

# Immunogold Localization of Nitrate Reductase in Maize Leaves<sup>1</sup>

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## ABSTRACT

Mature maize leaf tissue (*Zea mays* L.) was immunolabeled using a pre-embedding protocol with specific antibodies for nitrate reductase and protein A-colloidal gold. Immunogold label was found exclusively in the cytoplasm of mesophyll cells; no reaction was detected in bundle sheath cells. Chloroplasts, which were sliced open during cryosectioning, had no labeling. Thus, it appears nitrate reductase is localized exclusively in the cytoplasm of maize leaf mesophyll cells. No gold labeling was found on tissue sections embedded in L. R. White's or Lowicryl resin, indicating that previous chloroplast localization utilizing these protocols may be artifactual, resulting from shared epitopes or nonspecific antibody binding.

ical and immunocytochemical data indicate that one or the other protocol is in error.

Monospecific, polyclonal rabbit antisera and mouse ascites fluid for maize leaf NADH:NR were prepared in order to study its synthesis and regulation (2, 14). Using these antibodies to NR, we investigated the subcellular localization of the enzyme in mature maize leaf tissue. Mature maize leaf tissue appears to be an ideal system to use in these investigations because biochemical studies indicate that NR is found in the mesophyll cells and absent from bundle sheath cells (13). Thus, the bundle sheath cell can act essentially as an internal control. We present evidence here for the specific and exclusive localization of NR in the cytoplasm of corn leaf mesophyll cells.

## MATERIALS AND METHODS

**Plant Material.** Seeds of maize (*Zea mays* L. cv 'B 73 × Missouri 17') were planted in a 3:1 peat/perlite mix and grown for 14 d under greenhouse conditions. One day before sampling for microscopy, the samples were treated with 15 mM KNO<sub>3</sub> solutions to stimulate nitrate reductase activity. Samples from the same pot of plants used for microscopic analysis were also examined for NR protein by Western blotting (18) with the maize NR antibodies and antisera to *Chlorella* NR described previously (23). Reactions with all three antibody preparations revealed immunoreactive bands at 110 kD, in accordance with literature on maize (2, 14) and other data on NR subunit size (3, 8).

The initial step in nitrate assimilation by higher plants is catalyzed by nitrate reductase (NR<sup>2</sup>), which is a well characterized enzyme (3). While it has been clearly established that the second step in this assimilation pathway, which is catalyzed by Fd-nitrite reductase, takes place in the chloroplast stroma (1), the subcellular location of NR has yet to be established. Although early reports indicated NR activity was associated with chloroplasts (9), subsequent studies involving organelle isolation from leaves of spinach and tobacco indicated a cytosolic localization for NR (4). NR is readily obtained in the supernatant of leaf extracts as if it were a soluble enzyme or very easily solubilized from any membrane associations or organellar location (6).

Microscopic investigations of NR have presented a confusing picture of NR localization. In a histochemical investigation of etiolated barley leaves, Ekes (5) localized ferricyanide reduction, a partial activity of NR, in the plastid envelope and suggested NR was present in this compartment. Vaughn and co-workers (22, 23) found both histochemical and immunochemical evidence to support a cytoplasmic localization for NR in soybean cotyledons. However, Roldan *et al.* (16) found NR localized both in the cytoplasm and in chloroplast of spinach leaves using immunocytochemical localization. In addition, a recent report of immunogold localization of NR in spinach leaves showed NR exclusively associated with the chloroplast (7). NR was localized in the cell wall-plasmalemma region and the tonoplast of *Neurospora crassa* (15) and in the pyrenoid of nitrate-grown green algae (10, 11). These gross inconsistencies between the biochem-

**Antibodies.** Rabbit antiserum and mouse ascites fluid specific for NR were used from a previous study (2, 14). Specificity for NR was established using Western blotting of crude extracts of corn leaves where proteins were separated both by native and denaturing PAGE (2, 14). In addition, these antibodies for NR were specific in the enzyme-linked immunosorbent assay (2, 14).

