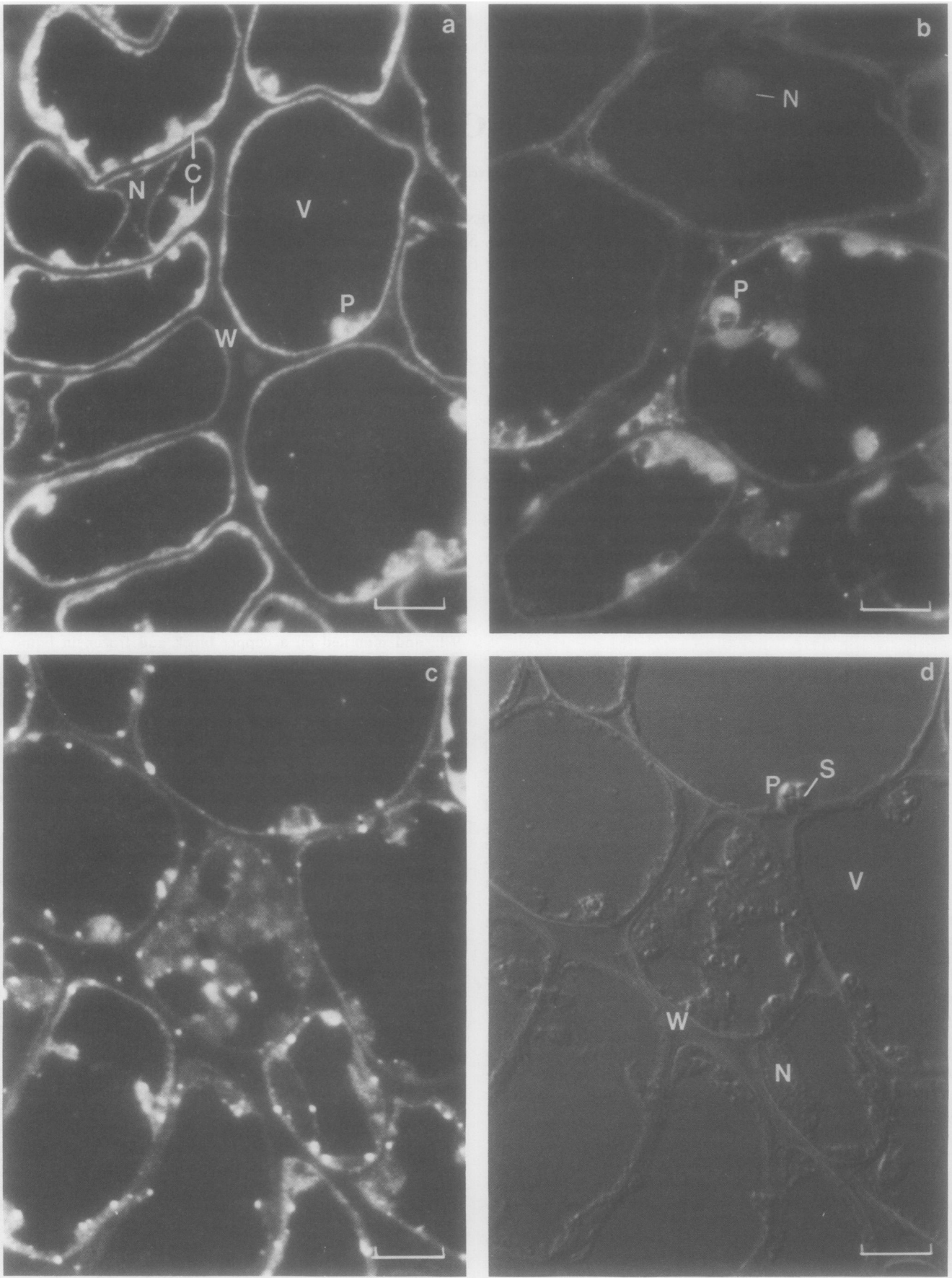

FIG. 1. Light micrographs of 1-μm thick cryosections of oat coleoptile tips. Immunofluorescence labeling with rhodamine. Bar is 10 μm. Abbreviations used are: C, cytoplasm; N, nucleus; P, plastid; V, central vacuole; S, starch; W, cell wall. a, Fluorescence image of a section of an etiolated plant showing a diffuse cytoplasmic distribution of antigen. b, Fluorescence image of a section of a white-light-grown plant showing a faint but significant fluorescence throughout cytoplasm. c, Fluorescence image of a section of an illuminated plant (etiolated + 5 min red light); a compartmentalization of antigen (phytochrome) in discrete parts of protoplast is seen on a low background cytoplasmic fluorescence. d, Interference contrast image of same field represented in c. Major cell organelles can be identified.

