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Identification of Porin-Like Polypeptide(s) in the Boundary Membrane of Oilseed Glyoxysomes

F. Javier Corpas [1], Luisa M. Sandalio [1], Michael J. Brown [2], Luis A. del Río [1] and Richard N. Trelease [2, 3]

[1] *Departamento de Bioquímica, Biología Celular y Molecular de Plantas, Estación Experimental del Zaidín, CSIC, Apdo. 419, E-18080 Granada, Spain*
[2] *Department of Plant Biology, P.O.B. 871601, Arizona State University, Tempe, AZ 85287-1601, U.S.A.*

A 36-kDa polypeptide of unknown function was identified by us in the boundary membrane fraction of cucumber seedling glyoxysomes. Evidence is presented in this study that this 36-kDa polypeptide is a glyoxysomal membrane porin. A sequence of 24 amino acid residues derived from a CNBr-cleaved fragment of the 36-kDa polypeptide revealed 72% to 95% identities with sequences in mitochondrial or non-green plastid porins of several different plant species. Immunological evidence indicated that the 36-kDa (and possibly a 34-kDa polypeptide) was a porin(s). Antiserum raised against a potato tuber mitochondrial porin recognized on immunoblots 34-kDa and 36-kDa polypeptides in detergent-solubilized membrane fractions of cucumber seedling glyoxysomes and mitochondria, and in similar glyoxysomal fractions of cotton, castor bean, and sunflower seedlings. The 36-kDa polypeptide seems to be a constitutive component because it was detected also in membrane protein fractions derived from cucumber leaf-type peroxisomes. Compelling evidence that one or both of these polypeptides were authentic glyoxysomal membrane porins was obtained from electron microscopic immunogold analyses. Antiporin IgGs recognized antigen(s) in outer membranes of glyoxysomes and mitochondria. Taken together, the data indicate that membranes of cucumber (and other oilseed) glyoxysomes, leaf-type peroxisomes, and mitochondria possess similar molecular mass porin polypeptide(s) (34 and 36 kDa) with overlapping immunological and amino acid sequence similarities.

Key words: Cucumber (*Cucumis sativa*) — Glyoxysomes — Immunogold — Peroxisomal membrane protein — Peroxisome — Porin.

Abbreviations: APX, ascorbate peroxidase; CCO, cytochrome *c* oxidase; CNBr, cyanogen bromide; PMP, peroxisomal membrane protein; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

Introduction

Peroxisomes are morphologically relatively simple. They are bounded by a single membrane and do not possess any internal membranous components or protein-synthesizing machinery, hence they acquire all of their membrane and matrix proteins from the cytosol. Metabolically, however, peroxisomes are incredibly complex due to their varied enzyme content depending on the developmental stage, and/or the tissue/cell type in which they reside (Huang et al. 1983, Van den Bosch 1992, Olsen and Harada 1995, del Río et al. 1998, Subramani 1998). Examples of this complexity are the presence in leaf peroxisomes of the NO-generating enzyme nitric oxide synthase (Barroso et al. 1999), all enzymatic components of the ascorbate-glutathione cycle (Jiménez et al. 1998), and a group of dehydrogenases with the capacity to regenerate the NADPH (Corpas et al. 1998, Corpas et al. 1999). The metabolic changeover from glyoxysomal metabolism (heterotrophic growth) to leaf-type peroxisomal metabolism (photoautotrophic growth) in oilseed cotyledons during postgerminative growth is another pertinent example of peroxisomal versatility in plants (Trelease 1984, Nishimura et al. 1996). Diverse metabolites constantly enter and exit peroxisomes through their boundary membrane (Mullen and Trelease 1996). For example, oilseed glyoxysomes acquire fatty acids from oil bodies, then small metabolites such as succinate, malate, coenzymes, etc., exit through the membrane for biosynthesis of carbohydrates and other compounds in the cytosol (Mettler and Beevers 1980, Donaldson et al. 1981, Doman and Trelease 1984, De Bellis et al. 1995, Escher and Widmer 1997). During growth in the light, small metabolites related to photorespiration enter and exit leaf (or leaf-type) peroxisomes through the boundary membrane (Liang and Huang 1983, Liang et al. 1984, Yu and Huang 1986, Reumann et al. 1995).

Peroxisomal metabolite transporters with properties similar to mitochondrial porins were described for mammalian (Lemmens et al. 1989, Kaldi et al. 1993) and yeast (Sulter et al. 1993) peroxisomes. More recently, physiological evidence was provided for the presence of porin-like channels in the membranes of spinach leaf peroxisomes (Reumann et al. 1995, Reumann et al. 1996, Reumann et al. 1998) and castor seed endosperm glyoxysomes (Reumann et al. 1997). Interestingly, data on single channel conductance, ion selectivity, and voltage dependence indicated that the porins in both types of plant peroxisomes were distinctly different from porins in mitochondria and plastids. Prior to our study, a plant peroxisomal porin had not been associated with a specific protein (polypeptide),

[3] Corresponding author: E-mail, trelease.dick@asu.edu; Fax, +1-480-965-6899.

although the activity of the castor seed glyoxysomal porin was related to an unidentified integral membrane protein in purified glyoxysomal membrane fractions (Reumann et al. 1997).

Most plant mitochondrial and plastid porins are composed of one type of polypeptide and range in molecular mass between 24 and 35 kDa (Fischer et al. 1994, Reumann et al. 1999). However, two porin isoforms were found in human and potato mitochondria (Blachly-Dyson et al. 1993, Ha et al. 1993, Heins et al. 1994). Only during the last 7 years have polypeptide sequences been determined for plant porins.

