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Genetics

Cell-type-specific gene expression and acatalasemic peroxisomes in a null *Cat2* catalase mutant of maize

(maize microbodies/mesophyll cells/bundle-sheath cells)

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ABSTRACT **Cell separation studies in conjunction with immunocytochemical studies indicate that mesophyll cells and bundle-sheath cells, the dimorphic photosynthetic cell types in the leaves of the C_4 plant *Zea mays*, differ in their catalase composition. In particular, catalase-2, the product of the *Cat2* gene, is found primarily in the bundle-sheath cells, whereas catalase-3, the product of the *Cat3* gene, is found primarily in the mesophyll cells. Electron microscopic observations reveal that bundle-sheath cells of A16, a mutant line lacking expression of the *Cat2* gene in all tissues examined, contain numerous peroxisomes, but they are acatalasemic as determined by staining with 3,3′-diaminobenzidine. The significance of this mutant in physiological studies is discussed.**

Four types of microbodies have been described in plants: *glyoxysomes*, characteristic of germinating fatty seeds (1, 2); *uricosomes*, characteristic of certain cells in root nodules of legumes (3, 4); *unspecialized peroxisomes*, characteristic of root tissue and most other achlorophyllous tissues (4, 5); and *peroxisomes*, characteristic of green leaves (6, 7). Peroxisomes are the organelles where the oxidative photosynthetic carbon cycle (i.e., photorespiration) occurs (8). In the green leaves of maize and other plants with low photorespiration (i.e., "C_4 plants") peroxisomes are primarily located in bundle-sheath (BS) cells and are particularly scarce in mesophyll (MS) cells (4). This observed distribution is in accord with the observation that in C_4 plants, ribulose-1,5-bisphosphate carboxylase/oxygenase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39], the key enzyme of photorespiration, is found exclusively in BS cells (9, 10). The harmful oxidant H_2O_2 is a by-product of photorespiration reactions and, for this reason, peroxisomes harbor large quantities of catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6) to decompose H_2O_2. Catalase activity is routinely used as a marker to identify microbodies in cell fractionation studies (11), and application of 3,3′-diaminobenzidine (DAB) to examine putative plant peroxisomes for catalase activity is often employed as the definitive test for identifying plant microbodies (4, 12).

Maize catalase is a tetrameric enzyme (13, 14) and is coded for by three distinct structural genes, *Cat1*, *Cat2*, and *Cat3*, which have been mapped to chromosome arms 5S, 1S, and 1L, respectively (15). The products of the three genes (CAT-1, CAT-2, and CAT-3, respectively) have been purified to homogeneity and characterized biochemically, and their antibodies, produced in rabbits, do not crossreact (16). Both CAT-2 and CAT-3 are clearly detectable in extracts of light-grown leaves of all inbred maize strains (e.g., W64A) examined by zymogram analysis (Fig. 1). However, it was not clear whether both CAT-2 and CAT-3 occur in the same cell types or whether each catalase is sequestered in a different leaf cell type (i.e., in MS or in BS cells).

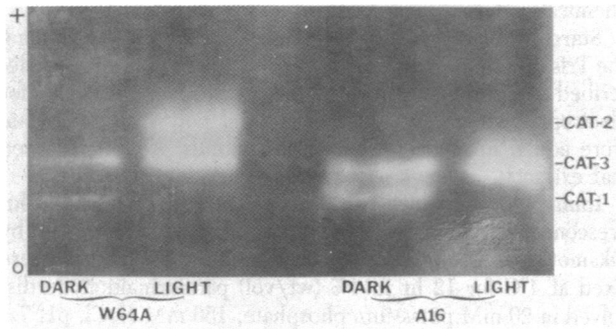

FIG. 1. Zymogram of catalase isozyme patterns in leaves of W64A and A16 after germination in dark or in light. In leaves of light-grown W64A, as in all light-grown maize lines examined to date, CAT-2 and CAT-3 are found, whereas in leaves of light-grown A16 only CAT-3 is present. In dark-grown seedlings of both lines CAT-3 and CAT-1 are found. Leaf extracts were prepared with glycylglycine buffer.

