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Recognition of Peroxisomes by Immunofluorescence in Transformed and Untransformed Tobacco Cells[1]

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We report the visualization of peroxisomes in tobacco (*Nicotiana tabacum*) leaves using fluorescently labeled antibodies to glycolate oxidase. In transgenic tobacco leaves the expression of isocitrate lyase was also visualized. In dual probing experiments both enzymes were shown to be present together in all peroxisomes in transgenic tobacco leaves.

Plant peroxisomes are classified according to their function into "unspecialized" peroxisomes, glyoxysomes, leaf peroxisomes, and peroxisomes for ureide metabolism. Leaf peroxisomes contain the enzymes of photorespiration including glycolate oxidase (Tolbert, 1981). Glyoxysomes are typically found in germinating oilseeds and contain the enzymes of β-oxidation and of the glyoxylate cycle. Isocitrate lyase is one of the unique enzymes of the glyoxylate cycle and is restricted to glyoxysomes (Weir et al., 1980). However, we have recently shown that the ectopic expression of isocitrate lyase in green leaves of tobacco leads to its accumulation in leaf peroxisomes (Onyeocha et al., 1993). The question therefore becomes is isocitrate lyase present in all the peroxisomes of the transformed tobacco leaf or is there a cellular subpopulation of leaf peroxisomes of limited function? In this paper we demonstrate unambiguously the co-localization of glycolate oxidase and isocitrate lyase in the leaf peroxisomes of transformed tobacco leaves by dual probing using fluorescently tagged antibodies.

MATERIALS AND METHODS

Plant Material

Tobacco (*Nicotiana tabacum* var Samson) was obtained from the stock of the Plant Sciences Department of the University of Cambridge, UK. The plants were maintained monoaxenically in Murashige and Skoog medium (Murashige and Skoog, 1962) in Magenta pots (Sigma) in a growth room. The growth room was maintained at a constant temperature of 25 ± 2°C with a light/dark cycle of 16/8 h.

The generation and analysis of transgenic plants is described in detail elsewhere (Onyeocha et al., 1993). Briefly, the cDNA encoding castor bean isocitrate lyase was inserted into the binary vector pROK-8 such that the open reading frame was under the control of the promoter of the small subunit of Rubisco. The resulting plasmid pIG204 and the corresponding vector pROK-8 were transferred into *Agrobacterium tumefaciens* LBA 4404 and from there into tobacco plants via the leaf disc co-cultivation method (Draper et al., 1988). Regenerated kanamycin-resistant plants were screened by western blotting for expression of isocitrate lyase. The vector-transformed plants were used as controls and subjected to the same analyses as the pIG204-transformed plants.

Preparation of Leaf Tissue

Tissue slices (2–3 mm) were cut from young expanding leaves avoiding the midribs. The tissue was fixed, embedded in PEG 1500 (Fisons), and sectioned as described by Marrison and Leech (1992), except that 7-μm-thick sections were laid onto dampened poly-L-Lys-coated slides (Sigma, prepared as recommended by the supplier). Sections were left to dry on a hot plate overnight at 40°C.

Antisera

The antiserum against castor bean isocitrate lyase was a gift from Prof. D.H. Northcote and is described by Martin and Northcote (1982). Spinach glycolate oxidase antiserum was obtained from Prof. C. Sommerville (Volokita and Sommerville, 1987). Rubisco antisera was a gift from Dr. R. Austin and was prepared at the AFRC Institute of Animal Physiology and Genetics Research, Cambridge, UK, using purified wheat Rubisco supplied by Dr. A.J. Keys (AFRC Institute of Arable Crops Research, Harpenden, UK) (Bird et al., 1982).

Immunolocalization

Immunolabeling and washing were carried out as described by Marrison and Leech (1992). All antisera were diluted in 0.5% (w/v) BSA/PBS. For single-labeling experiments the sections were incubated overnight at 4°C with 100 μL of

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Abbreviations: FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate.