The SDS-denatured polypeptide composition of peroxisomal membrane fractions has been described for several different plant species as well as for plants grown under varied conditions (e.g. Kruse and Kindl 1982, Chapman and Trelease 1992, Corpas et al. 1994, Jiang et al. 1994, López-Huertas et al. 1995, Yamaguchi et al. 1995a, Reumann et al. 1995). An *Arabidopsis* peroxisomal membrane protein, PMP22, of unknown function was identified and characterized as a plant homolog to a human PMP22 (Tugal et al. 1999), and a putative peroxin (Pex14p) was partially characterized (a 66 kDa protein) in sunflower glyoxysomes (López-Huertas et al. 1999b). Enzymatic activity has been attributed to several polypeptides recovered from membrane fractions of leaf peroxisomes and/or glyoxysomes, e.g. NAD(P)H:ferricyanide reductase (Luster and Donaldson 1987, Struglics et al. 1993), monodehydroascorbate reductase and ascorbate peroxidase (Bowditch and Donaldson 1990, Jiménez et al. 1998). Other functional activities attributed to plant PMPs include electron transport (Donaldson and Luster 1991) and production of superoxide radicals (del Río and Donaldson 1995, López-Huertas et al. 1997, López-Huertas et al. 1999a). However, up until now only three polypeptides have been localized in the peroxisomal membranes using immunoelectron microscopy: PMP30/31—an ascorbate peroxidase (APX) (Corpas et al. 1994, Yamaguchi et al. 1995b, Bunkelmann and Trelease 1996, Corpas and Trelease 1998); PMP73—immunorelated to molecular chaperones (Corpas et al. 1994, Corpas and Trelease 1997); and PMP28—unknown function (Yamaguchi et al. 1995a).

In this paper we provide several lines of evidence to show that the previously identified cucumber glyoxysomal 36-kDa polypeptide is a PMP (verified by electron microscopic immunogold localization) and that it likely is a porin polypeptide. This peroxisomal membrane porin appears to be structurally and immunologically similar to cucumber and other plant mitochondrial and plastid porins.

Materials and Methods

Plant material and growth conditions

Cucumber (*Cucumis sativus* L.) seeds were purchased from J.W. Jung Seed Company (Madison, WI, U.S.A.). They were soaked in deionized water with aeration for 30 min (30°C), then sown in water-saturated vermiculite in flats for germination and growth in the dark for 65–70 h at 30°C. Some flats with dark-grown seedlings were transferred for 1 d to continuous light, and others for 3 d in continuous light. For some experiments, flats with sown seeds were placed in the chamber for 8 d of continuous light without being previously germinated in the dark. Conditions for growth in continuous light are described by Corpas and Trelease (1998). Cotton (*Gossypium hirsutum* L. cv. Coker 100) seeds, castor (*Ricinus communis* L.) seeds, and sunflower (*Helianthus annuus* L.) achenes were germinated and grown in darkness as described by Corpas et al. (1994). Specifically, cotton seeds were imbibed in aerated water for 4 h then grown in paper scrolls for 48 h at 30°C. Castor beans with their seed coats removed were surface sterilized and grown for 72 h at 30°C. Sunflower achenes were imbibed in aerated water for 12 h, then grown at 30°C in vermiculite flats for an additional 48 h.

Isolation of organelles and preparation of integral membrane proteins

Details of procedures outlined in this section are given in Corpas et al. (1994). Glyoxysomes and mitochondria isolated from cotyledons of dark-grown cucumber seedlings, and glyoxysomes from cotton, castor bean, and sunflower cotyledons were isolated by centrifugation in linear sucrose density gradients (20–59%, w/w). Isolation of leaf-type peroxisomes was accomplished using slight modifications described by Corpas and Trelease (1998).

Gradient fraction analysis

Catalase (EC 1.11.1.6) activity was used as a marker for glyoxysomes (Ni et al. 1990) and CCO (EC 1.9.3.1) activity was used as a marker for mitochondria (Tolbert et al. 1968). The fractions with the three highest catalase activities were pooled, as were the fractions with the three highest CCO activities. Buoyant densities of gradient fractions were measured at room temperature with a Bausch and Lomb refractometer.

Gradient fractions (mitochondrial and glyoxysomal) obtained from the oilseeds listed above were diluted threefold with 50 mM K-phosphate (pH 7.2) and membranes were recovered by centrifugation (100,000×*g*, 45 min). The membrane pellets were mixed for 30 min (4°C) in 100 mM Na_2CO_3 (pH 11.5), then centrifuged for 45 min at 100,000×*g*. These membrane pellets were resuspended in 50 mM Tris-HCl, 630 mM aminocaproic acid, pH 7.2, 1% dodecyl-β-D-maltoside to solubilize the integral membrane proteins. The mixtures were centrifuged for 30 min at 100,000×*g*, and the supernatants contained the detergent-solubilized integral membrane proteins that were examined on the immunoblots presented in the Results section.

Purified *Zea mays* L. mitochondria, used as a control for the specificity of the CCO antiserum, were obtained from D.M. Rhoads (Arizona State University). Briefly, they were isolated from 3 d old seedlings grown as described by Lund et al. (1998) but without heat shock, and purified according to the methods described by Hayes et al. (1991) and Luethy et al. (1991).

SDS-PAGE and immunoblot analyses

Detergent-solubilized membrane proteins were heated at 95°C for 5 min in 62.5 mM Tris-HCl, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, and 10 mM DTT. Polypeptides were separated by SDS-PAGE in 12% mini gels (Fig. 2 panel B only) or in 10–15% linear gradient gels according to Corpas et al. (1994) using a Protean II apparatus with 18-cm plates. Gels were stained with silver according to the modifications described in detail by Jiang et al. (1994). Low molecular weight standards (BIO-RAD) were used to estimate the molecular mass of electrophoresed polypeptides. For immunodetections, SDS polypeptides were electroblotted to PVDF membranes at 1.5 mA cm^{-2} for 2 h using a semidry transfer cell (BIO-RAD). Antisera raised in rabbits against *E. coli*-expressed, potato mitochondrial 36-kDa polypeptides (Heins et al. 1994) and a porin purified from *Saccharomyces cerevisiae* were provided by U.K. Schmitz (Institut für Genbiol-

ogische Forschung, Berlin, Germany) and G. Schatz (University Biozentrum, Basel, Switzerland), respectively**.** The rabbit antiserum raised against cucumber glyoxysomal APX (PMP 67) was produced by Corpas et al. (1994). The rabbit antiserum raised against sweet potato CCO (whole enzyme) (Maeshima and Asahi 1991) was provided by M. Maeshima (Nagoya University, Japan). The rabbit anti-pumpkin seed catalase antiserum (Yamaguchi and Nishimura 1984) was provided by M. Nishimura (NIBB, Okazaki, Japan). For immunodetection of electroblotted polypeptides, membranes were immersed for 1 h at room temperature in 20 mM Tris-HCl, pH 7.8, 180 mM NaCl, 1.5% (w/v) non-fat dry milk, 0.2% sodium azide, then in antiserum dilutions in the same buffered medium for the same time period. Bound IgGs were observed via enhanced chemiluminescence with goat anti-rabbit horseradish peroxidase IgGs (Sigma) and a solution of 50 mM Tris-HCl, pH 8.6, 2 mM luminol, 45 mM of 4-iodophenol, and 0.005% H_2O_2 as described in detail by Bunkelmann and Trelease (1996).