Recently, we described a mutant line, A16, in which the *Cat2* structural gene is not expressed in any of its tissues (17–19), nor can CAT-2 be induced under light conditions (Fig. 1). Electrophoretic and immunological studies clearly show that the CAT-2 protein is not present in either an active or an inactive form in A16, and genetic analysis suggests that the absence of CAT-2 protein from A16 is due to a null allele at the *Cat2* structural gene locus (17, 18). Herein, we report on the differential expression of the *Cat2* and *Cat3* genes in the BS and the MS cells, respectively, and on the existence of acatalasemic peroxisomes in BS cells of A16.

MATERIALS AND METHODS

Plant Material. The two maize lines (i.e., W64A and A16) used in these studies are maintained in our laboratories. Seeds were surface sterilized for 10 min in 1% hypochlorite and soaked

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Abbreviations: DAB, 3,3′-diaminobenzidine; BS, bundle-sheath; MS, mesophyll; CAT, catalase; FITC, fluorescein isothiocyanate; P_i/NaCl, phosphate-buffered saline.

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in distilled water for 24 hr. The seeds were then placed in plastic containers on several layers of moistened germination paper. Germination and subsequent growth were carried out at 23°C in the dark. Alternatively, a 16-hr light, 8-hr dark photoperiod was used. After 10 days the plants were transplanted into flats containing Vermiculite and soil, and they were allowed to grow in the greenhouse at the same temperature and the same photoperiod. Leaves from 10-day-old seedlings were used for electron microscopy. Leaves from light-grown green plants 3–6 weeks old were used for immunocytochemistry and for the separation of MS cells from BS strands.

Materials. DAB was purchased from Serva (Heidelberg); FITC-conjugated goat antirabbit IgG was from Miles; CAT-2 and CAT-3 antibodies were prepared as described (16) and Driselase was from Kinki Yakult (Nishinomiya, Japan). All other chemicals were reagent grade.

Starch Gel Electrophoresis. Starch gel electrophoresis using the Tris/citrate buffer system was performed as previously described (13), except that during the preparation of samples for electrophoresis excess amounts of solid polyvinylpyrrolidone were added to absorb phenolic compounds present in green leaf extracts.

Immunofluorescence Microscopy. The indirect immunofluorescence technique employed was similar to that described by Okamoto and Akazawa (20). Pieces from 10-day-old leaves were fixed at 4°C for 12 hr in 4% (wt/vol) paraformaldehyde dissolved in 20 mM potassium phosphate, 150 mM NaCl, pH 7.4 (P_i/NaCl). Samples were washed overnight in P_i/NaCl with several changes and then incubated in a mixture of 15% (wt/vol) gelatin/10% (wt/vol) glycerol at 37°C for 24 hr. After the embedded samples were frozen they were sectioned to an 8- to 10-μm thickness with a cryostat. The sections were air-dried on microscope slides [precoated with 2% (wt/vol) gelatin] and then fixed for 10 min with 2% paraformaldehyde. After washing three times (15 min each) in P_i/NaCl, sections were incubated in a 1% bovine serum albumin solution for 30 min and washed again three times in P_i/NaCl. Sections were then incubated with rabbit anti-CAT-2 or anti-CAT-3 IgG (0.1 mg/ml) at 4°C overnight in a moist chamber, washed three times in P_i/NaCl, and then incubated similarly with fluorescein isothiocyanate (FITC)-conjugated goat antirabbit IgG (0.1 mg/ml). After three more washes in P_i/NaCl, cover glasses were mounted over the samples with Permount.