1:500 dilution of Rubisco antibody, 1:500 dilution of glycolate oxidase antibody, 1:2000 dilution of isocitrate lyase antibody, or 1:2000 dilution of preimmune serum and then visualized using FITC-conjugated goat anti-rabbit antiserum (Sigma, diluted as recommended by supplier). For dual detection of glycolate oxidase and isocitrate lyase in the same leaf section the tissue was incubated first with 1:500 antiglycolate oxidase overnight at 4°C and then with FITC-conjugated goat anti-rabbit antiserum for 1 h at room temperature. After washing, the sections were next incubated with 1:2000 anti-isocitrate lyase, again overnight at 4°C, and then reacted for 1 h at room temperature with TRITC-conjugated goat anti-rabbit antiserum (Sigma, diluted as recommended by the supplier). The sections were mounted in Citifluor (Agar Scientific Ltd.) and viewed using a Nikon Microphot FXA microscope with epifluorescence attachment and high-pressure mercury lamp. The filter combination Dichroic mirror 510, excitation filter 450 to 490 nm, barrier filter 515IF was used to excite FITC and the combination Dichroic mirror 580, excitation filter 546 nm, and barrier filter 590 was used to excite TRITC. Photomicrographs were taken using Kodak Ektachrome 400 color slide film with automatic exposure setting. Photomicrographs that were not immunolabeled were automatically exposed for twice the length of time compared with the immunolabeled samples.

RESULTS AND DISCUSSION

Localization of Glycolate Oxidase

Sections of young leaf tissue were taken from untransformed tobacco plants, from plants that had been transformed with the binary vector pROK-8, and from two plants transformed with plasmid pIG204: RD1 (the transformant analyzed in detail by Onyeocha et al., 1993) and 204^7 (an independent transformant from the same transformation experiment). Plasmid pIG204 is pROK-8 into which the open reading frame encoding isocitrate lyase (obtained from a castor bean endosperm cDNA library [Beeching and Northcote, 1987]) was inserted such that isocitrate lyase is expressed from the promoter of the small subunit of Rubisco (refer to Onyeocha et al., 1993, for details of the construction, plant transformation, and analysis).

The plant material was fixed, embedded, and sectioned as described by Marrison and Leech (1992). For each of the nine treatments at least 50 sections each containing approximately 100 cells, i.e. 5000 cells for each treatment, were examined. Photomicrographs were chosen to represent the characteristic appearance of the tissue after each of the treatments. In all cases, that is for vector-transformed and isocitrate lyase-transformed material, the immunolabeling was extremely uniform over large areas of leaf tissue.

To establish the antigenicity of the tobacco leaf samples, sections of untransformed tobacco leaf tissue were incubated with antibodies against Rubisco and visualized with FITC-conjugated secondary antiserum. After this treatment with the Rubisco antibody the fluorescent signal was specifically localized in all the chloroplasts of the mesophyll cells. The probed chloroplasts fluoresced yellow-green against a black background, as can be clearly seen in Figure 1a.

Untransformed leaf sections were next incubated with antispinach glycolate oxidase (Volokita and Somerville, 1987) and the enzyme was detected with FITC-conjugated goat anti-rabbit antiserum. Glycolate oxidase was localized specifically to the leaf peroxisomes that could be seen as fluorescent yellow-green spherical bodies against a background of the dull mustard yellow chloroplast autofluorescence (Fig. 1b). The peroxisomes were numerous and could always be located in every cell by altering the plane of focus, but, because of their position between the chloroplasts, the peroxisomes were sometimes not immediately visible. At least 20 to 30 peroxisomes are seen per cell frequently overlying or in close proximity to the chloroplasts. Typically, each peroxisome is present in the angle between the two convex surfaces of adjacent chloroplasts. Occasionally, when the section glances across the chloroplast surface just beneath the tonoplast, several peroxisomes can be clearly visualized in the same field of view (e.g. Fig. 1h). Tobacco peroxisomes are spherical, typically about 2 to 3 μm in diameter, which is in good agreement with the shape and size predicted from electron micrographs.

The peroxisomes in leaf tissue from a vector-transformed plant and two plants transformed with pIG204 (RD1 and 204^7) were also visualized using glycolate oxidase antibodies and FITC. The number per cell, size, shape, and location of the peroxisomes in the vector- and plasmid-transformed plants were similar to those previously observed in untransformed leaf tissue. The similarities are illustrated in Figure 1, c–e, which may be compared with the "normal" peroxisomes in Figure 1b.