Cyanogen bromide (CNBr) cleavage and amino acid sequencing

The method employed has been used to obtain internal sequences within several other putative PMPs (Corpas and Trelease 1997, Corpas and Trelease 1998). Specific procedures used for the 36-kDa polypeptide are described here. Polypeptides solubilized from the glyoxysomal membrane fractions were separated by SDS-PAGE in 10–15% linear gradient gels and stained with 0.1% Coomassie blue R-250 in 50% methanol and 0.1% acetic acid. Following destaining overnight in 10% methanol, the 36-kDa polypeptide bands were excised from gels and each band was electroeluted in a separate tube for 5 h at 400 V in a laboratory built apparatus. Polypeptides were collected in an Amicon Centricon 10 immersed in 2.5 mM TRIZMA (Sigma), 19 mM glycine, pH 8.3, 0.1% SDS. The concentrated polypeptides were precipitated overnight in 90% acetone (–20°C), and each pellet was washed twice with cold acetone and centrifuged at 3,000×*g* for 60 min (4°C). Cleavage of the polypeptides was accomplished according to Matsudaira (1990). Fragments were separated by 12% Tricine SDS-PAGE (Schäegger and von Jagow 1987), then electroblotted onto a PVDF membrane. The most prominent bands stained with Coomassie-Blue (in 50% methanol) were cut from the PVDF membrane. Determination of N-terminal sequences of several of these polypeptide fragments was attempted by automated Edman degradation as described by Chapman and Trelease (1992). A reliable sequence was obtained from the most prominent fragment. Sequence comparisons were accomplished with the basic local alignment search tool (BLAST).

Electron microscopy and immunocytochemistry

Processing of cotyledon segments (approximately 1 mm^2) for electron microscopy was as described by Corpas et al. (1994). Briefly, segments were fixed in 0.2% glutaraldehyde and 4% formaldehyde in 50 mM PIPES-KOH (pH 7.4) for 1 h (4°C), dehydrated in a graded series of ethanol (30–70% at room temperature, 70–100% at –20°C), embedded at –20°C in a graded series of ethanol and LR White resin, and then cold polymerized with catalyst at –20°C. Procedures for immunogold single labeling experiments were as follows. Thin sections on nickel grids were floated for 1 h on one of two alternative blocking solutions. One of them consisted of 2% (w/v) BSA (Sigma no. A3803), 5% (v/v) goat normal serum (GNS, British Bio Cell Int., Cardiff, U.K.) in TBST. TBST consisted of 10 mM Tris-HCl, pH 7.6, 0.9% (w/v) NaCl, 0.05% (v/v) Tween 20, and 0.02% (w/v) NaN_3. The other blocking solution was 5% (v/v) fetal calf serum (FCS) in TBST. The grids were floated for 3 h on drops of potato porin antiserum diluted 1 : 100 in TBST containing 2% BSA and 1% GNS, or 5% FCS. Preimmune serum was diluted 1 : 200 in the same solutions as the anti-porin antiserum. Following three washes in TBST (without blocking proteins), grids were floated for 1 h on goat anti-rabbit IgGs conjugated to 15-nm gold particles (British Bio Cell Int., Cardiff, U.K.) diluted 1 : 50 in TBST containing 2% BSA (without GNS) or 5% FCS. After one wash in TBST (without blocking proteins) and two washes in milliQ water, sections on grids were poststained for 5 min in 2% uranyl acetate prepared in 50% (v/v) ethanol. Finally, sections were examined with a Zeiss EM 10C electron microscope.

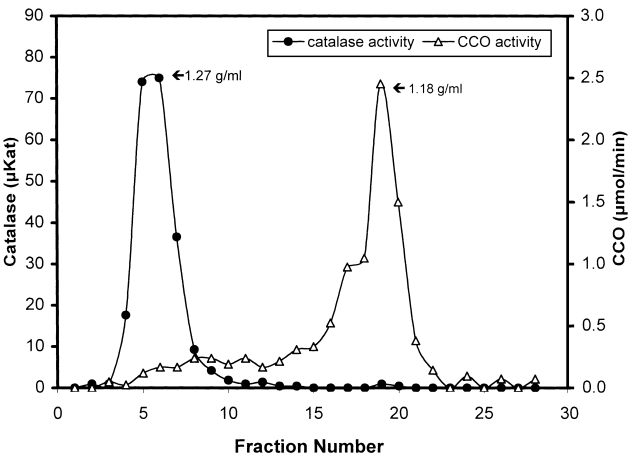

Fig. 1 Activities of glyoxysomal and mitochondrial marker enzymes in fractions from a representative sucrose density gradient.

Indirect, double immunogold labeling was accomplished by using both faces of thin sections. One face of the grid was incubated for 1 h in a 1 : 500 dilution of anti-pumpkin seed catalase (Yamaguchi and Nishimura 1984). The grids were rinsed three times in TBST and then the same face was incubated in a secondary antibody solution containing goat anti-rabbit IgGs conjugated to 5 nm gold particles (British Bio Cell Int., Cardiff, U.K.) diluted 1 : 50 in TBST and containing 5% FCS. The reverse faces of the grids were incubated for 3 h in a 1 : 100 dilution of anti-potato porin in TBST containing 5% FCS. The grids were washed three times in TBST and the reverse faces floated for 1 h on goat anti-rabbit IgGs conjugated to 15-nm gold particles (British Bio Cell Int., Cardiff, U.K.) diluted 1 : 50 in TBST containing 5% FCS. After one wash in TBST (without blocking proteins) and two washes in milliQ water, sections on grids were poststained and examined as described for single labeling.