Separation of MS Protoplasts and BS Strands. MS protoplasts and BS strands were isolated by the procedure of Kanai and Edwards (21) with minor modifications (10, 22). Briefly, the midribs of 3- to 6-week-old leaves were removed and several leaf halves were placed on top of each other and cut diagonally into strips 0.5–1.0 mm wide. The freshly cut material was immediately incubated in 0.05 mM $CaCl_2$/600 mM sorbitol, pH 5.5. After 20 min the material was transferred to a fresh sorbitol solution containing 0.5% (wt/vol) bovine serum albumin and 3% (wt/vol) Driselase. After 2–4 hr (depending on the age of leaves), the MS protoplasts and BS strands were collected by successive filtration through two sieves with pore diameters of 80 and 44 μm. BS strands were collected on the 80-μm sieve. They were washed in the sorbitol solution and any adhering MS protoplasts were detached by mild stirring of the cells on a Vortex mixer. The MS protoplasts passed through both sieves and were recovered in the filtrate. They were pelleted by centrifugation at 40 × *g* for 5 min in a swinging-bucket rotor. Excess cell debris was removed by centrifugation on a step gradient consisting of 1 ml each of a 15%, 10%, 6%, and 3% solution of dextran with a molecular weight of 18,000–20,000. Centrifugation was performed at 400 × *g* for 5 min in a clinical centrifuge with a swinging-bucket rotor. For electrophoresis the preparations of the two different cell types were resuspended in 0.05 M Hepes buffer, pH 7.5, containing 0.01 M dithiothreitol, and solid polyvinylpyrrolidone was added prior to a final centrifugation of 10,000 × *g* for 10 min. After this last centrifugation, the supernatant was used for electrophoresis. The yield of MS cells by this procedure is very low, whereas the yield of BS strands is very high.

Electron Microscopic Observations. Primary leaves (10-day-old) were fixed for 3 hr at room temperature in 0.05 M sodium cacodylate buffer, pH 7.2, containing 2.5% (wt/vol) glutaraldehyde and 2% (wt/vol) paraformaldehyde. After a postfixation in 1% osmium tetroxide at 4°C for 2 hr the specimens were dehydrated in an acetone series and propylene oxide and finally embedded in Spurr's epoxy resin. Semithin sections (500–800 nm thickness) of plastic-embedded tissue were prepared on a Reichert OM U_2 ultramicrotome and, after staining with 1% toluidine blue O in 1% borax solution, they were observed with a Zeiss Photomikroscop III. Ultrathin sections (70–100 mm thickness) were double stained with uranyl acetate and lead citrate and were examined and photographed with a JEOL 100-B electron microscope. For identification of catalase activity in

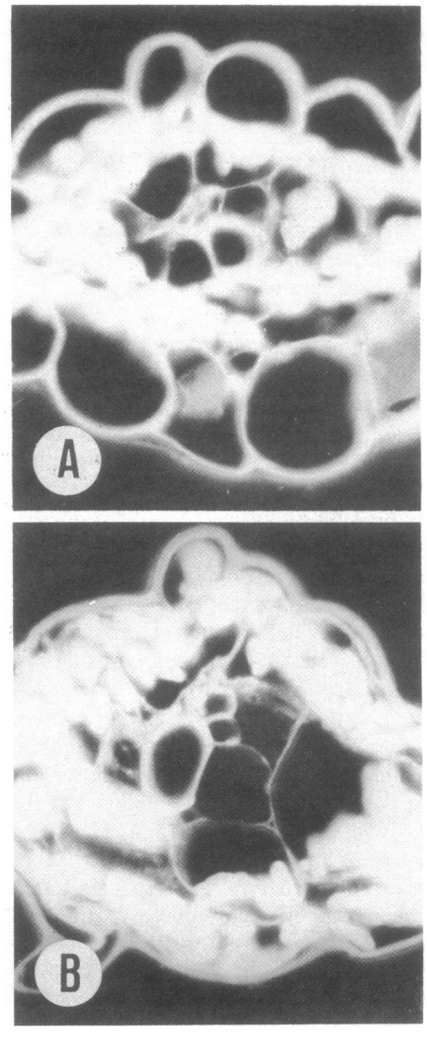

FIG. 2. Immunofluorescent localization of CAT-2 in the BS cells (*A*) and CAT-3 in the MS cells (*B*). An indirect immunofluorescent labeling technique was used in which thin sections of green maize leaves (W64A) were incubated with anti-CAT-2 or anti-CAT-3 IgG, respectively, followed by conjugation with the FITC-labeled goat anti-rabbit IgG. (×600.)

leaf microbodies, the standard incubation medium of Novikoff and Goldfischer (23) was used, and the procedure of Vigil (12) was followed.

RESULTS

The results of the immunofluorescence labeling of thin-sectioned 10-day-old W64A green leaf specimens are shown in Fig. 2. Treatment of the thin-sectioned leaf specimen with anti-CAT-2 IgG resulted in prominent fluorescence labeling of BS cells (Fig. 2*A*), whereas anti-CAT-3 IgG treatment gave prominent fluorescence labeling of MS cells (Fig. 2*B*). The freeze-sectioned leaf specimens treated with either only FITC-anti-rabbit IgG or nonimmune rabbit serum plus FITC-anti-rabbit IgG displayed a weak autofluorescence of the cell walls only (data not shown).