Localization of Isocitrate Lyase

The expression of isocitrate lyase is normally restricted to cotyledons and endosperm tissue of germinating oil seeds and is found in glyoxysomes in these tissues. When expressed under the control of the promoter of the Rubisco small subunit in tobacco leaves, the enzyme is found in leaf peroxisomes (Onyeocha et al., 1993). To determine whether we could visualize the transgene product by immunofluorescence, leaf sections from plants transformed with plasmid pIG204 were incubated with anti-isocitrate lyase (Martin and Northcote, 1982) or the corresponding preimmune serum. In leaf tissue from the two transformed plants, RD1 and 204^7, peroxisomes were clearly visible in similar positions in the cell and in all the mesophyll cells (Fig. 1, g and h). No peroxisomes were seen when sections from these transformed plants were treated with preimmune serum alone (Fig. 1, i–k). In contrast to the findings with glycolate oxidase antiserum, no peroxisomes could be visualized in untransformed leaf tissue (not shown) nor in tissue that had been transformed with the binary vector alone when these sections were probed with antiserum against isocitrate lyase (Fig. 1f). These observations confirm and extend the localization studies of isocitrate lyase in the peroxisomes of transformed leaf tissue using subcellular fractionation and immunoelectron microscopy (Onyeocha et al., 1993).