Sections were scanned at magnifications ranging from approximately 35,000 to 95,000 times to ascertain that organelles were clearly visible, then photographs were taken to ascertain the degree of authentic versus background labeling of organellar membranes by gold particles. Micrographs selected for presentation in Fig. 3 are representative of immunogold images observed in sections from two different blocks and four independent immunostaining sections, and were selected from at least 30 different micrographs.

Results

An analysis of sucrose gradient fractions (Fig. 1) shows that measurable catalase and CCO activity were resolved into narrow bands that were widely separated from each other in the gradient. The buoyant densities of the catalase and CCO peaks (1.27 and 1.18 g ml^{-1} respectively) are in agreement with previously published data (Corpas et al. 1994).

Table 1 Alignment of the amino acid sequence of the CNBr-cleaved fragment from cucumber PMP36 with porins of plants

Species (accesion number)	Organelle	Sequences	% Identities
Cucumis sativus PMP36	Glyoxysomes	1 GKGPGLYSDIGKRARDLLYLDYQS 24	—
Spinacia oleracea (U50900)	?	3 ....R.... 25	95
Solanum tuberosum 36 I, II (P42054)	Mitochondria	3 ......T....K......K..H. 25	86
Prunus armeniaca (AF139498)	Mitochondria	3 ......L....K......K.... 25	86
Solanum tuberosum 34 (P42055)	Mitochondria	2 .......TE...K......K.... 25	83
Mesembryanthemum crystallinum (U79765)	?	3 ......T....K......R...T 25	82
Pisum sativum (P42056)	Plastids	3 ..........K......R..V. 25	82
Arabidopsis thaliana (Z35367)	?	3 ......TE...K......R... 24	81
Triticum aestivum (P46274)	Mitochondria	3 ......G...K.K....R...T 24	77
Zea mays (3747046)	Plastids	3 .....TE...KT.....K...T 24	72

The search was done with the Basic Local Alignment Search Tool (BLAST) using the data bank of the National Institute of Health (NIH).

A prominent integral 36 kDa membrane polypeptide occurs in both mitochondrial and glyoxysomal fractions

Figure 2, panel A, shows a representative SDS PAGE, silver-stained polypeptide pattern of the mitochondrial and glyoxysomal gradient fractions (lanes A, B) and the detergent-solubilized proteins derived from alkaline carbonate-washed membranes recovered from these organelle fractions (lanes C, D). Lanes A and B show a diversity of polypeptides ranging in molecular mass from approximately 14 to 80 kDa in the mitochondrial and glyoxysomal preparations. The polypeptide diversity between these organelle fractions confirms the low level of cross-contamination of these two organelles as determined from comparisons of marker enzyme activities and immunoblots with CCO antiserum (panel B). Solubilized membrane fractions (lanes C, D) show fewer and more prominent silver-stained polypeptides as was expected. These polypeptides are considered to be integral membrane proteins because they remain associated with the membrane fractions following extensive washes in alkaline carbonate solutions. Among the diverse polypeptides, two prominent polypeptides of approximately 31 and 36 kDa existed in both membrane fractions (compare lanes C and D).

Purified glyoxysomal fractions are free of significant mitochondrial contamination

Figure 2, panel B, shows the Western blot analysis of cucumber organelles and their integral membrane proteins probed with anti-CCO antiserum. Lane A, a control, is purified corn mitochondria. The CCO antiserum recognizes three corn polypeptides with mol wt of 48, 38 and 31 kDa. These molecular masses correspond to subunit I of corn CCO, a cytochrome B subunit, and subunit II of CCO respectively (Hawkesford et al. 1989). A 48 kDa protein (presumably CCO subunit I) also was immunodetected in the pooled cucumber mitochondria fractions (lane B) as well as in mitochondrial membranes (lane D). In addition a 35 kDa polypeptide (probably CCO subunit II) was detected in the pooled cucumber mitochondrial fractions. No polypeptides were immunodetected with anti-CCO in the pooled cucumber glyoxysomal fractions (lane C), or in the glyoxysomal membranes (lane E).

Significant amino acid sequence identities exist between fragments of the glyoxysomal 36-kDa polypeptide and plant organellar porins

Meaningful amino acid sequence(s) were sought within the 36-kDa polypeptide recovered from the glyoxysomal fractions so that comparisons could be made with sequences in data bases. Our previous results with authentic cucumber glyoxysomal PMPs (e.g. PMP73 and PMP30) revealed that the N terminus of each was blocked (Corpas and Trelease 1997, Corpas and Trelease 1998). Taking these results into account, the 36-kDa polypeptide was subjected to CNBr cleavage to obtain sequences of cleaved, unblocked fragment(s). Table 1 shows the sequence of 24 amino acid residues that was obtained from a fragment that exhibited the most intense Coomassie blue staining (on a PVDF membrane). Comparisons of this sequence with those in data bases reveal high percent amino acid identities with sequences within several different plant porins. The highest percent identity, 95%, is with a spinach porin whose subcellular location is not known. The second highest identity, 86%, is with several mitochondrial porins located in apricot and potato mitochondria. Just over 80% sequence identity was observed for a porin in non-green pea root plastids. It also is apparent from the comparisons in Table 1 that non-identical amino acid residues in the cucumber protein generally are similar to those in other porins, e.g. serine/threonine, arginine/lysine, and leucine/lysine/arginine.