Upon digestion of leaf sections with cell wall-degrading enzymes, MS cells are easily released from the tissue and can be isolated as cell wall-deficient intact protoplasts (Fig. 3*A*). BS cells are less susceptible to cell wall-degrading enzymes and prolonged incubation is required to prepare protoplasts, but "strands" of cells still adhering to vascular elements after removal of MS cells can be isolated (Fig. 3*B*). Starch gel electrophoresis of these BS strands indicates only a strong CAT-2 band, whereas the small amount of pure MS cells usually obtained with this low-yielding separation procedure gave only a weak CAT-3 band (data not shown).

These results indicate that the *Cat2* gene is expressed primarily in the BS cells of W64A green leaf tissue. The leaf microbodies (peroxisomes) are also found primarily in the BS cells and catalase is known to be associated with them. Because the mutant line A16 lacks expression of the *Cat2* gene, it seemed possible that A16 BS cells possess acatalasemic peroxisomes. To test this possibility, electron micrographs were made of A16 BS cells from both dark- and light-grown leaves. As shown in Fig. 4, the A16 BS cells contain peroxisomes similar to those of W64A BS cells. To verify the lack of catalase activity in A16 peroxisomes, light-grown leaf sections from A16 were incubated in a medium containing DAB plus H_2O_2. As shown in Fig. 5, the dense reaction product attributable to catalase activity has permeated the leaf peroxisomes of W64A (Fig. 5*A*) but not those of A16 (Fig. 5*B*).

DISCUSSION

Two independent techniques, immunofluorescence labeling (Fig. 2) and cell separation (Fig. 3), have demonstrated that, in greening leaves, the *Cat2* catalase structural gene is primarily expressed in BS cells and the *Cat3* catalase structural gene in MS cells. Neither of the two techniques is accurate enough to determine if the expression of each gene is completely restricted to either one of the two cell types. With the first technique, it is difficult to distinguish if fluorescence is restricted to only one cell type. Both the BS and MS leaf cells are largely vacuolated, and their antigenic protoplasm is restricted to a thin zone at the periphery of the cells. Thus, although there is a clear difference in the distribution of fluorescence (Fig. 2) when anti-CAT-2 IgG is used (indicating that differential gene expression within the two cell types is occurring), it is difficult to determine whether fluorescence is also labeling the periphery of some MS cells. Difficulties also exist with the second technique. It has been estimated (9) that within any given cell type preparation there is about a 2% contamination of the other cell type. This fact, in addition to the low sensitivity of starch gel electrophoresis, makes it difficult to exclude the possibility of trace amounts of CAT-2 in MS cells or trace amounts of CAT-3 in BS cells. Overall, then, the two sets of data indicate that *Cat2* and *Cat3* are differentially expressed in the two cell types, but it is unclear whether or not their expression is completely restricted to only one cell type.

Other examples of differential enzyme localization between BS and MS cells have been described. Major leaf proteins such as phospho*enol*pyruvate carboxylase (9) and ribulosebisphosphate carboxylase/oxygenase (10) are differentially distributed between the two cell types. Herbert *et al.* (22) found such differences for a number of other enzymes as well, and Potter and Black (24) found significant differences in comparing the two-dimensional electrophoretic patterns of crabgrass, *Digitaria sangualis* Linnaeus (another C_4 plant), using MS and BS cell extracts. It can be envisioned that differential gene expression is the basis for these distinct enzyme localizations.