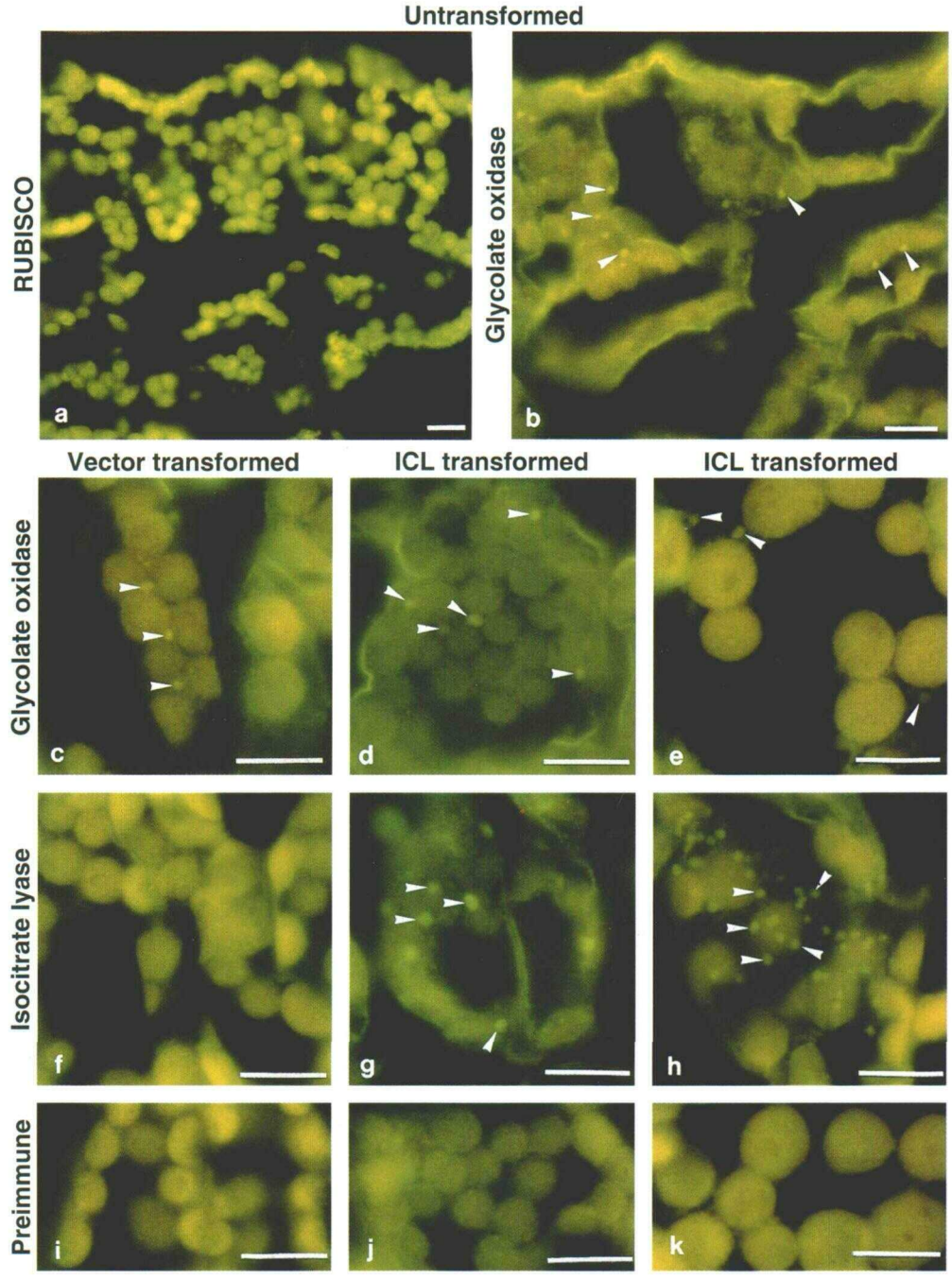

Figure 1. Immunolocalization of Rubisco, glycolate oxidase, and isocitrate lyase in transformed and untransformed tobacco leaf sections (7 μm thick). a, Untransformed tobacco leaf labeled with Rubisco antibody and FITC, showing chloroplast localization of Rubisco to establish the antigenicity of the tobacco leaf tissue. b, Untransformed tobacco leaf labeled with glycolate oxidase antibody and FITC. Arrows indicate peroxisome localization of glycolate oxidase. Vector-transformed control leaf (c) and two isocitrate lyase transformants (d and e) labeled with glycolate oxidase antibody and FITC. Arrows indicate peroxisome localization of glycolate oxidase in all the tissues examined. Vector-transformed control leaf (f) and two isocitrate lyase transformants (g and h) labeled with isocitrate lyase antibody and FITC. Arrows indicate peroxisome localization of isocitrate lyase only in isocitrate lyase-transformed tissue. Vector transformed control leaf (i) and two isocitrate lyase transformants (j and k) incubated with preimmune serum and FITC. In the preimmune controls no peroxisomes were visualized. Note: For photographic considerations, peroxisomes were brought into sharp focus because of the relative positions of peroxisomes and chloroplasts in the cell. This means that in some images the chloroplast profiles will inevitably be out of focus. Images f, i, j, and k had longer exposure times than the other images (see "Materials and Methods"). Images c, f, and i are from transformant ROK8; images d, g, and j are from transformant RD1; and images e, h, and k are from transformant 204[7]. Scale bar = 10 μm.

Co-Localization of Glycolate Oxidase and Isocitrate Lyase

The appearance of the peroxisomes in the transformed plants visualized using anti-isocitrate lyase was indistinguishable from the appearance of peroxisomes detected with the antiglycolate oxidase. It is important to establish whether the two enzymes are co-localized in each individual peroxisome of the cell population or whether subpopulations of peroxisomes contain either one enzyme or the other. To answer this question, leaf sections from the two transformants were double probed for both enzymes. Glycolate oxidase was labeled using fluorescein and isocitrate lyase was labeled using rhodamine. In all the pIG204-transformed tissue examined, all the peroxisomes contained both enzymes and no peroxisomes were identified that contained only glycolate oxidase or only isocitrate lyase.

The appearance of the peroxisomes is illustrated in the photomicrographs in Figure 2, b, c, e, and f. In the transformant RD1 (Fig. 2, b and e) the same peroxisomes are identifiable with each of the visually tagged antibodies (indicated by arrows) and resolved at both ×1080 and ×2160 (insets) magnifications. Clearly, glycolate oxidase and isocitrate lyase are both present in all the peroxisomes of the transformed tobacco leaf cells. Identical results were obtained for the 204[7] transformant, and the visual tagging of the peroxisomes in the transformed leaf tissue of this plant is illustrated in the photomicrographs in Figure 2, c and f (and insets).

To establish that all the rabbit epitopes of the glycolate oxidase antibody were saturated with the FITC-conjugated goat anti-rabbit antibody before the TRITC-conjugated goat anti-rabbit antibody was applied, the following control experiment was carried out. Vector-transformed tissue was tagged with antiglycolate oxidase and the peroxisomes were visualized as expected (Fig. 2a). Double labeling was then carried out by the addition of anti-isocitrate lyase, but as anticipated, peroxisomes were not seen (Fig. 2d) after this treatment. Clearly, no cross-labeling to either primary antibody is occurring during the procedure. Therefore, we can conclude that all the peroxisomes in the pIG204-transformed plants contain both glycolate oxidase and isocitrate lyase. This suggests that all the leaf peroxisomes and not just a specific subset of organelles remain competent to take up the glyoxylate cycle enzyme.