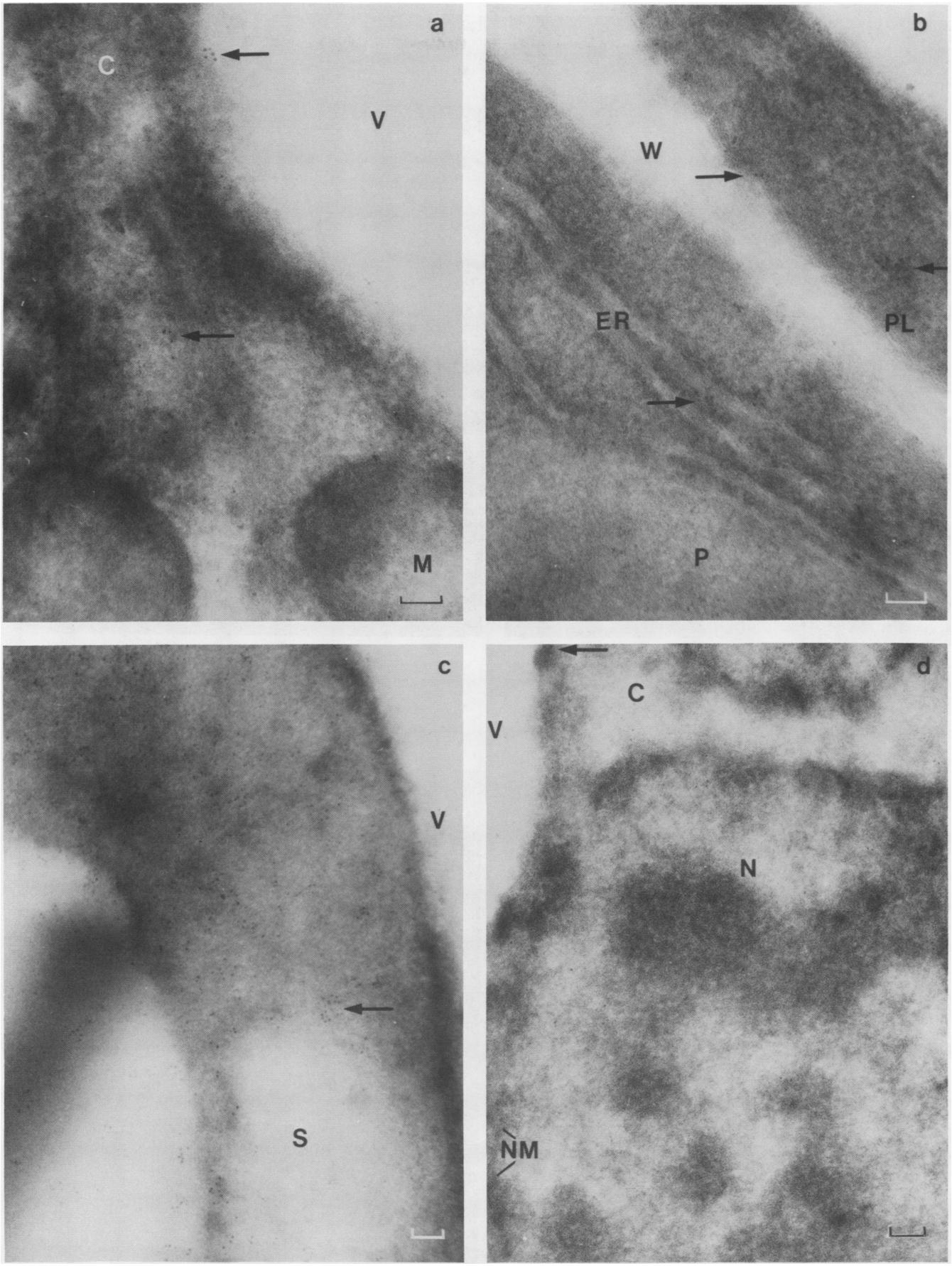

FIG. 2. Electron micrographs of ultrathin cryosections of etiolated oat coleoptile cells. Bar is 0.1 μm. Arrows indicate areas with typical ferritin labeling. Abbreviations as in Figure 1 except as follows: ER, endoplasmic reticulum; NM, nuclear membrane; M, mitochondrion; PL, plasmalemma. a, Part of cytoplasm with two mitochondria; labeling is especially evident in cytoplasm and at site of tonoplast. b, Parts of two neighboring cells with their common wall; labeling is found in cytoplasm and at site of plasmalemma and of ER. c, Part of a plastid, plastid stroma showing a high label density. d, Part of a nucleus: ferritin is virtually absent from this part of section.