We know of no other report of an acatalasemic microbody in higher plants. Our finding brings into question the physiological role that catalase plays within the leaf peroxisome, because the line A16 is fully viable even though it lacks catalase within

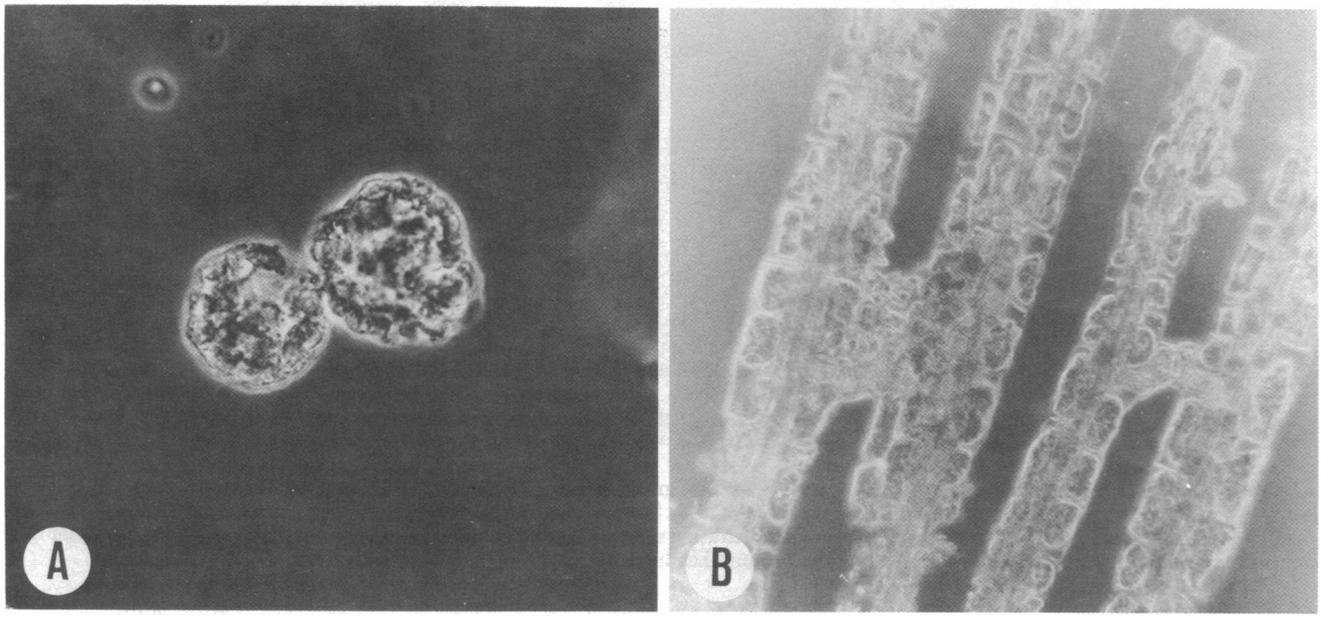

FIG. 3. Photosynthetic cell types of *Zea mays* isolated from W64A leaf tissue. (*A*) Light micrograph of isolated protoplasts from MS cells. (×500.) (*B*) Light micrograph of isolated BS strands composed of cells adhering to vascular elements. (×200.)