**Immunolabeling.** Pre-embedding immunolabeling of NR was performed according to protocols modified from Raikhel *et al.* (12) that allow satisfactory tissue preservation and immunological reactivity. Small leaf pieces (~3 mm<sup>2</sup>) of nitrate-treated maize were fixed in cold 2% (w/v) formaldehyde (prepared freshly from paraformaldehyde) in 0.10 M sodium phosphate buffer (pH 7.4) for 5 h at 4°C. The tissue was then incubated overnight in a solution of 0.4% (w/v) formaldehyde in 1.0 M sucrose containing 0.10 M phosphate buffer (pH 7.4). The molarity of sucrose was increased to 1.3 M for another 6 to 8 h before cryocutting. The tissue pieces were embedded in molten 3% gelatin/3% agar (~50°C) and affixed to chilled specimen stubs for cryocutting. Sections 10 μm thick were obtained with an AO Cryo-Cut freezing microtome. The sections were collected on a warm slide and transferred to a cold solution of PBS. Nonantigenic sites were blocked by incubation of the sections for 2 h at 4°C in 1% (w/v) PBS-BSA. The sections were then incubated overnight (12–18 h) in 1:20 dilution of rabbit or mouse antibodies for maize NR in PBS-BSA. After incubation in the antisera, the sections were

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<sup>2</sup> Abbreviations: NR, nitrate reductase (EC 1.6.6.1); PBS, phosphate buffered saline (0.02 M sodium phosphate [pH 7.2] with 0.50 M sodium chloride); RuBisCo, ribulose biphosphate carboxylase oxygenase.

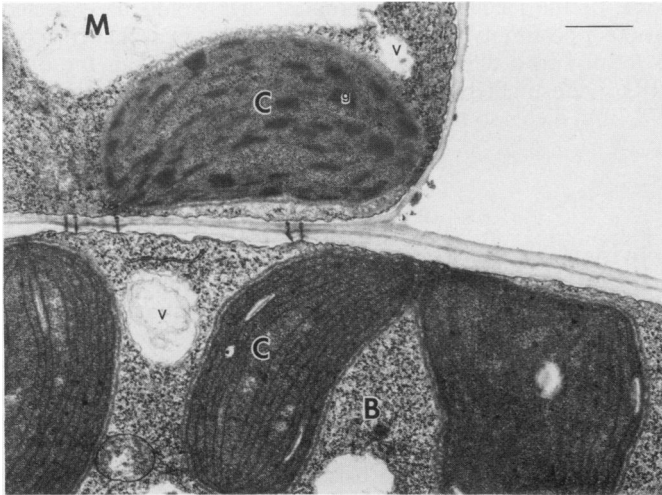


FIG. 1. Standard electron microscopy of the same leaf tissue used for immunogold staining reveals mesophyll cell (M) chloroplasts (C) with distinct grana lamellae. Chloroplasts in the bundle sheath (B) cells are essentially agranal. Vacuoles (V) are well developed in both cell types (bar = 1.0  $\mu\text{m}$ ).

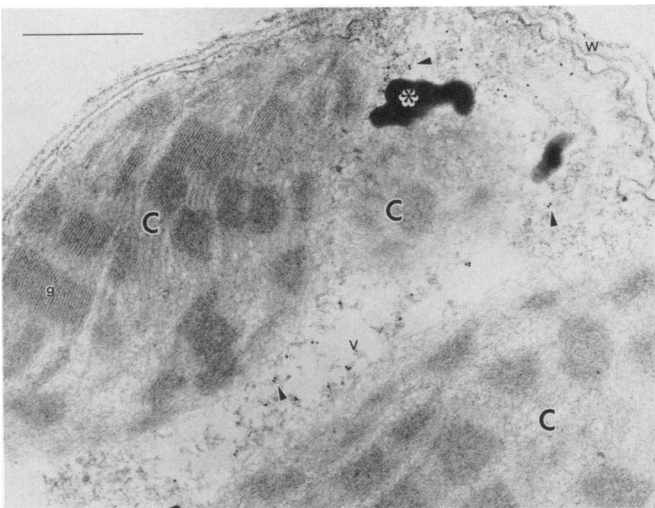


FIG. 2. Mesophyll cell after immunogold staining for NR. Colloidal gold particles (arrows) are found throughout the cytoplasm and along the edge of the vacuole. Chloroplasts (C) containing grana (g) are obvious but are unlabeled; w = wall. Asterisk denotes protein accumulation, probably due to the BSA used to block nonspecific reactions (bar = 1.0  $\mu\text{m}$ ).

washed in six changes of PBS-BSA (10 min each) and then transferred to a 1:25 dilution of 15 nm protein A-colloidal gold (EY Laboratories, San Mateo, CA) in PBS-BSA for 12 to 18 h. The sections were washed in PBS (6 times, 10 min each) and then post-fixed in 3% (v/v) glutaraldehyde in 0.05 M Pipes buffer (pH 7.4) for 2 h at 4°C. The remaining microscopic steps were as described by Vaughn (19).