36-kDa and 34-kDa polypeptides in cucumber glyoxysomal and mitochondrial membrane fractions are immunorelated to potato tuber mitochondrial porin

Lanes E–H in Fig. 2, panel A are representative immunob-

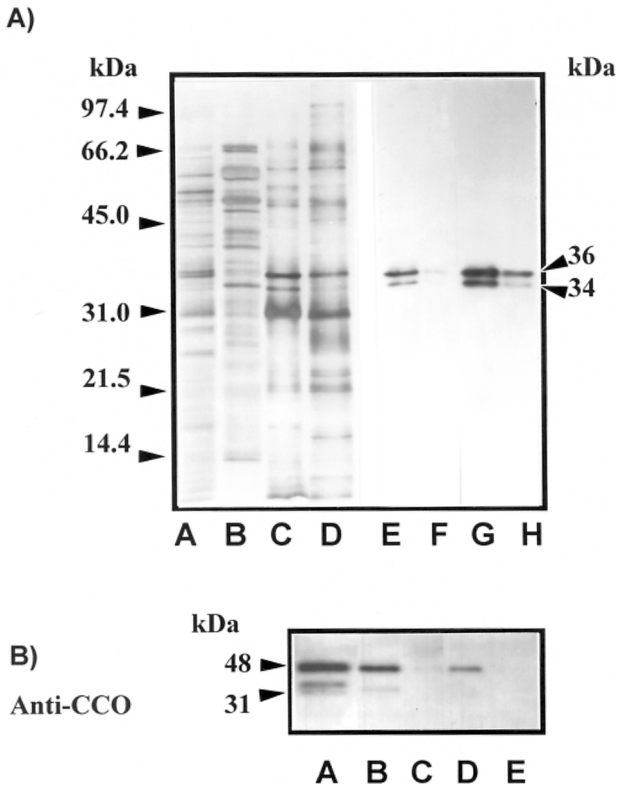

Fig. 2 (A) Silver-stained SDS gel (10–15% gradient) (lanes A–D; 10 μg protein per lane) and the corresponding immunoblot (lanes E–H; 20 μg protein per lane) probed with potato porin antiserum (1 : 500). Lanes A and E, mitochondrial fractions. Lanes B and F, glyoxysomal fractions. Lanes C and G, detergent-solubilized mitochondrial membrane protein fraction. Lane D and H, detergent-solubilized glyoxysomal membrane protein fraction. (B) Western immunoblot of a 12% SDS minigel (10 μg protein per lane), probed with sweet potato CCO antiserum (1 : 1,000). Lane A, corn mitochondria; lane B, pooled cucumber mitochondrial fractions; lane C, pooled cucumber glyoxysomal fractions; lane D, detergent-solubilized cucumber mitochondrial membrane proteins; lane E, detergent-solubilized cucumber glyoxysomal membrane proteins.

lot results obtained with antiserum raised against potato mitochondrial porin expressed in *E. coli*. The blotted samples are the same as those samples that were silver stained in lanes A–D. Two polypeptides, 34 and 36 kDa, in the cucumber mitochondrial fraction are recognized by IgGs in this potato porin antiserum (lane E). Neither these polypeptides, nor any others, were detected among the polypeptides that were electroblotted from the glyoxysomal fraction (lane F) even though the same amount of protein in the mitochondrial and glyoxysomal fractions was added to each lane of the SDS gels. However, this potato porin antiserum detects both polypeptides among those solubilized from the carbonate-washed membranes obtained from mitochondrial and glyoxysomal fractions (lanes G, H). The intensity of the immunoreactive bands was consistently greatest in the mitochondrial membrane fraction (lane G).

IgGs in the potato porin antiserum were affinity purified to 36-kDa polypeptides (bound to PVDF membranes) that were derived from the cucumber glyoxysomal membrane fractions. Application of these IgGs to electroblotted, carbonate-washed mitochondrial and glyoxysomal membrane polypeptides (similar to lanes G, H) resulted in virtually identical immunoreactive patterns as those shown in lanes G, H (data not shown). These results indicate that IgGs specific for the 36-kDa polypeptide also recognize the 34-kDa polypeptide. In other experiments, similar blots incubated with *Saccharomyces* mitochondrial porin antiserum did not result in any immuno-staining of polypeptide bands (data not shown).

Immunogold microscopy confirms localization of anti-porin polypeptide(s) in glyoxysomal and mitochondrial membranes

Figure 3 is a grouping of electron micrographs showing representative results of single immunolabeling experiments. In Fig. 3A, gold particles are selectively clustered over several mitochondria, a glyoxysome, and a non-green plastid (etioplast). In the inset showing a higher magnification view of a glyoxysome in another cell, gold particles decorate the boundary of the glyoxysome. Organelle boundary membranes are not visible in these micrographs because the formaldehyde-fixed cotyledons were not postfixed in osmium tetroxide; this step was omitted as a means to preserve antigenicity. Nevertheless, gold particles are most prevalent at the boundary of mitochondria and glyoxysomes, but not of plastids. In tangential sectional views of the organelles (panel A), some of the gold particles appear to be over the matrix of mitochondria and glyoxysomes. However, we interpret these images as gold particles marking antigens in obliquely cut boundary membranes that occur at the surface of the sections. Some gold particles are observed scattered over various cellular components, e.g. cell walls, cytosolic polyribosomes, protein bodies, plastid stroma, lipid bodies, etc., that almost certainly do not possess porin polypeptides.

Figure 3B is a representative micrograph from a control experiment wherein preimmune serum was applied to sections instead of porin antiserum. Clusters of gold particles do not appear over mitochondria or glyoxysomes as illustrated in Fig. 3A. However, a similar scatter of gold particles is observed over the non-porin containing cellular components. This scatter is considered as the background level of gold particle binding when BSA/GNS is used as the "blocking protein".

Solutions used for immunogold localizations vary considerably among immunogold studies. The particular blocking protein employed often has a significant positive or negative effect on the background level of gold particle binding. Bunkelmann et al. (1995) reported that inclusion of FCS as the blocking protein in the immunogold solutions resulted in a low background level of gold particle binding. As a positive control for the immunogold localizations obtained with BSA/GNS in antiserum solutions (panels A and B), cotyledon sections