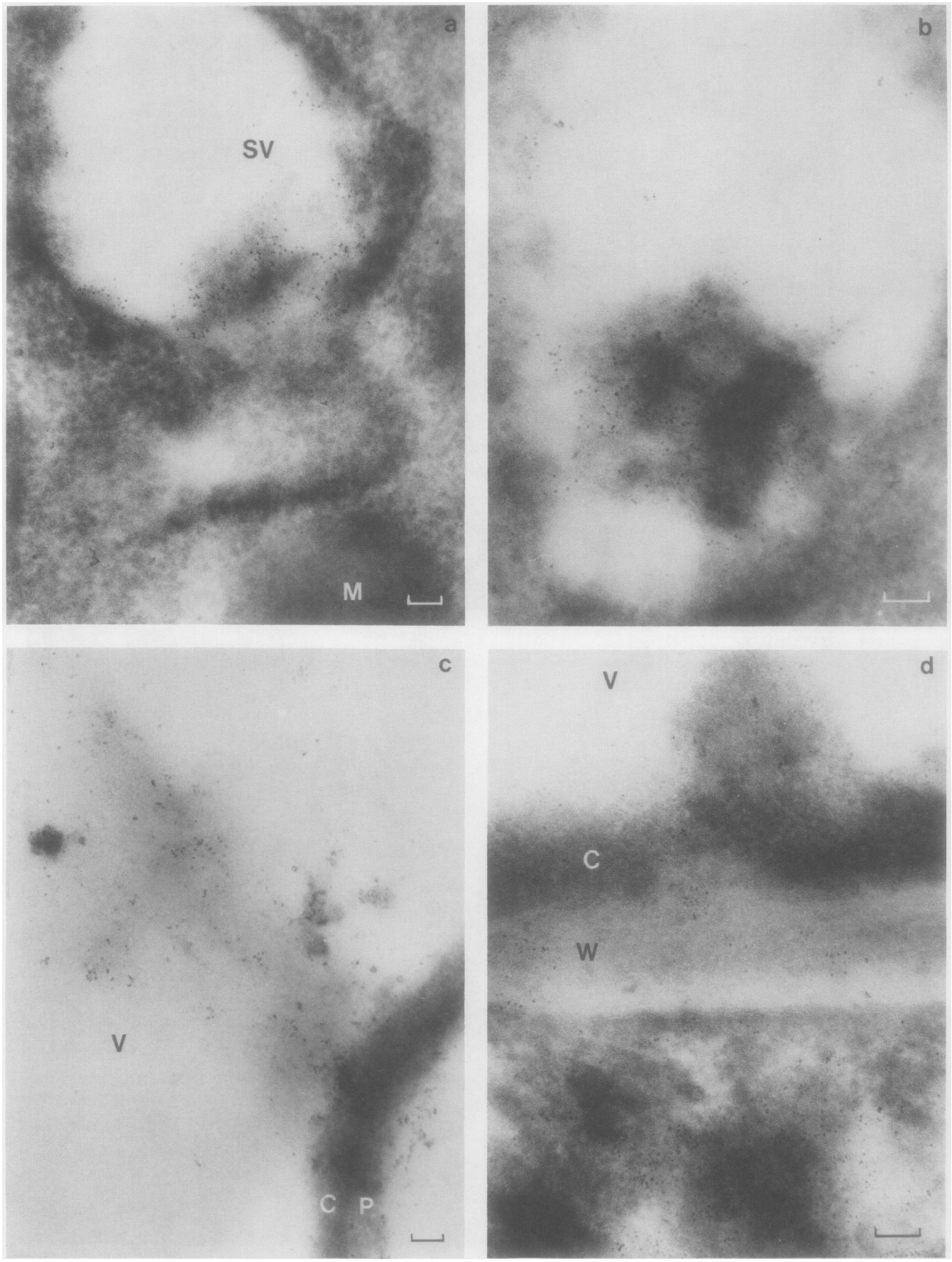

FIG. 3. Electron micrographs of ultrathin cryosections of oat coleoptiles irradiated for 5 min with red light immediately before fixation. Bar is 0.1 μm. Abbreviations as in Figures 1 and 2 except as follows: SV, small vacuole. a, Accumulation of ferritin-labeled material in a small vacuole. b, Similar to a, but at a higher magnification. c, Labeled protrusion into central vacuole, at border of a plastid, d, Ferritin present on a small protrusion into central vacuole and especially on an unidentified dense part of cytoplasm (see bottom of figure).

microscope and pictures were made on Kodak electron microscope film.

Control experiments for each assay were performed using non-immune immunoglobulins in place of specific antiphytochrome immunoglobulins.

RESULTS

All pictures were made from parenchyma or epidermis cells that were located close to a vascular bundle in order to maximize comparability of the data.

Light Microscope. In dark-grown seedlings, fluorescence was uniformly spread throughout the cytoplasm (Fig. 1a). Nuclei lacked any stain. Plastids showed a clear positive reaction although the fluorescence was hard to discern in amyloplasts due to the unfavorable ratio between the volumes of starch and of plastid stroma. After 5 min of red light, numerous highly fluorescent spots appeared in the cytoplasm, whereas the intensity of the general background stain appeared reduced (Fig. 1c). After 15 to 20 min of red light, these phytochrome aggregations were even more pronounced. However, they did not coincide with structures that can be identified by use of Nomarsky optics (Fig. 1d). Subsequent illumination with 5 min of far red light did not interfere with this phytochrome redistribution. Afterwards these phytochrome aggregations gradually faded away. They disappeared completely after either 90 min of red or white light, or 80 min after a red-far red cycle. They were, however, still visible in tissue irradiated for 5 min with red light and then incubated for 85 min in darkness. Thirty min after the onset of illumination (either 5 min or continuous red light) the nucleus started to show some phytochrome-specific stain. To a lesser extent, this phytochrome labeling was still present after prolonged illumination with white light. Cells of the coleoptile tip of naturally grown seedlings showed a faint but significant fluorescence after the immunocytochemical assay (Fig. 1b). The distribution of the fluorescing stain in these cells was similar to that of etiolated tissue, *i.e.* a general cytoplasmic stain that included the plastids. In this case, however, the nuclei were also faintly stained.