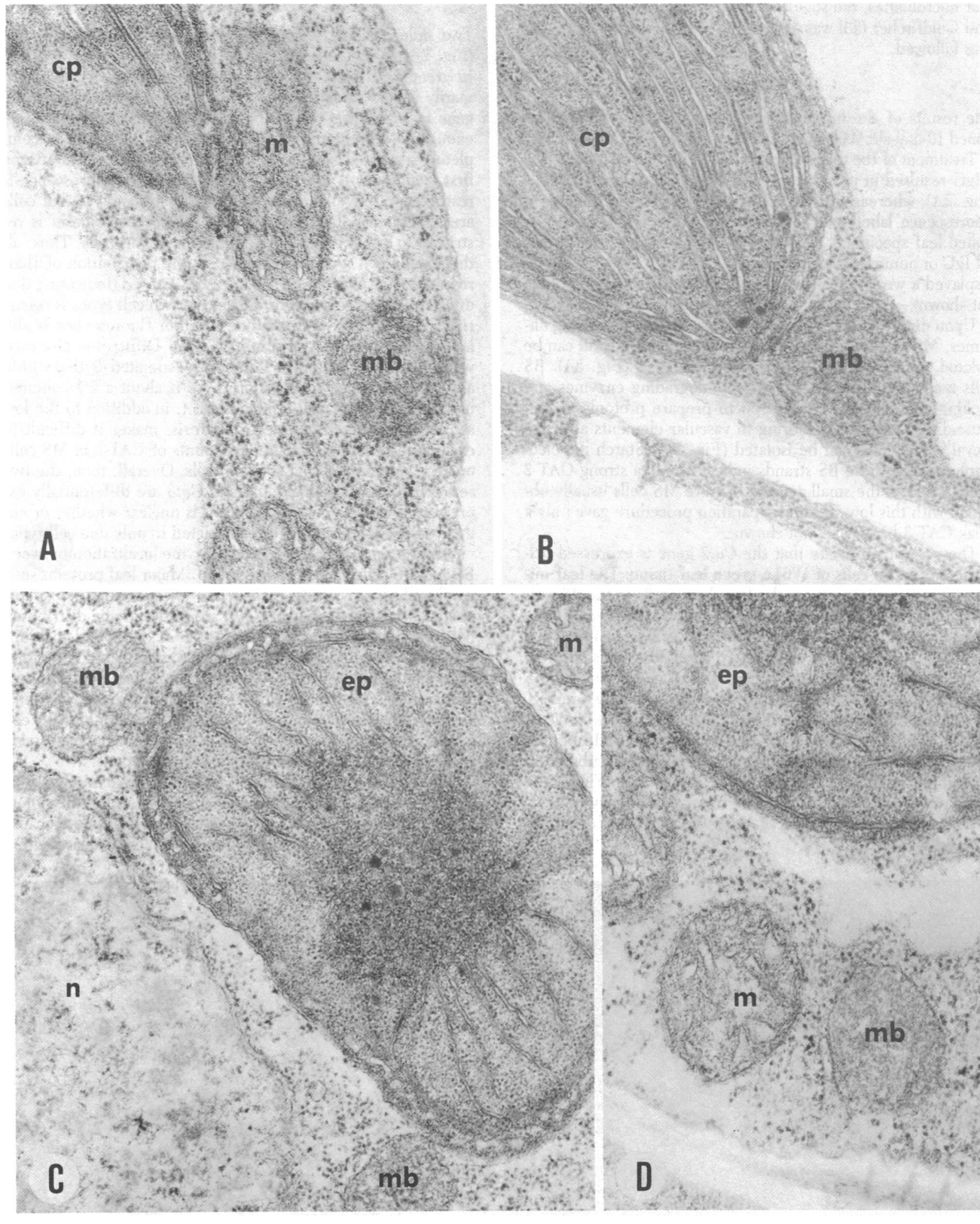

FIG. 4. Electron micrographs of portions of BS cells: (*A*) W64A leaf grown in light (×52,000); (*B*) A16 leaf grown in light (×46,000); (*C*) W64A leaf grown in dark (×40,000); and (*D*) A16 leaf grown in dark (×46,000). Leaves from 10-day-old seedlings grown under light (16 hr light and 8 hr dark) or dark conditions at 23°C were used in all these studies. Ultrathin sections were double stained with uranyl acetate and lead citrate for their observation with the electron microscope. cp, Chloroplast; m, mitochondrion; mb, microbody; ep, etioplast; n, nucleus.

these organelles. Apparently there must be other enzymes that can substitute for catalase in destroying the accumulated H_2O_2, which otherwise would be detrimental to the cell. If this is true, then why is catalase even necessary to the cell? It remains to be determined if the glyoxysomes (a microbody related to the peroxisome) of the scutellum from A16 are also acatalasemic.