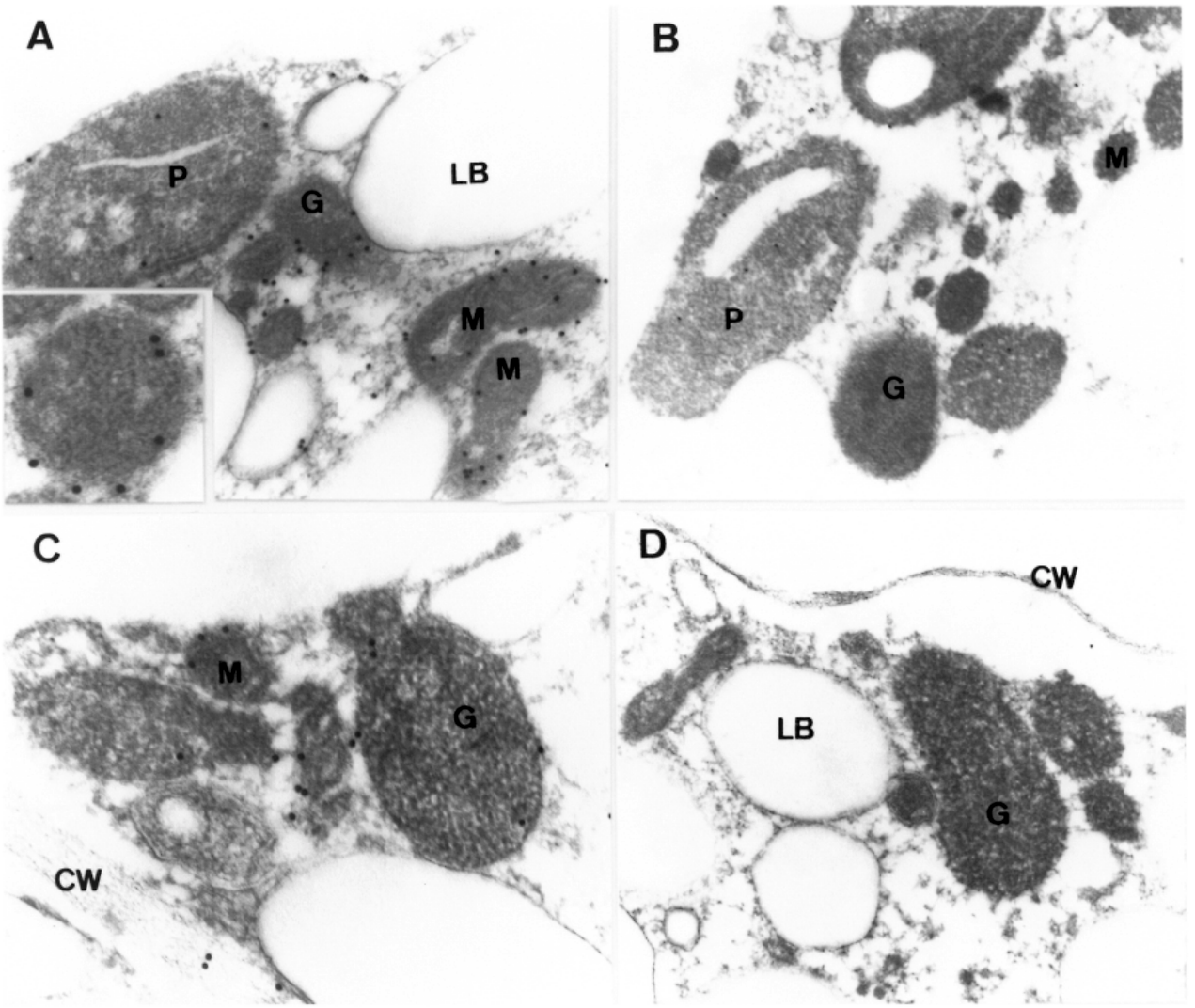

Fig. 3 Representative electron micrographs of cucumber cotyledon thin sections illustrating results of immunogold localization experiments with potato porin antiserum. (A) and (C) sections probed with porin antiserum diluted (1 : 100) in TBST containing 2% BSA/1% GNS (A), or in TBST containing 5% FCS (C). (B) and (D) sections probed with pre-immune serum diluted (1 : 200) in the same buffered solutions as A and C, respectively. CW, cell wall; G, glyoxysome; LB, lipid body; M, mitochondrion; P, plastid. The magnification are: 50,000× (A); 95,000× (inset); 26,000× (B); 70,000× (C); and 35,000× (D).

were incubated in solutions containing FCS instead of BSA/GNS. Figure 3C shows gold particles over glyoxysomes and mitochondria in sections incubated in porin antiserum with FCS. Figure 3D shows the results of applying preimmune serum with FCS in the antibody solution. Spurious gold particle binding is virtually non-existent under these conditions. Hence, binding of fewer gold particles per organelle as illustrated in panel C probably reflects more stringent, but more specific immunogold labeling than that shown in panel A. Indirect, postembedment immunogold electron microscopy was employed to determine whether immuno-reactive porin antigens existed within cucumber cotyledon glyoxysomes (and mitochondria). Figure 4 shows the results of a representative experiment confirming the identity of glyoxysomes shown in Fig. 3. The 15-nm gold particles, bound to anti-porin are at the periphery whereas 5-nm gold particles, bound to anti-catalase are throughout the matrix. The results clearly indicate that these organelles are glyoxysomes.

An anti-porin immunoreactive polypeptide is a constitutive component during the postgerminative conversion of glyoxysomes to leaf-type peroxisomes

Figure 5 shows the polypeptide composition (silver staining) and potato anti-porin immunoreactivity of solubilized anti-

gen(s) derived from peroxisomal membrane fractions of cucumber cotyledons at four stages of postgerminative growth. The silver-stained polypeptide profiles of glyoxysomal/leaf-type peroxisomal fractions (lanes A–D) reveal that some polypeptides disappear with time (e.g. 22 and 73 kDa) while others seem to become more prominent (e.g. 26 and 52 kDa). These are the same gel lanes that are illustrated by Corpas and Trelease (1998) in their Figure 1. Immunoblots of these polypeptides prepared with the potato porin antiserum reveal that an immunoreactive 36-kDa polypeptide is present in the glyoxysomal/leaf-type peroxisomal fractions at all the stages of postgerminative growth. This does not seem to be the case for the 34 kDa polypeptide. A substantially lighter band in the 34-kD region of the gels was observed in the dark-grown samples (2D) and those transferred to continuous light for 1 or 3 d (lanes E–G). Although the 34 kDa band is not visible in lane F of the figure, it was apparent when the film was exposed for longer times. However, a 34-kDa band was never observed in the leaf-type peroxisomal fractions obtained from seedlings maintained in continuous light for 8 d (lane H). Thus it seems that both porin polypeptides are present in the cucumber glyoxysomes, but that only the 36 kDa porin polypeptide persists in leaf-type peroxisomes. The reason for this is not known.