Control experiments in every case showed negligible fluorescence. Consequently, they are not shown.

Electron Microscopy. We focused our attention on the difference in localization between seedlings irradiated for 5 min with red light and completely etiolated material. In cells of dark-grown seedlings, labeling for phytochrome occurred throughout the cytoplasm, even in plasmodesmata and at the site of the cytoplasm boundaries, the plasmalemma and tonoplast (Fig. 2, a and b). No special arrangement or concentration of phytochrome-associated stain was found in or around dictyosomes or ER (Fig. 2b). The highest ferritin density in the cell was found in plastids (Fig. 2c). The nuclei of etiolated tissue never showed a significant amount of ferritin (Fig. 2d). Control sections showed that the staining of the starch grains which was observed in some pictures was completely nonspecific.

The distribution of phytochrome in coleoptiles fixed immediately after 5 min of red light was found throughout the cytoplasm and in various organelles but not in the nuclei. The major difference compared with completely etiolated tissue was found in and near the vacuoles. Frequently a high concentration of ferritin was observed in the lumen of small vacuoles (diameter approx. 1 μm) (Fig. 3 a and b). The label was generally coupled to the presence of an amorphous uranyl-stained material, showing no membranes or other identifiable cellular components. These protrusions also occurred in the large central vacuole but were then less obvious (Fig. 3c and d). In parallel with these findings, we observed an increase in label density in the cytoplasm lining the vacuoles. In one section we found a high label density on dense parts of the cytoplasm (Fig. 3d), but it is not clear whether this unique finding represents an artifact or whether these regions are related to the electron-dense protrusions into the vacuoles. The control sections showed negligible ferritin stain.

DISCUSSION

Our results are generally in agreement with previously published observations (1, 2, 4, 6). The immunocytochemical technique has been improved here by the use of ultrathin frozen sections and immunopurified, monospecific anti-phytochrome immunoglobulins. Thus, the resolution and sensitivity of the localization has been increased, which results in new observations and in a better knowledge of the changes in phytochrome content and localization in greening seedlings.

In etiolated shoots phytochrome is present at the plasmalemma, in the cytoplasm, in or around dictyosomes and ER, and at the tonoplast membrane. Plastids generally have a higher and mitochondria a lower density of stain than the cytoplasm. Phytochrome was not detected in the nuclei of dark-grown tissue, but was found there in tissue that had been exposed to light as was observed previously (4). In the cytoplasm, discrete areas of high phytochrome density appeared within five min of the onset of illumination. Afterwards, these aggregations of phytochrome disappeared and they were completely absent in seedlings illuminated for 1 to 2 h or in naturally grown plants of the same age. The complete disappearance of phytochrome labeling during deetiolation as described by Mackenzie *et al.* (4) was not observed in the present work. Their observation was probably due to the lower sensitivity of the assay that they used. The distinct labeling obtained with naturally grown (full sunlight prior to fixation) seedlings illustrates the sensitivity of the technique when using cryosections and purified immunoglobulin fractions.

The particulate localization observed after five min of red light, as observed with the light microscope, corresponds at the electron microscope level with phytochrome that is present in small vacuoles, and to a lesser extent, in the large central vacuole. This vacuolar localization of phytochrome after irradiation was also observed by Epel and Butler (unpublished results) using ferritin-labeled antibodies and electron microscopy. The vacuolar localization leads us to suggest that the redistribution of phytochrome into small highly sequestered regions, as described earlier (2, 5) might occur in order to transport phytochrome into the vacuolar system of the plant. Plant vacuoles are known to contain several hydrolytic enzymes and are considered to have lysosomal functions. The observations described here suggest that, upon illumination, excess phytochrome is transferred into vacuoles where it is destroyed. This hypothesis, which was suggested previously (5) as one of several possible interpretations of the data obtained by light microscopy, finds experimental support from the electron microscopy presented here.

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