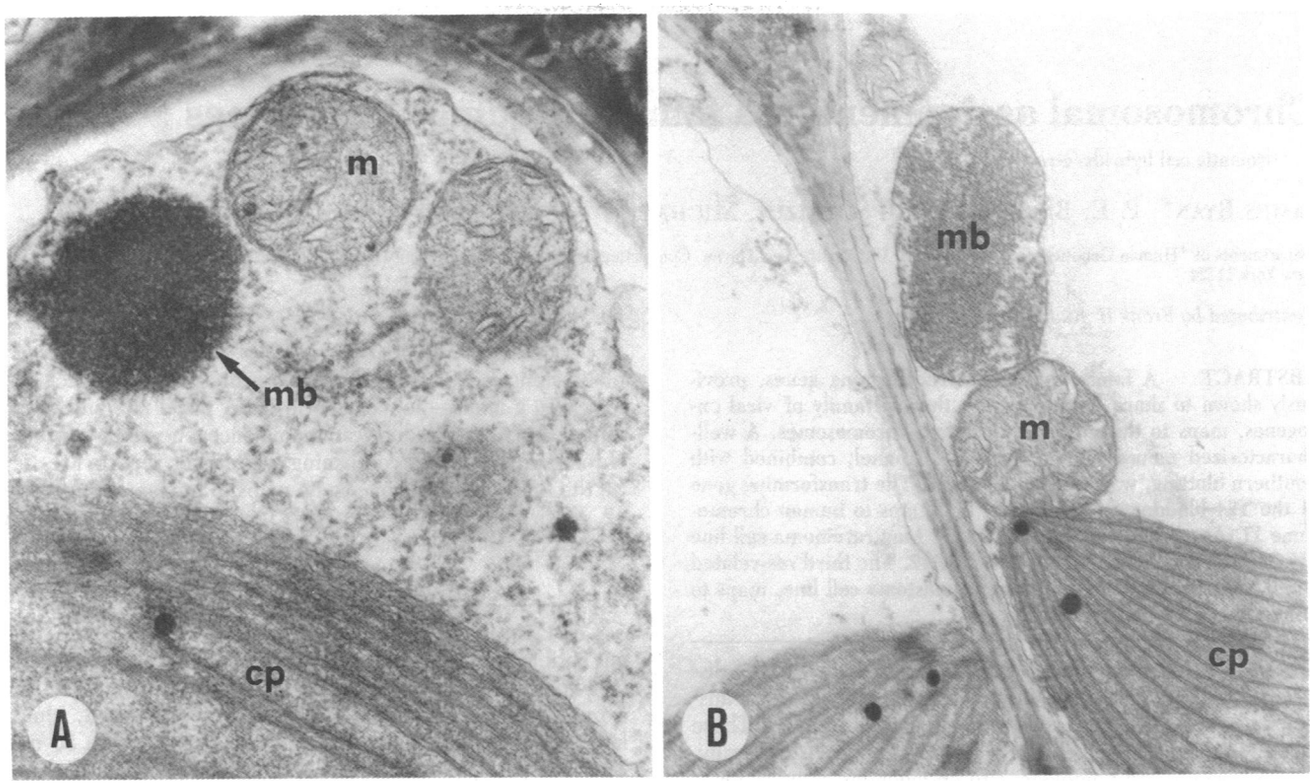

FIG. 5. Electron micrographs of portions of BS cells. (*A*) W64A leaf grown in light (×48,000); (*B*) A16 leaf grown in light (×29,000). The sections were incubated in a medium containing DAB and H_2O_2. Note that the dense reaction product attributable to catalase activity has completely permeated the leaf peroxisome of W64A, whereas no reaction is observed in the A16 peroxisome. cp, Chloroplast; m, mitochondrion; mb, microbody.

The results of such experiments should provide additional evidence concerning the importance of catalase in this organelle.

The absence of catalase from A16 peroxisomes renders this mutant a useful tool. First, because of its lack of catalase activity, it can be used to measure the amount of H_2O_2 normally produced in the BS cells under photorespirative conditions. Because maize is a C_4 plant this level may be very low due to the low levels of photorespiration and may explain why A16 is still viable even though it lacks catalase in the peroxisomes. Second, it can be used to investigate the possible role of catalase in H_2O_2 metabolism in maize. Finally, it can be used to measure the photorespiration rate in the absence of catalase.

Contrary to the viability of this peroxisomal catalase mutant (A16) several conditional lethal mutants have been developed in the crucifer *Arabidopsis thaliana* (a C_3 plant with high photorespiration) that lack other enzymes of the oxidative photosynthetic carbon cycle. Among these are mutants deficient for the enzyme serine–glyoxylate aminotransferase (EC 2.6.1.45) (25) and for the enzyme 2-phosphoglycolate phosphohydrolase (EC 3.1.3.18) (26). All these mutants have been developed in a high CO_2 atmosphere, which blocks photorespiration. When the mutants are placed in air and exposed to light, these mutations are all lethal (26). A16 seedlings should be examined for sensitivity to high-O_2 atmosphere (high photorespiration rate); also, it may be possible to create a truly acatalasemic maize line by eliminating the *Cat3* and *Cat1* genes from the A16 line by use of reciprocal translocations.

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