The results presented here demonstrate the sensitivity and degree of resolution attainable with dual probing by in situ immunofluorescence. In particular, the co-localization experiments provide a powerful means of analyzing the results of

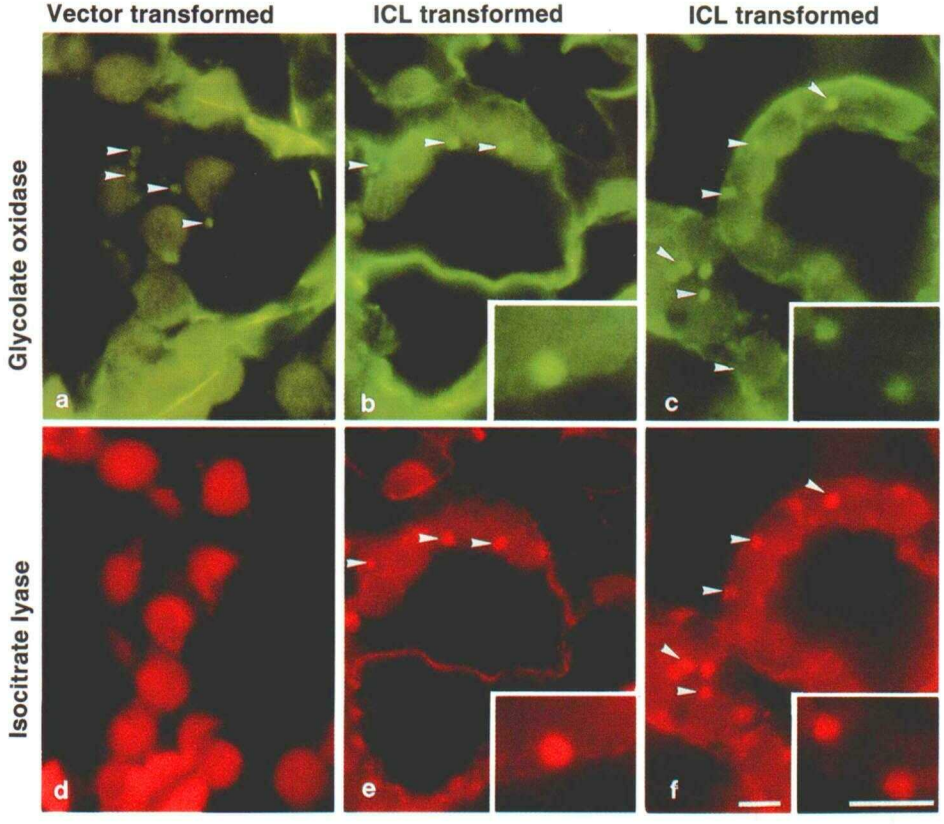

Figure 2. Co-localization of glycolate oxidase and isocitrate lyase in vector- and isocitrate lyase-transformed tobacco leaf sections (7 μm thick). Vector-transformed control leaf (a and d) and two isocitrate lyase transformants (b, e and c, f). Each section was double labeled with glycolate oxidase antibody and FITC (a–c) followed by isocitrate lyase antibody and TRITC (d–f). Images a and d are from transformant ROK8; images b and e are from transformant RD1; and images c and f are from transformant 204[7]. The four insets show peroxisomes at twice the magnification. Image d had a longer exposure time than the other images (see "Materials and Methods"). Scale bar = 5 μm.

gene manipulation and plant transformation. Specifically, in the context of protein sorting, the technique allows the subcellular localization of artificially created, in vivo-expressed gene products to be determined, thereby facilitating the analysis of targeting signals for peroxisomes and other compartments in the plant cell. Such an approach has been elegantly used in the laboratory of Subramani to dissect in vivo targeting of proteins into mammalian peroxisomes (Keller et al., 1987; Gould et al., 1989). The resolution of the techniques described here combined with recent advances in in vitro import systems for plant peroxisomes (Baker and Behari, 1993) should permit rapid progress in understanding protein targeting to peroxisomes in plants.

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