**Controls.** Substitution of nonimmune or no sera were used as controls. All the experiments were repeated four times with similar results. Approximately 12 samples from each experiment were examined microscopically.

**Other Microscopy.** Samples of the same leaf tissue used for immunolabeling were fixed as described previously (19). Another set of samples of the leaves used for immunolabeling was fixed and then embedded in Lowicryl or L. R. White's resin. Post-embedding labeling was performed as previously described (20, 21).

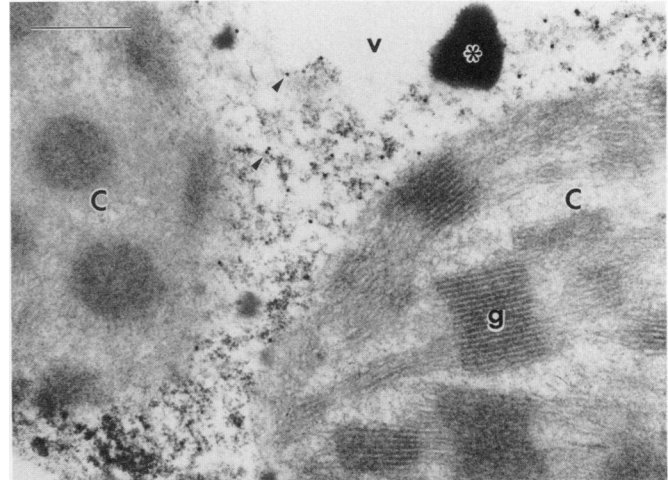


FIG. 3. Higher magnification details of NR immunogold staining. Reaction throughout the cytoplasm, along the tonoplast membrane, and along the chloroplast envelope. C = chloroplast; g = grana; v = vacuole; asterisk = protein accumulation (bar = 0.5  $\mu\text{m}$ ).

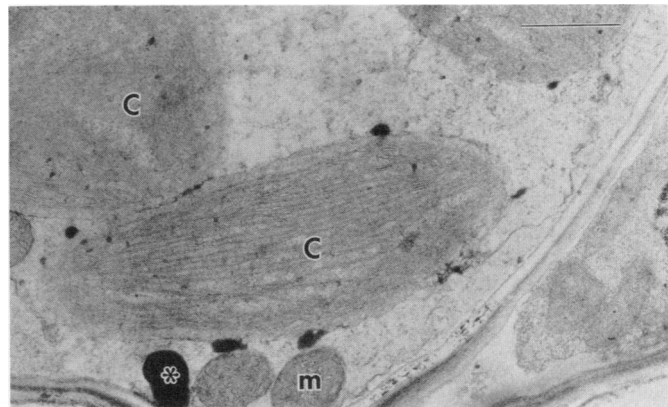


FIG. 4. Bundle sheath cell from same leaf section as the mesophyll cell in Figure 2. Although BSA deposits (\*) are found throughout the cell, no immunogold labeling is noted in these mature bundle sheath cells. C = chloroplast, m = mitochondrion (bar = 1.0  $\mu\text{m}$ ).

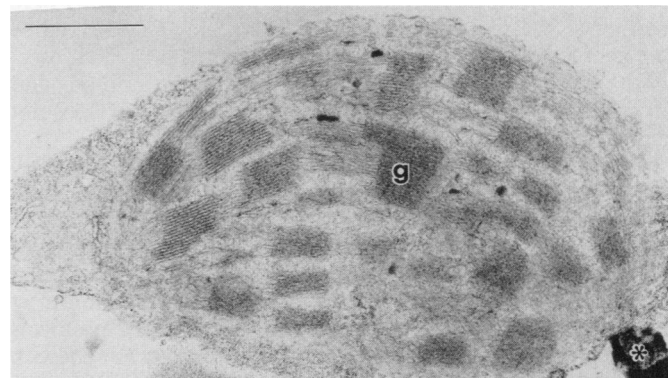


FIG. 5. Nonimmune control. Although this mesophyll cell is obviously permeable to protein, as indicated by the aggregate of BSA (\*), no immunogold staining is noted (bar = 1.0  $\mu\text{m}$ ).