Porin polypeptide(s) are common to glyoxysomes of several species

Figure 6, panel A, illustrates patterns in SDS gels of silver-stained membrane proteins in glyoxysomes from four different oilseed species. These data are similar to the ones presented by Corpas et al. (1994) in their Figure 5. The analysis of the polypeptide blots with potato porin antiserum (panel B) revealed the expected strong reaction with two polypeptides (34 and 36 kDa) in cucumber glyoxysomes, but an immunoreaction was apparent with only one polypeptide (34 kDa) in the membrane fractions from castor bean, sunflower, and cotton glyoxysomes. The immunoblot reaction was the weakest with the sunflower and cotton samples. The positive control shown in panel C illustrates on the same blotted membranes the immunological cross-reactivity of peroxisomal APX, a PMP that is common to the glyoxysomes in these four oilseed species. The molecular mass of these polypeptides is approximately 30 kDa.

Discussion

Biochemical and electron-microscopic immunogold evidence presented in this paper indicate that the 36 kDa polypeptide recovered from cucumber cotyledon glyoxysomal fractions is an authentic integral PMP (PMP36). Immunological and amino acid sequence data are consistent with the interpretation that PMP36 (and probably a 34 kDa polypeptide, PMP34?) is a porin molecule. Data showing that these molecules function as porin-type transporters are not included in this paper, but four studies by Reumann et al. (1995), Reumann et al. (1996), Reumann et al. (1997), Reumann et al. (1998) convincingly show that two types of plant peroxisomes (glyoxysomes and leaf peroxisomes) possess functional porins

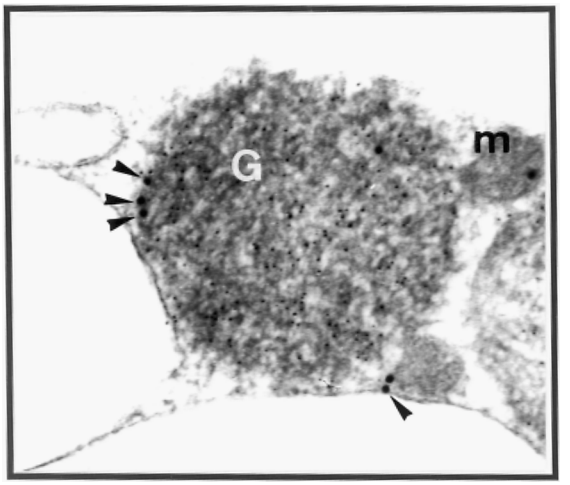

Fig. 4 A representative electron micrograph of cucumber cotyledon thin sections illustrating results of indirect, double immunogold labeling with potato porin antiserum and anti-pumpkin seed catalase. The 15-nm gold particles show the peripheral localization of porin. The 5-nm gold particles show the matrix localization of catalase. G, glyoxysome; m, mitochondrion. The magnification is 90,000×.

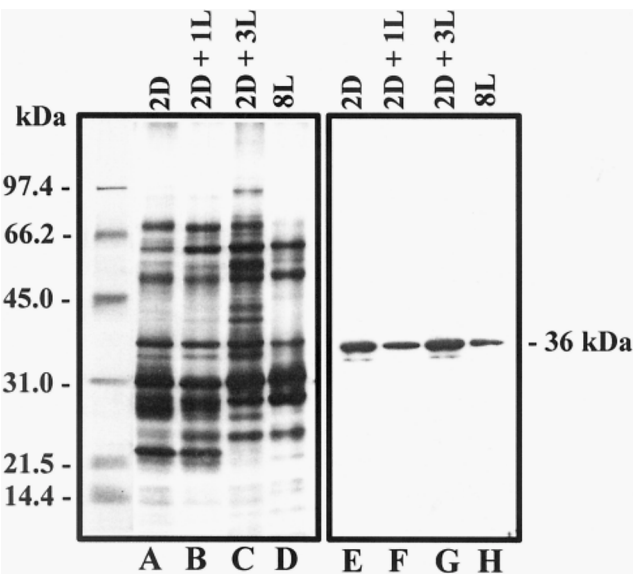

Fig. 5 Silver-stained SDS gel (10–15% gradient) of PMPs recovered from cucumber glyoxysomes at different stages of postgerminative growth (lanes A–D; 10 μg protein per lane) and the corresponding immunoblot (lanes E–H; 20 μg protein per lane) probed with potato porin antiserum (1 : 500). 2D, 2 d dark; 2D + 1L, 2 d dark plus 1 d continuous light; 2D + 3L, 2 d dark plus 3 d continuous light; 8L, 8 d continuous light.

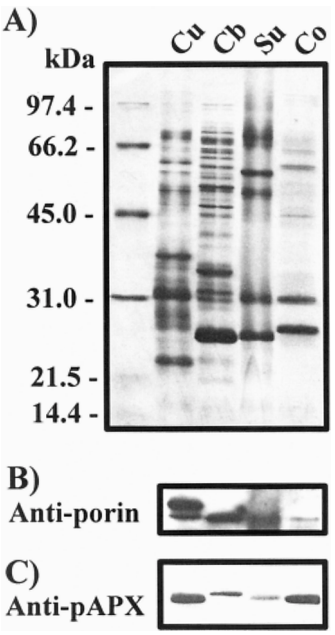

Fig. 6 Silver-stained SDS gel (10–15% gradient) of PMPs isolated from four oilseed species (A) and the corresponding immunoblots probed with potato porin antiserum (1 : 500) (B), or with cucumber peroxisomal APX antiserum (1 : 500) (C). Cu, cucumber; Cb, castor bean; Su, sunflower; Co, cotton.

in their boundary membranes. The boundary membrane of rat liver peroxisomes also exhibits properties that are characteristic of porin transport (Labarca et al. 1986, Van Veldhoven et al. 1987, Lemmens et al. 1989). In addition, a 31 kDa polypeptide purified from *Hansenula polymorpha* peroxisomes cross reacts with antibodies against the 31 kDa mitochondrial porin of *Saccharomyces cerevisiae* and exhibits pore forming properties in proteoliposomes (Sulter et al. 1993). Another yeast protein, PMP47 identified in *Candida boidinii*, exhibits significant structural homology with mitochondrial anion translocators (Jank et al. 1993). Thus, our interpretation that cucumber cotyledon glyoxysomes, and other oilseed glyoxysomes (Fig. 6), possess polypeptide(s) that likely function as porins is consistent with the ample evidence for the existence and function of peroxisomal membrane porins in diverse eukaryotes.

Reumann and co-workers reported that the pore-forming proteins of both glyoxysomes and leaf peroxisomes were distinctly different from mitochondrial and plastid porins. For example, the peroxisomal porins were characterized by substantially lower (10- to 20-fold) single channel conductance, a strong anion selectivity, and formation of smaller, specific transporter pores. Such functional differences suggest that the mitochondrial and peroxisomal polypeptides would be different, yet the data presented in this paper indicate similarities in immunological cross-reactivity, molecular mass, and amino acid sequences**.** The following discussion provides rationale for the apparent similarities and differences.

A common feature of porin monomers is their β-barrel secondary structure. In spite of this, there is a remarkable lack of close sequence homology. Why then were such high degrees of sequence identity found among PMP36 and the mitochondrial porins shown in Table 1? The fragment sequenced contained an apparent helical hydrophilic region (Pro4-Glu21). This motif has been recognized for many years to have high degree of sequence conservation near the N terminus of most mitochondrial porins, but not among bacterial porins (Nikaido 1992). Since CNBr cleaves just beyond Met residues, it is reasonable to suggest that the cucumber fragment was derived from the N terminus of the 36 kDa polypeptide. With Met (removed by CNBr action) in position 1, the helical portion would be Pro5 to Glu 22 in the cucumber polypeptide. This conserved N terminal region is not cleaved upon import (Heins et al. 1994). Thus, it is reasonable that this portion of PMP36 and plant mitochondrial (and plastid) porins would exhibit high degrees of amino acid sequence identities.

Immunological cross-reactivities among porins, especially within groups, i.e. plants and mammals, is not unusual even when functions are quite diverse (see references above). This may be due to the common epitopes in β-barrel regions. Another similarity among eukaryotic porins is the relatively narrow range of reported molecular mass of the polypeptides (30–36 kDa). However, this variation may be even less because estimates typically are made from migrations in SDS gels. For example, Heins et al. (1994) estimated one of the potato porins to be 36 kDa based on migration in SDS gels, but the deduced mass from the ORF encoding the polypeptide was 29.4 kDa. Taken together, this information provides some logical rationale for the apparent biochemical and immunological similarities between PMP36 (and PMP34) and mitochondrial porin polypeptides when the physiological characteristics of the membrane porins are quite different (e.g. Reumann et al. 1998).

A critical means for assessing and making distinctions about the subcellular localization(s) of polypeptides is immunogold electron microscopy. Ascertaining whether glyoxysomes actually possessed antigens specifically recognized by anti-porin IgGs was enhanced greatly by the internal positive control provided by probing the sections with the same IgGs that were known from immunoblot data to recognize mitochondrial porin(s). Gold particles consistently were clustered over mitochondria well above background levels whether BSA or FCS was used as the blocking protein. A measure of the reliability of the porin immunogold assessment would be the specific localization of gold particles at the mitochondrial outer membrane. Because the cucumber mitochondria were relatively small, it was not possible to make a reliable quantitative assessment of outer membrane gold labeling. However, our immunogold images of the mitochondrial porin localization are similar to the immunogold localizations of porin in the mitochondrial membranes of cultured COS7 cells (Yu et al. 1995) and muscle tissue (Lewis et al. 1994). These comparisons cou-

pled with our examination of numerous immunogold-labeled mitochondria convinced us that within the limits of resolution of the postembedment immunogold microscopy, porin(s) were localized to the outer membranes of cucumber cotyledon mitochondria. A similar conclusion was not reached for the immunogold labeling of cucumber plastids (etioplasts). Although gold particles consistently were observed over plastids, especially when BSA was employed as the blocking protein, the particles consistently were observed over the stroma, not over the boundary membranes of the plastids. Our interpretation is that the potato anti-porin IgGs do not recognize authentic cucumber etioplast porin(s) in the cotyledon sections.

Confidence in interpretations of glyoxysomal immunogold labeling with anti-porin IgGs was provided by the reliable, simultaneous immunogold localization of mitochondrial porin(s). However, assessment of authentic glyoxysomal membrane immunogold labeling could be hampered somewhat by encountering at a lower frequency suitable sectioned glyoxysomes with exposed membrane antigens, and by the apparent lower abundance of porin(s) in their boundary membrane compared to that in the cucumber mitochondria. The image presented in the inset of Fig. 3A illustrates the highest density, immunogold-decorated glyoxysome that was observed in this study. However, this image and a majority of the other immunogold images revealed a high preponderance of gold particles at the boundary of the glyoxysomes. This pattern was distinctly different from that observed over etioplasts. Our overall assessment is that gold particle decoration of glyoxysomes attributable to recognition of potato anti-porin IgGs is significantly greater than background gold particle binding to the sections. These results provide convincing evidence that show that porin polypeptide(s) (36 and/or 34 kDa) are associated with the cucumber glyoxysomal boundary membrane.

In summary, the evidence collectively indicates that at least one porin polypeptide is a common and constitutive membrane component of at least two types of plant peroxisomes, namely glyoxysomes and leaf-type peroxisomes. An anti-porin immunoreactive polypeptide, bearing sequences indicative of a porin (36 kDa, Table 1), is present in the peroxisome membrane fraction of cucumber cotyledons during both auto- and heterotrophic stages of postgerminative growth (Fig. 3), and an anti-porin immunoreactive polypeptide is present also in castor bean, sunflower and cotton glyoxysomal membrane fractions (Fig. 6). The common occurrence among oilseed glyoxysomes and the constitutive feature of PMP36 (PMP34) are known for only one other plant PMP, namely peroxisomal APX (Corpas et al. 1994, Yamaguchi et al. 1995a, Bunkelmann and Trelease 1997, Corpas and Trelease 1998).

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