## RESULTS AND DISCUSSION

Standard electron microscopy of the same tissue used for immunolabeling reveals that the tissues used had typical corn mesophyll and bundle sheath cell chloroplasts (Fig. 1). Mesophyll chloroplasts had well differentiated grana and stroma lamellae,

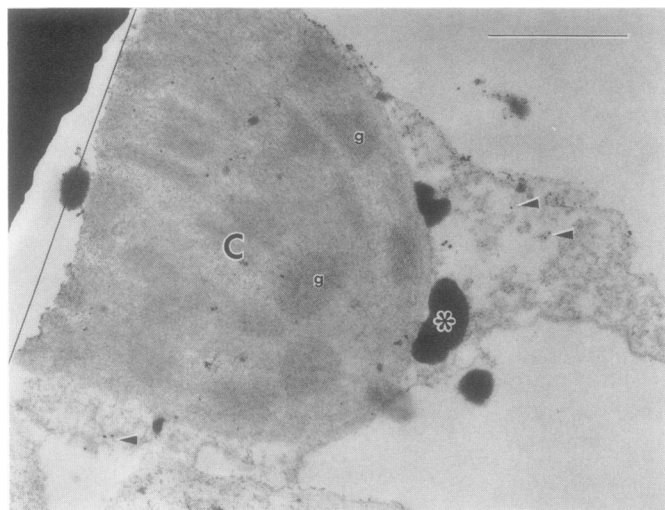


FIG. 6. Section of a mesophyll cell oriented to show the cut edge of the cryosection (thin line). The chloroplast (C) is clearly exposed to the antisera and colloidal gold, but only the cytoplasm has immunogold reaction (arrows) (bar = 1.0  $\mu$ m).

whereas the bundle sheath chloroplasts were agranal or nearly so (Fig. 1). Both cell types were highly vacuolate at this developmental stage.

Pre-embedding immunogold labeling was restricted to the mesophyll cytoplasm and was observed in tissues incubated in either mouse or rabbit antisera (Fig. 2). The distribution of gold particles did not appear to be random, however. Clusters of particles were found in the cytoplasm near the tonoplast membrane (Fig. 2) and around the chloroplasts (Fig. 3). In the cytoplasm, some small thread-like structures were found to be associated with the NR immunolabeling (Figs. 2 and 3) that may represent membranes that have not been well preserved during the protocols. Bundle sheath cells adjacent to labeled mesophyll cells were unlabeled (Fig. 4) as were nonimmune controls (Fig. 5).

Previous immunocytochemical studies have found that NR is localized in the chloroplast rather than the cytoplasm (7, 10, 11, 16). The cryostat sections obtained in this study also cut through chloroplasts. In the section shown in Figure 6, the block was oriented 90° from the direction in which the cryosections were obtained so that the cut ends of the section may be observed. A long profile of a mesophyll chloroplast opened during cryosectioning (but obviously unlabelled) verifies that the antibody and the protein A-gold had access to chloroplast sites, but does not label them.

In previous immunocytochemical protocols (7, 10, 11, 16), which involve on-grid staining, the chloroplast localization could have been obtained by the nonspecific binding of immunoglobulins to RuBisCo, a common problem in Western blotting protocols of leaf extracts (18), or due to contamination in the initial antigen so that antibodies to RuBisCo or another chloroplast antigen were produced. Attempt at on-grid labeling protocols using sera in this study revealed no labelling (data not shown), indicating the NR is such a sensitive and rare antigen that on-grid labeling protocols are not effective for it. Immunogold labeling of these same sections for the P700 protein, the chloroplast coupling factor, and RuBisCo, revealed specific labeling in the chloroplast in all cases (data not shown). On the contrary, RuBisCo is a relatively stable antigen, able to survive standard glutaraldehyde-osmium fixation, acetone dehydration, and embedding in Spurr's plastic (21). Thus, if some of the sera used by previous workers were produced to chloroplast antigens or if chloroplast antigens contained epitopes in common with NR,

some of the labelling of chloroplast sites would be expected. Another possibility, not eliminated in our study, is that corn NR is located in a different cellular compartment than the other NRs. Unfortunately, the corn antisera do not cross-react with NR from dicots so that this may be tested directly.

## CONCLUSIONS

In contrast to previous investigations of the immunocytochemical localization of NR in higher and lower plants (10, 11, 16), we found no evidence for NR in the chloroplast and localized it exclusively in the mesophyll cell cytoplasm of mature maize leaf tissue. These results indicate NR will probably not have a transit sequence on its N terminus because it is not targeted to an organelle. However, NR may be associated with membranes in the cytoplasm and, therefore, may have a membrane binding site as was previously suggested based on finding of a heavy metal binding site on the enzyme (17). In addition, the regulation of both the synthesis and turnover of NR is probably localized in the cytoplasm involving cytoplasmic and nuclear interactions, but the participation of plastids, especially in the biosynthesis of NR, must still be given careful consideration.

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